Formation of Hemoprotein–Alkylperoxide Complexes Demonstrated by ESR and Optical Absorption Spectroscopy

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Abstract

Reactions between alkylhydroperoxides and hemoglobin or myoglobin in the ferric form (met-Hb and -Mb) were investigated by means of simultaneous ESR and optical absorption measurements at 77 K. The optical absorption spectrum observed for the frozen solution, prepared by mixing whale met-Mb and t- or n-butylhydroperoxide (t- and n-BHPO) in the presence of tetramethylammonium hydroxide (TMAOH), shows characteristic absorption maxima at 415, 542 and 574 nm. On the other hand, the ESR spectrum recorded for the same frozen solution reveals the formation of a ferric low-spin complex (**B**: g_1 = 1.936, g_2 = 2.196, g_3 = 2.350). The results obtained from ESR titration suggest that complex **B** has t- or n-BHPO at the sixth coordinate of the heme chromophore. The observed absorption maxima of complex B are similar to the values of relevant t- and n-butylperoxide complexes of (5,10,15,20-tetraphenylporphyrinato)iron(III) (Fe(III)TPP). In addition, the observed g parameters of the complex agree well with those of the Fe(III)TPP–butylperoxide complex, which have the t- or n-butylperoxide anion at the axial position of Fe(III)TPP. Based on the crystal field analysis carried out for complex **B** and related Fe(III)TPP–peroxide complexes, the axial ligand set of complex **B** is assigned to be the deprotonated peroxide anion derived from t- or n-BHPO, and the nitrogenous ligand derived from the proximal histidine. The probable coordination structure of complex **B** is proposed to be the six-coordinate Fe(III)–hemoprotein–$^{-}$OO-butyl complex. The present complex **B** is a possible model for transient hemoprotein–peroxide complexes, and could be proposed as the reaction intermediate in the reaction processes of heme containing oxidases, such as P-450 and peroxidases.

Introduction

Several classes of heme enzymes, such as cytochrome P-450 (P-450) [1], catalase [2], peroxidases [3], heme oxygenase [4] and prostaglandin H synthase (PGH) [5], have shown multiple functions closely related to the metabolisms of oxygen and peroxides in biological systems. In both the reaction processes of activation and decomposition of peroxides, formation of the heme enzyme–peroxide complexes has been proposed in the early reaction stages. For example, the P-450–organic peroxide complex [6] was speculated in the monooxygenation process of P-450 utilizing organic peroxides. In addition, PGH–fatty acid peroxide adducts were also assumed in the processes of prostaglandin biosynthesis. The heme enzyme–peroxide complex could be one of the key intermediate species generated in the reaction processes of heme containing oxidases. To our present knowledges, however, the coordination and electronic structures of the heme enzyme–peroxide adducts have never been well identified at sub-molecular level, since these complexes have a very short lifetime and are rapidly converted to other species such as compound **I** [3].

Both the electronic and coordination structures of these heme enzyme–peroxide intermediates have attracted the intense interest of many workers. The coordination reactions between the synthetic iron porphyrin complexes and several peroxides, such as hydrogen peroxide, acylperoxide and alkylperoxide, have been extensively studied in order to characterize the structure and functions of the heme enzyme–peroxide complexes. Groves and Watanabe [7], for example, reported optical absorption spectra of the *m*-chloroperbenzoic acid complex of Fe(III)-TPP derivatives. The processes of higher valent iron complex formation through the iron–acylperoxide adduct were proposed on the basis of NMR and optical measurements. In addition, we have reported ESR and optical spectra of the Fe(III)TPP–butylperoxide [8, 9] and –hydrogen peroxide [10] complexes, having the butyl- or hydrogen peroxide anion at the axial position of Fe(III)TPP. The coordination structure of the peroxide complexes were concluded to be Fe(III)TPP($^{-}$OCH$_3$)($^{-}$OO–C-(CH$_3$)$_3$) and Fe(III)TPP($^{-}$OH)($^{-}$OOH), based on the results obtained from the simultaneous ESR and optical measurements carried out for frozen solutions. The rapid freezing and mixing method

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was indispensable for preparing the frozen solutions containing these heme–peroxide complexes, because these Fe(III)TPP–peroxide complexes are very unstable and the Fe(III)TPP is oxidatively decomposed to the non-heme iron species [10].

The rapid mixing and freezing methods were applied to investigate the coordination reaction between met-Hb or -Mb and t- or n-butylhydroperoxide (t- and n-BHPO), with the aid of simultaneous ESR and optical absorption measurements [9]. In the present paper, the formation of hemoprotein–butylperoxide adducts taking the ferric low-spin state is demonstrated. From comparison of the ESR and optical properties of complex **B** with related heme–peroxide complexes, complex **B** was classified into the six-coordinate heme–butylperoxide complex. The axial ligands of complex **B** were assumed to be the deprotonated form of t- or n-BHPO, and the nitrogenous ligand derived from proximal histidine. Finally, the important roles of the hemoprotein–peroxide complex, involved in the activation processes of naturally occurring heme enzymes, such as P-450 and prostaglandin H synthase (PGH) are discussed on the basis of the present observed results.

Experimental

Materials

Whale met-hemoglobin (met-Hb) and -myoglobin (met-Mb) were obtained from Sigma Co., Ltd. and used for measurements after purification by gel-filtration using Pharmacia G-75. Methanol solutions of tetramethylammonium hydroxide (TMAOH), choline and $NaOCH_3$ were obtained from Wako Pure Chemicals. t-Butylhydroperoxide (t-BHPO) was supplied from Nippon Oil & Fats Co., Ltd. n-Butylhydroperoxide (n-BHPO), cumylhydroperoxide (CHPO) and tetralinhydroperoxide (THPO) were synthesized in our laboratory by ordinary procedures [11]. The purity of t- and n-BHPO were checked by elemental analysis. Found (Calc. for $C_4H_{10}O_2$): t-BHPO: C, 53.03 (53.33); H, 11.33 (11.19); n-BHPO; C, 53.31 (53.33); H, 11.11 (11.19)%.

Optical Absorption Spectra and ESR Measurements

The absorption spectra of frozen solutions (77 K) were recorded in the wavelength ranging from 410 to 800 nm by a MCPD-100 spectrometer of Ohtsuka Electronic Co. Ltd. ESR spectra were recorded at 77 K by a JEOL FE2-XG X-band spectrometer operating with 100 kHz field modulation about 0.63 mT. The microwave frequency was monitored by an Advantest TR-5212 digital frequency counter. The magnetic field strength was calibrated by the hyperfine coupling constants (hfcc) of the Mn(II) ion doped in MgO powder (8.69 mT). The g values of the observed ESR spectra were estimated based on that of the Li-TCNQ radical salt (g = 2.0025) taken as a standard. The simultaneous ESR and optical measurements were carried out by the procedure described in our previous report [9, 10]. All the measurements were performed at the Advanced Instrumental Center for Chemical Analysis, Ehime University.

Preparation of Hemoprotein–Peroxide Complex B

Reactions between met-Mb or -Hb and t- or n-BHPO were carried out below 5 °C, to avoid the oxidative decomposition of hemoproteins induced by peroxides, as follow. The whale met-Mb dissolved in 10% NaCl solution (1.0 mM, 0.4 ml) was mixed with a methanol solution of TMAOH (0.1 M, 0.02 ml) and cooled below 5 °C. Then a water solution of t-BHPO (1.0 M, 0.02 ml) was rapidly added to the mixture and was frozen at 77 K within about 5 s. By monitoring the ESR signal intensity of complex **B**, the optimum mixing molar ratio of the met-Mb, TMAOH and t-BHPO was determined to be 1:5:50. By the same procedure, a frozen solution containing complex **B** derived from met-Hb was prepared.

In the same way, reactions between hemoprotein and cumyl(CHPO) or tetralinhydroperoxide (THPO) were also tested, but, no defined ESR signal ascribed to complex **B** was successfully recorded in these cases. It was found that CHPO and THPO were unable to generate complex **B** in the present reaction condition. Furthermore, reactions between t- or n-BHPO and water soluble iron porphyrin complexes, such as Fe(III)DPyTPPCl [12]* and hemin chloride, were studied by means of ESR spectroscopy. An aqueous solution of these iron porphyrins was mixed with t- or n-BHPO in the presence of TMAOH by the same mixing molar ratio as described above, however, no detectable amount of ESR signal assigned to the ferric low-spin species was recorded.

Results and Discussion

Coordination Reaction between Hemoproteins and Alkaline Reagents

As a reference, ESR measurements were carried out to clarify the coordination reaction occurring between the met-Hb or -Mb and methanol solution of tetramethylammonium hydroxide (TMAOH). Before addition of TMAOH, whale met-Mb (1.0 mM, 0.4 ml) dissolved in 10% NaCl solution gave the characteristic ESR signal ($g_\perp$ = 6 and $g_\parallel$ = 2) (Fig. 1(a)), which has an ESR lineshape typical for

*Fe(III)DPyTPPCl = chloro(1-dodecyl-4-(10,15,20-triphenyl-5-porphinyl)pyridinium bromide)iron(III).

TABLE 1. Optical absorption[a], ESR[b] and crystal field parameters of heme–peroxide complexes and related complexes

Complexes	Base	Peroxide	Solevent	Temperature[c]	Absorption (nm)	g_1	g_2	g_3	$\|R/\mu\|$	$\|\mu/\lambda\|$	Label	Reference
N–Fe–$^-$OR												
met-Mb(whale)–$^-OCH_3$	TMAOH	none	H_2O	298	412 559 593	1.886	2.177	2.535	0.52	7.2	1	this work
met-Mb(whale)–$^-OCH_3$	$NaOCH_3$	none	H_2O	298	413 560 595	1.882	2.170	2.530	0.51	7.3	2	this work
met-Mb(whale)–$^-OCH_3$	choline	none	H_2O	298	412 558 595	1.885	2.175	2.531	0.52	7.2	3	this work
met-Mb–$^-$OH	pH 10	none	H_2O	298		1.83	2.21	2.65	0.53	5.8	4	14
Fe(III)PPIXDME[d]($^-$OAr)–Im[e]		none	H_2O			1.84	2.19	2.61	0.51	6.2	5	15
N–Fe–$^-$OOR												
met-Mb(whale)–$^-$OOR	TMAOH	t-BHPO	H_2O	77	415 542 574	1.936	2.196	2.350	0.49	7.6	a	this work
met-Mb(whale)–$^-$OOR	TMAOH	n-BHPO	H_2O	77	418 546 576	1.937	2.192	2.349	0.51	7.7	b	this work
met-Hb(whale)–$^-$OOR	TMAOH	t-BHPO	H_2O	77	420 549 580	1.940	2.188	2.340	0.50	7.9	c	this work
met-Hb(whale)–$^-$OOR	TMAOH	n-BHPO	H_2O	77	421 548 581	1.933	2.190	2.343	0.50	7.7	d	this work
O^-–Fe–$^-$OOH												
Fe(III)TPP($^-$OH)–$^-$OOH	KOH	H_2O_2	DMF	77	420 562 605	1.962	2.157	2.264	0.45	9.8	6	10
O^-–Fe–$^-$OOR												
Fe(III)TPP($^-OCH_3$)–$^-$OOR	$NaOCH_3$	t-BHPO	CH_2Cl_2	77	420 543 573	1.952	2.157	2.316	0.49	9.0	7	9
Fe(III)TPP($^-OCH_3$)–$^-$OOR	$NaOCH_3$	n-BHPO	CH_2Cl_2	77	420 543 573	1.959	2.160	2.323	0.49	8.8	8	j
Fe(III)TPP($^-$OOR)$_2$	$NaOCH_3$	t-BHPO	CH_2Cl_2	77	424 547 578	1.964	2.154	2.243	0.38	10.1	9	9
Fe(III)TPP($^-$OOR)$_2$	$NaOCH_3$	n-BHPO	CH_2Cl_2	77	424 547 578	1.962	2.160	2.240	0.38	9.9	10	j
N–Fe–$^-$OOR												
Fe(III)TPP(Im)–$^-$OOR	TPA[f]	t-BHPO	CH_2Cl_2	158	418 546 582	1.940	2.185	2.324	0.47	8.1	11	j
Fe(III)TPP(Im)–$^-$OOR	TPA[f]	n-BHPO	CH_2Cl_2	158	418 553 588	1.935	2.194	2.352	0.50	7.6	12	j
Fe(III)TPP(4MeIm)[g]–$^-$OOR	TPA[f]	t-BHPO	CH_2Cl_2	158	421 548 582	1.943	2.183	2.323	0.48	8.1	13	j
Fe(III)TPP(4MeIm)[g]–$^-$OOR	TPA[f]	n-BHPO	CH_2Cl_2	158	418 549 588	1.935	2.195	2.344	0.48	7.6	14	j
met-Hb–$^-$OOH	pH 7	h	H_2O			1.967	2.148	2.265				16
met-Mb–$^-$OOH	pH 7	h	H_2O			1.941	2.176	2.296				16
HRP–$^-$OOH	pH 7	i	H_2O			1.95	2.16	2.31				17

[a]Experimental error in the values of λ_{max} was about ± 3 nm. [b]Experimental error in the g values recorded at 77 K was about ±0.003. [c]Temperature for optical absorption measurements. [d]ProtoporphyrinIX–dimethylester. [e]Im, imidazole; $^-$OAr, phenolate anion. [f]TPA, tri-n-propylamine. [g]4MeIm, 4-methyl-imidazole. [h]UV- or γ-irradiation to oxy-Hb and -Mb at 77 K. [i]γ-irradiation to oxy-horseradish peroxidase (HRP). [j]See footnote p. 216.

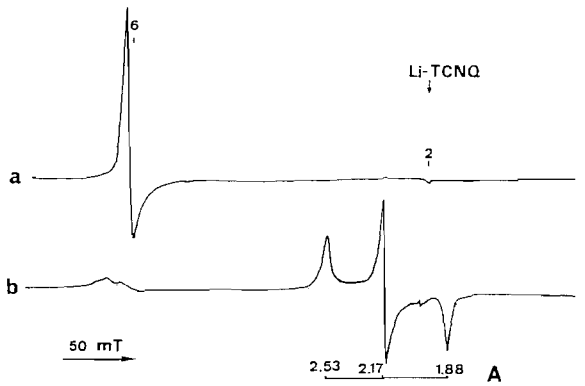


Fig. 1. ESR spectra observed at 77 K for the frozen solutions prepared by rapid mixing and freezing methods: (a) ESR spectrum recorded for the whale met-Mb (1.0 mM, 0.4 ml) dissolved in 10% NaCl solution at pH 7; (b) ESR spectrum recorded by addition of methanol solution of TMAOH (0.1 M, 0.02 M), by which the pH of the reaction mixture was raised to about 9 to 10. Mixing molar ratio of met-Mb and TMAOH was 1:5 and the solvent mixing ratio of H_2O and methanol was 400:20.

met-Mb taking the ferric high-spin state (S = 5/2) [13]. After addition of TMAOH (0.1 M, 0.02 ml) to the solution of met-Mb, the ESR spectrum (Fig. 1(b)) revealed the formation of a new ferric low-spin complex (denoted as complex A; g_1 = 1.886, g_2 = 2.177 and g_3 = 2.535) with a weak ESR signal due to the remaining ferric high-spin species. Similar ESR spectra were also recorded for met-Hb even by mixing methanol solutions of $NaOCH_3$ and choline instead of TMAOH. As summarized in Table 1, the observed g paramaters of complex A were less dependent on the structure of the cationic species involved in the alkaline reagents such as $(CH_3)_4N^+$, Na^+ and $(CH_3)_2N^+C_2H_4OH$. In our reaction system, the presence of about 5% methanol was indispensable to generate complex A. The results of ESR measurements indicate that the anion species commonly involved in these alkaline reagents could be the deprotonated $^-OCH_3$ anion rather than the ^-OH anion.

It was found that the g parameter of complex A was analogous to those of six-coordinate iron complexes having oxygenous and nitrogenous donors at the axial positions, such as hydroxy-Hb [14] (Table 1). From comparison of the g values of complex A with related ferric low-spin complexes, the axial ligand set of complex A was assumed to be the deprotonated methoxide anion and the nitrogenous ligand derived from the proximal histidine located at the fifth coordinate of met-Mb and -Hb. Therefore, the coordination structure of complex A was schematically assigned to be the six-coordinate Fe(III)–Mb– and –Hb–$^-OCH_3$.

Simultaneous ESR and Optical Measurements at 77 K

The coordination reaction between complex A and t-butylhydroperoxide (BHPO) was studied by means of simultaneous ESR and optical measurements [9]. The pre-cooled (0 °C) t-BHPO aqueous solution (1.0 M, 0.02 ml) was rapidly added to the solution of complex A, prepared by mixing whale met-Mb (1.0 mM, 0.4 ml) and TMAOH (0.1 M, 0.02 ml). Then the resulting mixture was rapidly frozen in liquid nitrogen as described in 'Experimental'. Upon addition of t-BHPO to complex A, the color of the frozen mixture changed to bright red. As shown in Fig. 2(a), characteristic absorption maxima were detected at 542 and 574 nm. The ESR spectrum recorded for the same frozen solutions (Fig. 3(a)) revealed formation of the ferric low-spin species (denoted as complex **B**; g_1 = 1.936, g_2 = 2.196 and g_3 = 2.350) with strong ESR signal height. A weak isotropic ESR signal was observed in the lower magnetic field at g = 4.3, which would be the non-heme type iron complex generated by oxidative decomposition of met-Mb reacting with t-BHPO. In addition, a strong ESR signal due to the free radical species was also detected in the free spin region (denoted as **R**; $g_{\parallel}$ = 2.033 and $g_{\perp}$ = 2.008). With reference to the previous reports [15], the free radical species **R** was assigned to be the t-butylperoxide radical derived from t-BHPO. It is noted here that the ESR signal of complex **B** was successfully detected, provided the optical spectra due to complex **B** was clearly recorded.

The ESR and optical measurements were continued for the frozen solution of complex **B** prepared by mixing whale met-Mb with n-BHPO instead of

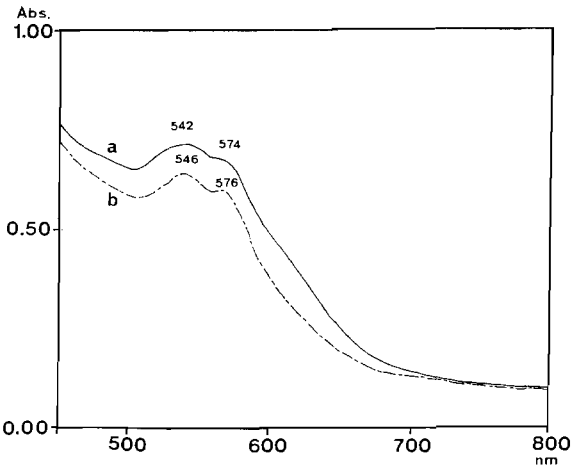


Fig. 2. Optical absorption spectra recorded for frozen solution (77 K) containing complex B prepared by mixing whale met-Mb (1.0 mM, 0.4 ml) with (a) t- and (b) n-BHPO (1.0 M, 0.02 ml) in the presence of TMAOH (0.1 M, 0.02 ml). The mixing molar ratio of met-Mb:TMAOH:peroxides was fixed at 1:5:50.

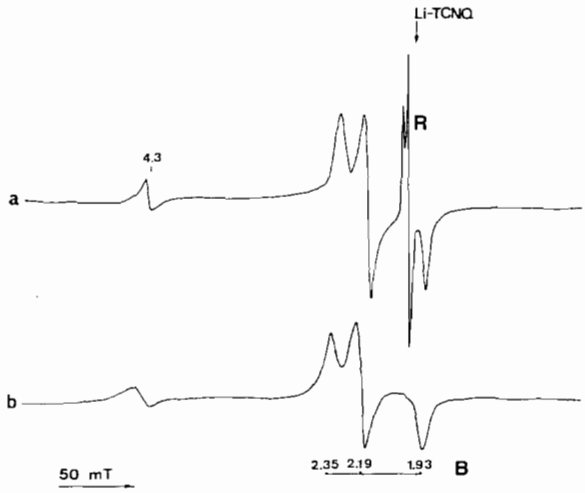


Fig. 3. ESR spectra observed for the same frozen solution supplied for the optical absorption measurements as shown in Fig. 2: (a) t-BHPO, (b) n-BHPO. The weak ESR signal appearing at g = 4.3 would be assigned as a non-heme type iron complex generated by oxidative decomposition of hemoprotein induced by t- or n-BHPO.

t-BHPO. As shown in Fig. 2(b), an analogous optical spectrum was also recorded at 77 K. The observed λ_{max} values (546 and 576 nm) agreed well with those obtained by addition of t-BHPO to whale met-Mb (542 and 574 nm), as mentioned above. ESR signals due to complex **B** (g_1 = 1.937, g_2 = 2.192 and g_3 = 2.349) was also detected for the same frozen solution, as shown in Fig. 3(b). In this case, however, the ESR signal due to the peroxide radical derived from n-BHPO was detected (g = 2.03) only as a weak ESR signal. This means that the lifetime of the n-butylperoxide radical is much shorter than that of the t-butylperoxide radical. Similar absorption and ESR spectra due to complex **B** were also recorded for whale met-Hb reacting with t- or n-BHPO by the same procedures.

As summarized in Table 1, ESR and optical parameters of complex **B** observed for met-Mb were comparable with those recorded for met-Hb. In addition, no important difference was recognized in the ESR and optical parameters of complexes **B** prepared by addition of t- and n-BHPO (Table 1). This means that the optical and ESR property of complex **B** was less dependent not only on the molecular structure of the alkylperoxides used, but also on the origin of the hemoprotein. The complex **B**, thus characterized by spectroscopic parameters (λ_{max} = 542 and 574 nm; g values: g_1 = 1.94, g_2 = 2.19 and g_3 = 2.35), is concluded to be the major iron complex derived from met-Mb and -Hb reacting with t- or n-BHPO in the presence of TMAOH.

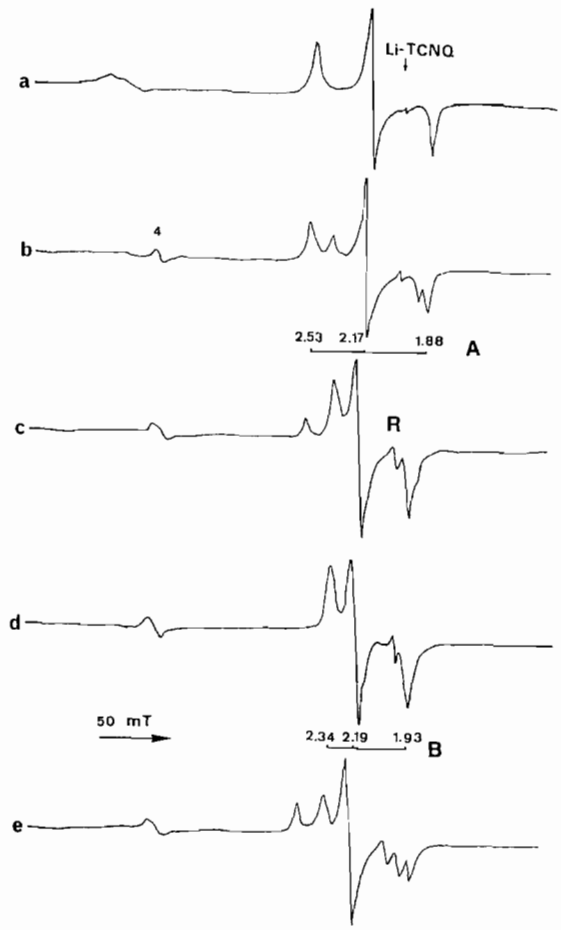


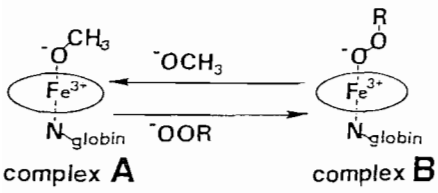
Fig. 4. ESR spectrometric titration performed by changing the mixing molar ratio of complex **B** and n-BHPO: (a) spectrum before addition of n-BHPO, with complex **A** prepared by addition of TMAOH (0.1 M, 0.02 ml) to a 10% NaCl solution of whale met-Mb (0.1 mM, 0.4 ml); (b) spectrum after addition of a 5 times excess amount of n-BHPO (0.1 M, 0.02 ml); (c) spectrum after addition of 25 times excess of n-BHPO (0.5 M, 0.02 ml); (d) spectrum after addition of 50 times excess of n-BHPO (1.0 M, 0.02 ml); during the ESR titration the mixing molar ratio of met-Mb: TMAOH was adjusted to 1:5; (e) spectrum recorded again after addition of TMAOH (0.1 M, 0.01 ml) to the reaction solution of (d); the final molar ratio of met-Mb, TMAOH and n-BHPO was changed to 1:55:50.

ESR Spectrometric Titration of Complex A with t- or n-BHPO

By monitoring the ESR signal intensity of complexes **A** and **B**, an ESR titration was carried out to understand the mechanism of the reaction between t- or n-BHPO and complex **A**. An aqueous solution of t-BHPO (0.1 M, 0.02 ml) was added to the cooled solution of complex **A** prepared by mixing met-Mb and TMAOH in the same molar ratio described above. As shown in Fig. 4(b), a weak ESR signal ascribed to complex **B** (g_1 = 1.94, g_2 = 2.19 and g_3 = 2.35)

was recorded. On the other hand, the ESR signal intensity of complex **A** was decreased by the addition of t-BHPO. The ESR signal height of complex **B** showed a concomitant increase depending upon the concentration of t-BHPO added, followed by a substantial decrease in the ESR signal intensity of complex **A** (Fig. 4(c)). When t-BHPO (1.0 M, 0.02 ml) was added to complex **A**, the ESR signal height of complex **B** reached a maximum (Fig. 4(d)), while the ESR signal of complex **A** almost disappeared. Significant changes in the ESR signal intensity and g parameters of complex **B** were never detected, even when about 250 times excess t-BHPO was added to complex **A**. The ESR signal height of complex **A** reversibly increased by addition of TMAOH (0.05 M, 0.02 ml) to the reaction mixture containing complex **B** prepared under optimum conditions, as shown in Fig. 4(e). A similar successive change in the ESR signal intensity of complexes **A** and **B** was also recorded for whale met-Hb.

ESR spectra (Fig. 4) observed during the titration of complex **A** with t- or n-BHPO gave important information for identification of the axial ligands of complex **B**. The ESR spectral change indicates that complex **A** is reversibly converted to complex **B** reacting with t- or n-BHPO in the presence of TMAOH. Recently, we [9] have reported a similar ESR spectral change observed for Fe(III)TPPCl titrated by t-BHPO in the presence of the $^{-}OCH_3$ anion in dichloromethane. The ESR spectrum due to Fe(III)TPP($^{-}$OO-t-butyl)$_2$ was detected when an excess amount of t-BHPO was added to Fe(III)-TPPCl under alkaline conditions. In the present case, however, a detectable amount of ESR signal ascribable to the heme–bis-butylperoxide complex was never observed. In our reaction system, the possibility of ligation of t- or n-BHPO at both axial positions of met-Mb or -Hb was safely ruled out. This implies that complex **B** will be generated by the axial ligand exchange reaction occurring at the fifth coordinate of complex **A**. Accordingly, the formation of complex **B** is explained to be the axial ligand exchange reaction between the $^{-}OCH_3$ anion and the t- or n-BHPO at the sixth coordinate of met-Mb or -Hb, as expressed in Scheme 1. The addition of t- or n-BHPO tends to shift the equilibrium to the right-hand side, and results in an increase of ESR intensity of complex **B**.



Scheme 1. Coordination chemistry occurring between met-Mb or -Hb ant t- or n-BHPO. Equilibrium reaction between complexes **A** and **B** depending upon the mixing molar ratio of t- or n-BHPO and the méthoxide anion derived from the methanol solution of TMAOH.

Possible Coordination Structure of Complex B

The observed optical and ESR parameters of complex **B** are compared with those of related heme–peroxide complexes, in order to assign the axial ligand of complex **B**. ESR and optical spectra ascribed to the six-coordinate heme–peroxide complexes, taking the ferric low-spin state, have frequently been observed for synthetic iron porphyrin complexes (Fe(III)TPP). For example, the ESR spectrum (g_1 = 1.962, g_2 = 2.157 and g_3 = 2.264) [10] was recorded for the frozen solution prepared by mixing Fe(III)TPPCl and hydrogen peroxide in the presence of aqueous KOH. The absorption maxima at 562 and 605 nm [10] were also detected for the same frozen solution (Table 1). The possible coordination structure of the ferric low-spin complex was proposed to be the six-coordinate Fe(III)TPP–hydrogen peroxide complex (Fe(III)TPP($^{-}$OH)($^{-}$OOH)) [10]. Furthermore, the ESR spectrum ascribed to the Fe(III)TPP–(t-BHPO) complex (g_1 = 1.952, g_2 = 2.157 and g_3 = 2.316) [9] was observed for the frozen solution containing six-coordinate Fe(III)-TPP($^{-}OCH_3$)($^{-}$OO-t-butyl). The characteristic absorption maxima were detected at 543 and 573 nm for the same frozen solution (Table 1) [9]. In addition, we have recently observed the ESR and optical spectra ascribable to the similar Fe(III)TPP-($^{-}$OO-t- or n-butyl) complexes having the nitrogenous ligand derived from the imidazole derivatives*. These complexes were prepared by the axial ligand exchange reaction between imidazole derivatives and the bis-butylperoxide complex, which possess two deprotonated peroxide anions ($^{-}$OO-t- or n-butyl) at the axial positions of Fe(III)TPP [9]. The coordination structure was assumed to be the six-coordinate Fe(III)TPP(imidazol)($^{-}$OO-t-butyl) complex, in which the nitrogen atom of the imidazole was ligated at the axial position of Fe(III)TPP($^{-}$OO-t-butyl). ESR and optical parameters of this complex

*To the cooled solution (−78 °C) composed of Fe(III)-TPPCl (1.0 mM, 0.4 ml) and tri-n-propylamine (TPA; 0.1 M, 0.05 ml), a precooled dichloromethane solution of t-BHPO (1.0 M, 0.06 ml) was mixed and rapidly frozen in liquid nitrogen. The frozen solution revealed formation of the ferric low-spin species (g_1 = 1.964, g_2 = 2.154 and g_3 = 2.243), which was safely assigned to be the bis-peroxide complex (Fe(III)TPP($^{-}$OO-t-butyl)$_2$. The reaction mixture was at once thawed in a dry ice–acetone bath (−78 °C), and a dichloromethane solution of imidazole (Im; 0.5 M, 0.02 ml) was added and again frozen. The ESR spectrum recorded for the frozen solution showed formation of a new ferric low-spin species (g_1 = 1.936, g_2 = 2.199 and g_3 = 2.347). Similar ESR spectra were successfully observed when imidazole derivatives, such as 4-methyl-imidazole, were added to the Fe(III)–bisperoxide complex prepared by mixing Fe(III)TPPCl and t- or n-BHPO in the presence of TPA.

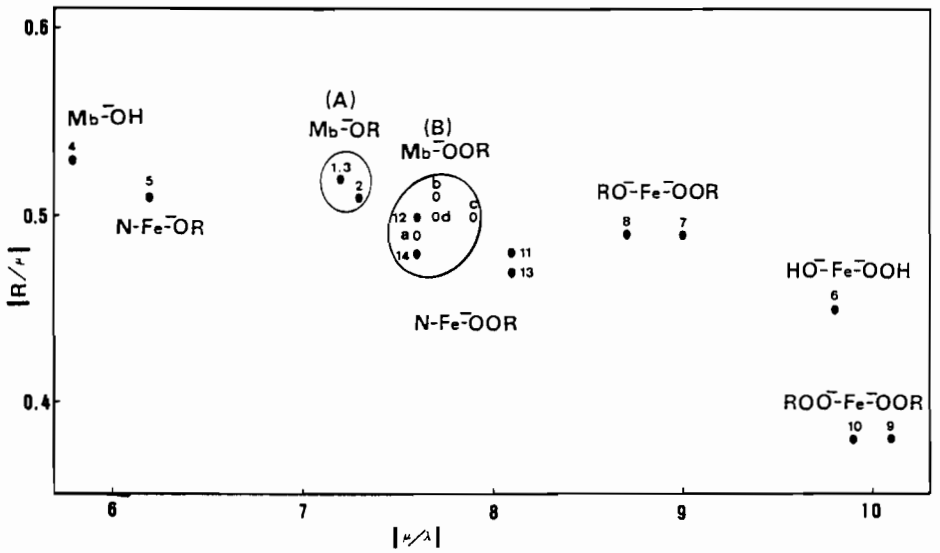


Fig. 5. Plots of rhombicity vs. tetragonality parameters calculated for complexes A, B and related heme–peroxide complexes.

were characterized to be $g_1 = 1.940$, $g_2 = 2.185$, $g_3 = 2.324$ and 420, 548, 584 nm, respectively (Table 1).

The observed g parameters of complex **B** ($g_1 = 1.936$, $g_2 = 2.196$ and $g_3 = 2.350$) are characterized by a small g anisotropy compared with the usual ferric low-spin complexes derived from met-Mb or -Hb, such as hydroxyl-Hb (Table 1). From comparison of the g value of complex **B** with related heme–peroxide complexes, it was found that the ESR parameters of complex **B** are analogous to those of the Fe(III)TPP($^-$OO-t- or n-butyl) complexes rather than those of the Fe(III)TPP–hydrogen peroxide complex (Table 1). Of interest is the fact that the observed g parameters of complex **B** showed excellent agreement with those of the Fe(III)TPP(imidazole)-($^-$OO-t- or n-butyl) complex (Table 1). The observed optical parameters of complex **B** are also comparable with those of the Fe(III)TPP($^-$OO-t- or n-butyl) complexes. The optical and ESR property of complex **B** is consistent with the six-coordinate Fe(III)TPP($^-$OO-t- or n-butyl) complexes, having the deprotonated peroxide anion at the axial position of heme. This indicates that the complex can be safely classified as a six-coordinate heme–butylperoxide complex, in which the deprotonated butylperoxide anion, derived from t- and n-BHPO, is located at the axial position of the heme chromophore.

The crystal field parameters [18], rhombicity (R/μ) and tetragonality (μ/λ), of complex **B** and relating peroxide complexes were calculated in terms of Bohan's proposal [19]. The calculated crystal field parameters (Table 1) of complexes **A**, **B** and Fe(III)-TPP($^-$OO-t- or n-butyl) and –hydrogen peroxide complexes are plotted in tetragonality versus rhombicity diagram (Fig. 5). The data points of complex **A**, having a deprotonated $^-OCH_3$ anion and proximal histidine at axial positions, fell into the small circle. The data points due to complexes **B** also make the small group, but they were distinctly separate from that of complex **A**. The plots of synthetic Fe(III)TPP($^-OCH_3$)($^-$OO–butyl) [11] and of Fe(III)TPP($^-$OH)($^-$OOH) [10] complexes were located far away from the circles of complexes **A** and **B**. Of interest is the fact that the plots of the Fe(III)TPP(imidazole)($^-$OO-n-butyl)* type complexes, having a nitrogenous ligand at the fifth coordinate instead of the $^-OCH_3$ anion, are partially overlapped with those of complex **B**. This means that the axially ligating donor set of the Fe(III)TPP-(imidazol)($^-$OO-t- and n-butyl) complexes are similar to that of complex **B**. The crystal field analysis (Fig. 5) supports the suggestion that complex **B** possesses the nitrogen donor and the deprotonated butylperoxide anion, derived from t- or n-BHPO, at the axial positions of the heme chromophore. The imidazole moiety of the proximal histidine is thought to be the most probable candidate for the origin of the nitrogenous ligand binding at the fifth coordinate of complex **B**. Based on the results obtained from the crystal field analysis, complex **B** is, therefore, concluded to be a six-coordinate Fe(III)–Hb– or –Mb–$^-$OO–butyl complex (Fig. 6), which possess the nitrogenous donor derived from the proximal histidine, and the deprotonated butyl peroxide anion at the axial positions of the heme chromophore. Ligation of the t- or n-butylperoxide anion will occur through the terminal oxygen atom of the peroxide moiety mostly for steric reasons, as is illustrated in Fig. 6.

*See footnote p. 216.

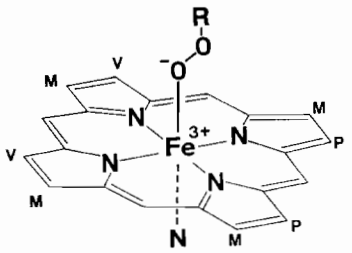


Fig. 6. Possible coordination structure of the hemoprotein–butylperoxide complex B; R, means the n-butyl or t-butyl moiety of t- and n-BHPO; N, means the nitrogen atom of imidazole derived from proximal histidine; V, means vinyl group; P, means propionic acid.

So far, ESR spectra ascribed to the hemoprotein–hydrogen peroxide complexes have frequently been observed for Hb, Mb and horseradish peroxidase (HRP). Symons *et al.* [16, 20], for example, reported ESR spectra due to the Fe(III)Hb– and Fe(III)Mb–$^{-}$OOH complex (g_1 = 1.967, g_2 = 2.148 and g_3 = 2.265), which were prepared by UV- or γ-irradiation to oxy-Hb or -Mb at 77 K. In addition, Gasyna [17] also observed analogous paramagnetic species ascribable to Fe(III)HRP–$^{-}$OOH (g_1 = 1.95, g_2 = 2.16 and g_3 = 2.31) after γ-irradiation to the oxygen complex of HRP (Table 1). As far as we know, however, complex **B** described herein may be the first example of ESR and optical spectroscopic detection carried out for met-Mb– and met-Hb–alkylperoxide complexes.

Complex B as a Model of Heme Enzyme–Peroxide Complex

The formation of heme enzyme–organic peroxide complexes has been proposed in the possible reaction processes of heme containing oxidases. These heme enzyme–organic peroxide complexes were regarded to be one of the important intermediate species to explain the detailed reaction mechanism of heme containing oxidases. For example, the heme enzyme–organic peroxide complex was proposed in the reaction processes of PGH and P-450. The former PGH exhibits conversion of fatty acid hydroperoxides to the corresponding alcohol derivatives [21]. Kulmacz *et al.* [22] have reported the ESR spectrum observed for purified PGH taking the ferric low-spin state. The presence of at least one nitrogen atom at the axial position was proposed according to the observed g parameters. However, no detailed description about the coordination structure of the intermediate PGH–peroxide complex were given. In the case of P-450, the heme–organic peroxide complex has often been speculated in the mono-oxygenation process of P-450, which catalyze or at least promote the mono-oxygenation in the presence of organic hydroperoxide. Blake and Coon [23] demonstrated the formation of the intermediate P-450–organic peroxide adducts by means of the accurate kinetic investigation carried out for the system composed of purified P-450 and cumyl- or benzylhydroperoxides. At present, however, the coordination structure of the P-450–organic peroxide complex is still equivocal, because the intermediate complexes were rapidly changed to the higher valent iron complexes. The hemoprotein–butylperoxide complex **B** could be the nobel model complex to understand the electronic and coordination structures of these heme enzyme–peroxide intermediates. These findings suggest that the six-coordinate hemoprotein–alkylperoxide complex probably takes the ferric low-spin state, provided that the axially ligating peroxide moiety prefers the deprotonated form in the heme pocket. The possible coordination structure of heme enzyme–peroxide complexes has been revealed by means of simultaneous ESR and optical measurements combined with the rapid mixing and freezing method. Further investigations to observe the ESR and optical spectra of heme enzyme–alkyl hydroperoxide complexes are now in progress.

Conclusions

The hemoprotein–alkylperoxide complex **B** generated by the reaction between met-Mb or -Hb and alkyl hydroperoxides, such as t- and n-butylhydroperoxide, is a six-coordinate met-Mb– and met-Hb–($^{-}$OO–t- or n-butyl) complex. The optical absorption spectra observed for complex **B** showed characteristic absorption maxima at 542 and 574 nm, which agreed well with those of related iron–peroxide complexes. ESR spectra recorded for the same frozen solution revealed the formation of a ferric low-spin complex with a small g anisotropy (g_1 = 1.936, g_2 = 2.196 and g_3 = 2.350). From comparison of the g values of complex **B** with relating heme peroxide complexes, complex **B** was assigned to be a hemoprotein–alkylperoxide adduct. The axial ligands of complex **B** were concluded to be the nitrogenous ligand derived from proximal histidine, and the deprotonated butylperoxide anion derived from t- or n-BHPO. The hemoprotein–peroxide complex **B** could be a possible model complex for intermediate species speculated in the processes involved in the reaction between alkylhydroperoxide and several classes of heme enzymes, such as peroxidases, catalases, cytochrome P-450, tryptophan dioxygenase and heme oxygenases.

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