Catalytic and Spectroscopic Properties of Imidazole(N)–Fe(III)porphyrin Complexes in Dioxygenation of Tryptophan Derivatives

Shigenori OKA,[a,1)] Yuji KITAMURA,[a] Masakazu NAGASAWA,[b] Minoru KASHIMOTO,[b] Kunihiko TAJIMA,[c] and Hiromu SAKURAI*,[a]

Department of Analytical and Bioinorganic Chemistry, Kyoto Pharmaceutical University,[a] Yamashina-ku, Kyoto 607–8414, Japan, Formulation Research Institute, Otsuka Pharmaceutical Co.,[b] Kawauchi-cho, Tokushima 771–0132, Japan, and Department of Polymer Science and Engineering, Kyoto Institute of Technology,[c] Sakyo-ku, Kyoto 606–0962, Japan.
Received April 16, 1998; accepted June 4, 1998

It has been proposed that a heme–peroxide complex is formed as an intermediate state in the enzymatic reaction. Since the axial position of the heme in tryptophan 2,3-dioxygenase (TDO) is occupied with a nitrogenous ligand from histidine residue, we studied the heme–peroxide complexes with an axial nitrogenous ligand at the fifth position in a chemical model for TDO. Addition of *N*-methyl-imidazole to a ferric high-spin porphyrin exhibited ESR spectrum due to a formation of ferric low-spin complex (complex A). Following addition of tetramethylammonium hydroxide to the reaction mixture, the signal due to a different ferric low-spin complex (complex B) appeared. When 3-methylindole (skatole; Sk) was added to the resulting solution followed by introduction of dry oxygen gas, a new ESR signal due to a ferric low-spin complex (complex C) was detectable. A similar ESR signal was observed when *N*-acetyl-tryptophan ethyl ester was used in place of Sk (complex D). Bohan plotting of the crystal-field parameters as a rhombicity–tetragonality diagram obtained from ESR *g*-values for the complexes (A, B, C, D) revealed that these heme iron complexes can be classified into three groups having N–Fe–N (complex A), N–Fe–O (complex B), and N–Fe–OO (complexes C, D) coordinations. Product analyses for the same reaction systems demonstrated the conversion of each substrate to the dioxygenated product.

Key words tryptophan 2,3-dioxygenase; chemical model; ESR; ternary complex ; dioxygenation mechanism

Tryptophan 2,3-dioxygenase (TDO) catalyzes the oxidative ring cleavage of tryptophan to formylkynurenin at the heme iron site.[2)] The reaction has been proposed to proceed by addition of O_2 across the 2,3-bond of the indole ring, in which the formation of an intermediate state, $EMSO_2$* (where EM is the resting form of TDO, S is the substrate tryptophan molecule, and O_2 is molecular oxygen) ternary complex has been proposed.[3)] The resting state (EM) of TDO with a ferrous heme group forms an EM–substrate (EMS) complex when the substrate molecule binds to the reaction site, which follows capture of O_2 at the axial position of heme to form an $EMSO_2$ complex. Then, O_2 is activated and inserted into the substrate molecule through the transient intermediate $EMSO_2$* complex.[3,4)] Both TDO and indoleamine 2,3-dioxygenase (IDO)[5)] catalyze the dioxygenation of L-tryptophan and its analogs. However, there are a few reports of model systems for evaluating the detailed reaction mechanisms, for instance, O_2 insertion to 3-substituted indoles to generate ring-opening products was examined with the (tetraphenylporphyrinato)irons, Fe(II)TPP[6)] and Fe(III)TPPCl,[7)] (tetraphenylporphyrinato)cobalt (Co(II)TPP),[8)] [bis(salicylidene)ethylenediaminonato]cobalt (Co(II)salen),[9)] manganese phthalocyanine (Mn(II)PC),[10)] or manganese chiral porphyrin.[11)] Previous studies on TDO and IDO have suggested that the substrate binds to the reaction site of the enzymes to form an enzyme–O_2–substrate ternary complex ($EMSO_2$*), which is an intermediate during catalytic cycle of the enzymes.[4,12)] However, the structures of the intermediate remain ambiguous due to lack of clear evidence on crystal structures of the enzymes; in contrast, those for cytochrome P450 (P450),[13)] catalase[14)] and peroxidases[15)] in their resting or substrate binding states have already been determined.

In O_2 or peroxide activations by P450,[16)] peroxidases[17)] and catalase,[18)] a heterolytic O–O bond cleavage in hydroperoxoiron(III) porphyrin complexes has been proposed to afford an oxoferryl porphyrin cation radical intermediate.[19)] A strong electron donation nature such as thiolate ligand in P450 is expected to destabilize the O–O bond and to facilitate its heterolytic cleavage to give an Fe(IV)=O porphyrin π-cation radical (compound I).[19)] However, lacking a strong donor, the ratio of the homolytic cleavage over heterolysis is increased to give a less reactive Fe(IV)=O porphyrin intermediate (compound II).

A possible model system of dioxygenation system composed of Fe(III)TPPCl, tetramethylammoniumhydroxide (TMAOH), 3-methylindole (skatole; Sk), and O_2 has been proposed.[7)] The product analysis demonstrated that Sk is effectively converted to *o*-formamidoacetophenone (*o*-FA), in which two atoms of molecular oxygen are incorporated in Sk. In addition, Fe(III)TPP–O_2–Sk ternary complex as a reactive ternary complex ($EMSO_2$*) and two kinds of free radical species (Sk neutral radical and Sk peroxide radical) were detectable in the reaction cycle.[20)] Since a native TDO has been proposed to have a protoporphyrin IX–iron complex (FePPIX) at the active site and a nitrogenous ligand from histidine residue at the fifth position,[3)] similar to those of hemoglobin, myoglobin, cytochromes, and some peroxidases,[21)] we expanded our work to construct a new biomimetic model of dioxygenase. We report here the detection and characterization of the heme–O_2–substrate (3-methylindole and *N*-acetyl-tryptophan ethyl ester) ternary complex ($EMSO_2$*) with an axial nitrogenous ligand at the fifth position of the FePPIX derivative as well as substrate dioxygenation activity, proposing a possible reactive intermediate of dioxygenase.

Experimental

Materials Iron(III)protoporphyrin dimethyl ester (Fe(III)PPIXDME) was prepared and purified as reported.[22)] TMAOH (10% solution in methanol) and *N*-acetyl-tryptophan ethyl ester (N-Ac-TrpE) purchased from

* To whom correspondence should be addressed.

Nacalai Tesque (Kyoto, Japan) were used without further purification. 3-Methylindole (Sk) was obtained from Wako Pure Chemicals (Osaka) and was used after recrystallization from toluene. 2,6-Di-*tert*-butyl-*p*-cresol (butylated hydroxytoluene; BHT) was of special reagent grade from Wako Pure Chemicals. *N*-Methylimidazole (N-MeIm) was obtained from Tokyo Kasei Kogyo (Tokyo). Toluene and methanol (Wako Pure Chemicals) were distilled before use.

Spectral Measurements Visible absorption spectra were recorded on a spectrophotometer MCPD-1000, Ohtsuka Electronic Co. (Tokyo) at 77 K with an ESR quartz tube (5 mm diameter). ESR spectra were measured with an ESR spectrometer RE-3X, JEOL (Tokyo), with 100 kHz field modulation. The microwave frequency applied to the sample at the power of 5.0 mW was monitored by digital frequency counter, Advantest R5372 (Tokyo). The magnetic field 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 77 K.

Preparation of Fe(III)PPIXDME(N-MeIm)($^{-}O_2R$) Ternary Complex Fe(III)PPIXDME (N-MeIm)($^{-}O_2R$) as a ternary complex was prepared in an ESR quartz tube at 195 K (dry ice–acetone bath). The sample solution was composed of Fe(III)PPIXDMECl (1 mM in toluene, 0.4 ml), N-MeIm (0.4 M in toluene, 0.02 ml), TMAOH (0.02 M in methanol, 0.02 ml), and Sk (0.2 M in toluene, 0.2 ml) or N-Ac-TrpE (0.2 M in methanol, 0.2 ml). A 100% oxygen gas was slowly introduced into the reaction mixture through a fine capillary at 195 K.[7,20] Instrument conditions for the measurements were as follows: magnetic field, 270±250 or 300±50 mT; modulation frequency, 0.63 mT; modulation amplitude, 100 kHz; output power, 5 mW; time constant, 0.1 s; sweep time, 4 min.

Product Analysis in the Reaction Mixture Sk (0.2 M in toluene, 0.4 ml) or N-Ac-TrpE (0.2 M in methanol, 0.4 ml) was added to a mixture (total volume, 1.28 ml) of Fe(III)PPIXDMECl (1.0 mM in toluene, 0.8 ml), N-MeIm (0.4 M in toluene, 0.04 ml) and TMAOH (0.02 M in methanol, 0.04 ml) to start the reaction by stirring under 100% O_2 atmosphere at 298 K. *o*-FA,[7,9,20] which is an oxygenated product of Sk, was determined by GLC method (Shimadzu GC-6AM (Kyoto), silicon OV-17 column), and the reaction was periodically monitored. The dioxygenation products of N-Ac-TrpE were determined by HPLC (column: COSMOSIL C18, detection: UV 280 nm) eluted with 40% (v/v) methanol–acetic acid.

Structure Determination of Dioxygenated Products from N-Ac-TrpE The dioxygenated products from N-Ac-TrpE were separated by a thin layer chromatoplate Silica gel 60 F_{254}, TLC aluminum sheet (Merck, U.S.A.) using a developing solvent, CH_3Cl : CH_3OH : CH_3COOH=75 : 20 : 5. The reaction mixture composed of Fe(III)TPPCl (1.0 mM in toluene, 0.8 ml), TMAOH (0.02 M in methanol, 0.04 ml), and N-Ac-TrpE (0.2 M in methanol, 0.4 ml) under O_2 gave three main products, their *Rf* values being 0.486, 0.852, and 0.876. The compound at *Rf*=0.852 was identified as N-Ac-TrpE by the control experiment. Other products were recovered from the TLC plates by extraction with ethyl acetate and the structures were determined by both ^{1}H-NMR and FAB-MS spectrum. The major product at *Rf*=0.876 was assigned to ethyl 2-acetoamido-3-(2-formamidobenzoyl)propionate (EAFP) by ^{1}H-NMR spectra recorded on a JEOL JNM-A500 spectrometer (Tokyo) with tetramethylsilane as an internal standard and FAB-MS spectra measured with a JEOL JMS-SX102A spectrometer (Tokyo). The purified EAFP separated from the model system was used as a standard for evaluation of the dioxygenation. The obtained NMR data for EAFP were as follows. ^{1}H-NMR (DMSO-d_6) δ 1.16 (t, 3H, J=7.1 Hz, CH_2CH_3), 1.83 (s, 3H, $COCH_3$), 3.48 (dd, 1H, J=17.9, 7.0 Hz, $COCH_2$), 3.52 (dd, 1H, J=17.9, 5.8 Hz, $COCH_2$), 4.08 (q, 2H, J=7.1 Hz, CH_2CH_3), 4.74 (ddd, 1H, J=7.0, 7.0, 5.8 Hz, $COCH_2CH$), 7.25 (ddd, 1H, J=8.0, 8.0, 1.0 Hz, ArH), 7.62 (ddd, 1H, J=8.0, 8.0, 1.4 Hz, ArH), 8.03 (dd, 1H, J=8.0, 1.4 Hz, ArH), 8.27 (br d, 1H, J=7.0 Hz, $NHCOCH_3$), 8.44 (br d, 1H, J=8.0 Hz, ArH), 8.46 (br s, 1H, CHO), 11.05 (br s, 1H, Ar-NH). The structure of a minor product at *Rf*=0.486 is unknown. When Fe(III)TPPCl was lacking in this system, a minor product was formed, suggesting decomposition of the substrate in the presence of TMAOH.

Results

Detection of Unstable Intermediate Species in the Ferric Low-Spin State during Dioxygenation of Sk a) ESR Spectra: Dioxygenation processes of Sk occurring in the reaction system were examined by ESR measurements. On addition of N-MeIm (0.4 M in toluene, 0.02 ml) to Fe(III)-PPIXDMECl (1 mM in toluene, 0.4 ml) at the ferric high-spin state ($g_\perp$=6 and $g_{//}$=2, data not shown) unaerobically, the ESR spectrum at 77 K exhibited the formation of a ferric low-spin complex (complex A: g_1=2.93, g_2=2.25, g_3=1.51) without a ferric high-spin complex (Fig. 1a). When TMAOH (0.02 M in methanol, 0.02 ml) was added to the resulting solution, a new ESR spectrum due to a ferric low-spin complex (complex B: g_1=2.59, g_2=2.20, g_3=1.86) was observed (Fig. 1b). The reaction mixture was annealed at 195 K, then Sk (0.2 M in toluene, 0.2 ml) was added to the reaction mixture; following slow introduction of dry O_2 gas through a fine capillary into the reaction mixture, the color of the solution gradually turned to bright red from dark red after the induction period of 3 min. The reaction mixture was frozen and the ESR spectrum was recorded at 77 K. As shown in Fig. 1c, ESR signal due to the ferric low-spin complex (complex C: g_1=2.320, g_2=2.193, g_3=1.939) was observed with disappearance of the signal due to complex B.

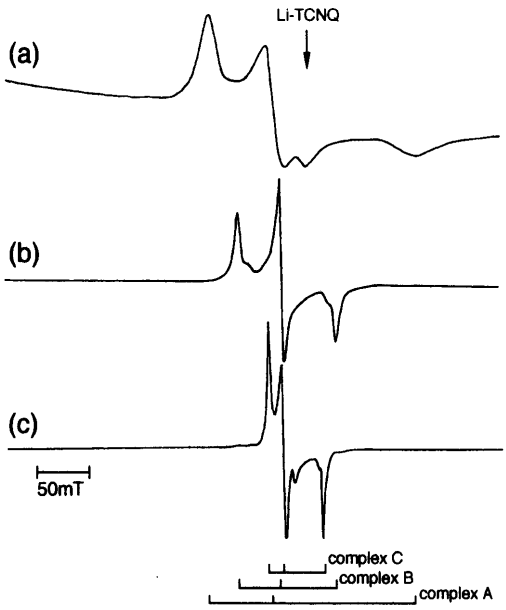

Fig. 1. ESR Spectra of TDO Model System at 77 K

The reaction mixture was composed of 0.62 mM Fe(III)PPIXDMECl (dissolved in toluene), 12.5 mM N-MeIm (dissolved in toluene), 0.62 mM TMAOH (dissolved in methanol) and 62 mM Sk (dissolved in toluene). The mixing molar ratio of chemicals was Fe(III)PPIXDMECl : N-MeIm : TMAOH : Sk=1 : 20 : 1 : 100, and the solvent ratio was toluene : methanol=31 : 1. Total volume=0.64 ml. (a) Fe(III)PPIXDMECl+N-MeIm; (b) (a)+TMAOH; (c) (b)+Sk+O_2.

b) Visible Absorption Spectra: Visible absorption spectra at 77 K for the same solution of ESR experiments composed of 0.61 mM Fe(III)PPIXDMECl, 12.2 mM N-MeIm, 0.61 mM TMAOH and 61 mM Sk were measured, the obtained absorption maxima being as follows: 409, 522, 550 (sh) nm for complex A, 408, 532, 565 nm for complex B, and 412, 535, 564 nm for complex C (Fig. 2).

Dioxygenation of Sk in the Model System and Effect of a Free Radical Scavenger Dioxygenation of Sk in the system of 0.61 mM Fe(III)PPIXDMECl, 12.2 mM N-MeIm, 0.61 mM TMAOH and 61 mM Sk (complete system I) was examined in toluene solvent under 100% O_2 atmosphere at 298 K. As shown in Fig. 3a, a time-dependent dioxygenation was observed. The product yield of *o*-FA on the basis of the substrate concentration in the complete system I was about 9% after 60 min of the reaction. However, no *o*-FA was detectable when Fe(III)PPIXDMECl was absent from the com-

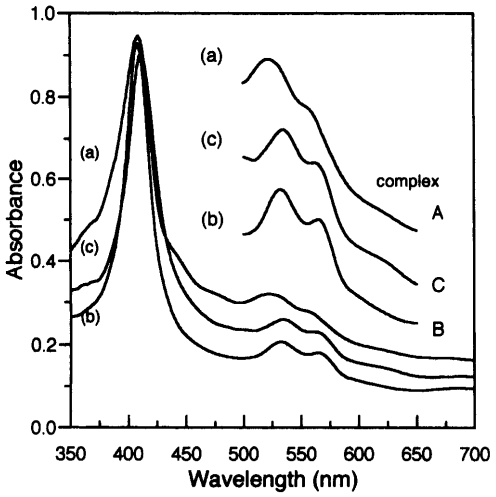

Fig. 2. Visible Absorption Spectra of TDO Model System at 77 K

Experimental conditions were the same as for Fig. 1. (a) Fe(III)PPIXDMECl+N-MeIm; (b) (a)+TMAOH; (c) (b)+Sk+O_2.

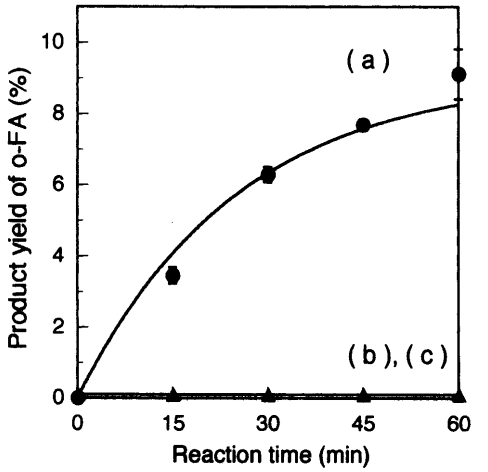

Fig. 3. Time-Dependent Dioxygenation of Sk in the TDO Model Reaction System

The reaction mixture contained 0.61 mM Fe(III)PPIXDMECl (dissolved in toluene), 12.2 mM N-MeIm (dissolved in toluene), 0.61 mM TMAOH (dissolved in methanol), 61 mM Sk (dissolved in toluene) and 0.61 mM BHT (dissolved in toluene). The mixing molar ratio of chemicals was Fe(III)PPIXDMECl : N-MeIm : TMAOH : Sk : BHT = 1 : 20 : 1 : 100 : 1, and the solvent ratio was toluene : methanol = 32 : 1. Total volume = 1.32 ml. The first-order kinetics was used.[20] (a) Fe(III)PPIXDMECl+N-MeIm+TMAOH+Sk+O_2 (complete system I); (b) complete system I−Fe(III)PPIXDMECl; (c) complete system I+BHT.

plete system I (Fig. 3b). Addition of a radical scavenger, BHT (0.61 mM) to the complete system I resulted in complete inhibition of the Sk dioxygenation (Fig. 3c).

Detection of Unstable Intermediate Fe(III)PPIXDME-(N-MeIm) ($^-O_2$N-Ac-TrpE) Ternary Complex by Visible Absorption and ESR Measurements at 77 K Visible absorption and ESR spectrometries at 77 K were used to know the dioxygenation processes of N-Ac-TrpE in the reaction system. An anaerobic reaction mixture composed of 0.61 mM Fe(III)PPIXDMECl, 12.2 mM N-MeIm, 0.61 mM TMAOH and 61 mM N-Ac-TrpE showed formation of a ferric low-spin complex (g_1=2.59, g_2=2.20, g_3=1.86 and 408, 532, 565 nm for absorption maxima). When these spectroscopic parameters were compared with those of the complex B described earlier, the observed low-spin complex was assignable as analogous to the complex B. When sufficient dry O_2 gas was introduced into the mixture at 195 K, an ESR signal due to

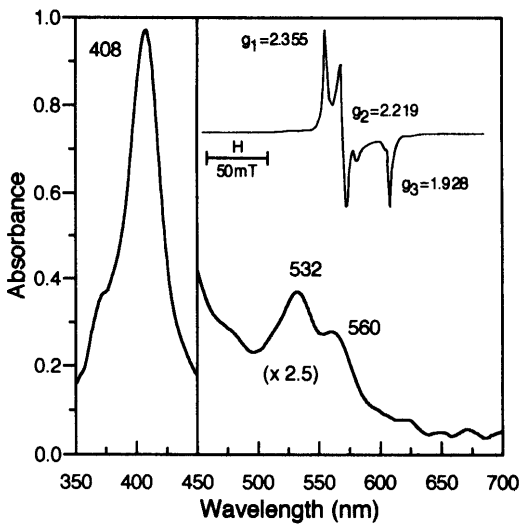

Fig. 4. Visible Absorption Spectrum of TDO Model System at 77 K

Experimental conditions were as for Fig. 1, except the solvent ratio, toluene : methanol = 22 : 11 and total volume, 0.64 ml. N-Ac-TrpE was used in the place of Sk. Inset: ESR spectrum at 77 K.

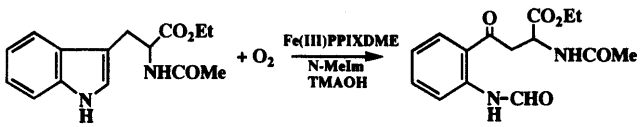

Chart 1. The Formulated Overall Reaction Profile in the TDO Chemical Model System Consisting of Fe(III)PPIXDMECl, N-MeIm, TMAOH, and N-Ac-TrpE under O_2

Table 1. Conversion on Sk or N-Ac-TrpE to the Respective Oxygenated Product (*o*-FA or EAFP) in the Model System

System	Conversion (%)
Complete system I[a]	9
Complete system I−Fe(III)PPIXDMECl	0
Complete system I+BHT	0
Complete system II[b]	1.2
Complete system II−Fe(III)PPIXDMECl	0
Complete system II+BHT	0

a) Fe(III)PPIXDME+N-MeIm+TMAOH+Sk, 1 h. *b*) Fe(III)PPIXDME+N-MeIm+TMAOH+N-ATrpE, 5 h

formation of a new ferric low-spin complex (complex D: g_1=2.355, g_2=2.219 and g_3=1.928) was observed (Fig. 4, inset). Visible absorption spectrum of complex D at 77 K showed absorption maxima at 408, 532, and 560 nm (Fig. 4). There was no spectral change of complex B measured by optical absorption and ESR when Sk or N-Ac-TrpE or dry O_2 gas was added to the complex B (data not shown).

Dioxygenation of N-Ac-TrpE in the Model System and Effect of a Free Radical Scavenger Dioxygenation of N-Ac-TrpE in the system consisting of 0.61 mM Fe(III)PPIXDMECl, 12.2 mM N-MeIm, 0.61 mM TMAOH and 61 mM N-Ac-TrpE (complete system II) was examined under 100% O_2 atmosphere at 298 K. A major product from N-Ac-TrpE was assigned as EAFP by HPLC analyses (Chart 1). Conversion of N-Ac-TrpE to the dioxygenated product EAFP was found to be 1.2% for 5 h (Table 1). Formation of other minor oxygenated products such as ethyl 2-acetoamido-3-(2-aminobenzoyl)propionate, ethyl 2-acetoamido-3-(2-isocyanobenzoyl)-propionate, and ring none-opening products have been re-

Table 2. ESR Parameters of Six-Coordinate Ferric Low-Spin Porphyrin Complexes

Complex	g_1	g_2	g_3	(R/μ)	(μ/λ)	Label	Ref.
N–Fe–N							
Fe(III)TPP(ImH)$_2$	2.92	2.30	1.56	0.65	3.2	1	28
Fe(III)TPP(Im) (ImH)	2.73	2.28	1.74	0.65	4.0	2	28
Complex A	2.93	2.25	1.51	0.56	3.3	3	a)
N–Fe–O							
Fe(III)PPIXDBE(py)(OAr)	2.61	2.19	1.84	0.52	6.2	4	26
Fe(III)PPIXDBE(OAr)(N-MeIm)	2.56	2.21	1.85	0.61	5.8	5	26
Fe(III)(basket-handle Porphyrin)($^-$OCR)(1 MeIm)	2.65	2.21	1.83	0.56	5.6	6	29
Complex B	2.59	2.20	1.86	0.55	6.2	7	a)
O–Fe–OOR							
Fe(III)TPP($^-$OMe)($^-$O$_2$ Bu)	2.316	2.157	1.952	0.59	9.0	8	30
Fe(III)TPP($^-$OMe)($^-$O$_2$ Sk)	2.311	2.160	1.954	0.57	9.1	9	20
Fe–(OOR)$_2$							
Fe(III)TPP($^-$O$_2$ Bu)$_2$	2.242	2.157	1.964	0.38	10.1	10	30
Fe(III)TPP($^-$O$_2$ Sk)$_2$	2.236	2.160	1.965	0.34	10.2	11	20
N–Fe–OOR							
Fe(III)Mb($^-$O$_2$ Bu)	2.350	2.196	1.936	0.49	7.6	12	31
Fe(III)Hb($^-$O$_2$ Bu)	2.340	2.188	1.940	0.50	7.9	13	31
Fe(III)TPP(Im)($^-$O$_2$ Bu)	2.324	2.185	1.940	0.47	8.1	14	31
Fe(III)TPP(4-MeIm)($^-$O$_2$ Bu)	2.323	2.183	1.943	0.48	8.1	15	31
Complex C	2.320	2.193	1.939	0.43	7.9	16	a)
Complex D	2.355	2.219	1.928	0.41	7.1	17	a)

Bu: *tert*-butyl. *a*) Present study.

ported.[11b] Since the possible products were found to be within trace amounts in our model system, the other products were not determined.

Discussion

Coordination Structures of Ferric Low-Spin Porphyrin Complexes in Dioxygenation Cycle ESR spectroscopy has been applied for many years as an useful tool to characterize the coordination structure of paramagnetic heme-iron complexes.[16e,23] Both ESR parameters and line-shape provide valuable information for identifying the axial ligands of the heme. In fact, several chemical model complexes with imidazole as an axial ligand of heme have been proposed to explain the coordination structure and reactivity of enzymes.[24–32] Comparison of ESR *g*-values of the ferric low-spin complexes formed in the dioxygenation cycles (complexes A, B, C, D) with those of previously reported complexes[24–32] shows that the ESR features of complexes C and D are characterized by their anomalously small *g*-anisotropy. As shown in Fig. 1a, the ESR spectrum observed for the system of Fe(III)PPIXDMECl and N-MeIm exhibited a formation of ferric complex in the low-spin state (complex A: g_1=2.93, g_2=2.25, g_3=1.51). A similar ESR spectrum was recorded in the system containing Fe(III)TPPCl in place of Fe(III)PPIXDMECl, as summarized in Table 2. An analogous ESR spectrum was reported by Ozaki and Yoshimura[27] and Quinn *et al.*[28] The ferric low-spin complex A is thus assignable to the six-coordinate complex, Fe(III)PPIXDME(N-MeIm)$_2$. Addition of TMAOH to the complex A resulted in formation of the ferric low-spin complex B (g_1=2.59, g_2=2.20, g_3=1.86) (Fig. 1b). Schaeffer *et al.* reported the observation of ESR spectra for the basket-handle porphyrin derivatives, in which O–Fe–N coordination mode was tightly locked at both axial positions of the iron chromophore.[29] Comparison of the ESR parameters with those of related complexes (Table 2) indicates that the complex B is assigned as the ferric low-spin six-coordinate Fe(III)PPIXDME(N-MeIm)($^-$OMe). As shown in Fig. 1c as well as Table 2, the ESR spectrum for complex C (g_1=2.320, g_2=2.193, g_3=1.939) with anomalously small *g*-anisotropy is assignable due to the formation of Fe(III)porphyrin–peroxide complex. We reported earlier[7] that the reaction of Fe(III)TPPCl, TMAOH, Sk, and O_2 resulted in the formation of Fe(III)TPP–O_2–Sk (Fe(III)TPP($^-$O$_2$Sk)) in the low-spin state which lacked the axial nitrogenous ligand. Being different from the results,[7,31] the coordination structure of the new complex C is proposed to be the six-coordinate Fe(III)-PPIXDME–peroxide with an axial nitrogenous ligand as Fe(III)PPIXDME(N-MeIm)($^-$O$_2$Sk).

Crystal-field analysis of ESR *g*-values for six-coordinate ferric low-spin porphyrin complexes have been developed by Griffith[33] and Kotani.[34] Then Blumberg and Peisach[24] followed by Bohan,[35] and Sakurai and Yoshimura[16e] proposed use of the two crystal-field parameters of rhombicity (R/μ) and tetragonality (μ/λ), calculated from *g*-values for various coordination types of ferric low-spin hemoproteins and their model complexes.[35] The crystal-field parameters of the present complexes were calculated according to Bohan's treatment.[35] The parameters for complexes A, B, and C are summarized in Table 2 together with those previously reported model heme complexes.[20,26,28–31] Using the crystal-field parameters for these complexes, a rhombicity–tetragonality diagram was plotted in Fig. 5. The heme complexes are classified into five axial coordination groups having N–Fe–N, N–Fe–O, O–Fe–OOR, Fe–(OOR)$_2$, and N–Fe–OOR. Each group is well separated to discriminate it in the diagram. The $|\mu/\lambda|$ value shows a stepwise increase with increase in the number of oxygenous donors ligating at the axial positions. In fact, the estimated $|\mu/\lambda|$ value of complex C (7.9 in N–Fe–OOR coordination mode, No.16) is the largest among the three complexes A (3.3 in N–Fe–N, No.3), B (6.2 in N–Fe–O, No. 7), and C, indicating that peroxide anion causes a strong axial perturbation compared with those by other ligands. In addition, $|\mu/\lambda|$ and $|R/\mu|$ values of complex C are

consistent with those of *met*-myoglobin (Fe(III)Mb)- and -hemoglobin (Fe(III)Hb)–peroxide complexes,[31] in which a nitrogenous donor from the histidine residue is retained at the axial position of the heme (Fig. 5). Coordination structures were also analyzed by visible absorption spectra for the complexes A, B, and C at 77 K. As summarized in Table 3, the optical parameters for these complexes are found to be slightly dependent on their coordination modes. Complex A exhibits the Soret absorption maxima at 409 nm with β- and α-maxima at 522 and 550 (sh) nm, respectively, while complex B does so at 408, 532, and 565 nm, and complex C at 412, 535, and 564 nm, the shift of β-band appearing to depend on the type of oxygen ligand, whether oxyanion or peroxyanion. The α- and β-absorption maxima for complexes B and C were very close, probably due to their similar axial perturbation from ligand for absorption. Since a dioxygenated product (*o*-FA) from Sk is periodically formed in complete system I (Fig. 3, Table 1), O_2 is incorporated into Sk through the complex formation. From the spectroscopic parameters as well as the product analysis, the structure of complex C was concluded to be imidazole(N)–Fe(III)-PPIXDME–O_2–substrate.

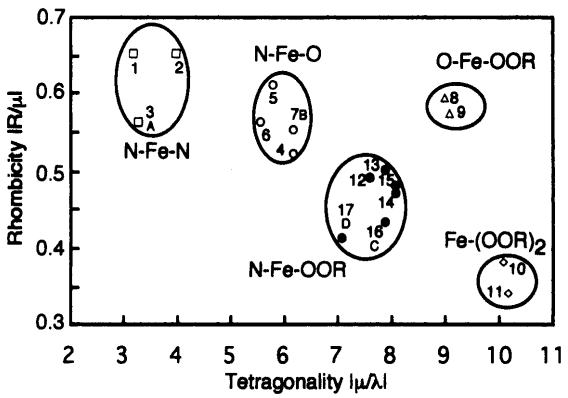

Fig. 5. Plot of Rhombicity $|R/\mu|$ *vs.* Tetragonality $|\mu/\lambda|$ Parameters for Six-Coordinate Ferric Low-Spin Porphyrin Complexes

Labels are as in Table 2.

On the basis of these results, we extended our work to use a tryptophan derivative as a substrate, as shown in Fig. 4 and Tables 1, 2 and 3. The ESR and visible absorption parameters of complex D indicate that Fe(III)PPIXDME(N-MeIm)-($^-O_2$ N-Ac-TrpE) is formed in the dioxygenation cycle. The coordination structure of complex D was also supported by the $|\mu/\lambda|$ and $|R/\mu|$ parameters (0.41 and 7.1, No.17 in Fig. 5) as calculated from *g*-values of the ESR spectrum (g_1= 2.355, g_2=2.219 and g_3=1.928) (Table 2). N-Ac-TrpE was found to be converted to EAFP (Table 1), during which the imidazole(N)–Fe(III)PPIXDME–O_2–substrate complex was formed. From the present results together with the previous data,[7,30,32] the coordination structure of complexes C and D as a ternary complex ($EMSO_2$*) is illustrated in Fig. 6. Complexes C and D can be expressed as Fe(III)PPIXDME–O_2–substrate ternary complex with an axial nitrogenous ligand at the fifth position, in which the central iron and substrate molecule are bridged by O_2. It is possible that O_2 is incorporated into substrate through the formation of such a ternary complex in native enzyme. The electronic structures of ternary complexes C and D are still controversial, and further investigations are now in progress.

Dioxygenation Mechanism In our efforts to determine the coordination structure and reactivity of intermediate species in the biomimetic model system for dioxygenase, the dioxygenation of substrate was found to proceed when a ternary complex such as complex C or D is detectable in the reaction cycle. The formation of complex C or D is indispensable for the dioxygenation of substrate.[20] We reported earlier that Fe(III)porphyrin–alkyl peroxide complex with the character of being reduced to a ferrous state under aerobic conditions catalyzes the heterolytic cleavage of iron–oxygen bond.[36] Based on these findings it is presumed that complexes C and D are reduced to the ferrous state, and thus dioxygenation of substrate proceeds. Moreover, the dioxygenation of substrates Sk and N-Ac-TrpE was found to be inhibited by addition of BHT to the system (Fig. 3, Table 1),

Table 3 Visible Absorption Spectra of Six-Coordinate Ferric Low-Spin Porphyrin Complexes

Complex	Substrate	Atmospheric	temp. (K)	λ_{max} (nm)				Ref.
N–Fe–N								
Fe(III)TPP($ImH)_2$	None	Air	298	416	456 (sh)	548	580 (sh)	28
Fe(III)TPP(Im) (ImH)	None	Air	298	418	444 (sh)	552	585 (sh)	28
Complex A	None	Air	77	409	522	550 (sh)		*a)*
N–Fe–O								
Fe(III)PPIXDBE(py) (OAr)	None	Air	296	411	530	557		26
Fe(III)PPIXDBE(N-MeIm) (OAr)	None	Air	296	411	528	558		26
Complex B	None	Air	77	408	532	565		*a)*
O–Fe–OOR								
Fe(III)TPP(-OMe) ($^-O_2$ Bu)	BuOOH	Air	77	420	543	573		30
Fe(III)TPP(-OMe) ($^-O_2$ Sk)	Sk	O_2	77	423	550	589		20
Fe–$(OOR)_2$								
Fe(III)TPP($^-O_2$ $Bu)_2$	BuOOH	air	77	424	547	578		30
Fe(III)TPP($^-O_2$ $Sk)_2$	Sk	O_2	77	423	550	589		20
N–Fe–OOR								
Fe(III)Mb($^-O_2$ Bu)	BuOOH	Air	77	415	542	574		31
Fe(III)Hb($^-O_2$ Bu)	BuOOH	Air	77	420	549	580		31
Fe(III)TPP(Im) ($^-O_2$ Bu)	BuOOH	Air	158	418	546	582		31
Fe(III)TPP(4-MeIm)($^-O_2$ Bu)	BuOOH	Air	158	421	548	582		31
Complex C	Sk	O_2	77	412	535	564		*a)*
Complex D	N-Ac-TrpE	O_2	77	408	532	560		*a)*

sh: shoulder, Bu: *tert*-butyl. *a)* Present study.

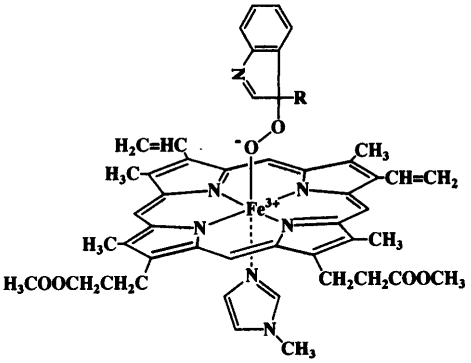

Fig. 6. Possible Coordination Structures of the Six-Coordinate Ferric Low-Spin Complex C, Fe(III)PPIXDME(N-MeIm)($^-O_2$SK), and Complex D, Fe(III)PPIXDME(N-MeIm)($^-O_2$N-Ac-TrpE)

R=Me in complex C and=$CH_2CH_2(CO_2Et)$(NHCOMe) in complex D.

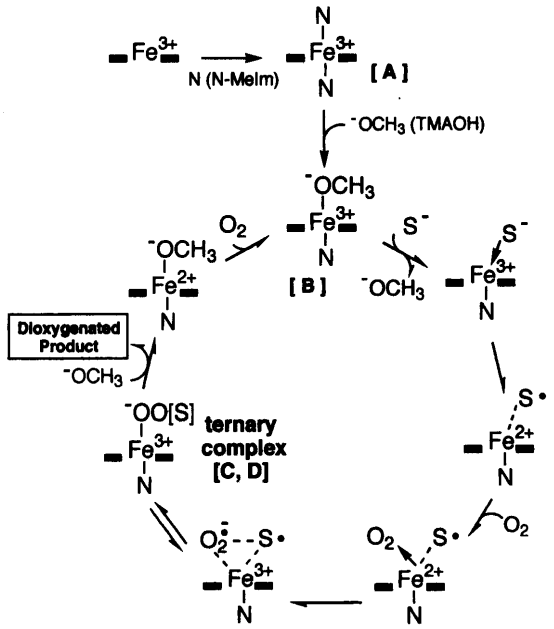

Chart 2. A Possible Reaction Mechanism for the Dioxygenation

S^- represents substrate anion. Substrate anion formed by substrate and methoxide anion acts an electron donor.[20]

suggesting that radical-dependent reactions participate in the dioxygenation of substrates.[20] On the basis of these results together with our previous observations,[7,20] a possible reaction mechanism of the dioxygenation by the present model system is proposed as shown in Chart 2.

Complexes C and D would be useful ternary complexes to evaluate the electronic structure and reactivity of the reactive intermediate of a dioxygenation system, since the imidazole(N)–heme–O_2–substrate ternary complex has been found to be essential in the reaction processes of the peroxide-dependent oxygenation of the substrate. Thus, the present ternary complex as a key intermediate may provide new insight to estimate the reactivity of the hemoproteins.

Acknowledgments This study was partially supported by grants from the Ministry of Education, Science, Sports and Culture of Japan to S. O. and H. S.

References and Notes

1) Present address: *Research Laboratory, Nagase Co., Ltd., 5–1, Nihonbashi-kobunacho, Chuo-ku, Tokyo 103—8355, Japan.*

2) *a*) Hayaishi O., Stanier R. Y., *J. Bacteriol.*, **62**, 691—709 (1951); *b*) Hayaishi O., Rothberg S., Mehler A. H., Saito, Y., *J. Biol. Chem.*, **229**, 889—896 (1957); *c*) Maezono K., Tashiro K., Nakamura T., *Biochem. Biophys. Res. Commun.*, **170**, 176—181 (1990); *d*) Ren S., Liu H., Licad E., Correia M. A., *Arch. Biochem. Biophys.*, **333**, 96—102 (1996).
3) *a*) Ishimura Y., Nozaki M., Hayaishi O., Nakamura T., Yamazaki I., *J. Biol. Chem.*, **245**, 3593—3602 (1970); *b*) Uchida K., Shimizu T., Makino R., Sakaguchi K., Iizuka T., Ishimura Y., Nozawa T., Hatano M., *ibid.*, **258**, 2519—2525 (1983).
4) Leeds J. M., Brown P. J., McGeehan G. M., Brown F. K., Wiseman J. S., *J. Biol. Chem.*, **268**, 17781—17786 (1993).
5) *a*) Tanaka T., Knox W. E., *J. Biol. Chem.*, **234**, 1162—1170 (1959); *b*) Sono M., Dawson J. H., *Biochim. Biophys. Acta*, **789**, 170—187 (1984); *c*) Kadoya A., Tone S., Maeda H., Minatogawa Y., Kido R., *Biochem. Biophys. Res. Commun.*, **189**, 530—536 (1992).
6) Yoshida Z., Sugimoto H., Ogoshi H., "Biomimetic Chemistry, Advances in Chemistry Series," No. 191, ed. by Dolphin D., Mckenna C., Murakami Y., Tabushi I., American Chemical Society, Washington, DC, 1980, pp. 307—326.
7) Tajima K., Yoshino M., Mikami K., Edo T., Ishizu K., *Inorg. Chim. Acta*, **172**, 83—91 (1990).
8) *a*) Dufour-Ricroch M. N., Crumbliss A. L., Johnston G., Gaudemer A., *J. Mol. Catal.*, **7**, 277—287 (1980); *b*) Fujii T., Ohta M., Kouno K., Ono Y., Ueda Y., *Chem. Pharm. Bull.*, **32**, 4252—4259 (1984).
9) *a*) Nishinaga A., *Chem. Lett.*, **1975**, 273—276; *b*) Nishinaga A., Ohara H., Tomita H., Matsuura T., *Tetrahedron Lett.*, **24**, 213—216 (1983).
10) Uchida K., Soma M., Naito S., Onishi T., Tamaru K., *Chem. Lett.*, **1978**, 471—474.
11) *a*) Ohkubo K., Ishida H., Sagawa T., *J. Mol. Cat.*, **53**, L5—L7 (1989); *b*) Ohkubo K., Sagawa T., Kuwata M., Hata T., Ishida H., *J. Chem. Soc. Chem., Commun.*, **1989**, 352—354; *c*) Ohkubo K., Sagawa T., Ishida H., *Inorg. Chem.*, **31**, 2682—2688 (1992); *d*) Sagawa T., Ishida H., Urabe K., Yoshinaga K., Ohkubo K., *J. Chem. Soc., Perkin Trans. 2*, **1993**, 1—5.
12) *a*) Makino R., Sakaguchi K., Iizuka T., Ishimura Y., *J. Biol. Chem.*, **255**, 11883—11891 (1980); *b*) Kobayashi K., Hayashi K., Sono M., *ibid.*, **264**, 15280—15283 (1989); *c*) Sono M., *Biochemistry*, **29**, 1451—1460 (1990).
13) *a*) Poulos T. L., Finzel B. C., Gunsalus I. C., Wagner G. C., Kraut J., *J. Biol. Chem.*, **260**, 16122—16130 (1985); *b*) Poulos T. L., Finzel B. C., Howard A. J., *Biochemistry*, **25**, 5314—5322 (1986).
14) Fita I., Rossmann M. G., *J. Mol. Biol.*, **185**, 21—37 (1985).
15) Finzel B. C., Poulos T. L., Kraut J., *J. Biol. Chem.*, **259**, 13027—13036 (1984).
16) For example: *a*) Ullrich V., *Top. Curr. Chem.*, **83**, 67—104 (1972); *b*) Sato R., Omura T. (eds.), "Cytochrome P-450," Academic Press, New York, 1978; *c*) Groves J. T., Haushalter R. C., Nakamura M., Nemo T. E., *J. Am. Chem. Soc.*, **103**, 2884—2886 (1981); *d*) Bruice T. C., Dichen C. M., Lu F., Nee M. W., *ibid.*, **107**, 5776—5789 (1985); *e*) Sakurai H., Yoshimura T., *J. Inorg. Chem.*, **24**, 75—96 (1985); *f*) Dawson J. H., Sono M., *Chem. Rev.*, **87**, 1255—1276 (1987); *g*) Traylor T. G., Xu F., *J. Am. Chem. Soc.*, **112**, 178—186 (1990); *h*) Higuchi T., Uzu S., Hirobe M., *ibid.*, **112**, 7051—7053 (1990); *i*) Tajima K., Edo T., Ishizu K., Imaoka S., Funae Y., Oka S., Sakurai H., *Biochem. Biophys. Res. Commun.*, **191**, 157—164 (1993); *j*) Ohkubo K., Sagawa T., Minamoto Y., *J. Mol. Cat.*, **85**, L7—L11 (1993); *k*) Newcomb M., Tadic-Biadatti Marie-Helene Le, Chestney D. L., Roberts E. S., Hollenberg P. F., *J. Am. Chem. Soc.*, **117**, 12085—12091 (1995); *l*) Raner G. M., Chiang E. W., Vas A. D., Coon M. J., *Biochemistry*, **36**, 4895—4902 (1997).
17) *a*) Dunford H. B., Stillmann J. S., *Cood. Chem. Rev.*, **19**, 187—251 (1976); *b*) Poulos T. L., *Adv. Inorg. Biochem.*, **7**, 1—36 (1988); *c*) Holzbaur I. E., English A. M., Ismail A. A., *J. Am. Chem. Soc.*, **118**, 3354—3359 (1996); *d*) Wagenknecht H. A., Woggon W. D., *Chem. Biol.*, **4**, 367—372 (1997).
18) *a*) Chance B., *Nature* (London), **161**, 914—917 (1948); *b*) Benecky M., Frew J. E., Scowen N., Jones P., Hoffman B. M., *Biochemistry*, **32**, 11929—11933 (1993); *c*) Khindaria A., Aust S. D., *ibid.*, **35**, 13107—13111 (1996).
19) For example: *a*) Dolphin D., Forman A., Borg D. C., Fajer J., Felton R. H., *Proc. Natl. Acad. Sci. U.S.A.*, **68**, 614—618 (1971); *b*) White R. E., Coon M. J., *J. Annu. Rev. Biochem.*, **49**, 315—356 (1980); *c*) Ogura T., Kitagawa T., *J. Am. Chem. Soc.*, **109**, 2177—2179 (1987); *d*)

Dawson J. H., *Science*, **240**, 433—439 (1988); *e*) Watanabe Y., Groves J. T., "The Enzymes," Vol. 20, ed. by Boyer P. D., Academic Press, Orlando, 1992, pp. 406—453; *f*) Yamaguchi K., Watanabe Y., Morishima I., *J. Am. Chem. Soc.*, **115**, 4058—4065 (1993); *g*) Farhangrazi Z. S., Sinclair R., Powers L., Yamazaki I., *Biochemistry*, **34**, 14970—14974 (1995); *h*) Matsui T., Nagano S., Ishimori K., Watanabe Y., Morishima I., *ibid.*, **35**, 13118—13124 (1996); *i*) Liu Y., Moenne-Loccoz P., Loehr T. M., Ortiz de Montellano P. R., *J. Biol. Chem.*, **272**, 6909—6917 (1997).

20) Oka S., Tajima K., Sakurai H., *Chem. Pharm. Bull.*, **46**, 377—383 (1998).
21) *a*) Antonioni E., Brunori M. (eds.), "Hemoglobin and Myoglobin in Their Reactions with Ligands," North-Holland, Amsterdam, 1971; *b*) Mathews F. S., Levine M., Argos P., *J. Mol. Biol.*, **64**, 449—464 (1972).
22) Adler A. D., Longo F. R., Varadi V., *Inorg. Synth.*, **16**, 213—220 (1976).
23) Walker F. A., Reis D., Balke V., *J. Am. Chem. Soc.*, **106**, 6888—6898 (1984).
24) *a*) Peisach J., Blumberg W. E., Adler A., *Ann. N.Y. Acad. Sci.*, **206**, 310—327 (1973); *b*) Peisach J., Mims W. B., *Biochemistry*, **16**, 2795—2799 (1977).
25) Tang S. C., Koch S., Papaefthymiou G. C., Foner S., Frankel R. B., Ibers J. A., Holm R. H., *J. Am. Chem. Soc.*, **98**, 2414—2434 (1976).
26) Ainscough E. W., Addison A. W., Dolphin D., James B. R., *J. Am. Chem. Soc.*, **100**, 7585—7591 (1978).
27) Ozaki T., Yoshimura T., *Inorg. Chim. Acta*, **36**, L421—L422 (1979).
28) Quinn R., Nappa M., Valentine J. S., *J. Am. Chem. Soc.*, **104**, 2588—2595 (1982).
29) Schaeffer C., Momenteau M., Mispelter J., Loock B., Huel C., Lhoste J.-M., *Inorg. Chem.*, **25**, 4577—4583 (1986).
30) Tajima K., Jinno J., Ishizu K., Sakurai H., Ohya-Nishiguchi H., *Inorg. Chem.*, **28**, 709—715 (1989).
31) Tajima K., *Inorg. Chim. Acta*, **169**, 211—219 (1990).
32) Tajima K., Oka S., Edo T., Miyake S., Mano H., Mukai K., Sakurai H., Ishizu K., *J. Chem. Soc., Chem. Commun.*, **1995**, 1507—1508.
33) Griffith J. S., *Nature* (London), **180**, 30—31 (1957).
34) Kotani M., *Suppl. Prog. Theor. Phys.*, **17**, 4 (1961).
35) Bohan T. L., *J. Mag. Reson.*, **26**, 109—118 (1977).
36) Tajima K., Mikami K., Tada K., Oka S., Ishizu K., Ohya-Nishiguchi H., *Inorg. Chim. Acta*, **194**, 57—65 (1992).