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A Model System of Cytochrome P-450: Hydroxylation of Aniline by Iron- or Hemin-Thiol Compound Systems

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Hydroxylation of aniline by several Fe(II)-thiol and hemin-thiol complexes was studied as a model system for cytochrome P-450 in liver microsomes. Cysteine, cysteine methylester, cysteamine, N-acetyl-cysteine, α-mercaptopropionic acid, β-mercaptopropionic acid, thiosalicylic acid and *o*-aminobenzenethiol were tested as thiolcompounds. In these systems, *p*- and *o*-aminophenol were the major products and *m*-aminophenol was not detected. The hydroxylation was affected by reaction time, pH, the type of thiol compound and the concentrations of thiol compound, aniline, Fe(II) and hemin. The aniline-hydroxylating activity of the hemin-thiol system was 6—20 times that of the Fe(II)-thiol system at pH 4. The Fe(II)-thiol and hemin-thiol systems may be useful chemical models for studies of the structure and function of cytochrome P-450.

Keywords——cytochrome P-450; monooxygenase; model system; Fe(II)-thiol complex; hemin-thiol complex; aniline hydroxylation; aminophenol; thiol compound

The liver microsomal monooxygenases are important drug-metabolizing enzymes.[2] These enzymes catalyze the hydroxylation of a wide variety of substrates. Cytochrome P-450, a monooxygenase containing protoheme, activates molecular oxygen and introduces one oxygen atom into a substrate.[3] Its activity is accompanied by spectroscopic changes which are unique among heme proteins.[3] The nature of the axial ligand at the heme may be an essential factor in the catalytic and spectroscopic properties.

The presence of a thiolate ligand in the fifth coordination position of heme in cytochrome P-450 has been postulated on the basis of EPR (electron paramagnetic resonance) and optical spectra[4–6] obtained from synthetic model systems.[7–10]

In order to elucidate the mechanisms of oxygen activation and hydroxylation by P-450, many chemical systems that hydroxylate organic compounds have been investigated.[11,12]

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An important feature of the model systems is whether the hydroxylation simulates typical enzymic properties such as specific hydroxylation of aliphatic compounds, the electrophilic hydroxylation of aromatic compounds, epoxidation of carbon-carbon double bonds and migration of substituents during aromatic hydroxylation, "NIH shift".[11] The Udenfriend system,[13] which is a well-known oxygen activation system consisting of a transition metal and reducing agent, does not cause epoxidation and NIH shift.[14] In the case of trifluoroperacetic acid or photolysis by aromatic N-oxides the occurrence of NIH shift has been reported.[15] However, many proposed model systems of cytochrome P-450[12] are suspect because the conditions in these systems are not physiological.

Ullrich has demonstrated that the Fe(II)-thiosalicylic acid-oxygen system hydroxylates several types of substrate.[16] This system hydroxylated several organic compounds in a manner similar to that of enzymic hydroxylation. It was reported that this system was also effective when Fe(II) was replaced by hemin.[17]

In a preliminary paper, we reported the hydroxylation of aniline with systems consisting of Fe(II) or hemin and various thiol compounds.[18] This paper deals with the hydroxylation of aniline with several Fe(II) or hemin-thiol compound-oxygen systems as a model for cytochrome P-450.

Materials and Methods

Thiosalicylic acid (TS), cysteine (Cys), cysteine methylester (CM), N-acetylcysteine (NAC), cysteamine (CA), α- and β-mercaptopropionic acid (α- and β-MPA) and *o*-aminobenzenethiol (ABT) were purchased from Nakarai Chemicals Co., Kyoto, or Wako Pure Chemicals Co., Osaka. Cristaline Fe(III)-protoporpyrin IX chloride (hemin chloride, bovine type I) was obtained from Sigma, St. Louis. Aniline, acetone, ether and chloroform were purified by distillation. Thiol compounds were dissolved in acetone, water or 0.1 M to 1.0 M sodium hydroxide immediately before use. The reaction mixture contained thiol compound (RSH), ferrous sulfate heptahydrate (Fe(II)) or hemin chloride and aniline in 10 ml of 80% aqueous acetone. The pH was adjusted with 1 M hydrochloric acid and 1 M sodium hydroxide. The reactions were carried out at 40° for various periods with vigorous shaking in air, and were stopped by addition of 0.5 ml of 2 M hydrochloric acid. Other experimental conditions are given in the legends to figures and tables. The reaction products were identified by two-dimensional thin-layer chromatography (TLC) and high-speed liquid chromatography (HLC). The products were separated on cellulose-silica gel G (5 : 2) plates with benzen: methanol: acetic acid (135 : 24 : 12) as the first developing solvent and chloroform: isopropanol: ammonium hydroxide (16 : 3 : 1) as the second developing solvent. Detection was carried out with iodine vapor. Separation of products by HLC was carried out at 22° on a Du Pont analytical column packed with Zipax SCX (1 m × 2.1 mm i.d.) The eluent was 0.1 M KH_2PO_4–0.1 M H_3PO_4 (pH 2.9). Eluted products were detected by their UV absorption at 254 nm in a flow cell. Quantitative determination of reaction products was achieved by HLC under the conditions mentioned above.[19]

Results

A model system consisting of Fe(II) and cysteine hydroxylated aniline to yield *p*-aminophenol (*p*-AP) and *o*-aminophenol (*o*-AP) as major products, as determined by TLC and HLC. *m*-Aminophenol (*m*-AP) was a minor product.[19] The yields of *p*-AP and *o*-AP were determined by HLC and the ratios of *p*-AP to *o*-AP (*p*/*o* ratio) were calculated.

Figure 1 shows the time course of aniline hydroxylation with the Fe(II)-thiosalicylic acid and the Fe(II)-cysteine systems. In the thiosalicylic acid system, maximum yields of

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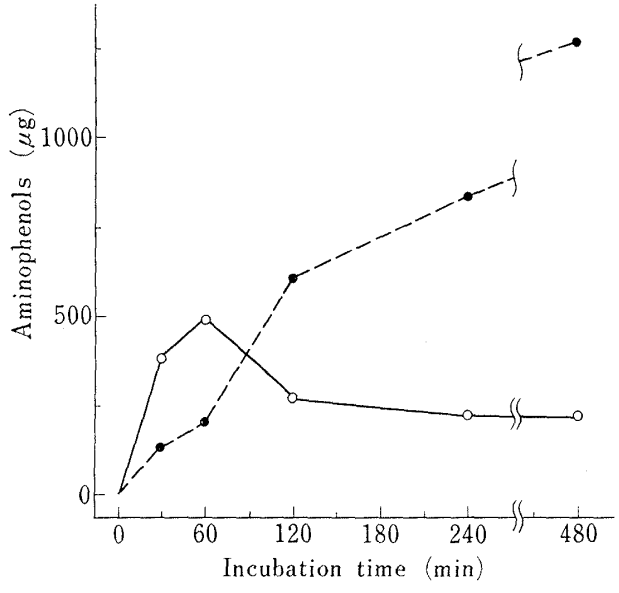
p-AP and *o*-AP were obtained after 1 hour. After 2 hours, the yield of *p*-AP was considerably decreased; however, the reason for this was not examined. The total yields of APs were nealy constant after 2 hours, and the *p*/*o* ratio was approximately one. In the cysteine system, the yields of both APs increased with reaction time. After 2 hours, the *p*/*o* ratio remained at about 2.5. The results of hydroxylation of aniline at various pH values are shown in Fig. 2. In both the Fe(II)-thiosalicylic acid and Fe(II)-cysteine systems the hydroxylations were enhanced in the range of pH from 4 to 7, but not in the alkaline range (pH 8 and 9), probably due to decomposition of the products or Fe(II)-thiol complexes. The yields of APs in the

Table I. Effect of Aniline Concentration on Its Hydroxylation by the Fe(II)-Thiosalicylic Acid (TS) and the Fe(II)-Cysteine (Cys) Systems

System	Mol ratio aniline/Fe(II)	Aminophenols (μg)	*p*/*o* ratio
Fe(II)-TS	5/1	120	1.07
	10/1	328	1.29
	50/1	395	1.16
Fe(II)-Cys	5/1	159	1.04
	10/1	251	1.73
	50/1	178	0.93

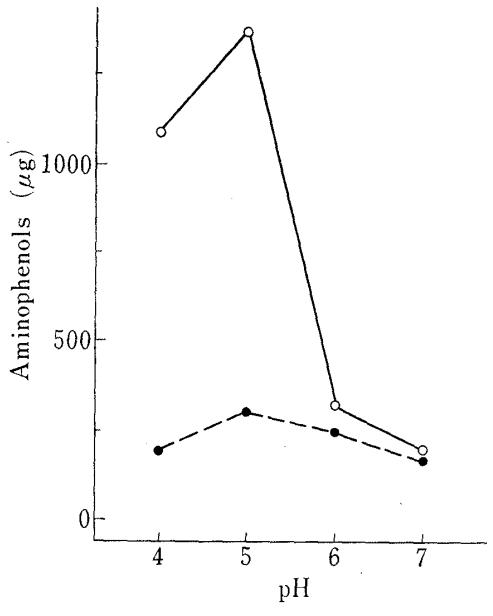
The reaction mixtures contained 10^{-2} M Fe(II), 10^{-1} M TS or Cys and 5×10^{-2}, 10^{-1} or 5×10^{-1} M aniline in 10 ml of aqueous acetone solution (acetone 80%) and were incubated for 45 minutes at pH 6. Each value is the mean of two experiments.



System	*p*/*o* ratio				
min	30	60	120	240	480
Fe(II)-TS	2.15	2.27	1.18	0.72	0.95
Fe(II)-Cys	1.78	1.35	2.55	2.58	2.44

Fig. 1. Effect of Incubation Time on the Hydroxylation of Aniline by the Fe(II)-Thiosalicylic acid (TS) and the Fe(II)-Cysteine (Cys) Systems

The reaction mixtures contained 10^{-2} M Fe(II), 10^{-1} M TS or Cys and 10^{-1} M aniline in 10 ml of aqueous acetone solution (acetone 80%), and were incubated at pH 6. ○—○; Fe(II)-TS system, ●—●; Fe(II)-Cys system. Each point respresents the mean of two experiments.



System	*p*/*o* ratio			
pH	4	5	6	7
Fe(II)-TS	1.27	1.03	1.29	1.00
Fe(II)-Cys	0.62	1.25	1.73	14.63

Fig. 2. Effect of pH on the Hydroxylation of Aniline by the Fe(II)-Thiosalicylic Acid (TS) and the Fe(II)-Cysteine (Cys) Systems

The reaction mixtures contained the same concentrations of reagents as in Fig. 1, and were incubated for 45 minutes. ○—○; Fe(II)-TS system, ●···●; Fe(II)-Cys system. Each point represents the mean of two experiments.

Table II. Effect of Fe(II) Concentration on the Hydroxylation of Aniline by the Fe(II)-Thiosalicylic Acid (TS) and the Fe(II)-Cysteine (Cys) Systems

System	Concentration of Fe(II)	Incubation time (hr)	Aminophenols (μg)	*p*/*o* ratio
Fe(II)-TS	10^{-2}M	1	490	2.27
		2	339	1.65
		4	296	1.28
	10^{-3}M	1	189	1.74
		2	175	1.30
		4	275	2.31
Fe(II)-Cys	10^{-2}M	1	215	1.44
		2	618	2.59
		4	846	2.62
	10^{-3}M	1	248	2.06
		2	638	1.65
		4	1201	1.66

The reaction mixtures contained the same concentrations of reagents as in Fig. 1 except for Fe-(II), and were incubated at pH 6. Each value is the mean of two experiments.

Table III. Effects of Thiol Compounds on the Hydroxylation of Aniline by the Fe(II)-Thiol and the Hemin-Thiol Systems

Thiol compound	Iron	pH	Aminophenols (μg)	*p*/*o* ratio
Cysteine	Fe(II)	4	162±12	1.92±0.36
		6	638±20	1.65±0.02
	Hemin	4	1599±100	1.02±0.07
		6	803±48	1.53±0.09
Cysteine methylester	Fe(II)	4	169±31	2.35±0.21
		6	160±34	3.28±1.24
	Hemin	4	1061±61	2.24±0.02
		6	1186±116	2.09±0.15
Cysteamine	Fe(II)	4	23	—
		6	0	—
	Hemin	4	452±71	5.79±0.92
		6	230±32	2.41±0.45
N-Acetylcysteine	Fe(II)	4	295±41	0.97±0.13
		6	—	—
	Hemin	4	1023±37	0.99±0.03
		6	—	—
α-Mercaptopropionic acid	Fe(II)	4	308±28	0.97±0.05
		6	288±34	1.09±0.07
	Hemin	4	1565±141	1.14±0.16
		6	353±25	1.47±0.18
β-Mercaptopropionic acid	Fe(II)	4	10±10	—
		6	613±108	1.46±0.13
	Hemin	4	1555±99	1.26±0.13
		6	351±20	2.25±1.05
Thiosalicylic acid	Fe(II)	4	1237±170	0.62±0.04
		6	175±16	1.30±0.14
	Hemin	4	737±104	0.39±0.07
		6	198±6	1.29±0.03
o-Aminobenzenethiol	Fe(II)	4	414±72	0.67±0.09
		6	231±35	0.54±0.09
	Hemin	4	862±62	1.01±0.03
		6	257±52	1.81±0.40

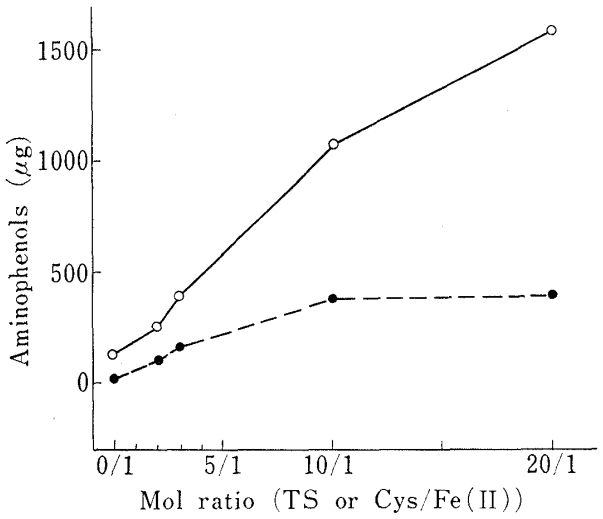
The reaction mixtures contained 10^{-3} M Fe(II) or hemin, 10^{-1} M thiol compound and 10^{-1} M aniline, and were incubated for 2 hours. Each value is the mean ± S.D. of four experiments except in the case of cysteamine (the mean of two experiments).

thiosalicylic acid system were pH-dependent, and the value of the *p/o* ratio was approximately one. On the other hand, the yields of APs in the cysteine system were pH-independent, and were less than those in the thiosalicylic system; the *p/o* ratio in the cysteine system ranged from 0.6 (pH 6) to 14.6 (pH 7).

Figure 3 shows the aniline-hydroxylation activities with various concentrations of thiosalicylic acid and cysteine. The yield of APs increased with the concentration of thiol compounds. The *p/o* ratios were approximately constant in both systems.

The presence of excess aniline with respect to Fe(II) was desirable to obtain maximum hydroxylation in both the thiosalicylic acid and cysteine systems (Table I). In the latter system, however, the formation of APs reached a maximum in the presence of a 10-fold molar excess of aniline to Fe(II).

The concentration of Fe(II) also affected the hydroxylation of aniline (Table II). Higher activity was obtained with 10^{-2} M Fe(II) than with 10^{-3} M in the thiosalicylic acid system. However, in the cysteine system 10^{-3} M Fe(II) hydroxylated aniline effectively.



System	*p/o* ratio				
	0/1	2/1	3/1	10/1	20/1
Fe(II)–TS	—	1.59	1.83	1.25	1.12
Fe(II)–Cys	—	1.27	1.23	1.04	0.79

Fig. 3. Effect of Concentration of Thiol Compound on the Hydroxylation of Aniline by the Fe(II)-Thiosalicylic Acid (TS) and the Fe(II)-Cysteine (Cys) Systems

The reaction mixtures contained 10^{-2} M Fe(II), 10^{-1} M aniline and 2×10^{-2}, 3×10^{-2}, 10^{-1} or 2×10^{-1} M TS or Cys in 10 ml of aqueous acetone solution (acetone 80%), and were incubated for 45 minutes at pH 4.
○—○; Fe(II)–TS system., ●···●; Fe(II)–Cys system.
Each point represents the mean of two experiments.

The hydroxylation activities with eight thiol compounds as ligands for Fe(II) are summarized in Table III. The hydroxylations were stimulated in the systems containing an aromatic thiol compound such as TS or ABT at pH 4, and in the systems containing an aliphatic thiol compound such as Cys or β-MPA at pH 6. The *p/o* ratios were larger than in the systems using aliphatic thiol compounds but smaller than in the systems with aromatic thiol compounds.

In order to study a model system resembling cytochrome P-450 more closely, hemin was used instead of Fe(II). As shown in Table III, in the presence of hemin the hydroxylations were promoted at pH 4 rather than pH 6 in all the systems investigated.

When methionine was used in place of the thiol compound, no aniline-hydroxylation activity was observed in the system containing Fe(II) or hemin.

Discussion

Based on the results for the system containing Cys, which is assumed to be an axial iron ligand in cytochrome P-450, the optimal reaction conditions giving the maximum yield of APs in the hydroxylation of aniline were as follows; 1) pH 6 for Fe(II) and 4 for hemin, 2) high concentration of cysteine (more than 10-fold molar excess relative to Fe(II)), 3) about 10-fold molar excess of aniline relative to Fe(II).

It is interesting that the presence of excess thiol compound promoted the hydroxylation of aniline in this model system. The finding that methionine was inactive in the hydroxylation of aniline indicates that the interaction of a thiol group with iron plays an essential role in the hydroxylation reaction in this model system. Therefore, it is assumed that the thiol group binds to Fe(II) or hemin (Fe(III)-porphyrin) to form an Fe(III)-S coordination, and

excess thiol readily reduces the hemin-thiol complex to Fe(II)-thiol complex.[20] Ullrich stated that the Fe(II)-thiosalicylic acid complex activated molecular oxygen with two-electron transfer.[16] Thus, the Fe(II) or hemin- thiol complex may play a major role in the activation of oxygen in the model systems, as illustrated in Chart 1.

In the hemin systems containing Cys, CM and CA the hydroxylations were enhanced at pH 4 rather than at pH 6. The activity at pH 4 was about 2—4 times that at pH 6. When Fe(II) was replaced with hemin the hydroxylation activity was enhanced approximately 6—20 times at pH 4.

In the hydroxylation of aniline with the Fe(II)- and hemin-thiol systems, a hydroxy group was in troduced into the *para*- and *ortho*-positions of the aromatic ring. The hydroxylation position (*para* or *ortho*) depended on the pH and reaction time. The results in Table III suggest that the systems with aliphatic thiol compounds containing an amino group, such as CM and CA, may show preferred *para* rather than *ortho* hydroxylation of aniline, whereas those with aromatic thiol compounds, such as TS and ABT, stimulate *ortho* rather than *para* hydroxylation.

Recently, it was found that some Fe(II)-thiol and hemin-thiol systems give reactions similar to those of monooxygenases[21] (the methyl migration during the hydroxylation of *p*-toluidine), and a hemin-thiol system containing cysteine or cysteine methylester required a dielectric constant in the range of 30—40 for the most effective hydroxylation of aniline.[22] It was also reported that the hemin-cysteine-pyridine complex resembles cytochrome P-450 in both optical and EPR spectral properties.[23] Thus, the Fe(II)-thiol and especially the heminthiol systems can be regarded as good chemical models for cytochrome P-450. The presence of a thiolate ligand, presumably a cysteinate residue, in the fifth coordination position of heme has been postulated to be an essential feature of cytochrome P-450.[4–10] Our model system, however, required an acidic pH region for optimal aniline-hydroxylation activity, which is not consistent with the thiolate structure proposed by many researchers,[4–10] since in the acidic pH region the thiol group of a low molecular compound is generally in the undissociated form.[24] Further studies are required on the activation of molecular oxygen by our model systems in the acidic pH region.

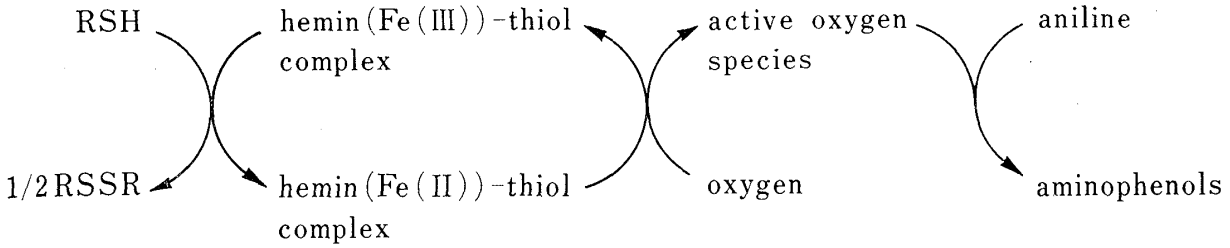


Chart 1

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