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UNUSUAL HYPERPORPHYRIN SPECTRUM BY BIS(GLUTATHIONE DIMETHYL ESTER)-HEMIN COMPLEX, A MODEL OF CYTOCHROME P-450-THIOLATE COMPLEXES

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Summary : A bis(thiolato) complex of iron-porphyrin exhibiting a "split Soret band" with peaks at 374 and 466 nm, classified as a "hyperporphyrin (HP) spectrum", was prepared with Fe(III)protoporphyrin dimethyl ester (FePPIXDME), glutathione dimethyl ester (GSHDME) and tetrabutylammonium hydroxide in acetone solvent. The structure of the complex was characterized by compairing their electronic absorption and electron spin resonance (ESR) spectra with those of thiolate adducts of cytochrome P-450 and their chemical model complexes. Under the same conditions as those for the formation of the HP spectrum, the generation of the free superoxide anions was detectable by ESR spectrometry at 77K. On the basis of the results, the possible reaction mechanism generating the superoxide anions by the bis(GSHDME)-FePPIXDME complex was proposed.

Cytochrome P-450 (P450), catalyzing various types of monooxygenase reaction (1), exhibits unusual spectroscopic properties (2). The unique Soret band at 450 nm of the reduced (ferrous) P-450-CO complex was identified by Hanson et al. (3) to be a part of unusual "hyperporphyrin (HP)" spectrum. The HP spectrum is characterized by the split intense "Soret band" with peaks in the near UV 350-380 nm region and in the visible 400-480 nm region, being due to a thiolate-ferrous heme-CO axial coordination mode (4). The HP spectra have thus

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Abbreviations : P450cam, cytochrome P-450 from *Pseudomonas putida* ; FePPIX, Fe(III)protoporphyrin ; FePPIXDME, FePPIX dimethyl ester ; GSHDME, glutathione dimethyl ester ; TBAOH, tetrabutyl ammonium hydroxide ; ESR, electron spin resonance ; TCNQ-Li, tetracyanoquinodimethane Li^+ salt ; TANOL, 4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl ; TGE, thioglycolate ethyl ester ; TN, thionalide ; ϕSH, thiophenol ; ϕCH_2SH, benzylmercaptane ; 4-$NO_2\phi SH$, 4-nitrothiophenol ; 4-$Cl\phi SH$, 4-chlorothiophenol ; nPrSH, n-propyl mercaptane ; DTT, dithiothreitol ; Ph_3P, triphenylphosphine ; TPP, tetraphenylporphyrinato ; OPE, octaethylporphyrinato ; THF, tetrahydrofurane ; DMSO, dimethylsulfoxide.

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been classified as a p-type HP spectrum (5), resulting from a mixture of two transitions : sulfur p' $\rightarrow$ porphyrin π^* and porphyrin $\pi \rightarrow \pi^*$, where p' is one of the two lone-pair sulfur orbitals (4).

Another unusual HP spectra due to bis(thiolato) iron- and cobalt-heme complexes have been found by exogenous addition of thiolate ligand at the sixth axial position of Fe(III)- (6) and Co(III)-substituted P450 (7), confirming that the occurrence of endogenous thiolate ligand at the fifth position of the heme in the resting state of the enzymes. Similar HP spectra were found for thiolate adducts of ferric chloroperoxidase, supporting also the presence of an endogenous cysteinate ligand on the heme of the enzyme (8).

The occurrences of these unusual HP spectra, exhibiting split Soret bands at the near UV 350-380 nm region and the visible 400-480 nm region, are currently limitted to the low molecular weight P450 model complexes (9-13). Previously we reported that glutathione (GSH)-heme complex is a good chemical model of P450 (14). The investigations have been continued to find the complex exhibiting HP spectrum using GSH, which is a tri-peptide ligand (γ-Glu-Cys-Gly). We report here the detection and characterization of the HP spectrum for bis(thiolato)-Fe(III)protoheme complex containing glutathione dimethyl ester (GSHDME) as the axial thiolate ligands. We also found the generation of free superoxide anion radicals, coupling with the formation of the HP spectrum.

MATERIALS AND METHODS

Glutathione (GSH) and FePPIX•Cl (type I, bovine) were obtained from Sigma Chemical Co. TANOL was a gift of Dr. Tazima of Ehime University. GSHDME•HCl was prepared in our laboratory, modifying the method reported previously (15). The structure and the purity of GSHDME•HCl were determined by proton NMR spectra and iodometry of the thiol group (85 %), respectively, under argon atmosphere. FePPIXDME•Cl was prepared from FePPIX•Cl according to the procedure described before (16). Electronic absorption spectra were measured with a Union SM-302 and a JASCO Ubest-50 UV-VIS spectrometers at room temperature. ESR spectra were recorded at 77K with a JES-FE1XG spectrometer (X-band) operated at 100 kHz modulation frequency. Magnetic field was calibrated with a Takeda Riken TR 5212 frequency counter. As standards, TCNQ-Li (g=2.00252) and Mn(II) doped in MgO (ΔH_{3-4}=86.9 G) were used. Crystal field parameters of axial distortion and rhombic distortion in the heme iron coordination sphere of the bis(GSHDME)-hemin complex in the low-spin state were computed from the three ESR g-values according to Bohan's method (17). Free superoxide anions generated by the model system were estimated using TANOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) radical.

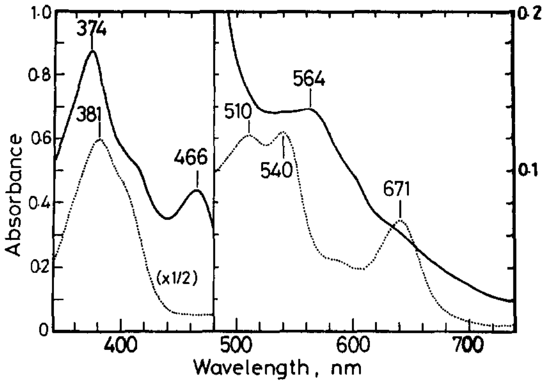

Figure 1. Hyperporphyrin spectrum of FePPIXDME-GSHDME-TBAOH system in acetone at room temperature. To a mixture (----) of FePPIXDME•Cl (12.5 μM) and GSHDME•HCl (5.0 mM) in acetone, TBAOH (2.3 mM in methanol) was added under air at room temperature (20 °C), and the absorption spectrum was recorded after 30 sec. (——).

RESULTS AND DISCUSSION

Addition of lipophilic alkaline regent (TBAOH) to a mixture of FePPIXDME•Cl and GSHDME•HCl in acetone solvent under aerobic conditions resulted in the appearance of a new split Soret band, with peaks at 374 and 466 nm, concomitant with an absorption band at 564 nm (Fig. 1).** The apparent half-life of this new spectrum under air was about 3 min. at the concentration ratio of GSHDME/FePPIXDME $\geq$ 100, monitored by each split Soret band. This spectrum is very similar to those of P450cam-thiol complexes (λ max : 370-380, 460-470 and -560 nm) (6,9) and bis(thiolato)-FePPIXDME complexes (λ max : 370-380, 450-470 and -560 nm) (9,10) (Table I). The ESR spectrum of FePPIXDME-GSHDME-TBAOH system prepared in acetone under air indicated the formation of a ferric complex in the low-spin state, concomitant with a formation of ferric high spin species (g=6.5), as shown in Fig. 2. The anisotropic ESR g-values (2.360, 2.235 and 1.944) as well as the crystal field parameters (tetragonality $|\mu/\lambda|=7.16$ and rhombicity $|R/\mu|=0.38$) of the ferric low-spin species were similar to those of the P450cam-thiolate adduct complexes (6b) and bis(thiolato)-ferric heme complexes (9,10), reported previously (Table II).

** FePPIXDME•Cl (12.5μM) in acetone shows absorption maxima at 381(105), 400^{sh}(82), 510(11), 540(11) and 641 nm (6). Addition of TBAOH (2.5mM) to FePPIXDME Cl results spectral changes, showing the maxima at 397(86), 425(50), 569(12) and 596 nm (11). The values in parentheses are molar extinction coefficient ($mM^{-1}cm^{-1}$).

Table I. Electronic Absorption Spectral Properties of Hyperporphyrin Spectra by Bis(thiolato)-Hemin Complexes

Heme	Axial thiolate ligand	Solvent	Absorption maxima, nm (ε : $mM^{-1}cm^{-1}$) λ_1	λ_1		Ref.
FePPIXDME	GSHDME	Acetone	374(69)	466(35)	564(11)	
	TGE	Acetone	371(66)	466(50)	562(10)	10a
	TN		372(75)	464(60)	561(12)	10a
	øCH_2SH	CH_2Cl_2	376	470	561	9b
	4-NO_2øSH		376	455	555	9b
P450cam	nPrSH	pH 9.1	377(69)	466(57)	559(12)	6b
	DTT	pH 7.0	374(69)	461(57)	557(12)	6b
	øCH_2SH		376(76)	464(58)	558(13)	6b
	4-CløSH		381(77)	463(50)	560(7)	6b

On the basis of these observations and results, the present new spectrum showing the intense split Soret bands as well as the anisotropic ESR signals is concluded to be attributable to a hyperporphyrin (HP) spectrum due to the formation of a bis(thiolato)-Fe(III)PPIXDME complex, in which two thiolate groups of GSHDME coordinate axially to the Fe(III)PPIXDME. These unusual split Soret bands for the bis(thiolato)-metalloporphyrin complexes, as well as the well-known ferrous P450-CO complex, were assigned to thiolate Sp $\rightarrow$ porphyrin π^* charge-transfer transitions, which mix with the porphyrin $\pi \rightarrow \pi^*$ transition (3). The HP spectra by FePPIXDME-GSHDME complex was not observed when benzene, dichloromethane or chloroform was used in place of acetone as a sol-

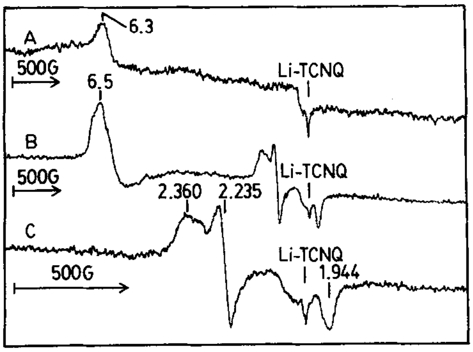

Figure 2. ESR spectra of FePPIXDME-GSHDME-TBAOH systems in acetone at 77K. (A) : FePPIXDME·Cl (0.20 mM) + GSHDME·HCl (2.38 mM), (B) : (A) + TBAOH (6.02 mM in methanol) and (C) : Expansion of spectrum for (B). These ESR spectra were recorded immediately after the preparation of samples.

Table II. ESR Spectral Properties of Bis(thiolato)-Hemin Complexes

Heme	Axial thiolate ligand	Solvent	ESR g-values				Crystal field parameters		Ref.
			g_1	g_2	g_3	g_1-g_3	$\lvert\mu/\lambda\rvert$	$\lvert R/\mu\rvert$	
FePPIXDME	GSHDME	Acetone	2.360	2.235	1.944	0.42	7.16	0.38	
	TGE	Acetone	2.289	2.228	1.957	0.33	8.01	0.22	10a
	TN		2.363	2.267	1.926	0.44	6.48	0.27	10a
	øCH$_2$SH	CH$_2$Cl$_2$	2.302	2.229	1.958	0.34	7.92	0.25	9b
	4-NO$_2$øSH		2.405	2.274	1.925	0.48	6.23	0.35	9b
P450cam	nPrSH	pH 7-9	2.36	2.25	1.93	0.43	6.72	0.32	6b
	DTT		2.38	2.24	1.95	0.43	7.07	0.42	6b
	øSH	pH 6-9	2.39	2.25	1.94	0.45	6.77	0.40	6b
	4-CløSH		2.39	2.25	1.93	0.46	6.60	0.39	6b

vent. Further, when low molecular weight thiol-ester compound such as cysteine methyl ester or cysteine ethyl ester was used instead of GSHDME, no HP spectrum was seen, indicating that more bulky and lipophilic thiol ligands might be needed for the appearance of HP spectrum.

On standing the solution of FePPIXDME-GSHDME-TBAOH in acetone for about 60 min. under air, the ESR spectrum at 77K showed an intense signal due to free radicals and the g-values ($g_{/\!/}$=2.095 and $g_{\perp}$=2.006 at 77K) provided evidence for the formation of free superoxide anions (Fig. 3). The g-values are very similar to those obtained by the model complexes of P450 (10b, 11a,b, 13) and the KO$_2$ systems (19, 20) (Table III). The concentration of the superoxide

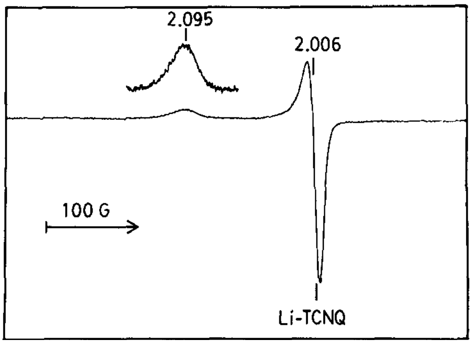

Figure 3. Generation of superoxide anions by FePPIXDME-GSHDME-TBAOH system in acetone. A mixture of FePPIXDME•Cl (0.20 mM), GSHDME•HCl (2.38 mM) and TBAOH (6.02 mM in methanol) was allowed to stand for about 60 min. in contact with air at room temperature (20 °C), and the ESR spectrum was measured at 77K.

Table III. ESR Parameters for Superoxide Anions Detected at 77K

System	Solvent	$g_{\parallel}$	$g_{\perp}$	Ref.
FePPIXDME-GSHDME-TBAOH	Acetone	2.095	2.006	
FeTPP-TGE-TMAOH	CH_2Cl_2	2.088	2.007	10b
CoTPP-TGE-TMAOH	CH_2Cl_2	2.084	2.006	11a,b
RhOEP-TGE-TMAOH	benzene	2.088	2.006	13
	THF	2.096	2.009	13
CoTPP-Ph_3P-TBAOH	Acetone	2.090	2.007	18
KO_2	DMSO	2.087	2.007	19
KO_2-Crown ether	DMSO/Toluene (1:1)	2.109	2.007	20

anions estimated with TANOL radical was approximately 0.01 mM just after 60 min. of the sample preparation, indicating that the superoxide anions were about 1/20 of the FePPIXDME concentration. The present results suggest that the bis(thiolato)-Fe(III)PPIXDME complex prepared at the 12:1 concentration ratio of GSHDME to FePPIXDME is reduced to the ferrous complex by the axial thiolate ligand, followed by reaction with molecular oxygen to form the superoxide anion. The generation of superoxide anions has previously been observed

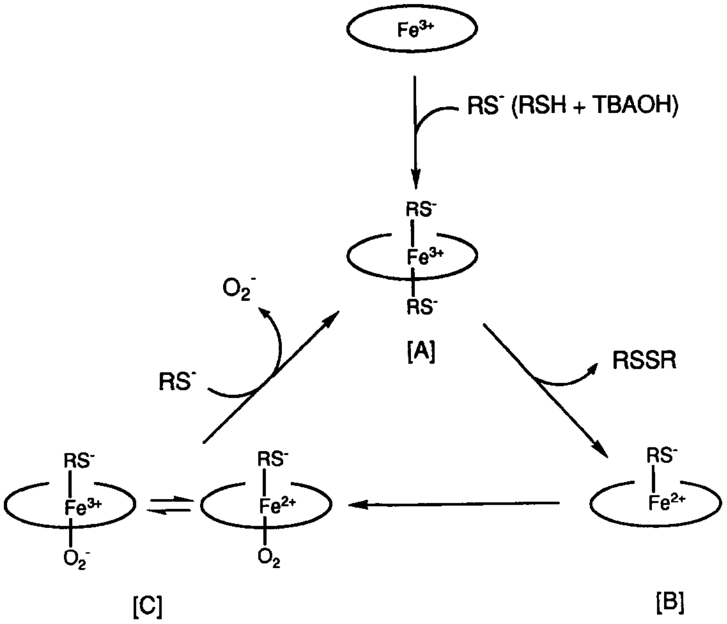

Chart 1. Superoxide Anion Generation by FePPIXDME-GSHDME/TBAOH System in Acetone.

Fe^{3+} : Fe(III)PPIXDME, RSH = GSHDME in the bis(thiolato)-metalloporphyrin complexes (10b, 11a,b, 13, 14). Considering our reactions in the Co- and Fe-containing metalloporphyrin complexes, the mechanism for generating the superoxide anions in the present complex is proposed as shown in Chart 1. (A) is the bis(GSHDME)-FePPIXDME complex with HP spectrum, which was deduced from the results of both optical and ESR spectra. (B) is speculated to be a penta-coordinate mono(GSHDME)-FePPIXDME complex, whose structure was previously estimated by ESR on the Co-substituted porphyrin complexes (11a,b). (C) is a hexa-coordinate oxygen adduct complex, which is an equilibrium mixture involving electron transfer from iron to oxygen to form an iron-bound superoxide anion. The similar complexes have been suggested to be involved as an intermediate in the moleculer oxygen activating process of P450 (1a). The generation of superoxide anions will continue until the available thiolate ligand is oxidized to the disulfide form in the system. Thus, the activation of molecular oxygen by the FePPIXDME-GSHDME complex in acetone is useful for studying the mechanism of the activation of molecular oxygen by P450, which is a thiolato-heme protein.

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