[Chem. Pharm. Bull. 26(5)1348—1352(1978)]

UDC 547.963.03.04 : 543.422.25.06

A Model of Cytochrome P-450; Optical and EPR Properties of a Thiol-containing Peptide-Hemin System and Its Activity of Aniline Hydroxylation

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(Received July 6, 1977)

The model system consisting of α-mercaptopropionylglycine (α-MPG), hemin and pyridine exhibited similar absorption and electron paramagnetic resonance spectra to cytochrome P-450. In the presence of an excess amount of pyridine, a low-spin state of heme iron was observed at both 293 and 77°K. A transition of the high-spin state of Fe(III) at 293°K to the low-spin state was detected at 77°K in the presence of trace amount of pyridine. When pyridine was omitted from the system, the high-spin state was produced. Under exposure of CO of this model system, the characteristic absorption band of 450 nm was observed, which is typical with cytochrome P-450. Two types of spectral change were obtained when aminopyrine (type I) or aniline (type II) was added to α-MPG-hemin. Spectral dissociation constant K_s of aniline with model system was compared with the reported value with a biological system. It was also discussed that the hydroxylating ability with model and liver microsomal systems.

Keywords——cytochrome P-450; cytochrome P-450 model system; α-mercaptopropionyl glycine; thiol-containing peptide; aniline hydroxylation; cytochrome P-450·CO complex

The unique spectral and electron paramagnetic resonance (EPR) properties of cytochrome P-450, a monooxygenase system containing protoheme, have attracted keen interest because its spectral characteristics differ greatly from those of other hemoproteins. Various P-450 model systems containing heme moiety coordinated with thiol-containing ligand have been investigated.[2-5] However, there are few studies on the hydroxylation of substrates with these systems.[6] We have already reported the hydroxylations of aniline and *p*-toluidine with systems consisting of hemin and various thiol compounds[7,8] which showed similar optical and EPR properties to cytochrome P-450.[9,10]

There are few reports on cytochrome P-450 models using a thiol-containing peptide as a ligand of heme iron. Considering the importance of the coordination of the mercaptide group of a cysteine redidue to the heme site of cytochrome P-450, peptides containing thiol, such as α-mercaptopropionylglycine (α-MPG) seem to be more useful than cysteine itself.

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This paper compares optical and EPR properties of a α-MPG-hemin (Fe(III)-protoporphyrin IX chloride) system with cytochrome P-450, and also reports on the hydroxylation of aniline with this model system and with liver microsomes.

Experimental

α-MPG was a gift from Santen Seiyaku Co. Hemin (Type I, bovine) was obtained from Sigma Chemical Co. Complexes were prepared by mixing α-MPG, hemin and pyridine in 1/15 M phosphate buffer of pH 7.2 in an atmosphere of air or carbon monoxide (CO) respectively. Absorption spectra of the complexes were measured in a Union spectrophotometer, Model SM-302 at 293°K and a Hitachi 356 recording spectrometer at 77°K (2 mm light path). When difference spectra were recorded, the same sample was divided into two cuvettes. Compounds, aniline HCl and aminopyrine dissolved in 1/15 M phosphate buffer, pH 7.2 were added to the sample cuvette and an equal volume of buffer was added to the reference cuvette. The spectral dissociation constant, K_s for aniline, was calculated according to the method of Schenkman.[11] EPR spectra were measured on a frozen glass at 77°K with a JOCO ME-3X spectrometer equipped with a gaussmeter and frequency-counter. Hydroxylation of aniline with the model systems was carried out as reported previously.[7] Hydroxylation of aniline with rat or mouse liver 9000 × *g* supernatant fraction was carried out by the method of Brodie *et al.*[12] The reaction products, *p*- and *o*-aminophenol (*p*- and *o*-AP), were determined by liquid chromatography.[13] Other detailed experimental conditions are given in the legends to Figures or Tables.

Results and Discussion

The optical and EPR spectral properties of the model complexes consisting of α-MPG, hemin and pyridine at physiological pH are summarized in Table I. When low concentra-

Table I. Optical and EPR Spectral Properties of Model Systems

System[a]	Absorption maximum (nm)										EPR spectrum (77°K)					
	293 °K					77 °K					*g*-value					Spin state
Hm-α-MPG	367		510 sh.			415				660	6.64	2.25				High+Low
Hm-α-MPG-Py[b]	365		515			410	541	570 sh.			2.380	2.245	(2.084)	1.946		Low
Hm-α-MPG-Py[c]	365	415	540			410	538	570 sh.		640 sh.	2.447	2.267		1.929		Low
											2.364			1.900		
Hm-α-MPG-Im[d]	367		510 sh.			415	537	560 sh.		640 sh.	6.21	2.26	1.91			High+Low
Hm-α-MPG-Im[e]	367		538	565		415	536	560 sh.			2.868	2.261	1.553			Low
Hm-Py	405				605		528	555			—					—
Hm-Im	400 sh.		435	543	567			540	560		2.902	2.242	1.549			Low
Cytochrome P-450[f]																
oxidivtc high-spin	393		505		650	417	535	566		649[g]	6.6	2.0				High
oxidized low-spin		415	535	565	650						2.41	2.25	(2.00)	1.91		Low

a) Additional abbreviations used; Hm: hemin, Py: pyridine, Im: imidazole.
b), *c*) The concentrations are the same as those shown in Fig. (b) and (c), respectively.
d), *e*) The concentrations of Im are $1.0 \cdot 10^{-3}$ M and $2.0 \cdot 10^{-3}$ M, respectively, and those of other compounds are shown in Fig. 1.
f) C.R.E. Jefcoate and J.T. Gayler, *Biochemistry*, **8**, 3464 (1969).
g) J. Peisach, C.A. Appleby and W.E. Blumberg, *Arch. Biochem. Biophys.*, **150**, 725 (1972).

tions of pyridine (2.25×10^{-3} M) were added to a mixture of α-MPG (6.54×10^{-3} M) and hemin (10^{-4} M) in 1/15 M phosphate buffer, pH 7.2, at 293 °K, absorption maxima at 365 and 515 nm appeared within 3 minutes. This spectrum resembled that of the ferric high-spin cytochrome P-450 which shows absorption maxima at 393 and 505 nm. Thirty minutes later, the maxima changed to 415^{sh}·, 528^{sh}· and 560^{sh}· nm, together with the disappearance of the EPR signals characteristic for oxidized heme. Therefore, the above spectrum corresponds

11) J.B. Schenkman, *Biochemistry*, **9**, 2081 (1970).
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to the reduced form of the complex, and the spectrum resembled that of the reduced cytochrome P-450. On the other hand, at 77 °K, this complex showed absorption maxima at at 410, 541 and 570$^{sh.}$ nm as shown in Fig. 1(b). This spectrum resembled that of the low-spin cytochrome P-450. The EPR spectrum of this complex showed the low-spin characteristics of a porphyrin, and the three well-defined g-values were close to those of oxidized low-spin cytochrome P-450 (Fig. 2(c)).[14]

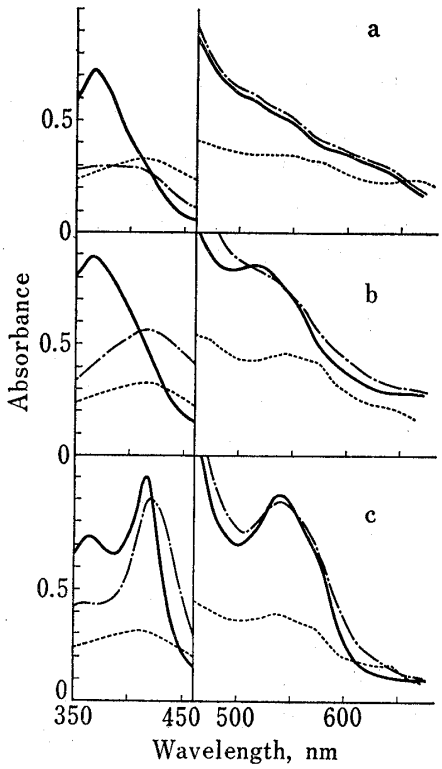

Fig. 1. Absorption Spectra of Model Complexes at pH 7.2

350—460 nm; hemin $2.0\cdot10^{-5}$ M, α-MPG $1.31\cdot10^{-3}$ M, pyridine
a: 0 M, b: $4.6\cdot10^{-4}$ M, c: $4.4\cdot10^{-2}$ M.
450—700 nm; hemin 10^{-4} M, α-MPG $6.54\cdot10^{-3}$ M, pyridine
a: 0 M, b: $2.25\cdot10^{-3}$ M, c: $2.20\cdot10^{-1}$ M.
Temperature; 293°K ———, 1 min after mixing,
———, 30 min after mixing,
77°K ---------, 40 sec after mixing.

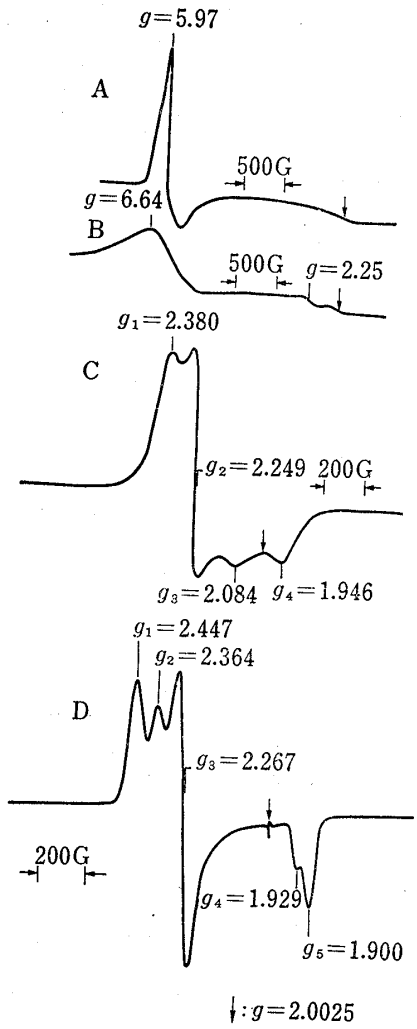

Fig. 2. EPR Spectra of Model Complexes at pH 7.2

A: hemin $5\cdot10^{-3}$ M, B: A+α-MPG $2.0\cdot10^{-1}$ M, C: B+pyridine $5\cdot10^{-2}$ M, D: B+pyridine 6.0 M
All samples were frozen (77°K) within one minute after mixing.

The addition of high concentrations of pyridine (2.20×10^{-1} M) resulted in a spectrum with absorption maxima at 415 and 540 nm at both 293 and 77 °K, corresponding to that of the typical ferric low-spin species of cytochrome P-450 (Fig. 1(c)). The EPR spectrum also showed the low-spin state of ferric porphyrin at 77 °K. However, in this case, two different types of low-spin state of ferric porphyrin (g-value; 2.447, 2.267, 1.900: 2.364, 2.267, 1.929) were observed (Fig. 2(D)).

14) V. Ullrich and Hj. Staudinger, "Concepts in Biochemical Pharmacology, Part 2, Handbook of Experimental Pharmacology," Vol. XXVIII, ed by B.B. Brodie and J.R. Gillette, Springer-Verlag, 1971, pp. 251—263.

In the system consisting of hemin and α-MPG without pyridine, the absorption maxima were observed at 367 and $510^{sh.}$ nm at 293 °K, suggesting the presence of a high-spin species of porphyrin (Fig. 1(a)). The EPR spectrum indicated that the complex was in mixed-spin state at 77 °K (Fig. 2(B)).

From these finding it can be concluded that the spin state of iron in porphyrin depended on the concentration of pyridine. Very similar absorption spectra to those of cytochrome P-450 in high- and low-spin states were obtainable with imidazole. However, a base such as pyridine rather than imidazole was necessary for the appearance of EPR signals similar to those of P-450 in our model system, the three g-values of low-spin species obtained with imidazole being different from those of P-450, suggesting complete ligand exchange of thiolate by imidazole. To obtain an estimate of this system as a model of P-450, we measured its absorption spectrum under an atmosphere of CO at pH 7.2 (Fig. 3). The α-MPG-hemin-pyridine system after the reaction with CO during 15 min showed an absorption maximum at 448 nm, using a mixture of α-MPG, hemin and pyridine as reference. It was considered that during 15 min ferric heme was reduced to ferrous form from the fact above mentioned. This is the typical absorption maximum of the ferrous P-450 CO complex. In the absence of any one of these four components there was no maximum at 450 nm. Hence, a combination of α-MPG, hemin and pyridine is essential for this absorption band. From this observation, the following reaction mechanism was assumed. The axial pyridine in α-MPG sulfur-heme iron-pyridine nitrogen structure may be substituted for CO. The reason for this phenomenon was not examined in detail, and further investigations are under way.

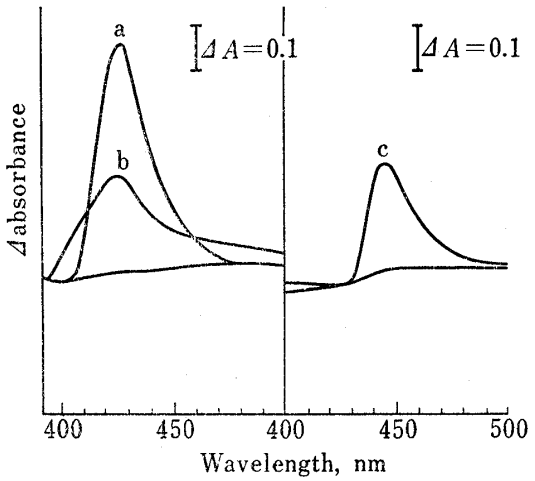

Fig. 3. Difference Spectra of Model Complexes under an Atmosphere of CO at pH 7.2

a : hemin (10^{-4} M)+pyridine ($4.4 \cdot 10^{-1}$ M).
b : hemin (10^{-4} M)+α-MPG ($1.31 \cdot 10^{-2}$ M).
c : a+α-MPG ($1.32 \cdot 10^{-2}$ M).
Difference spectrum was recorded after bubbling with CO during 15 min into the mixture of hemin, α-MPG and pyridine.

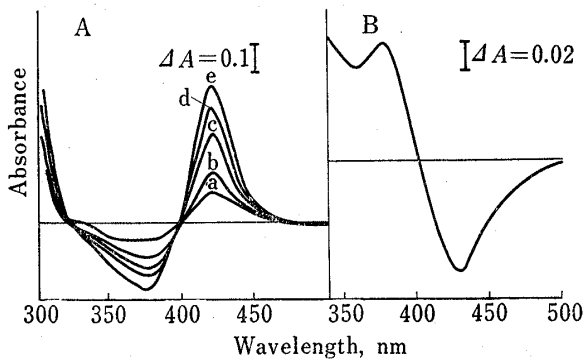

Fig. 4. Difference Spectra of Model Complex with Aniline (A) or Aminopyrine (B) at pH 7.2

hemin $4.29 \cdot 10^{-5}$ M, α-MPG $5.61 \cdot 10^{-3}$ M
A : aniline; a: $1.43 \cdot 10^{-3}$ M, b: $2.86 \cdot 10^{-3}$ M, c: $5.71 \cdot 10^{-3}$ M
d: $8.55 \cdot 10^{-3}$ M, e: $1.14 \cdot 10^{-2}$ M.
B : aminopyrine; $1.14 \cdot 10^{-2}$ M.

Two different types of the spectral changes are observed when substrates (Type I) and basic amines (Type II) interact with microsomal P-450.[11] Similar spectral changes were obtained with α-MPG-hemin instead of P-450 (Fig. 4). The type II-like spectral change was shown by the appearance of an absorption peak at 420 nm and the trough at 375 nm by aniline. Also, the type I-like spectrum was characterized by the appearance of an absorption peak at 378 nm and the trough at 430 nm when aminopyrine was added as a substrate. When cysteine was used instead of thiol-peptide, α-MPG, the type I-like spectrum was not

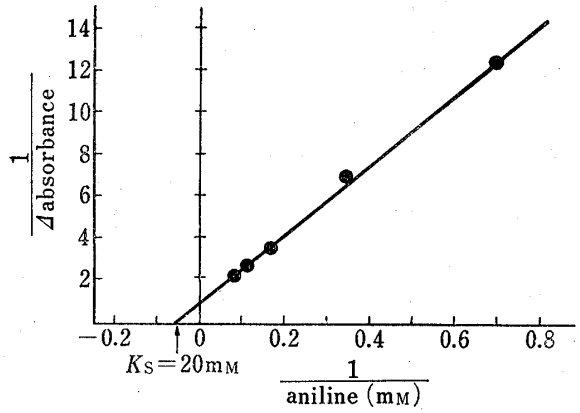

Fig. 5. Double-Reciprocal Plot of Aniline-Induced Spectral Change

obtained. Fig. 5 shows the double-reciprocal plot of the spectral change at various concentrations of aniline. A calculated spectral dissociation constant, K_s, for aniline was 20 mM. The K_s value for aniline with rat liver microsomes was reported as 0.3 mM[11]. It is indicated that the binding ability of aniline to the heme site of model system is very low compared with that of biological systems.

Table II shows the results of hydroxylations of aniline with hemin and Fe(II) systems containing α-MPG or α-MPA (α-mercaptopropionic acid) compared with those with liver microsomal systems. Hemin exhibited higher hydroxylation activity than Fe(II), and the activities of systems containing α-MPG were higher at pH 6 than at pH 4. Further, the peptide, α-MPG, was more effective in aniline hydroxylation than α-MPA. The hydroxylation yield by α-MPG-hemin system at pH 6 was approximately one seventieth of that by rat liver microsomes. It is interesting that different ratios of *p*-AP to *o*-AP (*p*/*o* ratios) were obtained in the products with the systems containing α-MPG and α-MPA. The *p*/*o* ratios obtained with the model systems were lower than those obtained with the microsome systems. This indicates that in model systems the hydroxylation is influenced by some factors, such as the solvent effect or polarity, tertiary structure of the peptide and steric interactions.

Table II. Hydroxylation of Aniline by Model and Biological Systems

System	pH	Incubation time (hr)	Exper. number	Product (μg) *p*-Ap	Product (μg) *o*-Ap	*p*/*o* ratio	Yield[a] (%)
FeII-α-MPG	4	2	7	12± 5	14± 3	0.82±0.16	0.024
	6	2	4	195±37	320±12	0.61±0.12	0.47
Hm-α-MPG	4	2	3	198±67	307±70	0.64±0.10	0.46
	6	2	4	157±71	360±19	0.45±0.20	0.47
FeII-α-MPA	6	2	4	150±14	139±21	1.09±0.07	0.26
Hm-α-MPA	6	2	4	209± 8	145±20	1.47±0.18	0.32
Rat liver[b] Microsome	7.4	0.5	8	161±36	25± 4	6.5 ±1.7	8.5
Mouse liver[b] Microsome	7.4	0.5	8	108±14	12± 3	9.4 ±2.1	5.5

a) Yield based on initial aniline concentration; model system: 1 mmol, biological system: 20 μmol.
b) Male ddY strain mice, weighing 20 g, and male Wister strain rats, weighing 120 g, were used. Each animal was treated with phenobarbital 50 mg/kg/day injected *i.p.* for 3 days. The reaction mixture, containing 25 mM phosphate buffer (pH 7.4), 100 μmol $MgCl_2$, 10 μmol G-6-P, 2 μmol NADPH and 9000 *g* supernatat (equivalent to 800 mg of liver) in a final volume of 8.0ml was incubated at 37° for 30 minutes.

This paper shows that α-MPG-hemin system resembles cytochrome P-450 both in spectral properties and aniline hydroxylation activity. The presence of a heme iron-sulfur bond is necessary, for the appearance of the unique spectral properties. Also iron thiol and hemin thiol systems are regarded as good chemical models of cytochrome P-450 monooxygenases.

Acknowledgement We thank Professor V. Ullrich, University of the Saarland, G.F.R. for helpful suggestions and a reading of the manuscript.