A Chemical Model of Tryptophan 2,3-Dioxygenase: Reactivity and Formation of a Reactive Intermediate in the Dioxygenation Processes

Shigenori OKA,[a,1)] Kunihiko TAJIMA,[b] and Hiromu SAKURAI*,[a]

Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University,[a] Yamashina-ku, Kyoto 607, Japan and Department of Polymer Science and Engineering, Kyoto Institute of Technology,[b] Sakyo-ku, Kyoto 606, Japan. Received September 18, 1997; accepted November 5, 1997

To understand the activation of molecular dioxygen by the heme-containing enzyme tryptophan 2,3-dioxygenase (TDO), a chemical model was constructed, which permitted an identification of reactive intermediates through the use of ESR, optical spectra, cyclic voltammetry, and oxygenated product analysis. The temperature-dependent ESR measurement for the chemical model, which consisted of tetraphenylporphine iron(III) chloride (Fe(III)TPPCl), 3-methylindole (skatole; Sk), tetramethylammonium hydroxide (TMAOH), and molecular dioxygen, revealed the generation of two kinds of free radical species, as well as the formation of two ternary complexes in the ferric low-spin state. The radical species R1, with an ESR value of $g=2.0045$, was determined to be a Sk neutral radical derived from the substrate by MO calculation. The radical species R2, with an ESR value of $g=2.015$ was indicated to be a Sk-peroxide radical, consisting of a dioxygen molecule bound to the Sk neutral radical. A comparison of the ESR parameters of the two ternary complexes with those of previously reported complexes suggested that the present complexes are six-coordinate ferric low-spin species: one which is bound with two dioxygenated Sk anions at the heme-iron site ([A]: $g_1=2.236$, $g_2=2.160$, $g_3=1.965$), and the other bound with a dioxygenated Sk anion and a methoxide anion ([B]: $g_1=2.311$, $g_2=2.160$, $g_3=1.954$). These ternary complexes are assumed to be possible models for the transient hemoprotein-peroxide complexes. The formations of R1 and ternary complexes were indispensable for the dioxygenation of the substrate. On the basis of these results, a possible reaction mechanism is proposed.

Key words tryptophan 2,3-dioxygenase; ternary complex; free radical; ESR; chemical model

Several classes of heme enzyme, such as cytochrome P450 (P450),[2)] heme oxygenase,[3)] tryptophan dioxygenase (TDO),[4)] catalase,[5)] and several peroxidases,[6)] have similar mechanisms by which, in the early catalytic stages, molecular dioxygen and peroxide are catalyzed and subsequently incorporated into endogenous or exogenous substrates. Consequently, it has been proposed that a heme–peroxide complex is formed as an intermediate state in these reactions.[7)] For example, the peroxide-dependent process in which P450 enzymes catalyze the activation of molecular dioxygen and insert one oxygen into a wide variety of organic substrates suggests the formation of a P450–peroxide complex.[8)] Similarly, a large number of studies focusing on metal iron-dependent oxygenations have been conducted using various chemical models of P450 to better understand the mechanism of enzymatic reactions.[9)]

On the other hand, during the process in which TDO[10,11)] catalyzes the oxidative cleavage of tryptophan to formylkynurenin at the heme iron site,[12)] it is assumed that the ternary complex TDO–dioxygen–substrate is formed as a transitory intermediate.[13)] The coordination and electronic structures of this reactive intermediate have been proposed with the model complexes Co(II)salen,[14)] Mn(II)Pc,[15)] Fe(II)tetraphenylporphine (Fe(II)TPP)–(pyridine)$_2$,[16)] Mn(III)TPP,[17)] and Co(II)TPP.[18)] Though no reactive intermediates which elucidate the structure and function of the enzyme have been identified, such intermediates involving the Fe(II) state are regarded as an important species in the catalytic reaction cycle of TDO, since the resting state of TDO is Fe(II). Further, Leeds *et al.* reported a possible reaction mechanism of TDO, as studied by isotope effects and alternative substrate reactivity,[19)] which suggests the occurrence of both a Fe(III) state and a free radical species derived from a substrate in the reaction cycle of TDO.

Recently, a possible model system of TDO composed of Fe(III)TPPCl, tetramethylammonium hydroxide (TMAOH), 3-methylindole (skatole; Sk), and molecular dioxygen was proposed.[20)] The product analyses by GLC and GC–MS revealed that Sk is effectively converted to *o*-formamidoacetophenone (*o*-FA), in which two oxygen atoms of molecular dioxygen are incorporated in Sk. The

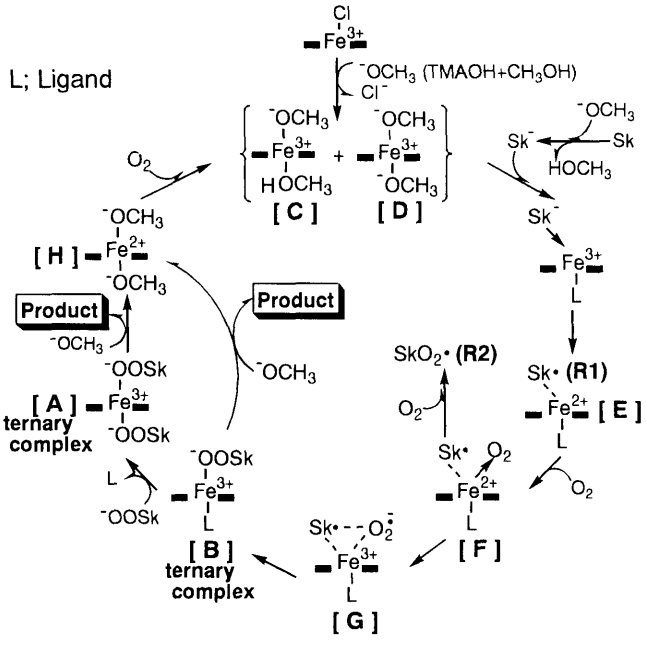

Chart 1

* To whom correspondence should be addressed.

simultaneous ESR and optical absorption measurements at 77 K indicated the formation of a transient intermediate, a ternary six-coordinate methoxide–Fe(III)TPP–Sk peroxide complex in the low spin state. Furthermore, ESR spectrum using $^{17}O_2$ indicated the formation of ferric low-spin complexes bound with two deprotonated 3-hydroperoxo-3-methylindolenin in the axial positions. We investigated in detail the characterization of both ternary complexes, as well as the dioxygenation reaction mechanism, focusing on the formation of the unstable intermediate species at the initial step of dioxygenation. The present study on the ternary complexes and unstable intermediates in our model helps to elucidate the reaction process of dioxygen activation and catalytic oxygenation, together with the active site structure of the enzyme. In this paper, we propose a chemical model system of TDO which illustrates a possible reaction mechanism of substrate dioxygenation, in terms of the formations of both an unstable ternary complex containing Fe(III) and a free radical species.

Experimental

Materials Tetraphenylporphyrin ($TPPH_2$) and its iron complex, Fe(III)TPPCl, were synthesized by the procedures described by Adler *et al.*[21] Tetrakis(2,4,6-trimethylphenyl)porphyrin (tetramesitylporphyrin; $TMPH_2$) and tetrakis(2,4-dimethylphenyl)porphyrin (tetraxylylporphyrin; $TXPH_2$) were prepared by the published method.[22] Iron was inserted into $TMPH_2$ and $TXPH_2$ to form Fe(III)TMPCl and Fe(III)TXPCl, respectively, by a standard method.[23] The structures of Fe(III)TPPCl and Fe(III)TMPCl were verified by elemental analyses as follows: Found (Calcd) for Fe(III)TPPCl ($C_{44}H_{28}N_4FeCl$): C, 74.56 (75.07); H, 4.14 (4.01); N, 8.11 (7.96), for Fe(III)TMPCl ($C_{56}H_{52}N_4FeCl$): C, 77.70 (77.10); H, 5.82 (6.01); N, 6.31 (6.42), for Fe(III)TXPCl ($C_{52}H_{44}N_4FeCl$): C, 77.46 (76.52); H, 5.61 (5.43); N, 6.41 (6.86). 3-Methylindole (Sk) was obtained from Wako Pure Chemicals and was used after recrystallization from toluene. Methanol solution of TMAOH was purchased from Tokyo Chemical Industry. Methanol solutions of sodium alkoxides (NaOMe, NaOEt, NaOPr) were prepared by addition of sodium metal to dry corresponding alcohols. Concentrations of alkaline reagents were estimated by pH titration method. 2,6-Di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene; BHT), 1,4-diazabicyclo(2,2,2)-octane (DABCO), ethanol (EtOH), *N*,*N*-dimethylformamide (DMF), dimethylsulfoxide (DMSO), ferrocene and toluene were a special reagent grade from Wako Pure Chemicals. Solvents were distilled before use. $^{17}O_2$ was purchased from Isotech Co.

Spectrophotometric Measurements Optical absorption spectra (wavelength range, 400—800 nm) at 173 K were recorded with a MCPD-1000 spectrophotometer, Ohtsuka Electronic Co. ESR spectra were measured with an ESR spectrometer RE-3X, JEOL, with 100 kHz field modulation. The microwave frequency applied to the sample at the power of 5.0 mW was monitored by an Advantest R5372 digital frequency counter. The magnetic field strength was calibrated by the hyperfine coupling constant (8.69 mT) of Mn(II) ion doped in MgO powder. The g values of the observed ESR spectra were estimated with Li-TCNQ (Li-tetracyanoquinodimethane) radical salt ($g=2.00252$) as standard. ESR spectra were obtained at 173 K and 77 K.

Preparation of Fe(III)TPP($^-O_2$Sk) Ternary Complex Fe(III)TPP($^-O_2$Sk) ternary complex was prepared in an ESR quartz tube (5 mm diameter) under N_2 atmosphere at 195 K (dry ice–acetone bath). The sample solution (total volume, 0.62 ml) was composed of Fe(III)TPPCl (1 mM in toluene, 0.4 ml), TMAOH (0.2 M in methanol, 0.02 ml), and Sk (0.2 M in toluene, 0.2 ml) in toluene. After 100% oxygen gas (10 ml) was slowly introduced for 10—20 s into the reaction mixture through a fine capillary at 173 K, ESR spectrum of the solution was recorded within 10 s at 173 K.

Product Analysis in the Reaction Mixture Sk (0.2 M in toluene, 0.4 ml) was added in a mixture (total volume of 1.24 ml) of Fe(III)–porphyrin (1.0 mM in toluene, 0.8 ml) and TMAOH (0.2 M in methanol, 0.04 ml) to start the reaction by stirring under 100% O_2 atmosphere at 298 K. *o*-FA, which is an oxygenated product of Sk, was determined by GLC method (Shimadzu GC-6AM, silicon OV-17 column), and the reaction was periodically monitored. The product yield of *o*-FA was expressed as $(100\times C_i/C_0)$ value, in which C_i and C_0 refer to the concentration of *o*-FA produced at i min and the initial concentration of Sk, respectively. *o*-FA as an authentic sample was prepared by modification of the procedure described by Nishinaga.[14] The IC_{50}-value represents the concentration producing 50% inhibition of dioxygenation in the presence of scavengers in the reaction mixture. The first-order rate constants were calculated from plots of $[\ln(C_0)/(C_0-C_i)]$ *vs.* time for the formation of *o*-FA from Sk.

McLachlan Hückel Molecular Orbital (HMO) Calculations Calculations were carried out using the McLachlan perturbation corrections to the HMO theory (without overlap), in which $\lambda=1.20$ was used.[24] Parameters used were $h_{NN}=0.5$ as coulombic integral of nitrogen atom and $h_{CC}=0.1$ as coulombic integral of carbon atom with methyl groups, in which the inductive model C–X for the methyl groups was used.

Formation of *tert*-Butylperoxyl Radical *tert*-Butylperoxyl radical was prepared in an ESR quartz tube (5 mm diameter) at 295 K. The sample solution was composed of Fe(III)TPPCl (1 mM, 0.4 ml) and tert-butyl hydroperoxide (0.2 M, 0.2 ml). ESR spectrum of the solution was recorded at 173 K.

ESR Measurement of Radical Species Prepared under $^{17}O_2$ Atmosphere The pre-degassed reaction mixture composed of Fe(III)TPP (1.0 mM in toluene, 0.4 ml), TMAOH (0.2 M in methanol, 0.02 ml), and Sk (0.2 M in toluene, 0.2 ml) was exposed to 49% enriched $^{17}O_2$ ($I=5/2$) at 195 K. ESR spectrum of the solution was immediately recorded at 173 K.

Electrochemical Measurements Cyclic voltammetry was carried out with a BAS 100B/W, with a three-electrode system. In dichloromethane (CH_2Cl_2), a Pt rod was used as the working electrode, a Pt wire as the auxiliary electrode, and Ag/Ag^+ electrode as the reference electrode. The reference electrode was separated from the bulk of the solution by a glass frit and was immersed in an acetonitrile solution containing 0.1 M tetra-*n*-butylammonium perchlorate. All experiments were carried out at 295 K in CH_2Cl_2 purged with N_2 gas. Potentials were recorded *vs.* Ag/Ag^+, and the cyclic voltammetry sweep rate was 50 mV/s. Half-wave potentials were evaluated by $E(i_{pc}/2)$, where i_{pc} is the peak current at the cathodic peak potential. In this TDO model system, E_{pa}, which represents the anodic peak potential, was clearly observed.

Results

Detection of Unstable Intermediate Species in the Ferric Low-Spin States at 158 K to 198 K during the Dioxygenation of Sk in the Fe(III)TPPCl–TMAOH–O_2 System An anaerobic reaction mixture composed of 0.64 mM Fe(III)-TPPCl, 6.4 mM TMAOH, and 64 mM Sk in toluene solvent showed no low-spin ESR signal at 158 K (data not shown). When sufficient O_2 gas was introduced into the mixture at 158 K, a bright red color (solution I) developed from an initial deep green. Additionally, ESR signals due to the formation of two types of ferric low-spin species were detected (Fig. 1), which was followed by measuring at 198 K ([A]; major: $g_1=2.230$, $g_2=2.145$, $g_3=1.973$ and [B]; minor at 173 K). These ferric low-spin species could not be detected above 198K, probably due to their relaxation times. The ESR spectrum at 77 K for the same solution in dichloromethane also showed two ferric complexes in the low-spin states ([A]: $g_1=2.238$, $g_2=2.160$, $g_3=1.964$; [B]: $g_1=2.316$, $g_2=2.169$, $g_3=1.952$).[20] The optical spectra of solution I at both 173 K and 77 K showed almost same absorption maxima at 421, 550, 589 nm and 423, 550, 589 nm, respectively (Figs. 2a, b). In a comparison of these spectroscopic parameters with those of several model complexes reported earlier (Table 1),[20,25] both ferric low-spin complexes [A] and [B] were assignable to be the six coordinate complexes as Fe(III)-TPP($^-O_2$Sk)$_2$ and Fe(III)TPP($^-$OCH$_3$)($^-O_2$Sk) as ter-

nary complexes, respectively.

Detection of Free Radicals Due to Sk in the Initial Reaction of Fe(III)TPPCl–TMAOH–Sk–O_2 ESR spectrometry was used to determine the dioxygenation processes of Sk in the reaction system at 173 K. ESR spectrum was recorded first under N_2 for the cooled solution (173 K) of 0.64 mM Fe(III)TPPCl, 6.4 mM TMAOH, and 64 mM Sk (Fig. 3a). Upon the slow introduction of dry O_2 (1 ml) through a fine capillary to the ESR silent reaction mixture (a) in Fig. 3a, the color of the solution gradually turned from deep green to bright red. As shown in Fig. 3b, ESR signals due to the ferric low-spin complexes ([A]: $g_1=2.230$, $g_2=2.145$, $g_3=1.973$); [B]: $g_1=2.301$, $g_2=2.145$, $g_3=1.964$) were observed with a concomitant formation of a free radical species (R1) at $g=2.0045$. The R1 signal with a hyperfine splitting of 0.6 mT exhibited a very short half life ($t_{1/2}=10$—15 s at 173 K) and was converted to another type of free radical species (R2) at $g=2.015$. Upon the introduction of dry O_2 (10 ml) to the ESR silent reaction mixture (a), however, a new radical species at $g=2.015$, which has the same feature as R2, appeared together with ESR signals due to the complexes [A] and [B] (Fig. 3c). ESR signal intensities due to the complexes [A] and [B] increased with further introduction of O_2, showing saturation of the signals after addition of 10 ml O_2, but the signal due to (R1) disappeared.

Dioxygenation of Sk in the Model Systems and the Effects of Free Radical Scavengers The dioxygenations of Sk in the system of 0.61 mM Fe(III)TPPCl, 6.1 mM TMAOH, and 61 mM Sk were examined in toluene solvent under 100% O_2 atmosphere at 298 K. As shown in Fig. 4a, a time-dependent oxygenation was observed. The product yield of *o*-FA on the basis of the substrate concentration in the complete system was about 40% after 60 min of the reaction. However, no *o*-FA was detectable when there was a lack of Fe(III)TPPCl or TMAOH in the system. The addition of a radical scavenger, BHT to the complete model system resulted in inhibition of the Sk oxygenation (Fig. 4b). These results were also supported by ESR measurements. ESR spectrum for the toluene solution of the complete system (Fe(III)TPPCl/TMAOH /Sk/O_2) at 173K showed the characteristic ESR signal pattern ascribable to the formation of ferric low-spin complexes ([A]: $g_1=2.230$, $g_2=2.145$, $g_3=1.973$; [B]: $g_1=2.301$, $g_2=2.145$, $g_3=1.964$) and two kinds of free radical species

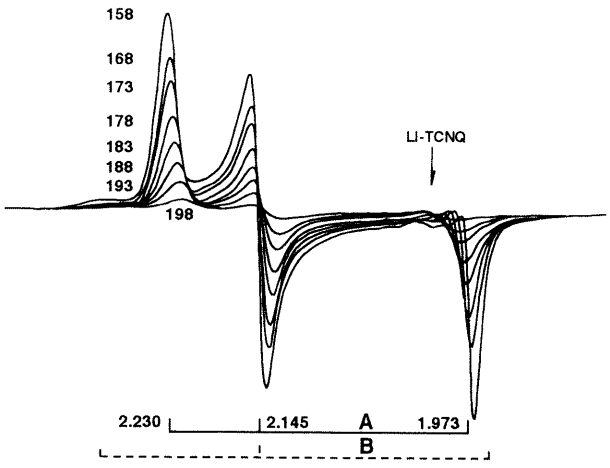

Fig. 1. Temperature-Dependent ESR Spectral Change of the Reaction Mixture

The reaction mixture was composed of 0.64 mM Fe(III)TPPCl (dissolved in toluene), 6.4 mM TMAOH (dissolved in methanol) and 64 mM Sk (dissolved in toluene). The mixing molar ratio of chemicals was as follows: Fe(III)TPPCl: TMAOH : Sk = 1 : 10 : 100. The resulting soluent ratio was toluene : methanol = 30 : 1. Total volume = 0.62 ml. The range of the temperature change was from 158 K to 198 K.

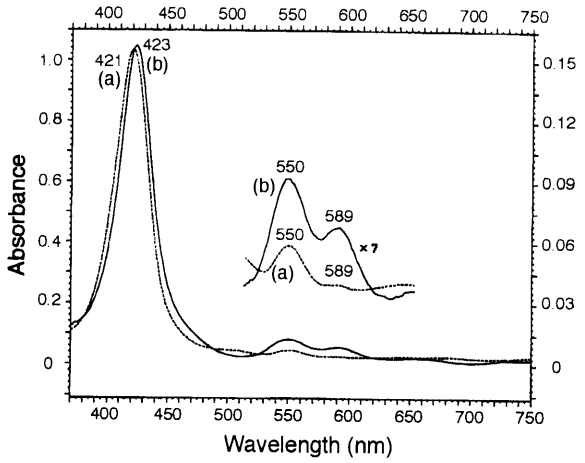

Fig. 2. Optical Absorption Spectra of the Reaction Mixture

The reaction mixture contained 0.64 mM Fe(III)TPPCl (dissolved in toluene), 6.4 mM TMAOH (dissolved in methanol) and 64 mM Sk (dissolved in toluene). The resulting solvent system was toluene : methanol = 30 : 1. Total volume = 0.62 ml. (a) recorded at 173 K; (b) recorded at 77 K.

Table 1. ESR and Optical Parameters of Fe(III)TPPCl–Dioxygen–Sk Ternary Complex and Relating Ferric Low-Spin Complexes

Complex	Solv.	Temp. (K)	ESR parameter			Temp. (K)	Optical parameter				Ref.
			g_1	g_2	g_3		λ_{max} (nm)				
Solution I, complex A	T	173	2.230	2.145	1.973	173	421	550	589		e)
complex B	T	173	Ferric low-spin			173	421	550	589		e)
Solution I, complex A	T	77	2.236	2.160	1.965	77	423	550	589		e)
complex B	T	77	2.311	2.160	1.954	77	423	550	589		e)
TPP(-OMe)$_2$[a]	D[b]	77	2.487	2.167	1.915	198	438	550	597	638	25
	T[c]	77	2.490	2.167	1.915	173	418	550	595	644	e)
TPP($^-$OMe)($^-$O$_2$R)[d]	D	77	2.316	2.157	1.952	198	425	542	575		25
TPP($^-$O$_2$R)$_2$	D	77	2.242	2.157	1.952	198	425	542	575		25
TPP($^-$OMe)($^-$O$_2$Sk)	D	77	2.316	2.169	1.952	198	421	550	586		20
TPP($^-$O$_2$Sk)$_2$	D	77	2.238	2.160	1.964	198	421	550	586		20

a) TPP: Fe(III)TPP. *b*) dichloromethane. *c*) toluene. *d*) R: *tert*-butyl. *e*) present study.

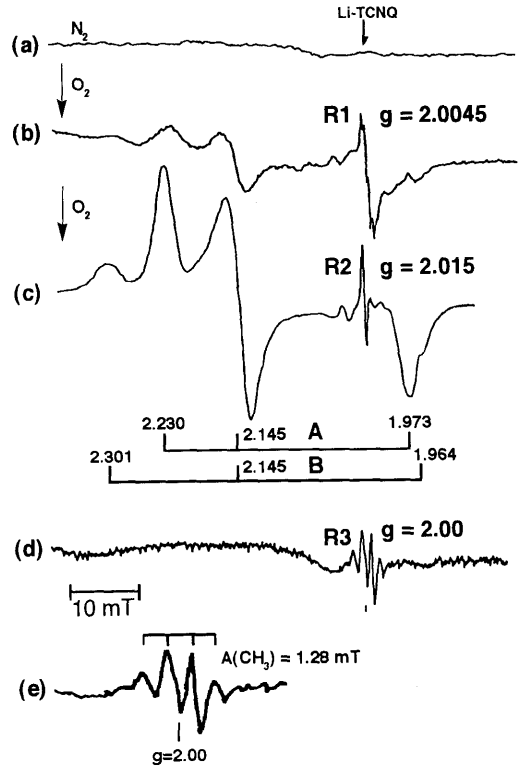

Fig. 3. ESR Spectra of TDO Model Systems in the Initial Reaction at 173 K

The reaction mixture was composed of 0.61 mM Fe(III)TPPCl (dissolved in toluene), 6.1 mM TMAOH (dissolved in methanol) and 61 mM Sk (dissolved in toluene). The mixing molar ratio of chemicals was Fe(III)TPPCl : TMAOH : Sk = 1 : 10 : 100, and the solvent ratio was toluene : methanol = 30 : 1. Total volume = 0.62 ml. (a) Under N_2; (b) after O_2 bubbling; (c) under O_2; (d) with BHT (0.61 mM, dissolved in toluene); (e) magnification of (d).

(R1 = 2.0045; R2 = 2.015), as shown in Figs. 3b and c. When BHT[26] was added to the system, a different ESR signal pattern was observed (Fig. 3d), in which two ferric low-spin complexes ([A]: Fe(III)TPP($^-O_2$Sk)$_2$; [B]: Fe(III)TPP($^-$OCH$_3$)($^-O_2$Sk)) disappeared, but a new ESR signal (R3) consisting of a 1 : 3 : 3 : 1 quartet with three identical hyperfine splittings (A(CH$_3$) = 1.28 mT) was detected (Fig. 3e). This new ESR signal (R3) was assumed to be a free radical species due to 2,6-di-*tert*-butyl-4-methylphenoxyl as judged by the lineshape, *g*-value (g = 2.0), and hyperfine splitting constant.[27]

The effects of radical scavengers on the dioxygenation of Sk were examined with an alkyl- and alkylperoxy-radical scavenger BHT,[26] the singlet oxygen quencher DABCO,[28] and hydroxyl radical scavengers such as EtOH,[29] DMF, and DMSO. Concentration-dependent inhibition of Sk dioxygenation was observed when BHT was used (Fig. 5). The effects of radical scavengers or singlet oxygen quencher were evaluated by the concentration producing 50% inhibition of Sk dioxygenation (IC_{50} value) (Table 2). BHT was found to be the strongest radical scavenger (IC_{50} = 210 μM) among the compounds examined, suggesting that BHT scavenges both R1 and R2 radicals. The scavenging ability of the compounds for the Sk dioxygenation for 1 h reaction was found to be in the following order: BHT ≫ DMSO, DABCO, DMF > EtOH, indicating essentially no contribution of hydroxyl radicals or singlet oxygen in the reaction.

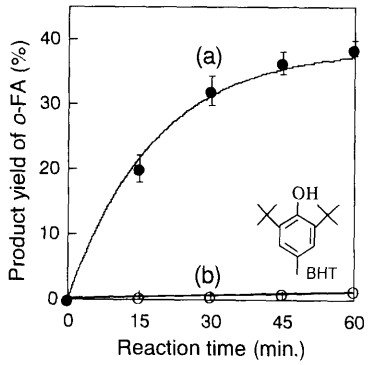

Fig. 4. Time-Dependent Dioxygenation of Sk in the TDO Model Reaction System

The reaction mixture was composed of 0.61 mM Fe(III)TPPCl (dissolved in toluene), 6.1 mM TMAOH (dissolved in methanol), 61 mM Sk (dissolved in toluene) and 0.61 mM BHT (dissolved in toluene), and it was stirred under oxygen atmosphere at 298 K. The mixing molar ratio of chemicals was as follows: Fe(III)TPPCl : TMAOH : Sk : BHT = 1 : 10 : 100 : 1. Total volume = 1.24 ml. (a) In the absence of BHT; (b) in the presence of BHT.

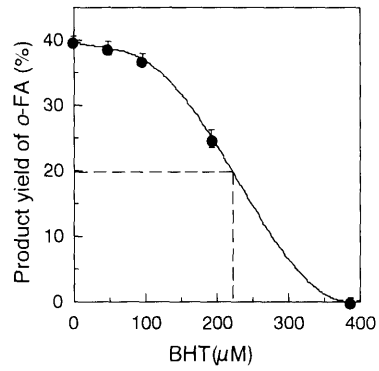

Fig. 5. Dose-Dependent Scavenging Activity of BHT on the Formation of *o*-FA

The reaction mixture was composed of 0.61 mM Fe(III)TPPCl (dissolved in toluene), 6.1 mM TMAOH (dissolved in methanol), 61 mM Sk (dissolved in toluene) and *x* mM BHT (dissolved in toluene), and it was stirred under oxygen atmosphere at 298 K. The mixing molar ratio of chemicals was Fe(III)TPPCl : TMAOH : Sk : BHT = 1 : 10 : 100 : *x*. Total volume = 1.28 ml. Data are the means ± standard deviations of 4 runs.

Fe(III)–Porphyrin Structure-Dependent Dioxygenation of Sk The dioxygenations of Sk in the system of 0.61 mM three types of Fe(III)–porphyrin, 6.1 mM TMAOH, and 61 mM Sk were examined in toluene solvent under 100% O_2 atmosphere at 298 K. As shown in Fig. 6, Sk-dioxygenation yields catalyzed by Fe(III)–porphyrin complexes for 1 h were found in the following order: Fe(III)TPP (38.6%) > Fe(III)TXP (22.2%) > Fe(III)TMP (5.20%). The first-order rate constants (k) for the substituted Fe(III)–porphyrins are summarized in Table 3.

Structure Analyses of Free Radicals (R1 and R2) Detected during the Reaction Cycle HMO calculation was carried out to determine the structure of R1. The radical species R1 is ascribable to the Sk neutral radical, in which an unpaired electron is localized highest at the 3-carbon atom of Sk and followed by nitrogen atom (Fig. 7). R2, on the other hand, is assignable to the Sk–peroxide radical by comparison of the *g*-values of R2 and *tert*-butylperoxyl radical (Figs. 8a, b) or cumene peroxyl radical (spectrum of cumene peroxyl radical not shown). This *g*-value (g = 2.015) is a typical parameter for the peroxyl radical,

Table 2. Effect of Free Radical Scavenger on Sk Dioxygenation in the Model Reaction

Antioxidant	IC_{50}	Molar ratio of (antioxidant/Fe(III)TPPCl) at IC_{50} value
BHT	210 μM	0.34
EtOH	>1.2 M	>2130
DMSO	190 mM	310
DMF	310 mM	500
DABCO	>62 mM	>100[a]

a) Saturated solution.

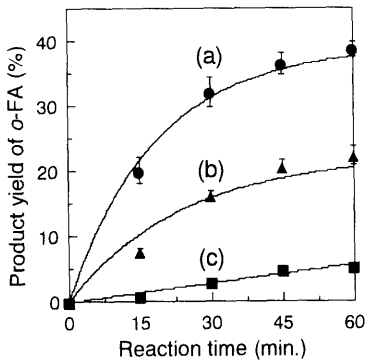

Fig. 6. Fe(III)–Porphyrin Structure- and Reaction Time-Dependent Dioxygenation of Sk in the TDO Model Reaction System

The reaction mixture was composed of 0.61 mM Fe(III)–porphyrin (dissolved in toluene), 6.1 mM TMAOH (dissolved in methanol), 61 mM Sk (dissolved in toluene), stirred under oxygen atmosphere at 298 K. The mixing molar ratio of chemicals was as follows: Fe(III)–porphyrin : TMAOH : Sk = 1 : 10 : 100. Total volume = 1.24 ml. (a) Fe(III)TPPCl; (b) Fe(III)TXPCl; (c) Fe(III)TMPCl.

Table 3. Substituent Effect on First-Order Rate Constant for Sk Dioxygenation in the Model Reaction

Fe(III)–porphyrin	10^3 k (min^{-1})
Fe(III)TPP	96
Fe(III)TXP	47
Fe(III)TMP	0.94

in which molecular dioxygen covalently binds at the tertiary carbon atom. In addition, when 49% enriched $^{17}O_2$ (I=5/2) was used in place of $^{16}O_2$, the linewidth (ΔHpp; peak-to-peak linewidth) of the signals due to R2 was slightly broadened (ΔHpp = 0.172 mT in $^{16}O_2$ and ΔHpp = 0.203 mT in $^{17}O_2$) (Fig. 8c).

Electrochemical Properties of Model Porphyrins Cyclic voltammetry was performed with the model porphyrins in CH_2Cl_2, and the obtained half-wave potentials expressed as $E(i_{pc}/2)$ were as follows: −580 mV for FeTPP, −760 mV for FeTXP, and −870 mV for FeTMP (Table 4). Such data were not obtained when toluene was used in place of CH_2Cl_2. Therefore, the half-wave potentials of iron-porphyrin complexes obtained in CH_2Cl_2 were used. The potentials of both reference electrodes Ag/Ag^+ and SCE were calibrated by the half-wave potential of ferrocene, and the following equation was obtained: $E(Ag/Ag^+) = E(SCE) - 248$ mV. The redox potential $E(i_{pc}/2)$ of the Fe(III)TPPCl was found to be analogous to the reported values.[30]

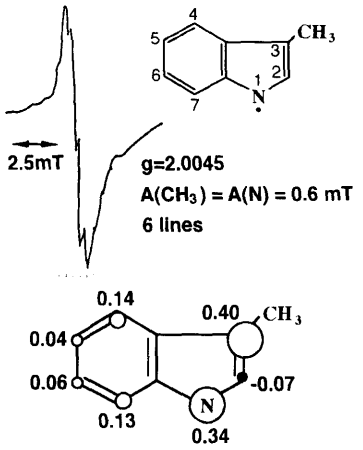

Fig. 7. ESR Signal Due to the Free Radical Species (Sk·) Detected at $g = 2.0045$ (R1) at 173 K and the Result by McLachlan HMO Calculation

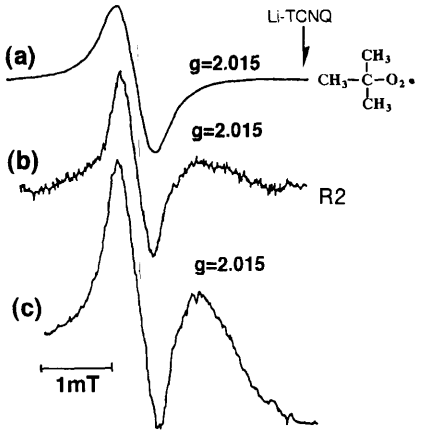

Fig. 8. ESR Signal of the Free Radical Species (SkO_2·) Detected at $g = 2.015$ (R2) at 173 K

(a) *tert*-Butylperoxide radical; (b) SkO_2· radical under $^{16}O_2$; (c) SkO_2· radical under $^{17}O_2$.

Table 4. Half-Wave Potentials of Central Metal Reduction of Fe(III)–Porphyrin Complexes

Complex	Solvent	Potential (mV)	Ref.
Fe(III)TPP	CH_2Cl_2	−580	c)
	CH_2Cl_2	−340[a] (−588)[b]	30
Fe(III)TXP	CH_2Cl_2	−760	c)
Fe(III)TMP	CH_2Cl_2	−870	c)

a) V *vs.* SCE. b) V *vs.* Ag/Ag^+. c) Present study.

Discussion

The system consisting of Fe(III)–porphyrin complexes, alkaline reagents, Sk, and molecular dioxygen was examined for the formations of the Sk neutral radical (R1), Sk peroxide radical (R2), and ternary complexes, as well as the production of *o*-FA, in order to elucidate the process by which Sk is dioxygenated. The results are summarized in Table 5. When Fe(III)TPPCl or Fe(III)TXPCl was used, all four formations were observed. But when Fe(III)-TMPCl was used in place of Fe(III)TPPCl or Fe(III)-TXPCl, no formations of radicals, ternary complexes or

Table 5. Reaction Conditions in TDO Model Systems

Porphyrin	Base	Oxidant	Inhibitor	R1[a]	R2[b]	Ternary complex	Product (GLC)
Fe(III)TPP	TMAOH	O_2	None	Detected	Detected	Detected	*o*-FA
Fe(III)TXP	TMAOH	O_2	BHT	—	—	—	—
	NaOMe	O_2	None	Detected	Detected	Detected	*o*-FA
	NaOMe	O_2	BHT	—	—	—	—
	NaOEt	O_2	None	Detected	Detected	Detected	*o*-FA
	NaOPro	O_2	None	Detected	Detected	Detected	*o*-FA
Fe(III)TMP	TMAOH	O_2	None	—	—	—	—
	NaOMe	O_2	None	—	—	—	—
	NaOEt	O_2	None	—	—	—	—
	NaOPro	O_2	None	—	—	—	—
None	None	PbO_2	None	—	—	—	—
	TMAOH	ROOH	None	—	—	—	—
	TMAOH	NiPo	None	—	—	—	—

a) Free radical detected at $g=2.0045$. *b*) Free radical detected at $g=2.015$.

o-FA were seen, probably due to the steric hindrance of the substituents on the porphyrin ring. In the systems containing oxidants such as PbO_2, ROOH, and nickel peroxide (NiPo), neither intermediates nor *o*-FA were produced, indicating that the formations of the Sk neutral radical (R1) and ternary complexes are indispensable for the dioxygenation of Sk. Interaction between substituent groups of porphyrin ring and the substrate, probably due to the stacking effect between them, influenced the Sk-dioxygenation by preventing the approach of both O_2 and substrate at the active site of the heme. The relative Sk-dioxygenation yield catalyzed by Fe(III)–porphyrin complexes for 1 h was Fe(III)TPP > Fe(III)TXP > Fe(III)-TMP (Fig. 6 and Table 3), which corresponds well with their redox-potentials, Fe(III)TPP (-580 mV) > Fe(III)-TXP (-760 mV) > Fe(III)TMP (-870 mV) (Table 4). The catalytic activity for dioxygenation of the Fe(III)–porphyrin has been found to depend on both its reducibility and the steric effect of the porphyrin, in which the dioxygenation is more rapidly promoted by the Fe(III)–porphyrin complex presenting the least steric structure and possessing the highest redox-potential. On the basis of these observations, a possible reaction mechanism for the dioxygenation process of Sk is proposed as depicted in Chart 1.

Addition of TMAOH to Fe(III)TPPCl results in the formations of two ferric complexes ([C]: Fe(III)TPP-$(HOCH_3)(^-OCH_3)$ and [D]: Fe(III)TPP$(^-OCH_3)_2$), which are very stable under the aerobic conditions described earlier.[31] Since the dioxygenation of Sk did not proceed without an alkaline reagent, the formations of the six-coordinate complexes can be considered essential. In the six-coordinate Fe(III)TPP complexes ([C], [D]), strong donors such as methoxide anion occupy axial positions of the heme. A Fe(III)TPP$(piperidine)_2$ complex, which has strong donors in the axial positions, is known to be autoreduced to the corresponding ferrous low-spin complex, as estimated by NMR and ESR.[32] In addition, the Fe(III)TPP$(^-OCH_3)_2$ complex [D] has been reported to be autoreduced to the Fe(II)TPP species in DMSO containing methanol.[31] From these observations, we suggest that reduction would proceed in the present complexes ([C], [D]). A Sk anion formed by deprotonation of Sk with methoxide anion acts as an electron donor. The one electron transfer from the Sk anion to the Fe(III)TPP complexes results in the formations of both R1 and Fe(II)TPP complexes ([E] in Chart 1). In fact, R1 was detected by ESR measurements (Fig. 3b), in which this radical species was assumed to be a precursor at the initial dioxygenation step. When [E] reacts with O_2, the complex [F] will be formed, which in turn produces $SkO_2\cdot$ as a form of the complex [G]. In the system of the present study, R2 was observed with introduction of a large amount of O_2 (Fig. 3c). R2 is thus assumed to be formed by binding of R1 and O_2.

The generation of the free superoxide (O_2^-) in the reaction of the six-coordinate bis(thiolate)–Fe(III)TPP complex with molecular dioxygen was previously observed.[33] However, in the present system, the free superoxide was not detected by the direct ESR measurements at 77 K. In addition, superoxide generated from KO_2 was indicated that O_2^- did not react with Sk, and thus the dioxygenation product of Sk was not detected by GLC (data not shown). Judging from these results, superoxide is not likely to be generated in the present system. After an electron transfer from Fe(II) to $SkO_2\cdot$, the resultant SkO_2^- coordinates to the Fe(III) and the ternary complex [B] will be formed. [A] will be partially formed by the binding of SkO_2^- at both axial positions of porphyrin. We reported before that Fe(III)TPP–alkyl peroxide complex has a tendency to be reduced to ferrous state under aerobic conditions by heterolytic cleavage of iron—oxygen bond.[34] It can similarly be presumed that both [A] and [B] are reduced to ferrous state ([H]). When dioxygen is present in the system, the forms ([C], [D]) are re-formed by binding of the methoxide anion ($^-OCH_3$) or methanol, which is abundantly present in the system.

In conclusion, O_2 is incorporated into Sk through the formation of ternary complexes [A] and [B]. Since the present ternary complexes and free radical species formed at the initial step of dioxygenation are very unstable and their detections are difficult in the native enzyme system, this proposed reaction mechanism might provide the tool with which the reaction mechanism of the dioxygenation process in the enzyme can be understood. The ternary complexes are key intermediates to determine the reactivity of hemoproteins, such as di-oxygenases. Further investigations are under way.

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