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The Active Site Structure as Thiolate-Hemin-Hydroxyl Group Coordination Mode in Cytochrome P-450. An Approach from Model Investigations

HIROMU SAKURAI*

Faculty of Pharmaceutical Sciences, University of Tokushima, Sho-machi 1, Tokushima 770, Japan

and TETSUHIKO YOSHIMURA

The Environmental Science Institute of Hyogo Prefecture, Suma-ku, Kobe, Hyogo 654, Japan

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During the last five years the concept of the active site structure of cytochrome P-450 has emerged, based on the physico-chemical studies on this protein and its model complexes. The active site of P-450$_{LM}$ contains an iron-protoporphyrin IX complex in the hydrophobic cleft of the protein. The iron is present in hexa-coordinate form in the resting state of protein, four of the ligands being contributed by the planar, tetradentate porphyrin ring. The fifth ligand has been thought of as a non-replaceable thiolate ligand contributed by a cysteine residue of the polypeptide [1, 2]. For the sixth ligand, two types of candidates have been proposed and these are oxygen-ligating species such as serine, threonine [3], tyrosine [4] or water [5–7], and imidazole-ligating species such as histidine residue [8]. Thus the identification of the sixth ligand remains controversial. This paper presents evidence in favour of the oxygen coordination, but against the imidazole ligation at the sixth position in cytochrome P-450, based on our model studies.

The purified cytochrome P-450$_{LM_2}$ [9] and P-450$_{CAM}$ [10] in low-spin state have the distinct α- and β-bands as well as the Soret band at 417–8 nm, and the relative intensity ratio of α-band to β-band (α/β ratio) in both proteins is approximately 1 or more. These characteristic spectra have been simulated by the chemical models having 'thiolate *trans* oxygen' ligation mode [3] and also by the ligand replacement reaction of high-spin, ferric P450$_{LM_2}$ with n-butanol [11]. The ligation of an imidazole at the sixth position has not resulted in the distinct α-band.

In the previous paper we reported the formation and characterization of a new hyperporphyrin complex having dithiolate-hemin coordination mode, as well as the ligand exchange reaction with imidazole at room temperature [12]. During our studies on the hyperporphyrin complexes, we found that a hydroxyl group such as methanol can coordinate to the *trans* position of the thiolate-hemin complex.

The addition of methanol to the hyperporphyrin complex which was formed from FePPIXDME, TGE and tetramethylammonium hydroxide in acetone solvent at room temperature causes a new Soret band at 407 nm and distinct β- and α-bands at 530 and 560 nm respectively in the methanol:acetone (80:20) solvent system (Fig. 1). The α/β ratio of this spectrum has been found to be 1. The characteristics of this spectrum were compared with other systems (Table I). The EPR g-values characteristic of ferric low-spin state also shifted from those of hyperporphyrin complex (g = 2.29, 2.23, 1.95), depending on the methanol:acetone ratio (Table II). The representative spectrum in 82% methanol is shown in Fig. 2 and the g-values are compared with other systems (Table III). Similar results were obtained with other thioglycolic acid esters.

On the other hand, when the high-spin complex (g = 5.83) consiting of FePPIXDME, methanol and tetramethylammonium hydroxide (under this conditions methanol is not deprotonated) was titrated with TGE, then a low-spin complex first was obtained in which the EPR signals were observed at g = 2.345, 2.248, 1.940 and then gradually changed to those with g-values of hyperporphyrin complex by increasing TGE concentration. The former low-spin complex showed the same g-values as the resulting complex

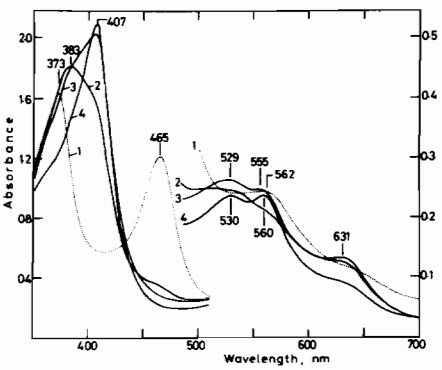


Fig. 1. Spectrophotometric titration of Hemin-Dithiolate complex with methanol at room temperature (20°C). The hyperporphyrin complex consisting of FePPIXDME Cl 0.025 m*M*(dissolved in acetone), TGE 12.5 m*M* and tetramethylammonium hydroxide 0.73 m*M* was titrated with methanol in acetone solvent at room temperature (20°). Acetone:Methanol = (1) 100:0, (2) 87:13, (3) 60:40, (4) 20:80.

*Author to whom correspondence should be addressed.

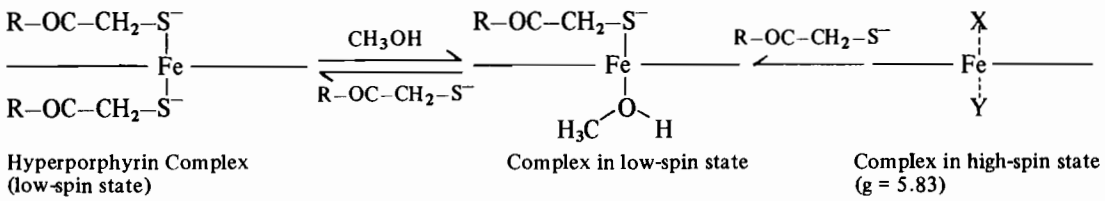
Abbreviations: EPR, electron paramagnetic resonance; TGE, thioglycolic acid ethyl ester; FePPIXDME, Fe(III)-protoporphyrin IX dimethyl ester complex; FeOEP, Fe(III)-octaethylporphyrin complex; FeTPP, Fe(III)-tetraphenylporphyrin complex; 2MeTHF, 2-methyltetrahydrofuran; THF, tetrahydrofuran; DMF, dimethyl formamide; P-450$_{LM}$, cytochrome P-450 form liver microsomes; P-450$_{CAM}$ cytochrome P-450 from *Pseudumonas putida*.

TABLE I. Electronic Spectra of Iron-Porphyrin Complexes in Ferric Low-Spin State.

Heme	Axial Ligands		Absorption Maxima, nm				
	5th position	6th position	Soret	β	α	α/β Ratio	Reference
FePPIXDME	$^{-}SC_6H_4NO_2$	2MeTHF	418	535	566	1.1	13
FePPIXDME	$^{-}SC_6H_4NO_2$	DMF	420	533	567	1.1	13
FePPIXDME	$^{-}SC_6H_4Cl$	2MeTHF	422	536	566	1.0	13
FeOEP	$^{-}SC_6H_4NO_2$	2MeTHF	407	526	556	1.2	13
FePPIXDME	$^{-}SC_6H_4NO_2$	CH_3OH	417	534	566	1.0	3
FePPIXDME	$^{-}SCH_2COOC_2H_5$	CH_3OH	407	530	560	1.0	present work
FePPIXDME	$^{-}SCH_2COOC_8H_{17}$	CH_3OH	407	530	559	1.0	present work
FePPIXDME	$^{-}SCH_2COOC_{18}H_{37}$	CH_3OH	407	530	559	1.0	present work
$P\text{-}450_{CAM}$			417	535	571	1.1	10
$P\text{-}450_{LM_2}$			418	535	568	1.1	9

derived from the hyperporphyrin complex by titration with methanol.

The new complex with the distinct α- and β-bands as well as the Soret band at 407 nm and the anisotropic low-spin EPR signals, therefore, has a thiolate form of TGE at the fifth coordination position and a methanol at the sixth position (the hemin complex in the presence of methanol has absorption bands at 397, 481 and 596sh nm). Thus the reaction sequence can be depicted as follows:



Hyperporphyrin Complex (low-spin state) — Complex in low-spin state — Complex in high-spin state (g = 5.83)

The axial ligands in the high-spin complex have not yet been identified.

TABLE II. The g-value Variations caused by Addition of Methanol to Hyperporphyrin Complex consisting of Fe-PPIXDME and Thioglycolic Acid Ethyl Ester*.

Acetone:Methanol	g-values at 77 K		
	g_1	g_2	g_3
83:17	2.299	2.231	1.952
71:29	2.304	2.234	1.949
63:37	2.310	2.238	1.948
39:61	2.312	2.241	1.949
18:82	2.321	2.242	1.945
4:96**	2.245	2.248	1.940

*The concentrations of the components are the same as in Fig. 2 except for **.

**FePPIXDME Cl 9.43 m*M*(dissolved in chloroform), TGE 0.94 m*M* and tetramethylammonium hydroxide 84.62 m*M*.

The other alcohols, such as ethanol, n-butanol or n-propanol, were also used in place of methanol but the distinct α-band in the optical spectrum at room temperature was not observed. With EPR spectroscopy the anisotropic low-spin signals similar to the methanol complex were detected (Table III), probably due to the use of higher concentration in EPR measurement than in optical measurement. Under the same conditions, phenol does not coordinate to the *trans* position of heim, based on the result of optical spectrum. Furthermore, with various types of imidazoles spectra always showed an absorption shoulder at an α-band different from that of the native cytochrome P-450.

From our present study and other observations [1–3, 13], there is no doubt about the thiolate nature of the fifth ligand of cytochrome P-450 in the resting state. With regard to the sixth ligand in hexacoordinated cytochrome P-450, the presence of a hard ligand such as alcoholic hydroxyl group can be suggested, in good agreement with our previous result [3], although the Soret band at 407 nm in our present model is shifted to much shorter wavelength than the native proteins [9, 10].

The α/β ratio in the spectrum of cytochrome P-450 can be postulated, because the spectrum having approximately 1 or more than 1 value of α/β ratio can be simulated only with the thiolate-hemin-hydroxyly group coordination mode. As a conclusion, a hydroxyl group contributed either from a seryl or threonyl residue or water is the most probable candidate for the *trans* sixth ligand of cyto-

TABLE III. EPR Parameters of Iron-Porphyrin Complexes in Ferric Low-Spin State.

Heme	Axial Ligands		g-values at 77 K			Reference
	5th position	6th position	g_1	g_2	g_3	
FePPIXDME	$^-SC_6H_4NO_2$	2MeTHF or THF	2.36	2.26	1.94	13
FePPIXDME	$^-SC_6H_4NO_2$	DMF	2.46	2.28	1.90	13
FePPIXDME	$^-$S-Cys(Ac)NHMe	DMF	2.37	2.24	1.95	13
FePPIXDME	$^-SCH_2C_6H_5$	DMF	2.36	2.24	1.95	13
FePPIXDME	$^-SCH_2C_6H_5$	THF	2.29	2.22	1.97	13
FeOEP	$^-SC_6H_5$	DMF	2.42	2.27	1.92	13
FeTPP	$^-SC_6H_5$	THF	2.34	2.27	1.94	13
FePPIXDME	$^-SC_6H_4NO_2$	CH_3OH	2.411	2.273	1.926	3
FePPIXDME	$^-SC_5H_4NO_2$	C_2H_5OH	2.404	2.273	1.927	3
FePPIXDME	$^-SCH_2COOC_2H_5$	CH_3OH	2.321	2.242	1.945	present work
FEPPIXDME	$^-SCH_2COOC_2H_5$	C_2H_5OH	2.312	2.238	1.945	present work
FePPIXDME	$^-SCH_2COOC_2H_5$	n-C_3H_7OH	2.312	2.238	1.950	present work
FePPIXDME	$^-SCH_2COOC_2H_5$	n-C_4H_9OH	2.312	2.237	1.950	present work
P-450$_{CAM}$			2.45	2.26	1.97	14
P-450$_{LM}$			2.41	2.26	1.91	15

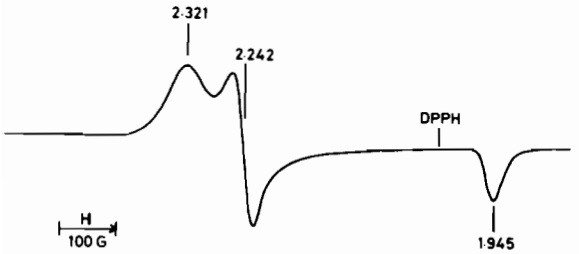


Fig. 2. EPR spectrum of Thiolate-Hemin Methanol complex at 77 K. The complex was formed with FePPIXDME Cl 8.55 m*M*(dissolved in chloroform), TGE 87.2 m*M* and tetramethylammonium hydroxide 76.7 m*M*. Acetone:Methanol = 18:82.

chrome P-450 in resting state. Furthermore, it should be noted that the ligation of hydroxyl group decreases with increasing length of carbon chain in alcohol, as indicated by our optical spectra of model compounds. This result indicates that a hydroxyl group is the most favourable candidate, probably water also, in the *trans* sixth position of thiolate-hemin complex or cytochrome P-450.

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