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A Model of the Heme Site of Cytochrome P-450: Characterization of a Sulfhydryl- and Imidazole-containing Peptide-Heme System in Solution

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The absorption and electron paramagnetic resonance (EPR) spectral properties of model systems consisting of sulfhydryl- and imidazole-containing peptides (mercaptoacetylhistidine, β-mercaptopropionylhistidine, mercaptoacetylglycylhistidine and mercaptoacetylalanylhistidine) and hemin were studied at pH 7.2. In these systems, mixed spin states of ferric heme were observed, and analysis of the EPR data indicated the formation of three different types of complex. Addition of pyridine to these systems caused a spin transition to a single type of low spin state ferric heme, indicating the formation of –S⁻–Fe(III)–N coordination. The ligand-field parameters of low spin species of the model systems were calculated and compared with those of cytochrome P-450. A possible reaction sequence and proposed structures of the model complexes formed in the reaction between hemin and peptides in solution are presented.

Keywords——cytochrome P-450; model system; sulfhydryl containing peptide; absorption spectrum; EPR spectrum; ligand field parameter

Cytochrome P-450, which catalyzes various monooxygenase reactions, is the only reasonably well-substantiated class of proteins having a heme iron-sulfur bonding. This protein exhibits unusual spectroscopic properties. The spectroscopic properties[2,3] and the monooxygenase-like activities[4] of model heme complexes ligated with mercaptide sulfur mimic those of cytochrome P-450. In these studies, simple thiol compounds such as *n*-butylmercaptan, benzenethiol or cysteine derivatives have generally been used as axial sulfur ligands to heme. Very recently, we reported on a cytochrome P-450 model using the thiol-containing peptide, α-mercaptopropionylglycine.[5] In this work we suggested the usefulness of a thiol-containing peptide as a possible ligand to heme iron.

The amino acid compositions of the *Pseudomonus putida* and rabbit liver microsomal cytochrome P-450 are known.[6] The coordination mode in the oxidized state of cytochrome P-450 is probably Cys–S–Fe(III)–L, where L=His, Lys, Cys–SH, Met or Asn.[6] The occurrence of histidine and cysteine in the peptide moiety of cytochrome P-450 is particularly striking.[6a]

1) Location: *a) Scho-machi 1, Tokushima 770, Japan; b) Kyoto 606, Japan; c) Ehime, 790, Japan.*
2) *a)* S.C. Tang, S. Koch, G.C. Papaefthymiou, S. Foner, R.B. Frankel, J.A. Ibers, and R.H. Holm, *J. Am. Chem. Soc.*, **98**, 2414 (1976); *b)* J.P. Collman, T.N. Sorrell, and B.M. Hoffman, *J. Am. Chem. Soc.*, **97**, 913 (1975).
3) *a)* C.K. Chang and D. Dolphin, *J. Am. Chem. Soc.*, **98**, 1607 (1976); *b)* J.O. Stern and J. Peisach, *FEBS Lett.*, **62**, 364 (1976).
4) *a)* H. Sakurai and S. Ogawa, *Biochem. Pharmacol.*, **24**, 1257 (1975); *b)* H. Sakurai and M. Kito, *ibid.*, **24**, 1647 (1975); *c) Idem, ibid.*, **25**, 2113 (1976).
5) H. Sakurai, S. Shimomura, K. Fukuzawa, and K. Ishizu, *Chem. Pharm. Bull.* (Tokyo), **26**, 1348 (1978).
6) *a)* R.L. Tsai, I.C. Gunsalus, and K. Dus, *Biochem. Biophys. Res. Commun.*, **45**, 1300 (1971); *b)* K. Dus, W.J. Litchfield, A.G. Miguel, I.A. van der Hoeven, D.A. Haagen, W.L. Dean, and M.J. Coon, *ibid.* **60**, 15 (1974); *c)* B.L. Vallee and W.E.C. Wacker, "The Proteins," Vol. V, 2nd ed., H. Neurath, Ed., Academic Press, New York, 1973, Chapter III.

In view of the presence of both cysteine and histidine as axial ligands of heme iron in oxidized cytochrome P-450, we present here and characterize a model system for oxidized cytochrome P-450 using several peptides having both thiol and imidazole groups, such as mercaptoacetylglycylhistidine (MAH), β-mercaptopropionylhistidine (MPH), mercaptoacetylglycylhistidine (MAGH) and mercaptoacetylalanylhistidine (MAAH). The optical and EPR data for the hemin complexes with all peptides investigated exhibited mixed-spin states of ferric iron at pH 7.2, and the data for the low-spin state complexes were very similar to those for cytochrome P-450. It is interesting to note the occurrence of a spin transition from mixed-spin states to the low-spin state on adding pyridine to the peptide-hemin system. The possible reaction sequence and the structures of the model complexes are discussed.

Materials and Methods

Hemin chloride (protoporphyrin IX chloride, bovine, type I) was purchased from Sigma Chemical Co. and was used without further purification. Mercaptoacetylhistidine (MAH), β-mercaptopropionylhistidine (MPH), mercaptoacetylglycylhistidine (MAGH) and mercaptoacetyl-β-alanylhistidine (MAAH) were systhesized according to the reported method.[7] Complexes were prepared by mixing peptide, hemin and/or pyridine in 0.1 M phosphate buffer, pH 7.2, in air. Optical spectra of the complexes were measured with a Union SM-302 spectrometer at room temperature (22°) using a hemin concentration of 2.0×10^{-5} or 10^{-4} M. Electron paramagnetic resonance (EPR) spectra were measured on frozen glass at 77°K with a JES-ME-3X spectrometer operating at 100 KHz. EPR operating frequencies were measured with a Takeda-Riken microwave frequency counter, and g values were determined by taking Li-TCNQ ($g=2.0025$) as a standard. The magnetic fields were calibrated based on the splitting of Mn(II) in MgO ($\Delta H_{3-4}=86.9$ gauss). The tatragonal splitting (μ) and rhombic splitting (R) parameters for t_{2g} orbitals of the ground state of the ferric ion in the one-electron formalism were estimated with λ (spin-orbit coupling constant) obtained from the g values of EPR spectra in the low-spin complexes according to the method of Weissbluth.[8] Other experimental conditions are given in the legends to figures and tables.

Results

When MPH was added to a solution of hemin in 0.1 M phosphate buffer, pH 7.2, at room temperature, the absorption spectrum exhibited maxima at 367, 410 and 532 nm within 3 minutes, suggesting mixed spin states of ferric iron (Fig. 1). The absorption maximum at 600—650 nm due to the presence of high spin species was not observed in this system, probably owing to the presence of mixed spin states of hemin. In the presence of pyridine, the solution showed absorption maxima at 412, 538 and 565^{sh} nm with disappearance of the maximum at 367 nm (Fig. 1). In both solutions the absorption maxima changed to 415, 525 and 553 nm after 30 minutes, and the EPR signals disappeared, indicating the reduction of ferric heme. Therefore, the spectra correspond to the reduced from of heme. The other peptides showed similar behavior. The optical properties of the model systems in the oxidized form at room temperature and at pH 7.2 are summarized in Table I. In

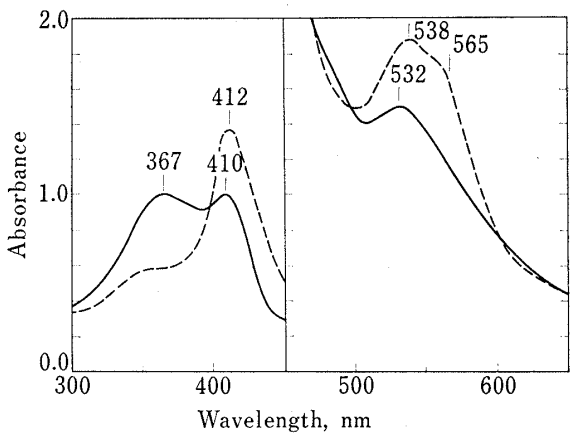

Fig. 1. Absorption Spectra of β-MPH-Hemin Complex (—) and β-MPH-Hemin-Py Complex (----) at Room Temperature and at pH 7.2

300—450 nm; hemin=2.0×10^{-5} M, β-MPH=3.5×10^{-3} M, Py=7.6×10^{-2} M.
450—650 nm; hemin=1.0×10^{-4} M, β-MPH=1.7×10^{-2} M, Py=3.8×10^{-1} M.
All spectra were measured within 3 minutes after mixing.

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8) *a*) M. Weissbluth, *Struct. Bonding (Berlin)*, **2**, 1 (1967); *b*) T. Yoshimura, T. Ozaki, and Y. Shintani, *J. Inorg. Nucl. Chem.*, **39**, 185 (1977).

Table I. Optical Spectral Properties of Model Systems with and without Pyridine at Room Temperature[a)]

Compound	Number of carbons between S and N	λ_{max} (nm) Hemin			λ_{max} (nm) Hemin+Py				$\Delta\lambda$ ($=\lambda'_3-\lambda_3$) (nm)
		λ_1	λ_2	λ_3	λ_1'	λ_2'	λ_3'	λ_4'	
MAH	9	366	416	532	—	417	539	570sh	7
MPH	10	367	410	532	—	412	538	565sh	6
MAGH	13	366	411	533	—	413	538	560sh	5
MAAH	14	365	414	538	—	416	540	570sh	2

a) The concentrations were the same as in Fig. 1.

the case of cytochrome P-450, absorption maxima were observed at 394, 517, 540[sh] and 647 nm and at 417, 534 and 568 nm in the oxidized high spin and low spin states, respectively.[9,10] In the absence of pyridine all the model systems showed mixed high and low spin states of hemin, based on the absorption spectra. However, the addition of pyridine caused the spin state transition to the low spin state only. The effect of pyridine on the $\Delta\lambda$ value (Table I) of the optical spectra of the hemin-peptide systems decreased with increasing number of carbon atoms between the sulfur and imidazole nitrogen atoms of the peptides at the 532—540 nm absorption band (α-band), but there was no correlation at the Soret bands.

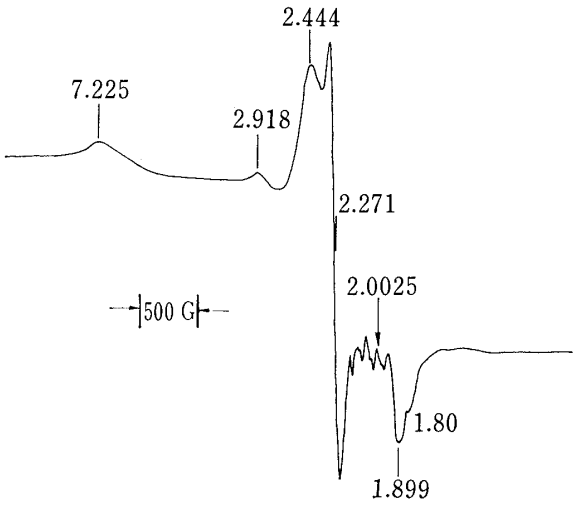
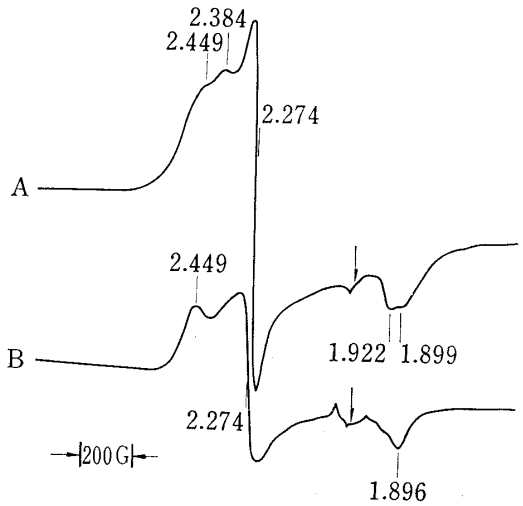


Fig. 2. EPR Spectrum of Hemin Complex with β-MPH at Liquid Nitrogen Temperature and at pH 7.2

Hemin=5.0×10^{-3} M, β-MPH=2.0×10^{-1} M.
The sample was frozen (77 °K) within one minute after mixing. The EPR spectrum was recorded at a microwave frequency of 9.20 GHz at a microwave power of 20 mW, and with a modulation amplitude of 4 G.



| : $g=2.0025$

Fig. 3. EPR Spectra of Low Spin Hemin-MAAH Complex without Pyridine (A) and with Pyridine (B) at Liquid Nitrogen Temperature and at pH 7.2

Hemin=5.0×10^{-3} M, MAAH=2.0×10^{-1} M, Py=5.0 M.
The samples were frozen (77 °K) within one minute after mixing.

The EPR spectrum of the hemin-MPH system at pH 7.2 exhibited signals at $g=7.3$ indicating the presence of high spin species and at $g=2.444$, 2.271 and 1.899 due to low spin species (Fig. 2). This spectrum was in accord with the optical spectrum, in which the presence

9) R.W. Estabrook, J. Baraon, J. Peterson, and Y. Ishimura, *Biochem. Soc. Symp.*, No. 34, 159 (1973).
10) *a*) J. Paisach and W.E. Blumberg, *Proc. Natl. Acad. Sci.*, U.S.A., **67**, 173 (1970); *b*) T.A. van der Hoeven and M.J. Coon, *J. Biol. Chem.*, **249**, 6302 (1974).

of mixed spin states of ferric heme was suggested. When MAAH was used instead of MPH, it was observed that two different types of low spin signals of heme at $g=2.449$, 2.274 and 1.899 and at $g=2.384$, 2.274 and 1.922 (Fig. 3A) contributed to the EPR spectrum of this system concomitant with the high spin signals. However, as pyridine was added to this system, only one type of ferric low spin state of heme was obtained (Fig. 3B) and no high

TABLE II. Principal g Values of EPR Spectra of Model Systems at 77 °K and at pH 7.2[a]

System	g values			
	High spin	Low spin		
Hemin-MAH	7.27	2.499	2.271	1.889
		2.381		1.926
Hemin-MPH	7.27	2.444	2.271	1.899
Hemin-MAGH	7.21	2.456	2.273	1.888
Hemin-MAAH	7.23	2.449	2.273	1.899
		2.384		1.926

a) The concentrations and the preparation of samples were the same as in Fig. 3.

TABLE III. Principal g Values, Energy Differences between t_2g Orbitals and Coordination Modes of Low Spin Type Complexes

System	g value			Tetragonal splitting	Rhombic splitting		Coordination mode
	g_x	g_y	g_z	$\mu(\lambda)$[a]	$R(\lambda)$[a]	μ/R	
Hemin-MAH	1.889	2.271	2.499	4.878	4.112	1.18	
	1.926	2.271	2.381	4.613	5.405	0.85	
Hemin-MPH	1.898	2.271	2.444	4.688	4.570	1.03	
Hemin-MAGH	1.888	2.273	2.456	4.591	4.408	1.04	
Hemin-MAAH	1.899	2.273	2.449	4.683	4.535	1.03	
	1.926	2.273	2.384	4.538	5.333	0.85	
Hemin-MAH-Py	1.897	2.270	2.441	4.659	4.575	1.02	
Hemin-MAGH-Py	1.896	2.270	2.440	4.639	4.575	1.01	
Hemin-MAAH-Py	1.896	2.274	2.449	4.623	4.514	1.02	
Rabbit liver microsomes[b]	1.912	2.256	2.436	5.132	4.713	1.09	
Rat liver microsomes[b]	1.914	2.244	2.412	5.262	4.917	1.07	
Rat liver microsomes + Aniline	1.912	2.251	2.438	5.256	4.687	1.12	
Hemin[c]-($^-SC_6H_4NO_2$)-N-MeIm	1.91	2.27	2.43[d]	4.77	4.77	1.00	
Hemin[c]-($^-SC_6H_5$)-N-MeIm	1.92	2.26	2.42[d]	5.06	4.93	1.03	
Hemin[c]-($^-$S-Cys(Ac)NHMe)-N-MeIm	1.94	2.24	2.37[d]	5.51	5.63	0.98	$-S^-$–Fe–N
Hemin[c]-($^-SC_6H_5$)-Py	1.92	2.27	2.41[d]	4.77	5.04	0.95	
Hemin[c]-($^-$ S-Cys(Ac)NHMe)-n-$PrNH_2$	1.93	2.23	2.40[d]	5.84	5.16	1.13	
Hemin[c]-(n-$C_4H_9S^-$)$_2$	1.958	2.227	2.310[e]	5.64	6.76	0.83	
Hemin[c]-($^-SCH_2C_6H_5$)$_2$	1.959	2.228	2.302[e]	5.70	6.91	0.82	
Hemin[c]-($^-SC_2H_5$)$_2$	1.936	2.262	2.385[e]	5.01	5.45	0.92	$-S^-$–Fe–S^-
Hemin[c]-(p-$NO_2C_6H_4S^-$)$_2$	1.925	2.274	2.405[e]	4.73	5.15	0.92	
P-450-($C_6H_5CH_2SH$)	1.94	2.25	2.37[e]	5.24	5.65	0.93	
Hemin-Im_2	1.549	2.242	2.982[b]	3.51	1.96	1.79	
Hemin[c]-N-$MeIm_2$	1.57	2.29	2.90[d]	3.32	2.02	1.63	N–Fe–N
Fe(OEP)[f]-N-$MeIm_2$	1.53	2.25	2.96[d]	3.47	1.87	1.86	

a) λ, spin-orbit coupling constant.
b) H. Sakurai, unpublished data.
c) Fe(III)-protoporpyrin IX dimethyl ester.
d) See reference [2a].
e) See reference [11].
f) Fe(III)-octaethylporpyrin.

spin species. In the case of MAH, the same tendency was seen. The principal g values of the EPR spectra of the model complexes at pH 7.2 and at 77 °K are summarized in Table II. The tetragonal (μ) and rhombic (R) splitting parameters of the model systems, representing the distortion in the direction of the axial ligands and the distortion of the heme skeleton in the ferric low spin complex, were estimated using the λ (spin orbit coupling constant[8]) together with the results calculated from the reported g values of other synthetic model complexes[2,11] (Table III). The ratio, μ/R, was introduced to describe the relative distortion around the ferric ion in heme between microsomal cytochrome P-450 and the model systems. The μ and R values and μ/R ratio of liver microsomal P-450 were in the region of 5.1 λ, 4.7—4.9 λ and 1.09—1.12, respectively. Those of the peptide-containing model systems were found to be in the range of 4.5—4.9 λ, 4.1—5.4 λ and 0.85—1.18, respectively. Those of model complexes whose coordination modes were confirmed to be $-S^{-}-$Fe(III)–N,[2a] $-S^{-}-$Fe(III)–$S^{-}-$[13] and N–Fe(III)–N[2a] were in the range of 4.7—5.8 λ, 4.7—5.6 λ and 0.95—1.13, 4.7—5.7 λ, 5.1—6.9 λ and 0.82—0.93, and 3.3—3.5 λ, 1.9—2.0 λ and 1.79—1.86, respectively.

Discussion

In the ferric porphyrin with one axial ligand in the high spin state, the coordination polyhedron is known to be square pyramidal, while that of ferric porphyrin with two axial ligands is octahedral in the low spin state.[12] Even in ferric porphyrin or ferric hemoprotein with two axial ligands, the ferric ion can be in the high spin state provided one ligand bound to iron is long and very weak.[13]

In the case of the model systems presented here, the absorption and EPR spectra suggest the simultaneous formation of 5- and 6-coordinated complexes on complex formation between hemin and peptide. Recently we reported that a high spin species was observed in the absence of pyridine in the hemin-cysteine system by EPR spectroscopy at 77 °K, probably due to the formation of a 5-coordinated complex, and also that the transition of the high spin state of porphyrin observed at 293 °K to the low spin state at 77 °K occurred in the presence of a small amount of pyridine.[14,5] The high spin species was observed even at 77 °K, in spite of the presence of a histidine residue in the peptide presented here. The g values of high spin species were 7.21—7.27 in all the hemin-peptide systems (Table II); these values are very similar to those (g=7.2) of the synthetic 5-coordinated compound which had a thiolate-Fe (III) bond.[2a] Thus, it is suggested that the high spin species in the EPR spectra was a 5-coordinated complex with coordination between hemin and a thiolate group, not an imidazole group, of the peptide.

Distinct differences in the structure of the oxidized complexes of low spin state formed in solution were indicated by comparison of thr ratio, μ/R, calculated from the μ and R values in the EPR spectrum. Ratios of 1.18, 1.03, 1.04 and 1.03 for the MAH, MPH, MAGH and MAAH systems, respectively, are consistent with the $-S^{-}-$Fe(III)–N coordination mode, whereas values of 0.85 in the MAH and MAAH systems correspond to the $-S^{-}-$Fe(III)–$S^{-}-$ coordination mode. After the addition of pyridine, all of the systems exhibited ratios of 1.01—1.02 indicating the formation of the $-S^{-}-$Fe(III)–N coordination mode, and not

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13) W.S. Caughey, *Inorg. Biochem.*, Vol. 2, G.L. Eichhorn Ed., 1973, Chap. 24.
14) H. Sakurai, S. Shimomura, and K. Ishizu, *Chem. Pharm. Bull.* (Tokyo), **25**, 199 (1977).
15) V. Ullrich, H. Sakurai, and H.H. Ruf, Scientific Conference, Cytochrome P-450, Structure and functional aspects, Eberswalde, DDR (1978), and H. Sakurai, H.H. Ruf, and V. Ullrich, *Hoppe-Seyler's Z. Pysiolog. Chem.*, **359**, 1140 (1978).

N–Fe(III)–N coordination. Further, the absorption and EPR spectra of the pyridine-containing system were very similar to those of a six-coordination complex, Fe(III)-protoporphyrin IX dimethylester-4-nitrobenzenthiolate-imidazole.[15] Based on these data, it is also suggested that the coordination mode in the oxidized low spin species of the peptide-containing system was $-S^{-}$–Fe(III)–N (Im or Py) except in the case of the dithiolate complex.

It should be emphasized that judging from the μ and R values in Table III the symmetry around Fe in the oxidized low spin state of microsomal cytochrome P-450 and the model systems presented here is very low. The observation that the effect of pyridine decreased with increasing number of carbon atoms between sulfur and imidazole nitrogen in the peptides indicates that peptides of higher molecular weight form the $-S^{-}$–Fe(III)–N(Im) coordination more easily than lower molecular weight peptides.

Analysis of the g values of the EPR spectrum suggested the simultaneous formation of the following two kinds of complex in all the model systems; a five-coordinated complex of high spin state $-S^{-}$–Fe(III) and a six-coordinated complex of low spin state $-S^{-}$–Fe (III)–N(Im). In MAH or MAAH systems, an additional six-coordinated complex of low spin state $-S^{-}$–Fe(III)–S^{-}– was formed. The dithiolate–hemin complex was characterized as a hyperporphyrin from its absorption spectrum.[11] However, in the system containing MAH

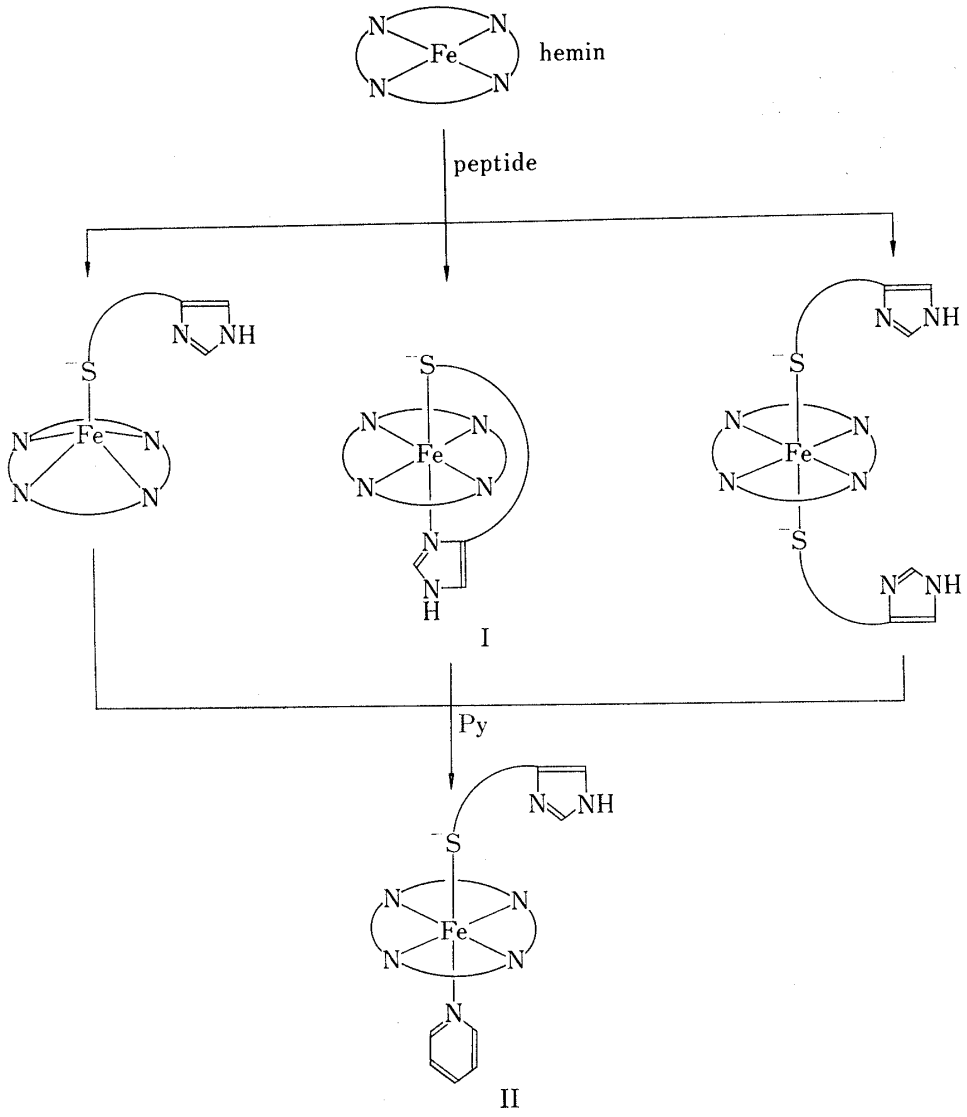

Chart 1

or MAAH, the typical hyperporphyrin spectrum was not observed, indicating the formation of only a small quantity of this complex in solution. Further, a trace amount of dinitrogen complex was detected at $g=2.92$ in the EPR spectrum (Fig. 2); the coordination mode (Im)N–Fe(III)–N(Im) could virtually be excluded, because this type of complex exhibited a very different type of absorption spectrum (absorption maxima of the hemin-$(Py)_2$ complex at pH 7.2 were at 405, 525 and 555 nm).

Pyridine brought about a transformation of mixed spin states of hemin to a single low spin state of hemin (Table I). This indicates the coordination of pyridine to the sixth position of the five-coordinated complex, and ligand exchange of one peptide or coordination group in the six-coordinated complexes, $-S^-$–Fe(III)–S^-– or $-S^-$–Fe(III)–N(Im), respectively, by pyridine to form the $-S^-$–Fe(III)–Py complex.

From the above discussion, the possible reaction sequence and the structures of the complexes formed in solution can be depicted as in Chart 1. In the reaction of hemin and peptide at pH 7.2, a five-coordinated monothiolate high spin state complex and a six-coordinated low spin state complex (I) are formed simultaneously. In the case of MAH and MAAH systems, a small amount of six-coordinated dithiolated complex is formed in addition. When pyridine is added to these systems, a single six-coordinated low spin state complex (II) is formed. We propose here the structures (I) and (II) as possible coordination modes of oxidized low spin state cytochrome P-450, based on our peptide-containing model.

Recently, Chevion *et al.* proposed imidazole of a histidine residue as a candidate for the sixth ligand after an investigation of the EPR ligand field parameters of various thiolate-hemin complexes and cytochrome P-450.[16] In contrast, pulse relaxation NMR studies have been interpreted as favoring a water molecule,[17] R–OH, $R-NH_2$ or $R-CONH_2$[18] at the heme site. However, identification of the true coordination core requires determination of the amino acid sequence of cytochrome P-450 and three-dimensional structure analysis.

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