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Cytochrome P-450-Butyl Peroxide Complex Detected by ESR

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Summary: ESR measurements were performed for the reaction systems composed of t- or n-butylhydroperoxide and cytochrome P-450 (UT-2), purified from rat liver microsomes. On addition of n-butylhydroperoxide to the P-450 at pH 7.4, ESR signal due to a ferric low-spin species (g_1=2.29, g_2=2.24 and g_3=1.96) was recorded. The observed g-parameters agreed well with those of a model thiolate-heme-iron(III)-peroxide complex, Fe(III)TPP($^-$S-TGE)($^-$OO-t-butyl) (g_1=2.285, g_2=2.198 and g_3=1.959). In terms of the g-parameters, the new P-450 complex was concluded to be a P-450-butyl peroxide adduct, in which a butyl peroxide anion ligates at the sixth position of heme iron(III).

Cytochrome P-450s[1,2] occur widely in most animal tissues and organelles and in plants and microorganisms, and are well established to function chiefly in mono-oxygenations of lipophilic substances, such as drugs, endogenous steroids and fatty acids. P-450 activates molecular di-oxygen [3,4] coupled with a P-450 reductase. Kadlubar et al.[5] discovered that P-450 in microsomes also catalyzes or promotes the mono-oxygenation at the expense of

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Abbreviations:
ESR, electron spin resonance; P-450, cytochrome P-450 mono-oxygenase; BHPO, butylhydroperoxide; TGE, thioglycolate ethyl ester; Fe(III)TPPCl, iron (III)-tetraphenylporphyrin chloride; Li-TCNQ, lithium-tetra-cyanoquinodimethane.

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peroxides. Since then, the reactions occurring between P-450 and organic peroxides,[6] such as cumen- and benzyl-hydroperoxide, have attracted intense interests of many chemists. P-450 peroxide complexes have frequently been assumed to be one of the transient intermediate complexes in the processes of the per oxide-dependent mono-oxygenations. However, the detail coordination and electronic structures of the complexes are still unknown, in spite of the importance for understanding the peroxide-dependent mono-oxygenations by P-450. We report here with the first characterization for P-450-peroxide adduct on the basis of the results by ESR and electronic spectra, supported by the model complexes.

Experimental:

Materials. An aqueous solution of P-450 (UT-2; 0.122 mM) in phosphate buffer (pH 7.4, 0.1M), containing sodium chloride (0.01M) and glycerol (20%), was purified from rat liver microsomes by a method as previously reported[7]. Fe(III)TPPCl was prepared by the method reported by Adler et al.[8] n-BHPO was synthesized in our laboratory according to the procedures as described by Williams and Mosher.[9] Both 70 % aqueous solution of t-BHPO (Nacalai Tesque, Kyoto Japan), and TGE (Tokyo-Kasei, Tokyo Japan) were used for measurements after dryness and distillation twice under reduced pressure.

Methods. ESR spectra were recorded at 77 K, with JEOL FE1XG and FE2XG X-band spectrometers, operated with 100 kHz field modulation (6.3 gauss). The frequency of microwave (5.0 mW) was monitored by an Advantest TR-5211 digital frequency counter. The magnetic field strength was calibrated by hyperfine coupling constants (86.9 gauss) of Mn(II) ion doped in MgO matrix. g-Values were estimated on the basis of the g-value (g=2.0025) of Li-TCNQ as a standard.

Results and discussion: ESR measurements at 77 K were carried out for an aqueous P-450 (UT-2; 0.122 mM, pH 7.4) solution, containing 0.1% sodium phosphate, 0.01% sodium chloride and 20 % glycerol. As shown in Fig. 1-a, ESR spectrum due to a ferric low-spin species (denoted as complex A; g_1=2.41, g_2=2.24 and g_3=1.93) was observed. ESR signals around g=2.1 region may be from residual components of the paramagnetic species, such as Mn(II) or Cu(II). Similar ESR spectra have been recorded for the resting state of P-450 (g_1=2.436, g_2=2.256 and g_3=1.912) in liver microsomes.[10] The recent X-ray analysis made for the resting form of P-450[11] demonstrated the presence of a water molecule at the sixth-coordinate position of heme iron. Both axial ligands in the complex A is thus assigned to be the thiolate anion from cysteine residue of the protein, and an oxygen of water molecule.

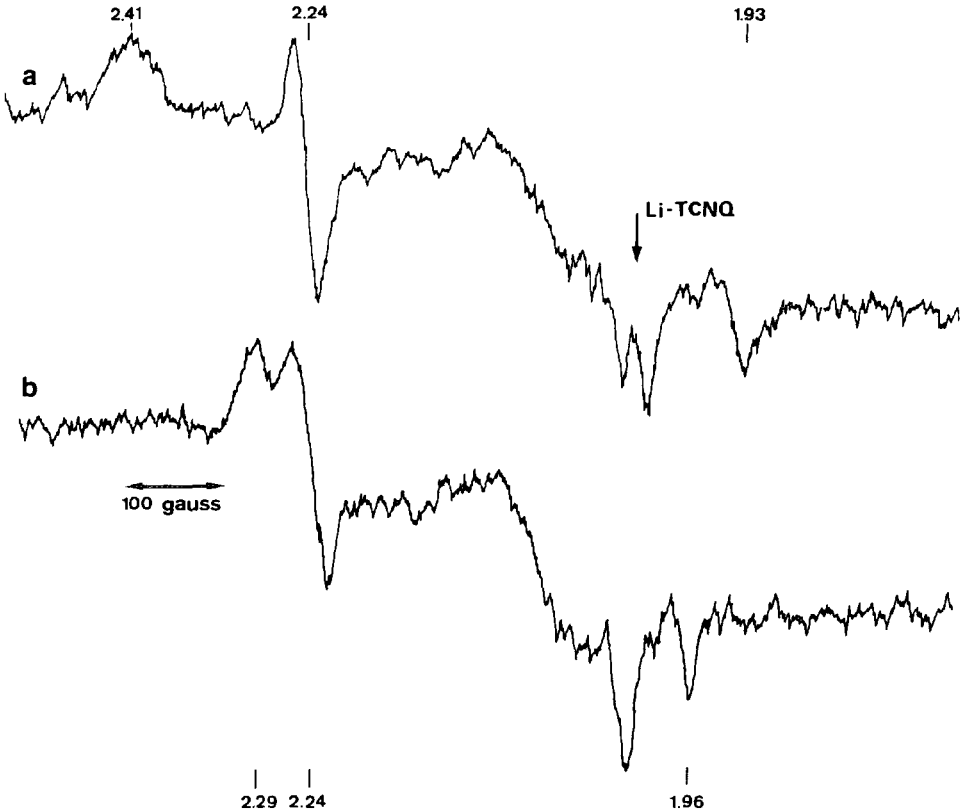


Fig. 1. ESR spectra observed for cytochrome P-450 at 77 K.
a)spectrum for P-450 (UT-2; 0.122 mM, pH 7.4; complex A).
b)spectrum after addition of n-BHPO (2.4 mM) to P-450 (complex B).

The solution of P-450 was then thawed at 277 K and n-BHPO (2.44 mM) was added and immediately frozen at 77 K. The observed ESR spectrum (Fig. 1-b) showed a formation of a new ferric low-spin species (denoted as complex B; g_1=2.29, g_2=2.24 and g_3=1.96), and the ESR signal due to complex A was completely disappeared. Analogous ESR spectrum (g_1=2.28, g_2=2.25 and g_3=1.96) was also recorded, when t-BHPO was added to P-450 instead of n-BHPO. These results indicate that g-parameters of complexes B are independent on the molecular structure of butyl peroxide used. The ESR spectral changes, observed before and after addition of n-BHPO, suggest that the axially coordinate water molecule in the complex A changes to a new axial ligand derived from t- or n-BHPO. When the reaction mixture was annealed at 277 K for about 30 sec, no significant spectral changes were observed, as judged by both g-values and signal intensities of the complex B.

Recently, we[12] have reported that addition of t- or n-BHPO to met-Mb resulted in formation of a ferric low-spin complex

(g_1=2.340, g_2=2.188 and g_3=1.940), at physiological pH regions. The g-parameters of the complex showed a good agreement with those observed for a model complex, Fe(III)TPP(imidazole)($^-$OO-t- or -n-Butyl) complex (g_1=2.352, g_2=2.185 and g_3=1.941), having a peroxide anion and an imidazole at both axial positions. Thus, the complex was assumed to be a six coordinate met-Hb-peroxide complex, in which a deprotonated form of t- or n-BHPO binds at the axial position of heme iron. These results indicate that the t- or n-BHPO easily exchange with the water in solution, suggesting that the peroxide anion possess a higher affinity toward heme iron(III) chromphore than that of water molecule. Thus the peroxide anion derived from t- or n-BHPO is expected to be a candidate for the sixth ligand in complex B. To support the idea, we constructed a new model complex for P-450-peroxide complex, in which both thiolate and peroxide anions coordinate to the axial positions of heme iron(III).

An axial ligand exchange reaction between Fe(III)TPP($^-$OO-t-butyl)$_2$ and TGE was performed to obtain a model complex, by monitoring simultaneous ESR and optical spectra. Fig. 2-a shows ESR spectrum due to the Fe(III)TPP($^-$OO-t-butyl)$_2$ complex (denoted as complex C: g_1=2.239, g_2=2.141 and g_3=1.965), which was prepared by addition of t-BHPO (43.48 mM) and sodium ethoxide (43.48 mM) to a dichloromethane solution of Fe(III)TPPCl (0.87 mM). The reaction mixture (solution (1)) gave the absorption maxima at 419, 542 and 572 nm, as shown in Fig. 3-a. The obtained optical and ESR parameters of the complex C were consistent with those of Fe(III)TPP($^-$OO-t-butyl)$_2$ complex (Table 1), previously reported by us.[13] On addition of a dichloromethane solution of TGE (2.08 mM) to the solution (1), the resulted mixture (solution (2)) showed a weak ESR signal of a new ferric low-spin species (denoted as complex D; g_1=2.285, g_2=2.198 and g_3=1.959), as shown in Fig. 2-b. Maximum ESR signal intensity due to complex D was observed for a solution (3), which was prepared by mixing TGE (4.00 mM) to the solution (2). A red-shifted optical absorption spectrum (Fig. 3-c; 435, 546 and 594 nm) was observed for the solution (3), in which the complex D is exclusively formed, these absorption maxima being distinctively different from those for complex C (Fig. 3-b). Similar ESR and optical spectra for the complex D were also recorded for the frozen solution prepared by n-BHPO and complex C (Table 1). Excess addition (solution (4)) of TGE (6.90 mM) to the solution (3) lead formation of the ferric low-spin species (denoted as complex E; g_1=2.347, g_2=2.251 and g_3=1.943) (Fig. 2-

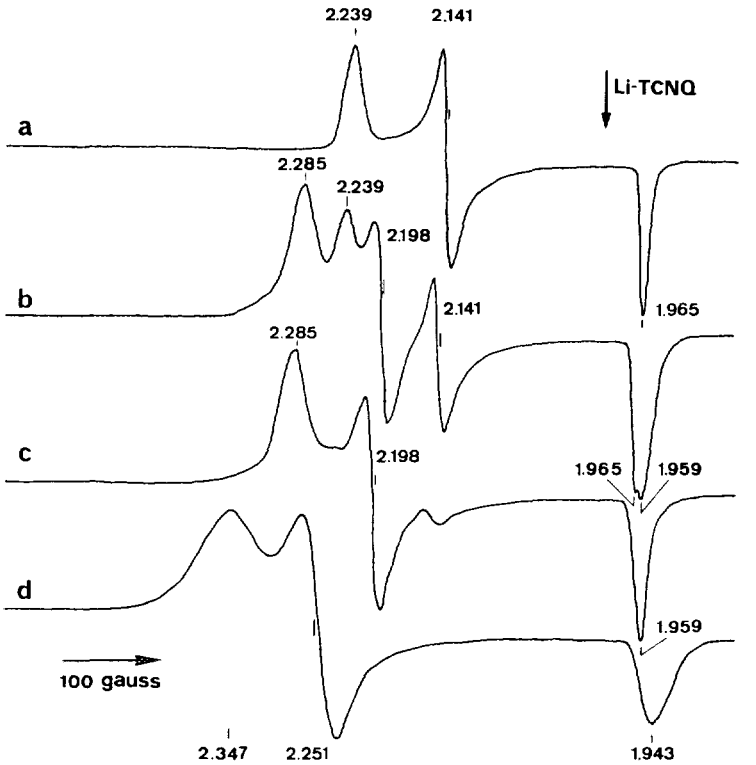


Fig. 2. ESR spectra (77 K) observed for Fe(III)TPP($^{-}$OO-t-butyl)$_2$ complex during the titration with TGE.
a) spectrum for the solution (1) composed of dichloromethane solutions of Fe(III)TPPCl (0.87 mM), t-BHPO (43.48 mM) and ethanol solution of sodium ethoxide (43.48 mM). The molar ratio of Fe-(III)TPPCl : t-BHPO : sodium ethoxide is 1 : 50 : 5.
b) spectrum for the solution (2) after addition of TGE (2.08mM) to the solution (1). The molar ratio of Fe(III)TPPCl : TGE is 1 : 2.5.
c) spectrum for the solution (3) after addition of TGE (4.00 mM) to the solution (1). The molar ratio of Fe(III)TPPCl : TGE is 1 : 5.
d) spectrum for the solution (4) after addition of TGE (6.90 mM) to the solution (1). The molar ratio of Fe(III)TPPCl : TGE is 1 : 10.
All solutions were prepared at 213 K under air.

d), along with the disappearance of ESR signals due to the complexes C and D. The g-parameters of the complex E agree well with those for the bis-thiolate-heme iron complex[14] (Table 1), which we have already assigned to be Fe(III)TPP($^{-}$S-TGE)$_2$.[15] This complex is well characterized by a d-type hyperporphyrin optical spectrum,[14-16] exhibiting a split Soret band at 360 and 455 nm for the frozen solution (3) (Fig. 3-c). It is interesting to note here that ESR signals of complexes C and D were again detected by excess addition of t- or n-BHPO to the bis-thiolate-Fe(III)TPP complex E.

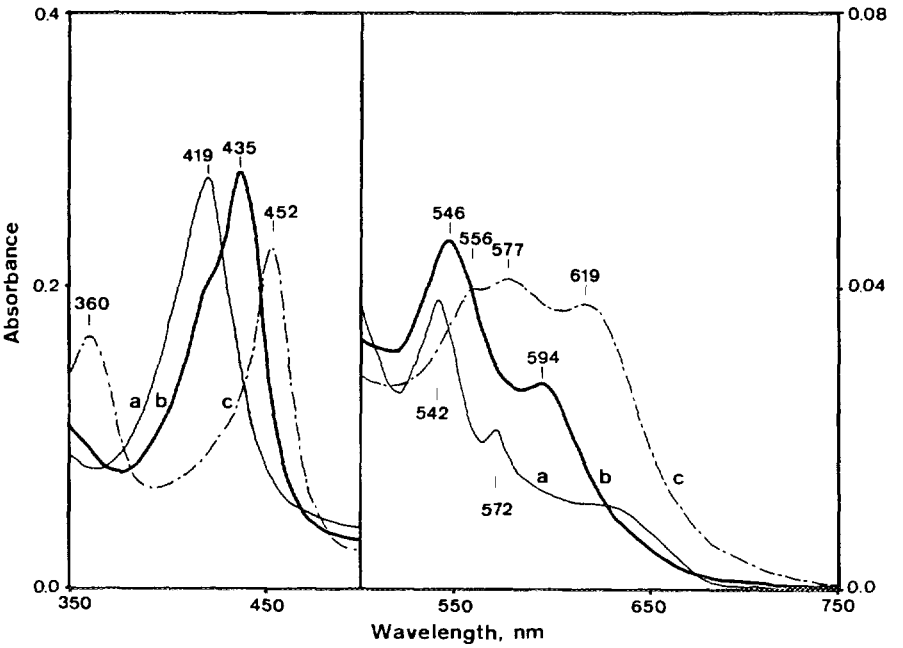


Fig. 3. Optical absorption spectra at 77 K observed for the same solutions supplied for ESR measurements.
a) spectrum for the solution (1) composed of dichloromethane solutions of Fe(III)TPPCl (0.87 mM), t-BHPO (43.48 mM) and ethanol solution of sodium ethoxide (43.48 mM). The molar ratio of Fe(III)TPPCl : t-BHPO : sodium ethoxide is 1 : 50 : 50.
b) spectrum for the solution (3) after addition of TGE (4.00 mM) to the solution (1). The molar ratio of Fe(III)TPPCl : TGE is 1 : 5.
c) spectrum for the solution (4) after addition of TGE (6.90 mM) to the solution (1). The molar ratio of Fe(III)TPPCl : TGE is 1 : 10.
All solutions were prepared at 213 K under air.

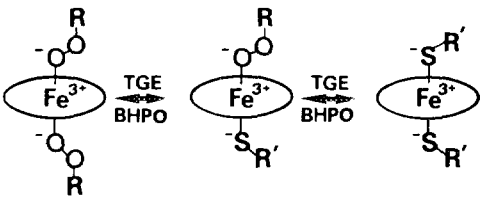
Table 1: ESR and optical parameters of P-450-peroxide complex and relating complexes

complex	peroxide	thiol	g_1	g_2	g_3	λ_{max}(nm)	ref.
Complex A							
Fe(III)P450-	none	none	2.41	2.24	1.93	---	*
(H_2O)	none	none	2.436	2.256	1.912	---	10
Complex B							
Fe(III)P450-	t-BHPO	none	2.28	2.25	1.96	---	*
($^-$OO-butyl[a])	n-BHPO	none	2.29	2.24	1.96	---	*
Complex C							
Fe(III)TPP-	t-BHPO	none	2.239	2.141	1.965	419 542 572	*
($^-$OO-butyl[a])$_2$	n-BHPO	none	2.243	2.151	1.964	420 542 573	*
"	t-BHPO	none	2.242	2.150	1.964	420 543 571	13
Complex D							
Fe(III)TPP							
($^-$TGE[b])-	t-BHPO	TGE	2.285	2.198	1.959	435 546 594	*
($^-$OO-butyl)	n-BHPO	TGE	2.290	2.219	1.958	436 553 590	*
Complex E							
Fe(III)TPP	t-BHPO	TGE	2.347	2.251	1.943	360 452 556 577 619	*
($^-$TGE)$_2$							
"	n-BHPO	TGE	2.347	2.254	1.940	360 455 554 570 622	*
"	none	TGE	2.322	2.228	1.951	375 460 562	14

*present study; [a]t- or n-butyl moiety of t- or n-BHPO;
[b]deprotonated form of TGE.

On the basis of the results on ESR- and optical absorption-spectrometric titrations, a stepwise axial ligand exchange reaction between the thiolate anion derived from TGE and peroxide anions ligated to the axial positions of the heme iron(III) complexes, is proposed as shown in Scheme 1. Stepwise addition of TGE to the complex C forms the complex D and E. On the other hand, when the complex E was titrated with t-BHPO, the complexes C and D are formed stepwisely. The complex D is proposed here to be an intermediate species in the process of the axial ligand exchange reaction between the bisperoxide-heme iron and the bis-thiolate-heme iron complexes. The complex D is concluded to be the six coordinate Fe(III)TPP($^-$S-TGE)($^-$OO-t- or -n-butyl) complex, having the peroxide and thiolate anions at the axial positions of Fe(III)TPP (Scheme 1). To our present knowledges, the complex D is a new complex as well as the first model for P-450-organic peroxide complex, characterized by ESR and optical absorption spectroscopies.

The g-values of the complex B (g_1=2.29, g_2=2.24 and g_3=1.96) showed an excellent accordance with those of the complex D (g_1=2.285, g_2=2.198 and g_3=1.959), as summarized in Table 1. Thus, the complex B is safely classified into the six coordinate heme-peroxide complex, having the peroxide anion at the sixth position. The fifth coordinate of the complex B is still occupied by the thiolate anion of cystein residue of the protein. Therefore, the complex B is concluded to be the six coordinate P-450-butyl peroxide complex, in which both thiolate anion of cysteine and deprotonate form of t- or n-BHPO bind at the axial positions of heme iron. The ligation of the butyl peroxide anion to complex B will occur through the terminal oxygen atom of the peroxide moiety, mostly for the steric reason. P-450-peroxide complex B



Complex C Complex D Complex E

Scheme 1. Possible axial ligand exchange reactions between Fe(III)TPP($^-$OO-t- or n-butyl)$_2$ and TGE. $^-$OOR is a butylperoxide anion derived from t- or n-BHPO, in the presence of sodium ethoxide, and $^-$SR' is a deprotonated form of TGE.

will be one of the intermediate species in the reaction processes of the peroxide-dependent mono-oxygenation of P-450. Further investigations focusing on the chemical reactivity of the complex are now in progress.

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References:

1) Ishimura, Y. (1978) "**Cytochrom P450**" Sato, S. and Omura, T. (eds.) Kodansha & Academic, Tokyo, pp. 209-227.
2) Gunsalus, I. C. and Sligar, S. G. (1978) "**Adv. Enzymology**" 47, 1-44.
3) White, R. E. and Coon, M. J. (1980) **Ann. Rev. Biochem.**, 49, 315-356.
4) Coon, M. J., White R. E. and Blake, R. C. (1982) "**Oxidases and Related Oxidation Systems**" King, T. E., Mason, H. S. and Morrison, M. (eds), 857-886, Oxford, Nerw York, Tront, Sydeny, Paris and Frankfurt.
5) Kadlubar, F. F., Morton K. C. and Ziegler, D. M. (1973) **Biochem. Biophys. Res. Commun.**, 54, 1255-1261.
6) Balke, R. C. and Coon, M. J. (1980) **J. Biol. Chem.**, 4100-4111.
7) Funae, Y., and Imaoka, S. (1985) **Biochem. Biophys. Acta**, 842, 119-132.
8) Adler, A. D., Longo, F. R., Vardi, V. (1976) **Inorg. Synth.**, 16, 213-220.
9) Williams, H. R., and Mosher H. S. (1954) **J. Am. Chem. Soc.**, 76, 2984-2989.
10)Tsai, R. C., et al. (1960) **Proc. Nat. Acad. Sci. U. S.**, 66, 1157-1163.
11)Poulos, T. C., Finzel, B. C. and Howard, A. J. (1986) **Biochemistry**, 25, 5314-5322.
12)Jinno, J., Shigematsu, M., Tajima K., Sakurai, H., Ohya-Nishiguchi, H. and Ishizu, K. (1991) **Biochem. Biophys. Res. Commun.**, 176, 675-681.
13)Tajima, K, Jinno, J., Ishizu, K., Sakurai H. and Ohya-Nishiguchi, H. (1989) **Inorg. Chem.**, 28, 709-715.
14)Sakurai, H., Ishizu, K. and Okada, K. (1984) **Inorg. Chim. Acta**, 91, L9-11.
15)Sakurai, H. and Yoshimura, T. (1985) **J. Inorg. Biochem.**, 24, 75-96.
16)Gouterman, M. (1978) "**The Porphyrins**" Dolphin, D. (ed.) Academic Press, New York, San Francisco, London, Vol. 3, 69-74.