FEBS Letters 528 (2002) 90–94

FEBS 26487

Functional modulation of a peroxygenase cytochrome P450: novel insight into the mechanisms of peroxygenase and peroxidase enzymes

Isamu Matsunaga[a,*], Tatsuo Sumimoto[b], Minoru Ayata[a], Hisashi Ogura[a]

[a] *Department of Virology, Osaka City University Medical School, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan*
[b] *Osaka Prefectural Institute of Public Health, 1-3-69 Nakamichi, Higasinari-ku, Osaka 537-0025, Japan*

Received 5 June 2002; revised 7 August 2002; accepted 8 August 2002

First published online 23 August 2002

Edited by Stuart Ferguson

Abstract **Cytochrome $P450_{BS\beta}$ is a peroxygenase that catalyzes the α- or β-hydroxylation of myristic acid by utilizing H_2O_2. The wild-type enzyme not only hydroxylated myristic acid, but oxidized 3,5,3′,5′-tetramethylbenzidine (TMB), a peroxidase substrate, in a myristic acid-dependent reaction. Study of inhibition of hydroxylation of myristic acid by TMB indicates these two substrates compete for the same highly reactive intermediate during the course of their respective reactions. When deuterated myristic acid was used as a substrate to decrease hydroxylation activity, the rate of TMB oxidation increased. This increased rate of TMB oxidation was greatly enhanced when the R242K mutant enzyme bound with deuterated myristic acid was used. These results suggest that there are critical structural elements at the distal active site which determine whether this enzyme acts as a peroxygenase or a peroxidase.**

Key words: Cytochrome P450; Fatty acid; Peroxygenase; Peroxidase

1. Introduction

Cytochrome P450 (P450) enzymes are heme-thiolate proteins and comprise a very large enzyme superfamily. Many P450 enzymes catalyze monooxygenation of various hydrophobic compounds such as fatty acids by introducing an oxygen atom derived from molecular oxygen [1]. This reaction usually requires electron transfer from NAD(P)H through a reductase and subsequent activation of molecular oxygen at the heme active site. P450 enzymes act most efficiently when the P450, a reductase, NAD(P)H and in some cases, an iron–sulfur protein and/or cytochrome *b*5 are reconstituted in a functional electron transfer chain. In contrast to these reactions, some P450s can use peroxides as oxygen donors and in this case, a reductase and NAD(P)H are not required. This constitutes the so called peroxide shunt pathway that generally proceeds more slowly than the usual P450 reaction which is supported by a reductase. Moreover, the highest activity is obtained only at an unphysiological concentration of peroxide and the affinities for peroxides are usually low. Thus, this type of reaction supported by peroxides in a P450 would not normally be regarded as physiological significant.

We have recently identified a very unusual P450 enzyme from *Bacillus subtilis* ($P450_{BS\beta}$ as a trivial name; CYP152A1 in systematic nomenclature [2]), which efficiently utilizes H_2O_2 to hydroxylate fatty acids [3]. The turnover in the reaction of this P450 enzyme is extremely high, and its apparent affinity for H_2O_2 (as judged by the K_m value for H_2O_2) is considerable, in comparison to other P450s (e.g. for P450 2B4, K_m for H_2O_2 was reported to be 5 mM [4]). These properties are very unusual compared to other P450s. Moreover, we could not reconstitute its activity by NADH, ferredoxin, ferredoxin reductase, and the peroxygenase P450 or NADPH, NADPH-P450 reductase, and the peroxygenase P450 (unpublished observation). Therefore, this enzyme appears to have an essential requirement for H_2O_2, and it is perhaps more appropriate to classify it as a peroxygenase rather than a monooxygenase.

Other unique features of this peroxygenase P450 is the unusual regiospecificity [3] displayed during hydroxylation of fatty acid substrates. The known P450s hydroxylate positions close to the ω-end of the fatty acid [5], whereas the peroxygenase $P450_{BS\beta}$ hydroxylates the carbon atom adjacent to the carboxyl moiety. Our recent study of $P450_{BS\beta}$ by site-directed mutagenesis revealed that the Arg residue at position 242 on the distal helix of $P450_{BS\beta}$ can interact with the carboxylate of the substrate close to the heme iron at the distal side of the heme molecule and this determines its regioselectivity [6]. The Arg residue adjacent to the heme iron is reminiscent of the architecture of horseradish peroxidase (HRP), whereas the carboxylate group is more typical of chloroperoxidase (CPO).

In contrast to the monooxygenase P450s, HRP, one of the archetypal heme-containing peroxidases, usually catalyzes one- or two-electron oxidations of various organic compounds using H_2O_2 [1]. Although peroxygenase activity of HRP was reported, this was much lower than its peroxidase activity [7]. Molecular engineering of HRP, however, was reported to strengthen the peroxygenase activity, when the His residue at its distal site, which is critical for the peroxidase activity, was replaced with Glu [8]. The functional Glu residue at the distal side of the heme is found in CPO which shows significant peroxygenase activity as well as peroxidase activity [9].

Based on these findings, we hypothesized that $P450_{BS\beta}$

*Corresponding author. Fax: (81)-6-6645 3912.
E-mail address: matsunagai@med.osaka-cu.ac.jp (I. Matsunaga).

Abbreviations: CPO, chloroperoxidase; HRP, horseradish peroxidase; P450, cytochrome P450; $P450_{BS\beta}$, fatty acid α- or β-hydroxylating-cytochrome P450 from *Bacillus subtilis*; TMB, 3,5,3′,5′-tetramethylbenzidine

0014-5793/02/$22.00
PII: S0014-5793(02)03261-1

might behave as a peroxidase by modulating the distal environment of its heme group. In this study, we investigated H_2O_2 utilization and peroxidase activity of $P450_{BS\beta}$ and discuss mechanistic features of the peroxygenase and peroxidase reactions. Especially, we present evidence for direct competition between peroxidase and peroxygenase chemistry in the active site of $P450_{BS\beta}$: this appears to involve competition for a common intermediate during the catalytic turnover.

2. Materials and methods

2.1. Materials

Myristic acid and α-hydroxylauric acid were purchased from Sigma-Aldrich Japan (Tokyo, Japan). 9-Anthryldiazomethane and *d27*-myristic acid were purchased from Funakoshi Co. Ltd. (Tokyo, Japan). 3,5,3′,5′-Tetramethylbenzidine (TMB) was purchased from Dojindo laboratories (Kumamoto, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Preparation of the recombinant form of $P450_{BS\beta}$ and its R242K mutant enzyme and assays for fatty acid-hydroxylating activity and peroxidase activity

Expression, purification, and site-directed mutagenesis of the recombinant form of $P450_{BS\beta}$ were described previously [6].

The standard reaction mixture contained 0.1 M potassium phosphate buffer (pH 6.7), 0.1 mM H_2O_2, 0.2 mM myristic acid, and 5 pmol of the enzyme preparation in the total volume of 0.2 ml. Myristic acid was dissolved in ethanol and 1 μl of this solution was added to the reaction mixture. When *d27*-myristic acid was used as a substrate, it was dissolved in *d6*-ethanol. All reactions were performed at 37°C. For determining fatty acid-hydroxylating activity, the reaction was terminated by the addition of 20 μl of 2 N HCl and then, 5 μl of 1 mM α-hydroxylauric acid was added to the reaction mixture to act as an internal standard. Extraction, derivatization with 9-anthryldiazomethane, and determination of the reaction products by high performance liquid chromatography were carried out as described previously [5,6].

H_2O_2 consumption was measured by transferring a 0.1 ml aliquot of the reaction mixture to a new tube containing 0.3 ml of TMB solution {167 mM acetate buffer (pH, 5.0), 0.833 mM TMB, 33 μg/ml HRP}. The solution was incubated at 37°C for 3 min and the optical absorbance at 450 nm ($\varepsilon_{450} = 5.9 \times 10^4$ M^{-1} cm^{-1}) was measured.

For determining peroxidase activity, TMB was added to the reaction mixture at a final concentration of 0.7 mM. TMB was dissolved in N,N′-dimethylformaldehyde. After the reaction, 12.5 μl of 6 N H_2SO_4 and 187.5 μl of distilled water were added to the reaction mixture and the optical absorbance at 450 nm was measured.

3. Results and discussion

TMB, a substrate for HRP, is a chromogen and a sensitive indicator of HRP activity. One electron oxidation of TMB gives the TMB radical (blue product) and a further one electron oxidation gives the diimine (yellow product) which has a strong absorbance at 450 nm and is stable in acidic pH [10,11]. Since one molecule of the diimine is non-enzymatically formed from two molecules of the TMB radical at acidic pH and both the high valent oxo-ferryl and hydroxy-iron intermediates (compound I and II, respectively) in the catalytic cycle of HRP [1] can oxidize TMB, one molecule of diimine is formed by one molecule of H_2O_2. This reaction can be used for measuring H_2O_2 concentration and evaluating peroxidase activity. As shown in Fig. 1A, a catalytic amount of $P450_{BS\beta}$ was able to oxidize TMB in the presence of the fatty acid substrate. These spectra of diimine formation were typical

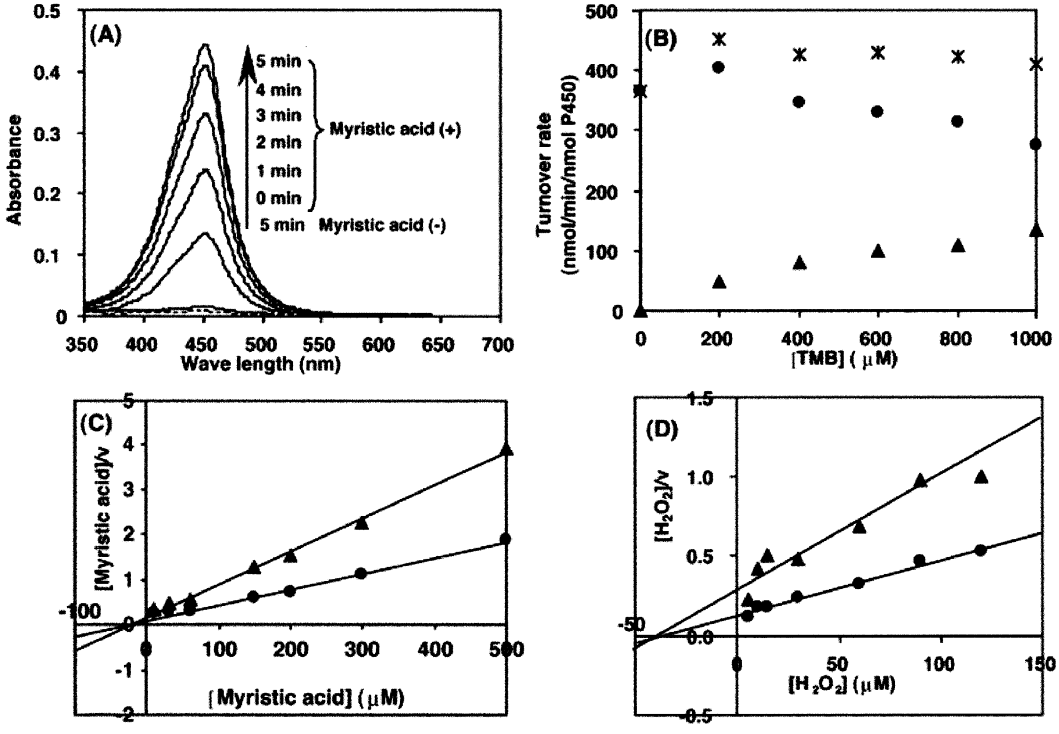


Fig. 1. TMB oxidation by wild-type $P450_{BS\beta}$. A: Spectra of diimine formed by acidification after the reaction with $P450_{BS\beta}$, myristic acid, and TMB. The reaction times are indicated in the panel. A dotted line indicates the spectrum in the absence of myristic acid. B: Effects of TMB concentration in the reaction mixture on turnover rates of TMB oxidation (closed triangles), myristic acid hydroxylation (closed circles), and total product formation (asterisks). The reaction time was 2 min. C: Kinetic study on TMB oxidation (closed triangles) and myristic acid hydroxylation (closed circles) when the concentrations of myristic acid were varied. The reaction time was 2 min. D: Kinetic study on TMB oxidation (closed triangles) and myristic acid hydroxylation (closed circles) when the concentrations of H_2O_2 were varied. The reaction time was 2 min.

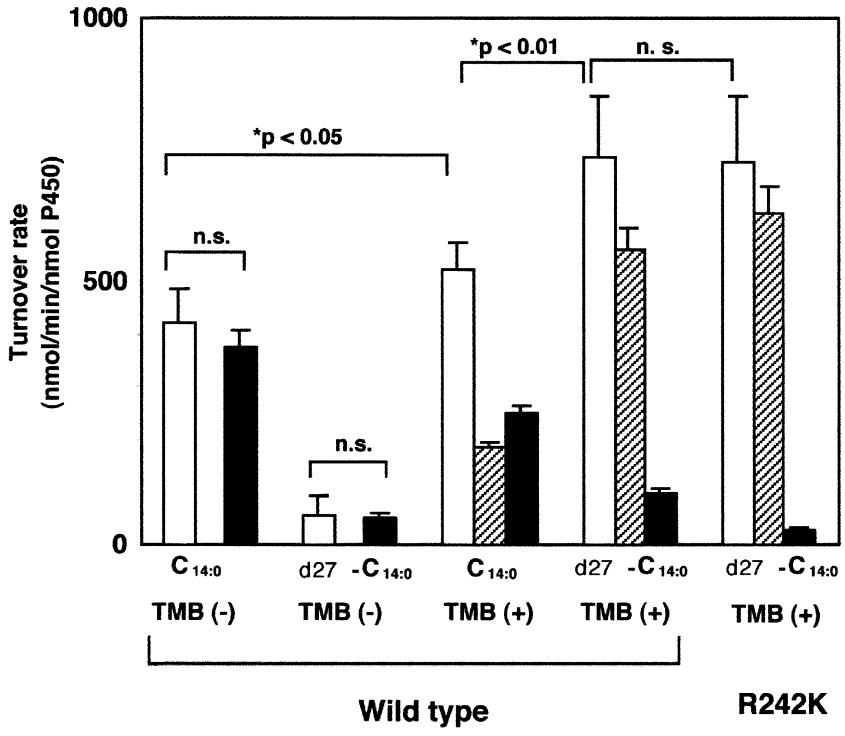


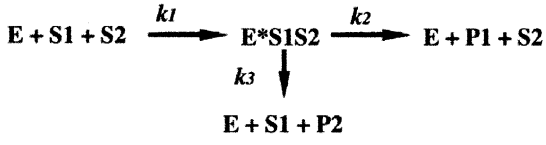
Fig. 2. Peroxygenase and peroxidase activities of the wild-type and R242K mutant $P450_{BS\beta}$. Open, hatched, and closed bars indicate turnover rates of H_2O_2 consumption, TMB oxidation, and myristic acid hydroxylation, respectively. Each result is expressed as the mean and standard deviation of five experiments. The reaction times for the wild-type and the mutant enzymes were 2 min and 1 min, respectively. Asterisks indicate significant differences as indicated the *P*-values estimated by the Student's *t*-test. n.s., not significant.

of the ones previously reported [10,11]. Electrophilic compound I and II-like intermediates have been postulated in the catalytic cycle of $P450_{BS\beta}$ [12]. However, in the case of $P450_{BS\beta}$, production of these compounds requires binding of the fatty acid, in contrast to HRP which cleaves H_2O_2 to form compound I without any organic substrate. No $P450_{BS\beta}$ catalyzed TMB oxidation was observed in the absence of the fatty acid substrate {see Fig. 1A, myristic acid (−)}. In this case, TMB could bind nearby the heme iron of the enzyme, because we observed small, but significant spectral changes including decrease of intensity of the Soret band, increase of the absorbance around 390 nm, and appearance of a charge transfer band when TMB was added to the enzyme preparation (data not shown). Therefore, it is speculated that TMB oxidation is linked to fatty acid-metabolizing reaction pathway of $P450_{BS\beta}$.

To further clarify this point, we investigated the effect of TMB on myristic acid hydroxylation, and performed kinetic studies. Myristic acid hydroxylation was inhibited by TMB oxidation in a TMB concentration-dependent manner (Fig. 1B). The K_m value (~26 μM) for myristic acid in the formation of hydroxyl products was the same as that in the TMB oxidation (Fig. 1C). Furthermore, the K_m value (~35 μM) for H_2O_2 in hydroxyl product formation coincided with that in TMB oxidation (Fig. 1D). In addition, the optimum pH values of myristic acid hydroxylation and TMB oxidation were identical (data not shown). These results strongly suggest that both myristic acid hydroxylation and TMB oxidation involve a common highly reactive intermediate of $P450_{BS\beta}$. TMB can apparently donate electrons to this intermediate produced during the normal reaction pathway of fatty acid hydroxylation by $P450_{BS\beta}$ as shown in Scheme 1. It was noted that the highly reactive intermediate could be produced only in the presence of a fatty acid substrate and thus, fatty acid hydroxylation can occur even when the enzyme is saturated with TMB (see Fig. 1B).

Moreover, the rate of consumption of H_2O_2 in the reaction of the wild-type $P450_{BS\beta}$ is almost the same as the hydroxylation rate {see Fig. 2, TMB (−)}. This result indicates that one oxygen atom of H_2O_2 is utilized for the hydroxylation of myristic acid and the other is used to form a water molecule, as seen in the peroxide shunt pathway of P450 enzymes. It should be emphasized that the leakage of decomposed H_2O_2 was negligible in this reaction and thus, this enzyme has evolved to efficiently utilize H_2O_2 to hydroxylate the fatty acid substrate. As described above, TMB as an additional substrate intercepted the hydroxylation of myristic acid. In this reaction, the overall rate of H_2O_2 decomposition appeared to slightly increase (Fig. 2).

Based on these findings, we thought that the function of $P450_{BS\beta}$ could be modulated to display only peroxidase activity if the hydroxylation event was prevented. We have observed that deuterium-labeled fatty acids decrease the overall



Scheme 1. Reaction pathways of TMB oxidation and myristic acid hydroxylation. E, S1, S2, P1, and P2 indicate $P450_{BS\beta}$, myristic acid, TMB, hydroxymyristic acid, and TMB radical, respectively. E* is the activated enzyme consisting of the highly reactive intermediate and k_1, k_2, and k_3 are kinetic constants at each step. In this scheme, it is considered that the enzyme–substrate complex is actually saturated with H_2O_2.

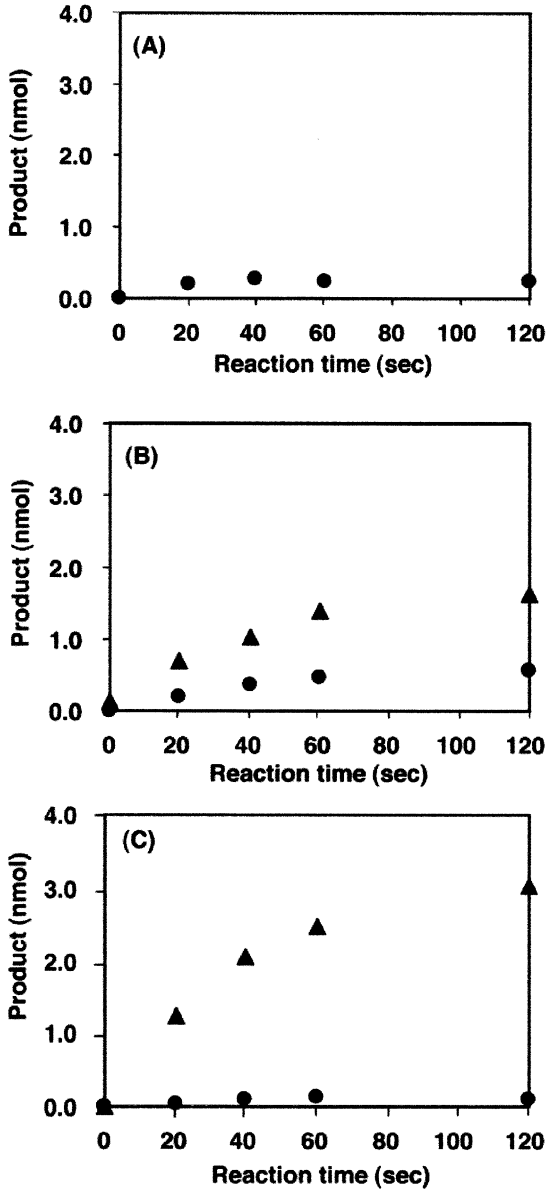


Fig. 3. Time course study of TMB oxidation and myristic acid hydroxylation using the R242K mutant $P450_{BS\beta}$. Closed triangles and closed circles indicate the amounts of diimine and hydroxymyristic acid, respectively. A: The reaction was carried out with myristic acid and without TMB. B: The reaction was carried out with myristic acid and TMB. C: The reaction was carried out with *d6*-myristic acid and TMB.

turnover rate of the hydroxylation reaction catalyzed by $P450_{BS\beta}$ where abstraction of a hydrogen atom from the α- or β-carbon of the substrate is affected ([12] and Fig. 2). The abstraction of the hydrogen atom is promoted by a highly reactive intermediate, possibly an oxo-ferryl π-cation radical intermediate which corresponds to a compound I of HRP. Therefore, it was expected that the TMB oxidation rate would increase if fatty acid hydroxylation could be inhibited due to an isotope effect. As expected, the turnover for TMB oxidation was greatly increased when *d27*-myristic acid was used as a substrate (Fig. 2). In addition, the turnover for decomposition of H_2O_2 was also increased as compared to that with non-deuterated myristic acid, indicating that the kinetic constant for TMB oxidation is somewhat larger than that for myristic acid hydroxylation (in Scheme 1, $k_3 > k_2$).

The Arg 242 residue of $P450_{BS\beta}$ is thought to be important for binding the fatty acid substrate and a mutation at this residue to Lys decreases the hydroxylation activity of the enzyme [6]. Rapid inactivation of this mutant enzyme, possibly due to leakage of reactive oxygen species, appears to be one of the reasons why this mutant enzyme lost its activity (Fig. 3A). The fatty acid substrate interacting with the shorter side chain of the Lys residue may be unable to dock at an appropriate position in the active site. In this situation, the intermediate is unable to effectively attack the substrate and the formation of reactive oxygen species can lead to enzyme inactivation. The addition of TMB, however, rescues such inactivation at least in part (Fig. 3B). In this case, leakage of reactive oxygen may decrease so that the highly reactive intermediate is used for oxidation of TMB which binds as it is independent of residue 242. Interestingly, a R242K mutant bound to *d27*-myristic acid predominantly showed peroxidase activity (Figs. 2 and 3C).

Here, we demonstrate peroxidase activity of $P450_{BS\beta}$. This property appears to be fundamentally based on intercepting the highly reactive intermediate produced in the normal catalytic cycle of this peroxygenase P450. To our knowledge, however, there are no examples of a P450 enzyme which can oxidize TMB during their normal catalytic cycle, although a high concentration of P450s can non-enzymatically oxidize TMB: e.g. heme staining [13]. Presumably this would depend on whether TMB could be positioned within the area that electrons can be transferred even when a substrate is bound to the enzyme.

We have previously proposed that the fatty acid substrate supports O–O bond cleavage of H_2O_2 on the heme molecule of $P450_{BS\beta}$ to produce a highly reactive and electrophilic intermediate [12]. Proton supply is required for the cleavage of the O–O bond. $P450_{BS\beta}$, however, does not have the machinery necessary to deliver a proton from the solvent as is the case with monooxygenase P450s [14], because $P450_{BS\beta}$ lacks the amino acid residues operating such proton delivery [3,6]. We had previously speculated that a water molecule associated with the carboxylate of the substrate may be involved in this O–O bond cleavage by analogy to P450eryF [15]. However, it would also be possible for the carboxylate of the substrate to act directly as an acid–base catalyst as in the proposed mechanism of CPO [16]. In either case, the carboxylate positioned in close proximity to a heme iron appears to be a key functional group.

Although hydroxylation of myristic acid was greatly inhibited in the reaction of the R242K mutant enzyme bound with TMB and deuterated myristic acid, the decomposition rate of H_2O_2 was comparable with that in the wild-type enzyme in the same condition, indicating substitution of Arg242 for Lys does not affect O–O cleavage (Fig. 2). We can see these results from another aspect: if the carboxylate is set up at an appropriate position and if a peroxidase substrate is positioned within the area that electrons can be donated to the highly reactive oxygen species, this enzyme can apparently behave as a peroxidase. The different mechanisms of P450 and HRP have been extensively reviewed in the literature. The importance of the proximal ligand, the thiolate of Cys in P450 and imidazole nitrogen of His in HRP, has been emphasized [1]. However, the existence of CPO which has a proximal thiolate

ligand, but predominantly acts as a peroxidase, poses some intriguing questions concerning its mechanism [17]. Recent evidence suggests that the distal machinery of both P450s and peroxidases are important in determining whether these enzymes act as an oxygenase or a peroxidase [8,9,18]. Here, we provide further evidence that the distal machinery including a bound substrate is critical. The proximal ligand appears to be less important than previously expected. P450, HRP, and CPO have no relation to each other in terms of gene evolution, but have similarity in their reaction mechanisms: e.g. they appear to use an oxo-ferryl intermediate in their reactions. Thus, differences in how to use this highly reactive intermediate might not be a result of diversity of a particular ancient gene, but rather depend on which machinery these enzymes select in their active site and what substrates position themselves within the area that the active oxygen can react.

Acknowledgements: We thank Kayo Tatsuta and Eiko Uenaka for technical assistance. This work was in part supported by a Grant for Scientific Research (14580616) from the Ministry of Education, Science, Culture and Technology of Japan to I.M.

References

[1] Sono, M., Roach, M.P., Coulter, E.D. and Dawson, J.H. (1996) Chem. Rev. 96, 2841–2888.
[2] Nelson, D.R., Kamataki, T., Waxman, D.J., Guengerich, F.P., Estabrook, R.W., Feyereisen, R., Gonzalez, F.J., Coon, M.J., Gunsalus, I.C., Gotoh, O., Okuda, K. and Nebert, D.W. (1993) DNA Cell Biol. 12, 1–51.
[3] Matsunaga, I., Ueda, A., Fujiwara, N., Sumimoto, T. and Ichihara, K. (1999) Lipids 34, 841–846.
[4] Shumyantseva, V.V., Bulko, T.V., Alexandrova, S.A., Sokolov, N.N., Schmid, R.D., Bachmann, T. and Archakov, A.I. (1999) Biochem. Biophys. Res. Commun. 263, 678–680.
[5] Sawamura, A., Kusunose, E., Satouchi, K. and Kusunose, M. (1993) Biochim. Biophys. Acta 1168, 30–36.
[6] Matsunaga, I., Ueda, A., Sumimoto, T., Ichihara, K., Ayata, M. and Ogura, H. (2001) Arch. Biochem. Biophys. 394, 45–53.
[7] Savenkova, M.I., Newmyer, S.L. and Ortiz de Montellano, P.R. (1996) J. Biol. Chem. 271, 24598–24603.
[8] Tanaka, M., Ishimori, K., Mukai, M., Kitagawa, T. and Morishima, I. (1997) Biochemistry 36, 9889–9898.
[9] Yi, X., Mroczko, M., Manoj, K.M., Wang, X. and Hager, L.P. (1999) Proc. Natl. Acad. Sci. USA 96, 12412–12417.
[10] Bos, E.S., Van der Doelen, A.A., Van Rooy, N. and Schuurs, A.H.W.M. (1981) J. Immunoassay 2, 187–204.
[11] Josephy, P.D., Eling, T. and Mason, R.P. (1982) J. Biol. Chem. 257, 3669–3675.
[12] Matsunaga, I., Yamada, A., Lee, D.S., Obayashi, E., Fujiwara, N., Kobayashi, K., Ogura, H. and Shiro, Y. (2002) Biochemistry 41, 1886–1892.
[13] Henne, K.R., Kunze, K.L., Zheng, Y.M., Christmas, P., Soberman, R.J. and Rettie, A.E. (2001) Biochemistry 40, 12925–12931.
[14] Meunier, B. and Bernadou, J. (2000) in: Metal Oxo and Metal Peroxo Species in Catalytic Oxidations, Struct. Bonding (Meunier, B., Ed.), Vol. 97, pp. 1–35, Springer-Verlag, Heidelberg.
[15] Cupp-Vickery, J.R., Han, O., Hutchinson, C.R. and Poulos, T.L. (1996) Nat. Struct. Biol. 3, 632–637.
[16] Sundaramoorthy, M., Terner, J. and Poulos, T.L. (1998) Chem. Biol. 5, 461–473.
[17] Sundaramoorthy, M., Terner, J. and Poulos, T.L. (1995) Structure 3, 1367–1377.
[18] Tanaka, M., Ishimori, K. and Morishima, I. (1996) Biochem. Biophys. Res. Commun. 227, 393–399.