A Possible Model of a Hemoprotein–Hydrogen Peroxide Complex

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Abstract

Simultaneous ESR and optical measurements have been carried out for the frozen DMF solution of the Fe(III)TPP–hydrogen peroxide complex prepared by mixing chloro(5,10,15,20-tetraphenylporphyrinato)-iron(III), (Fe(III)TPPCl), with hydrogen peroxide in the presence of alkaline reagents such as tripropylamine (TPA) or KOH. The optical spectrum observed for the complex at 77 K is very similar to heme–peroxide complexes such as Fe(III)TPP–alkylperoxo complexes. The ESR spectra recorded at 77 K demonstrate that the complex was in the ferric low-spin state with anomalously small g anisotropy (g_1 = 1.962, g_2 = 2.157 and g_3 = 2.264), being well consistent with those of Fe(III)hemoglobin–$^-$OOH and Fe(III)horseradish peroxidase–$^-$OOH. It is concluded that the coordination structure of this complex takes a six coordinate geometry written as Fe(III)TPP-($^-$OH)($^-$OOH). The present complex is the first example of a possible model complex of the hemoprotein–hydrogen peroxide complexes.

Introduction

Mechanisms of the reaction between ferric porphyrin complexes and hydrogen peroxide are of much interest with respect to the functions of several classes of heme enzymes. For example, peroxidases such as horseradish peroxidase (HRP) [1] and cytochrome C peroxidase (CCP) [2] catalyze the oxidation of several substrates in the presence of hydrogen peroxide. Catalase [3] is closely related to these enzymes, which readily decompose hydrogen peroxide into water and molecular oxygen. In the early reaction stages of their catalytic reactions, the formation of a heme–hydrogen peroxide complex has often been speculated. In addition, cytochrome P-450 [4] enzymes utilize a NADPH dependent cytochrome P-450 reductase and molecular oxygen in oxidative metabolism of a wide variety of organic substrates. P-450 also catalyzes a peroxide-dependent oxygenation of chemicals [5] in the presence of oxidizing agents such as hydrogen peroxide, alkylperoxides and acylperoxides. At present, however, it is not clear whether these reactive intermediates formed in the P-450 system via NADPH and O_2 are identical to those of the hydrogen peroxide pathway, in which the formation of heme protein peroxide complexes are also presumed. The heme peroxide complexes formed in these reactions have been thought to have a very short lifetime and be quickly converted to the high valence oxo–iron complexes, followed by cleavage of the oxygen–oxygen bond in the axially ligating peroxide moiety.

The electronic structures of these heme protein peroxide complexes were investigated by using synthetic iron porphyrin complexes and alkyl- or acyl-peroxides instead of hydrogen peroxide. Groves and Watanabe [6] prepared five coordinate heme–acylperoxo complexes in a reaction system composed of *m*-chloroperbenzoic acid and Fe(III)TPP derivatives. The stepwise conversion of the iron porphyrin–acylperoxo complex to the oxo–iron species was monitored by optical spectral measurements. On the other hand, we [7] have reported a model for a heme–alkylperoxo complex prepared by mixing Fe(III)TPPCl and t-butylhydroperoxide in the presence of alkaline reagents such as $^-OCH_3$. On the basis of the results obtained by ESR and optical measurements, the coordination structure was postulated to be six coordinate Fe(III)TPP($-OCH_3$)($^-$OOC-$(CH_3)_3$) in the ferric low-spin state [8]. To our present knowledge, however, models of heme hydrogen peroxide intermediates have not been reported, because the reactions between synthetic iron porphyrins and hydrogen peroxide are too fast and vigorous to detect the intermediate species by means of conventional spectroscopic measurements.

We have, therefore, tried a rapid mixing and freezing method [8] to prevent decomposition of the iron porphyrin complex and found that this method is effective for investigating rapid reactions between the heme complex and peroxides. By using this technique coupled with simultaneous ESR and optical

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measurements [8, 9], we have succeeded in detecting the heme-hydrogen peroxide complex in the frozen DMF solution system consisting of Fe(III)TPPCl*, hydrogen peroxide and alkaline reagents such as tripropylamine or potassium hydroxide. In this paper a possible coordination structure of the Fe(III)TPP–hydrogen peroxide complex is proposed. Finally, resemblance in the coordination structure of the model complex with that of heme–hydrogen peroxide adducts speculated in the catalytic cycles of naturally occurring heme enzymes will be discussed.

Experimental

Materials

The free base of $TPPH_2$ and its iron complex, Fe(III)TPPCl, were synthesized by the usual procedures described by Adler *et al.* [10]. The purity of Fe(III)TPPCl was checked by ^1H NMR and elemental analysis. Fe(III)TPPCl ($C_{44}H_{28}N_4FeCl$): Found (calc.): C, 74.56 (75.06); H, 4.14 (4.01); N, 8.11 (7.96%). Hydrogen peroxide (30.0% aqueous solution) was obtained from Wako Pure Chemicals and a diluted 0.01 M solution was prepared by using the $KMnO_4$ titration method. Tripropylamine (TPA) and *N,N*-dimethylformamide (DMF) were obtained from Wako Pure Chemicals and used after drying and distillation under reduced pressure or nitrogen atmosphere. An 0.02 M aqueous solution of potassium hydroxide was prepared and stored under argon atmosphere. Sample solutions were prepared just before use in each measurement.

Preparation of the Heme–Hydrogen Peroxide Complex (C)

In order to prevent the vigorous chemical reaction occurring between Fe(III)TPPCl and hydrogen peroxide, all sample preparations were carried out in a dry ice–CCl_4 bath at −45 °C. The DMF solution composed of Fe(III)TPPCl (0.5 mM, 0.4 ml) and TPA (1.0 M, 0.01 ml) was frozen once at 77 K and thawed at −45 °C. A previously cooled (5 °C) water solution of hydrogen peroxide (1.0 M, 0.02 ml) was added and the reaction mixture was again frozen at 77 K within 10 s. The ESR signal intensity of the complex **C** depended on the reaction time at −45 °C. When the mixture was standing for about 3 min at this temperature, no ESR signal due to complex C was detected. The mixing ratio of water and DMF was very important for observing the ESR signal of complex **C** in the reaction system. After try-and-error procedures monitoring the ESR signal intensity of complex **C**, the optimum mixing ratio of water and DMF was found to be DMF:H_2O = 410:20. Complex **C** was also prepared by using a water solution of KOH instead of TPA. Preparation of the peroxide complex **C** has been tried in numerous organic solvents such as DMSO, CH_2Cl_2, CH_3OH and toluene, however, these solvents failed to generate complex **C**. It is noteworthy that the formation of complex **C** was recognized only in DMF containing about 5.0% water.

ESR and Optical Absorption Spectral Measurements

The absorption spectra at 25 °C were measured by a JASCO UV-1000 spectrophotometer. The absorption spectra of the frozen solutions were recorded at 77 K using an Ohtsuka Electronic Co. Ltd. MCPD-100 spectrometer in wavelengths ranging from 450 to 800 nm. ESR spectra were recorded at 77 K by a JEOL FE2-XG X-band spectrometer operating with 100 kHz field modulation of about 6.3 Gauss. 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 (86.9 Gauss). The g values of the observed ESR spectra were estimated based on the g value of the Li–TCNQ radical salt (g = 2.0025).

Simultaneous ESR and optical measurements were carried out for the frozen solutions prepared in 5.0 mm diameter ESR quartz tubes using a JEOL quartz dewer. All the measurements were performed at the Advanced Instrumental Center for Chemical Analysis, Ehime University.

Results and Discussion

Reaction between Fe(III)TPPCl and Hydrogen Peroxide in the Presence of Alkaline Reagent

The optical absorption spectra observed at 25 °C for the DMF solution of Fe(III)TPPCl (denoted as complex A) (0.5 mM, 0.4 ml) showed characteristic absorption maxima at 420, 508, 570 and 688 nm [10] (Fig. 1a), due to the five coordinate iron complex. The ESR spectrum of complex A at 77 K showed a typical line shape ($g_\perp$ = 6 and $g_\parallel$ = 2) (Fig. 2a) corresponding to the ESR of the ferric high-spin state [11]. When an aqueous solution of hydrogen peroxide (1.0 M, 0.02 ml) was added to the DMF solution of complex A at 25 °C in the presence of TPA (1.0 M, 0.01 ml), the color of the resulting mixture changed to pale yellow within a few minutes. As shown in Fig. 1b, the observed absorption spectrum showed weak absorption maxima at 415, 565 and 620 nm, and the characteristic absorption maximum due to Fe(III)TPPCl disappeared. This means that the conjugated π-system of the porphyrin ring

*Abbreviations: ESR, electron spin resonance; ENDOR, electron nuclear double resonance; Fe(III)TPPCl, chloro(5,10,15,20-tetraphenylporphyrinato)iron(III); TPA, tripropylamine; CCP, cytochrome C peroxidase; HRP, horseradish peroxidase; cytochrome P-450, P-450.

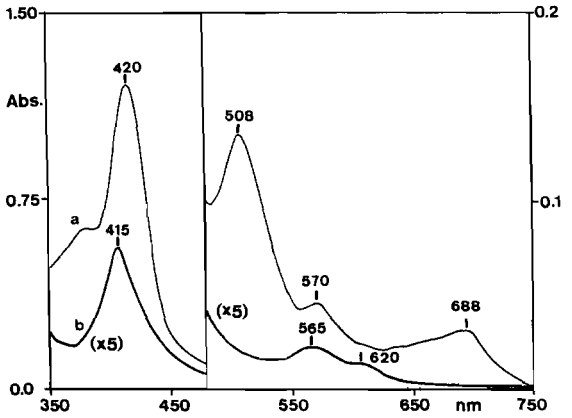

Fig. 1. Optical spectra of Fe(III)TPPCl observed before and after addition of hydrogen peroxide at 25 °C. (a) Before addition of hydrogen peroxide. Optical spectrum was recorded for DMF solution of the Fe(III)TPPCl (0.5 mM, 0.4 ml) using 0.1 mm optical cell. (b) Optical spectrum recorded within 5 min after addition of aqueous solution of hydrogen peroxide (1.0 M, 0.02 ml) in the presence of DMF solution of TPA (1.0 M, 0.01 ml). The molar ratio is Fe(III)-TPPCl:hydrogen peroxide:TPPA = 1.0:50:100, and content of water was about 5.0%.

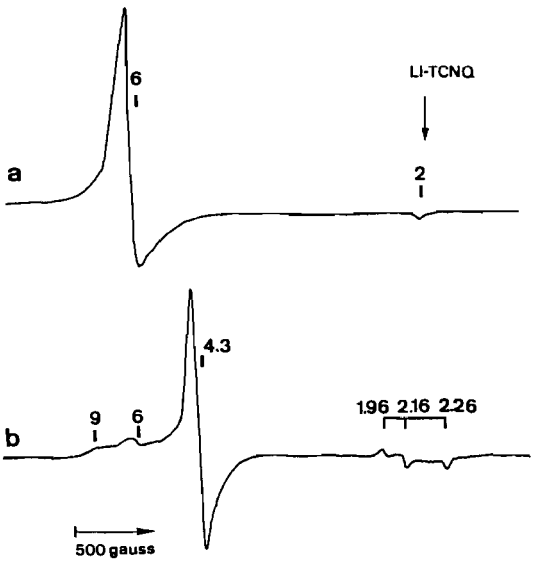

Fig. 2. ESR spectra observed at 77 K for the solution of Fe(III)TPPCl in the absence and presence of hydrogen peroxide. (a) ESR spectrum observed in the absence of hydrogen peroxide for DMF solution of Fe(III)TPPCl (0.5 mM, 0.4 ml). (b) ESR spectrum recorded within 5 min after addition of aqueous hydrogen peroxide (1.0 M, 0.02 ml) in the presence of DMF solution of TPA (1.0 M, 0.1 ml). The molar ratio and solvent mixing ratio were same to that of optical measurements as in Fig. 1.

moiety was disordered by reaction with hydrogen peroxide. The ESR spectrum recorded for this solution at 77 K (Fig. 2b) showed that the ESR signal

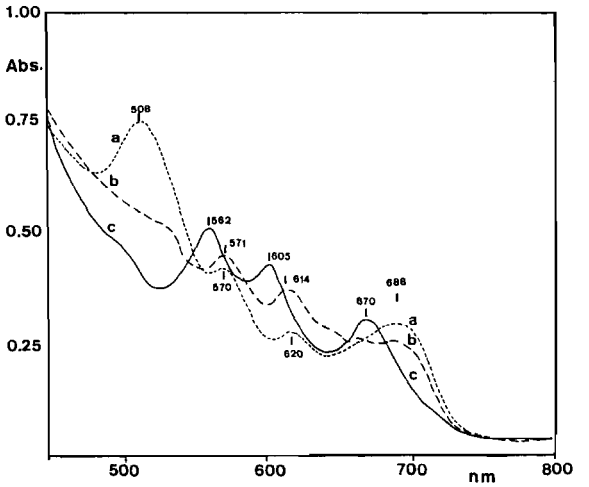

Fig. 3. Optical spectra (77 K) of the frozen solutions prepared by rapid mixing (−45 °C) and freezing method. (a) Optical spectrum observed for frozen DMF solution of Fe(III)TPPCl (0.5 mM, 0.4 ml) before addition of TAP and hydrogen peroxide. (b) Optical spectrum recorded for the frozen DMF solution of Fe(III)TPPCl (0.5 mM, 0.4 ml) and TPA (1.0 M, 0.01 ml). The molar ratio is Fe(III)TPP:TPA = 1:50. (c) Optical spectrum (77 K) observed after addition of aqueous solution of hydrogen peroxide (1.0 M, 0.02 ml) to the mixture of Fe(III)TPPCl and TPA. The molar ratio is Fe(III)TPPCl:TPA:HOOH = 1:50:100, and content of water is about 5.0%.

height due to Fe(III)TPPCl at $g = 6$ remarkably diminished, while a new ESR signal was detected at $g = 9$ and 4.3 (denoted as complex **B**). The observed line shape and g values of complex **B** agreed well with those of non-heme iron complexes in the ferric high-spin state such as the Fe(III)EDTA complex. These observations suggest that the porphyrin ring of Fe(III)TPPCl is decomposed to open-chain tetrapyrrol derivatives [12]. Interestingly, weak ESR signals due to formation of the low-spin ferric complex (denoted as complex **C**: $g_1 = 1.96, g_2 = 2.16$ and $g_3 = 2.26$) were usually detected (Fig. 2b) in the present reaction system.

In order to specify the coordination structure of complex **C** involved in the heme breakdown reaction, ESR and optical measurements were continued with the combining rapid mixing and freezing method. As a reference, the coordination reaction between Fe(III)TPPCl and TPA was studied by means of simultaneous ESR and optical measurements at 77 K. The optical (Fig. 3a) and ESR (Fig. 4a) spectra of Fe(III)TPPCl were first recorded (77 K) in the absence of TPA. The optical spectral parameters of Fe(III)TPPCl recorded at 77 K agreed well with that recorded at 25 °C, as summarized in Table 1. After addition of TPA (1.0 M, 0.01 ml), the absorption maximum at 508 nm due to Fe(III)TPPCl almost disappeared and Q-band absorption maxima showed

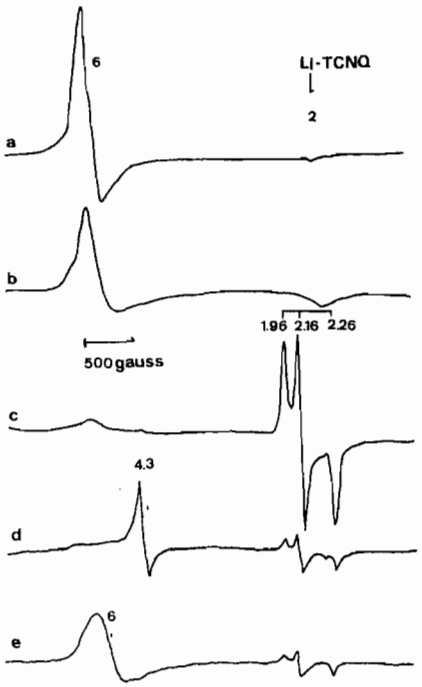

Fig. 4. ESR spectra (77 K) of the frozen solutions prepared by rapid mixing (−45 °C) and freezing method. (a) DMF solution of Fe(III)TPPCl (0.5 mM, 0.4 ml). (b) DMF solution composed of Fe(III)TPPCl (0.5 mM, 0.4 ml) and TPA (1.0 M, 0.01 ml). (c) ESR spectrum observed after addition of aqueous hydrogen peroxide (1.0 M, 0.02 ml) to the mixture of Fe(III)TPPCl and TPA. The mixing molar ratio and solvent mixing ratio were same to those of optical spectral measurements as cited in Fig. 3. (d) The frozen mixture was thawed at −45 °C and standing at −5 °C for 30 s. Then the resulting mixture was frozen at 77 K and ESR spectrum was again recorded at 77 K. (e) ESR spectrum observed for the frozen DMF solution of Fe(III)TPPCl (0.5 mM, 0.4 ml), TPA (1.0 M, 0.01 ml) and hydrogen peroxide (1.0 M, 0.02 ml), which was prepared by dilution of concentrated hydrogen peroxide with dry DMF. The mixing molar ratio was Fe(III)TPPCl:TPA:hydrogen peroxide = 1:50:100, and content of water was about 0.5%.

a slight shift to 571 and 614 nm, as shown in Fig. 3b. The ESR spectrum (Fig. 4b) observed for the solution showed formation of a high-spin ferric complex with a broad line shape compared with that observed before addition of TPA. Such broad ESR spectra have been reported for six coordinate high-spin ferric complexes, such as Fe(III)TPP–($(CH_3)_2SO$)($^-OCH_3$) or –($HOCH_3$)($^-OCH_3$) [13], in which weak ligands bind to both axial positions of Fe(III)TPP. As summarized in Table 1, the optical parameters of these six coordinate complexes agreed well with that observed for the mixture of Fe(III)TPPCl and TPA. Therefore, the iron complex featured by the absorption maxima at 571 and 614 nm is assigned similar to a six coordinate high-spin ferric complex, in which DMF and TPA weakly bind at the axial positions of Fe(III)TPP. It is concluded that the Fe(III)TPPCl complex was changed to the six coordinate high-spin ferric species in the presence of TPA.

The DMF solution consisting of Fe(III)TPPCl and TPA was then treated with an aqueous solution of hydrogen peroxide (1.0 M, 0.02 ml), as described in 'Experimental'. After addition of hydrogen peroxide, the color of the reaction mixture clearly changed to a bright red, similar to that seen for oxy-Hb or -Mb. Figure 3c shows the optical spectrum recorded for the bright red solution at 77 K. The Q-band absorption maxima shifted from 571 and 614 nm to 562, 605 nm in addition to 670 nm (Fig. 3c), respectively. The ESR spectrum observed for this frozen solution showed formation of complex **C** ($g_1 = 1.962$, $g_2 = 2.157$ and $g_3 = 2.264$) with a strong signal intensity as shown in Fig. 4c. The ESR spectrum of complex **C** was detected everywhere when the pair of absorption maxima (562 abd 605 nm) was detected. These observations demonstrated that complex **C** detected by ESR is identical to the iron complex as featured by the pair of absorption maxima at 562 and 605 nm.

It was noted here that the pair of absorption maxima (562 and 605 nm) was not observed in the absence of alkaline reagents such as TPA or aqueous KOH, but, the absorption maximum at 670 nm was still observed even in the absence of the alkaline reagent. The absorption maximum at 670 nm is very close to that of the high valent iron complexes produced by mixing Fe(III)TPP derivatives and *m*-chloroperbenzoic acid [6]. At present, the accurate geometry of the iron species characterized by the absorption maxima at 670 nm is still equivocal, however, this species can be one of the transient intermediates involved in the decomposition of the Fe(III)TPPCl complex, since the formation of such high valent iron complexes was often speculated in the processes of heme breakdown reactions [14].

ESR measurements combined with the thaw-and-freeze treatment were carried out to characterize the role of complex **C** in the processes of the heme breakdown reaction. The ESR spectrum recorded for the frozen solution, prepared by the same procedure described above, showed the formation of complex **C** with a strong ESR line intensity (Fig. 4c). Then the frozen mixture was thawed at −5 °C for about 30 s and rapidly frozen in liquid nitrogen, and the ESR spectrum was again recorded at 77 K. As shown in Fig. 4d, observed ESR signals intensity of complex **C** abruptly decreased, followed by a concomitant formation of complex **B** at $g = 4.3$. The ESR spectrum observed after the thaw-and-freeze treatment was analogous to that observed for the solution prepared by mixing Fe(III)TPPCl, TPA and hydrogen peroxide at 25 °C, as shown in Fig. 2b. In addition, the optical absorption spectrum recorded for the mixture at 25 °C was consistent with that illustrated in Fig. 1b. It is found that the lifetime of the

TABLE 1. Results of ESR[a] and optical[b] absorption measurements for iron peroxide complexes and related complexes

Complex	Solvent	Base	Temperature[c]	Peroxide	Abs. maxima (nm)					g value			Reference
Fe(III)TPPCl	DMF		298	none	420	508	570	620	688	6.0	2.0		d
Fe(III)TPPCl	DMF		77	none	421	508	570	620	688	6.0			d
Fe(III)TPPCl	DMF	TPA	77	none			571	614	686	6.0			d
Fe(III)TPPCl	DMF	KOH	77	none			574	620	684	6.0			d
Fe(III)TPP($^{-}OCH_3$)($HOCH_3$)	DMSO	$NaOCH_3$	298	none	420		580	625		6.0			13
Fe(III)TPP($^{-}OCH_3$)DMSO	DMSO	$NaOCH_3$	298	none	422		575	615		6.0			13
Fe(III)TPP($^{-}OCH_3$)$_2$	CH_2Cl_2	$NaOCH_3$		none						1.91	2.17	2.49	13
Fe(III)TPP(Im)$_2$[e]	CH_2Cl_2			none						1.56	2.30	2.92	15
Fe(III)TPP(^{-}OH)(^{-}OOH)	DMF	TPA	77	H_2O_2	420		562	605		1.962	2.157	2.264	d
Fe(III)TPP(^{-}OH)(^{-}OOH)	DMF	KOH	77	H_2O_2	420		563	607		1.961	2.159	2.266	d
Fe(III)TPP($^{-}OCH_3$)($^{-}OOC(CH_3)_3$)	CH_2Cl_2	$NaOCH_3$	77	t-BHPO	424		543	571		1.952	2.157	2.316	8
Fe(III)Hb–^{-}OOH	H_2O			O_2[f]						1.96	2.14	2.25	17
Fe(III)Mb–^{-}OOH	H_2O			O_2[f]						1.94	2.16	2.28	17
Fe(III)HRP–^{-}OOH	H_2O			O_2[g]						1.95	2.16	2.31	19
Fe(III)BLM–^{-}OOH	H_2O	pH 8		H_2O_2						1.93	2.17	2.25	16

[a]ESR spectra were recorded at 77 K, and experimental error in g value of ferric low-spin species were within about 0.003. [b]Experimental error in absorption maxima were within about 3 nm. [c]Temperature for the optical measurements. [d]Present work. [e]Im, means imidazole. [f]UV- or γ-irradiation. [g]γ-Irradiation.

complex **C** is very short at −5 °C and complex **C** is readily decomposed to the non-heme type iron complex **B** ($g = 4.3$). Thus complex **C** was characterized to be one of the intermediate complexes formed in the processes of the heme degradation reaction. The whole reaction mechanism of the Fe(III)TPP breakdown processes involving complex **C** and the high valence iron complex appearing at 670 nm is under investigation and the results will be reported elsewhere.

Essential Conditions for Formation of Complex C

The ESR and optical absorption measurements were continued in order to understand the reaction conditions necessary to generate complex **C**. As a trial, the reaction occurring between Fe(III)TPPCl and hydrogen peroxide in the presence of aqueous KOH was carried out by the same procedure. The frozen mixture was prepared by mixing hydrogen peroxide (1.0 M, 0.02 ml) and the cooled solution composed of Fe(III)TPPCl (0.5 mM, 0.4 ml) and aqueous KOH (0.1 M, 0.02 ml). The optical (λ_{max} 563, 607 nm) and ESR spectra ($g_1 = 1.961$, $g_2 = 2.159$ and $g_3 = 2.266$) ascribable to complex **C** were also observed for this frozen mixture. As summarized in Table 1, both parameters of the ESR and optical spectra of complex **C** were less dependent on the alkaline reagents used, and complex **C** has never been observed in the absence of alkaline reagents. This finding justifies the fact that these alkaline reagents, TAP and KOH, play an important role in generating the deprotonated hydrogen peroxide anion, $^{-}$OOH, species in the present reaction system.

Interestingly, ESR and optical absorption parameters of complex **C**, prepared in the presence of aqueous KOH, showed excellent agreement with that observed for the system consisting of TPA, as summarized in Table 1. Therefore, axial ligation of the nitrogeneous group of TPA can be safely ruled out for complex **C**. In both reaction systems, chemical species such as DMF, HOOH, H_2O, $^{-}$OOH and $^{-}$OH can be considered to be the axial ligand of complex **C**. In the above chemical species, however, the deprotonated $^{-}$OOH and $^{-}$OH anions would be more likely candidates for the axial ligand of complex **C**, because, the presence of alkaline reagents (KOH or TPA) was indispensable for generating complex **C**.

The effect of the H_2O content on the generation of complex **C** was investigated by monitoring the ESR signal intensity of complex **C**. A solution of hydrogen peroxide (1.0 M), which was prepared by dilution of 30% aqueous hydrogen peroxide with dry DMF, was used for measurements. The ESR spectrum was recorded for the frozen solution prepared by mixing hydrogen peroxide (1.0 M, 0.02 ml) with the pre-cooled dry DMF solution consisting of Fe(III)TPPCl (0.5 mM, 0.4 ml) and TPA (1.0 M, 0.01 ml). Under these reaction conditions, the content of water was below about 0.5%. As shown in Fig. 4e, a weak ESR signal due to complex **C** was recorded for this reaction mixture. In this case, however, the observed ESR signal height of complex **C** was about one half of that recorded in the presence of 5.0% water (Fig. 4c). This frozen solution was thawed at −45 °C, and 0.02 ml water was added to the mixture, by which the water content increased to about 5.1%. After the addition of water, the color of the resulted frozen solution turned to bright red, and the ESR signal of complex **C** was detected with a strong line intensity as good as that shown in Fig. 4c. The ESR signal intensity of complex **C** was strongly dependent on the water content involved in the reaction mixture, indicating the fact that the presence of about 5% H_2O in DMF is very important for generating complex **C** in this reaction system. The $^{-}$OH anion derived from water is more probably the axial ligand of complex **C** rather than the H_2O molecule, because alkaline reagents were indispensable to produce complex **C**. Based on the observation results described in this section, the possible axial ligand set of complex **C** was speculated to be the $^{-}$OH and $^{-}$OOH anions.

Coordination Structure of Complex C

The ESR parameters of the complex **C** are characteristic of anomalously small g anisotropy compared with those of the usual low-spin ferric iron complexes such as Fe(III)TPP($^{-}$OCH$_3$)$_2$ [13] or Fe(III)TPP-(imidazole)$_2$ [15] (Table 1). Low-spin ferric complexes analogous to complex **C** have often been observed for some six coordinate iron peroxide complexes. For example, an anticancer non-heme iron complex of bleomycin, (Fe(III)BLM) [16], forms a six coordinate Fe(III)BLM–$^{-}$OOH ($g_1 = 1.93$, $g_2 = 2.17$ and $g_3 = 2.25$) species in the presence of hydrogen peroxide under alkaline conditions above pH 8.2. The hydroperoxide adduct of iron–BLM thus formed was regarded to be a key intermediate in the DNA cleavage reaction. In addition, Symons and Petersen [17] have reported the ESR spectra of [Fe(II)–Hb-OO]$^{-}$ ($g_1 = 1.96$, $g_2 = 2.14$ and $g_3 = 2.25$), generated by γ- or UV-irradiation to the ferrous heme–oxygen complexes at 77 K. The exposure to X-irradiation resulted in the formation of one-electron inserted oxygen complexes such as Fe(II)–O_2^{-} or Fe(III)–O_2^{2-} species. Their recent ENDOR study [18] demonstrated that formation of a hydrogen bond between the latter Fe(III)–O_2^{2-} and NH proton of distal histidine was important for generating the paramagnetic species. Accordingly, the coordination structure of the ferric low-spin species was tentatively formulated to be the six coordinate Fe(III)–$^{-}$OOH complex. ESR spectra ascribable to be the Fe(III)–HRP–$^{-}$OOH complex [19] ($g_1 = 1.95$, $g_2 = 2.16$ and $g_3 = 2.31$) were also

observed after γ-irradiation to the oxygen complex of HRP. The satisfactory resemblance seen in the g parameter between complex **C** and those of six coordinate heme–hydrogen peroxide adducts (Table 1), suggests that complex **C** can be classified as a heme–hydrogen peroxide adduct.

ESR spectra of six coordinate iron–hydrogen peroxide complexes have been reported frequently (Table 1), however, few optical absorption spectra corresponding to such iron–peroxide complexes have been reported, to our present knowledge. Recently, we [8] have proposed the formation of Fe(III)TPP–alkylperoxide complexes demonstrated by means of simultaneous ESR and optical absorption spectroscopy. Based on ESR spectrometric titration, the coordination structure of the complex was speculated to be six coordinate Fe(III)TPP($^-OCH_3$)($^-$OO–tert-butyl); the methoxide and tert-butylperoxide anion were located at the axial position. The g parameter of the complex ($g_1 = 1.952$, $g_2 = 2.157$ and $g_3 = 2.316$) and characteristic absorption maxima 543 and 571 nm) were analogous to those of complex **C** recorded at 77 K, as summarized in Table 1. In addition, similar features were recognized in the reaction conditions used to prepare these Fe(III)-TPP–alkylperoxide and –hydrogen peroxide complexes. No detectable amount of ESR signal due to the former alkylperoxide complex has been observed in the absence of the methanol solutions of $NaOCH_3$, which behave not only as an alkaline reagent to promote deprotonation of tert-butyl–hydroperoxide, but also as an oxygenous donor binding at the axial position of heme. In the case of the latter, the presence of about 5.0 %H_2O and alkaline reagents, such as TPA or KOH, is essential to form complex **C**. The deprotonated $^-$OH anion will be a more preferable axial ligand for complex **C**, analogous to the case of Fe(III)TPP–alkylperoxide complexes having the $^-OCH_3$ anion at the axial position. Therefore, the probable coordination structure of complex **C** was schematically concluded to be six coordinate Fe(III)-TPP($^-$OH)($^-$OOH), as illustrated in Fig. 5. The presence of a similar heme–hydrogen peroxide complex was speculated based on the kinetic studies carried out for the decomposition processes of hydrogen peroxide occurring in the presence of water-soluble Fe(III)TPP derivatives [20]. However, no detailed spectroscopic description has been given for the intermediate heme–hydrogen peroxide complexes. To our present knowledge, complex **C** described here is the first example of a heme–hydrogen peroxide complex directly detected by ESR and optical absorption spectroscopy.

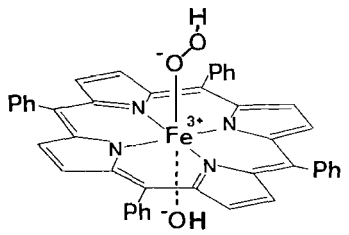

Fig. 5. The proposed coordination structure of the Fe(III)-TPP hydrogen peroxide complex C, Fe(III)TPP($^-$OH)-($^-$OOH).

The formation of heme enzyme–hydrogen peroxide adducts [1–3] has often been postulated in the initial stages of their enzymatic reactions. The high resolved X-ray analysis carried out for catalase [21], CCP [22] and P-450 [23] demonstrated that the endogenous ligands located at the fifth position of heme were characterized to be the O^-, S^- and N donors derived from tyrosine, thiolate and histidine, respectively. The coordination structure of these heme enzymes–hydrogen peroxide adducts can be tentatively formulated to be six coordinate O^-–, S^-– or N–Fe–$^-$OOH. Complex **C** takes a six coordinate structure similar to these heme enzymes–hydrogen peroxide intermediates, but the fifth position is occupied by the $^-$OH anion derived from the water molecule. Nevertheless, complex **C** will be a more practical model for the heme enzymes–hydrogen peroxide complex. Further investigations on the formation of the Fe(III)TPP–$^-$OOH complex having a phenolate anion, thiolate anion and imidazol as the fifth coordinate are now in progress.

In conclusion, a possible intermediate iron complex observed in the heme breakdown processes is proposed to be the six coordinate low-spin ferric Fe(III)TPP–($^-$OH)($^-$OOH) complex with anomalously small g anisotropy; $g_1 = 1.962$, $g_2 = 2.157$ and $g_3 = 2.264$. The optical spectrum of the hydrogen peroxide complex shows absorption maxima at 562 and 605 nm. The hydrogen peroxide complex **C** will provide useful information about the coordination structure of the intermediate species formed in the processes of reactions occurring between hydrogen peroxide and several classes of heme enzymes.

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