# Aromatic Hydroxylation of Acetanilide and Aniline by Hemin–Thiolester Complex as a Cytochrome P-450 Model

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Received February 4, 1983

*Acetanilide and aniline hydroxylations with a hemin–thiol compound and Cu(II)-containing systems were studied under various conditions as models of cytochrome P-450 monooxygenases. Thereby the determination method of acetaminophenols was established using liquid chromatography. The reactions were characterized by the yield of hydroxylated products and the product ratio. The model system containing thioglycolate ester simulated the acetanilide hydroxylation with liver microsomal fraction in both conversion of the substrate and product ratio (p-:m-:o-acetaminophenol). Hydroxylation of acetanilide and aniline was enhanced upon addition of additional reducing agents or thiol compounds to the hemin–thioglycolate ester complex, suggesting an involvement of the similar reaction profile of cytochrome P-450 monooxygenases requiring NAD(P)H as a reducing agent. The hemin–thioglycolate ester complex is thus concluded to be a good chemical model of cytochrome P-450-dependent monooxygenases.*

## Introduction

Cytochrome P-450 is a heme-protein capable of activating dioxygen for its insertion into biomolecules and xenobiotics [1]. Unusual optical and EPR properties of cytochrome P-450 were observed by Omura and Sato [2] and Hashimoto *et al.* [3], respectively. These properties are attributed to the non-replaceable thiolate ligand, peptide cysteinate, to heme-iron in this enzyme [4]. A thiolate ligand in the 5-th coordination position of the heme has been postulated to be essential on the basis of EPR [5–7] and optical [7–9] studies with model complexes. In our preceding papers on aniline- and *para*-toluidine hydroxylation [10–15], we demonstrated that hemin–thiol complexes retaining sulfur-heme iron linkage operated like cytochrome P-450 monooxygenases, indicating that the sulfur-heme binding was also essential for the function of cytochrome P-450.

During investigations on the ability of hemin–thiol complexes which may serve as models of cytochrome P-450, we found that a hemin–thioglycolate ester complex mimics both the spectroscopic properties and hydroxylation activities on cytochrome P-450. This paper reports the results of hemin–thiolester activities in hydroxylation of aromatic substances such as acetanilide and aniline compared with those of Cu(II)-containing model systems and liver microsomal systems. This paper also presents a new type of cytochrome P-450 model where aromatic hydroxylation was enhanced by the addition of electron donating compounds, simulating the reaction of NAD(P)H-dependent cytochrome P-450 monooxygenases.

## Experimental

### *Materials*

Hemin (Type I, bovine) and *dl*-isocitrate dehydrogenase were obtained from Sigma Chemical Co. NADPH, $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were products of Oriental Yeast Co. *dl*-Isocitric acid trisodium salt and cysteine ethyl ester·HCl were purchased from Nakarai Chemicals. Thioglycolate esters were from Tokyo Kasei KK. Cysteine and thioglycolic acid were products of Wako Chemicals.

### *Preparation of Liver 105,000 × g Microsomal Fractions*

Male rats of Wister strain, weighing 174–188 g, were used. The animals were fasted overnight prior to being killed, but were given tap water *ad lib.* Pretreatment of the animals with sodium phenobarbital was performed by intraperitoneal injection at a daily dose of 50 mg per kg body wt. for 4 consecutive days. The

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Abbreviations used: Hm, hemin; Cys, cysteine; CysM, cysteine methyl ester; CysE, cysteine ethyl ester; Pen, penicillamine; TG, thioglycolic acid; TGE, thioglycolate ethyl ester; TGB, thioglycolate n-butyl ester; TGO, thioglycolate n-octyl ester; TGEH, thioglycolate 2-ethyl-hexyl ester; TGOD, thioglycolate octadecyl ester; Asc, ascorbate; GSH, glutathione; *p*-AP and *o*-AP, *para*- and *ortho*-aminophenol; *p*-, *m*- and *o*-AAP, *para*-, *meta*- and *ortho*-acetaminophenol; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance.

0020-1693/83/$3.00

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livers, removed and chilled on ice, were homogenized in 3 volumes of 1.15 *M* KCl and centrifuged at 800 × g for 10 min. The supernatant was further centrifuged at 9000 × g for 10 min. The post-mitochondrial fraction was centrifuged at 105,000 × g for 60 min to obtain the microsomal fraction. The pellet was resuspended in 1.15 *M* KCl. The protein concentration was determined by the method of Lowry *et al.* [16] using bovine serum albumin as standard. The cytochrome P-450 content was estimated as described by Omura and Sato [17].

*Analysis for Aminophenol and Acetaminophenol Isomers*

The reaction products, *para(p)-*, *meta(m)-* and *ortho(o)*-aminophenol, were determined by liquid chromatography as reported previously [10, 18]. The separation of *p-*, *m-* and *o*-acetaminophenol was carried out on a Chemco $5C_{18}$ column ($\phi$ 4.0 mm × 200 mm) mounted on an Altex 330 high performance liquid chromatograph operated at 254 nm, with a *M*/30 $Na_2HPO_4$–*M*/30 $H_3PO_4$ buffer containing 20% (v/v) methanol (pH 6.0) as mobile phase at a flow-rate of 0.8 ml $min^{-1}$ and room temperature (22 °C). Samples of 10 $\mu$l were injected.

*Assay Method using Model Systems*

The standard hydroxylation system consisted of 1 m*M* hemin, 100 m*M* thiol compound, 100 m*M* substrate, buffer solution (pH 3.0–7.0) and acetone. Hydroxylation of substrate with the model systems was carried out as reported previously [10]. Other detailed experimental conditions are given in the legends.

*Assay Method using Rat Liver 105,000 × g Microsomal Fractions*

In the standard reaction mixture for the assay of microsomal acetanilide-hydroxylation activity, the following three methods were applied. The first system (A) contained, in a final volume of 5.0 ml, microsomes (10 mg of protein), 20 m*M* acetanilide, 100 m*M* phosphate buffer (pH 7.4), 0.1 m*M* EDTA and an NADPH-generating system with 0.33 m*M* $NADP^+$, 8 m*M* glucose-6-phosphate, 6 m*M* $MgCl_2$ and 0.5 unit of glucose-6-phosphate dehydrogenase [19]. The second system (B) contained, in a final volume of 5.0 ml, microsomes (10 mg of protein), 50 m*M* Tris-HCl buffer (pH 7.5), 20 m*M* acetanilide and an NADPH-generating system consisting of 1 m*M* $NADP^+$, 5 m*M* *dl*-isocitrate, 0.55 unit of *dl*-isocitrate dehydrogenase and 5 m*M* $MgCl_2$ [20]. The third system (C) contained in a final volume of 5.0 ml, microsomes (25 mg of protein), 2 m*M* NADPH, 2 m*M* $NADP^+$, 0.1 *M* phosphate buffer (pH 7.4) and 20 m*M* acetanilide [21]. The reaction was carried out aerobically at 37 °C for 30 min and the hydroxylated products were determined as described above.

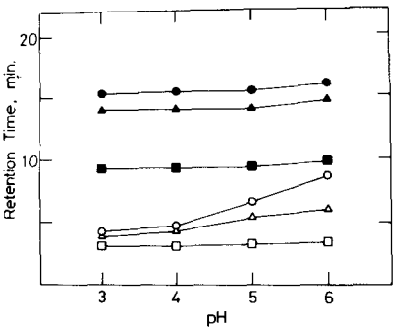


Fig. 1. pH Effect of mobile phase on retention time of acetaminophenol and aminophenol isomers. Eluent, *M*/30 $Na_2HPO_4$–*M*/30 $H_3PO_4$ containing 20% (v/v) methanol; flow-rate, 0.8 ml $min^{-1}$. –□–, *p*-aminophenol; –△–, *m*-aminophenol; –○–, *o*-aminophenol; –■–, *p*-acetaminophenol; –▲–, *m*-acetaminophenol; –●–, *o*-acetaminophenol.

## Results

*Detection of Acetaminophenol and Aminophenol Isomers*

The optimal conditions for detecting three acetaminophenol and three aminophenol isomers as the reaction products were examined; they were simultaneously separated using the mobile phase of *M*/30 $Na_2HPO_4$–*M*/30 $H_3PO_4$ buffer solutions containing 0–20% (v/v) methanol on a Necleosil $5C_{18}$ column at pH 3.0–6.0. The time required for separation was shortened with increased methanol content. When the pH of the eluting solution was lowered the resolution was inadequate. When the pH of the buffer system was elevated, the separation was improved, although the retention time was not significantly changed (Fig. 1). Hence, the *M*/30 $Na_2HPO_4$–*M*/30 $KH_2PO_4$ buffer containing 20% methanol at pH 6.0 was chosen as an optimal condition for further study. The amounts and peak-heights of the three acetaminophenols were well correlated over the ranges studied (0–10 nmol for *p*-acetaminophenol, 0–20 nmol for *m*-acetaminophenol and 0–40 nmol for *o*-acetaminophenol). The average recovery of the compounds was higher than 90%. Under these experimental conditions, it was confirmed that acetanilide was hydroxylated by the model system, the products being *p-*, *m-* and *o*-acetaminophenols. The deacetylated product and subsequently hydroxylated products were not detected.

*Aniline Hydroxylation by Model Systems*

Our previous study [10] showed that aniline, known as a type II substrate [22], was hydroxylated by a hemin and thiol compound at pH 5.0 in 50% acetone solution. For the present system consisting of hemin and thioglycolate ester, the assay condition was tested and a pH of 4.0 in 80% acetone was found to be optimal (Fig. 2). Therefore, aniline hydroxyla-

TABLE I. Hydroxylation of Aniline by Various Systems.

| System[a] | pH | Acetone (%) | Total Yield (μmol) | Conversion (%/2 hr) | Product Ratio *p*-AP:*o*-AP |
|---|---|---|---|---|---|
| Hm–Cys | 5 | 50 | 10.51 ± 0.19 | 1.05 | 35.6:64.4 |
| –CysM | 5 | 50 | 3.44 ± 0.17 | 0.34 | 41.0:59.0 |
| –CysE | 5 | 50 | 2.85 ± 0.17 | 0.29 | 42.1:57.9 |
| –Pen | 5 | 50 | – | – | |
| –TG | 4 | 50 | 2.72 ± 0.20 | 0.27 | 64.3:35.7 |
| –TGE | 4 | 80 | 10.17 ± 0.12 | 1.02 | 43.4:56.6 |
| –TGB | 4 | 80 | 10.51 ± 0.21 | 1.05 | 44.0:56.0 |
| –TGO | 4 | 80 | 10.43 ± 0.57 | 1.04 | 48.1:51.9 |
| –TGEH | 4 | 80 | 9.49 ± 0.60 | 0.95 | 45.4:54.6 |
| –TGOD | 4 | 80 | 6.99 ± 0.20 | 0.70 | 41.9:58.1 |
| Hm alone | 4 | 80 | – | – | |
| Hm–TGEH (10 m*M*) | 4 | 80 | 0.86 ± 0.12 | 0.09 | 58.1:41.9 |
| (25 m*M*) | 4 | 80 | 1.86 ± 0.35 | 0.19 | 58.1:41.9 |
| (50 m*M*) | 4 | 80 | 8.23 ± 0.23 | 0.82 | 46.9:53.1 |
| (100 m*M*) | 4 | 80 | 9.49 ± 0.88 | 0.95 | 42.3:57.7 |
| (200 m*M*) | 4 | 80 | 9.39 ± 0.61 | 0.94 | 45.0:55.0 |
| Hm–TGEH–Asc | 4 | 80 | 13.83 ± 0.78 | 1.38 | 37.8:62.2 |
| Hm–TGEH–Cys | 4 | 80 | 17.24 ± 0.40 | 1.72 | 41.9:57.2 |
| Hm–TGEH–GSH | 4 | 80 | 3.06 ± 0.19 | 0.31 | 42.8:57.2 |
| Hm–TGEH–$Na_2S_2O_4$ | 4 | 80 | 1.92 ± 0.07 | 0.19 | 62.5:37.5 |
| Post-mitochondrial fraction | | | | | |
| Rat liver[b] | 7.4 | | | 8.5[c] | 86:14 |
| Mouse liver[b] | 7.4 | | | 5.5[c] | 90:10 |

[a]The concentration of components and conditions were as for Fig. 2. Data are means ± S.D. for 3–4 experiments. [b]See reference [13]. [c]Conversion for a reaction time of 30 min.

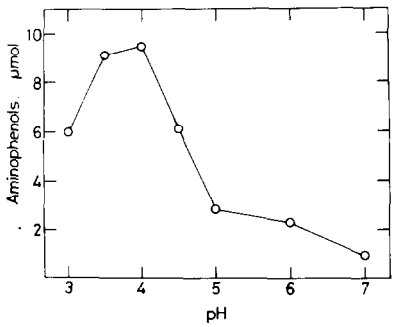


Fig. 2. pH-Dependency on aniline hydroxylation by hemin–TGEH model system. The reaction mixture contained 1 m*M* hemin, 100 m*M* TGEH and 100 m*M* aniline in 10 ml of buffer–acetone solvent (80% acetone). The buffer systems used; pH 3.0–3.5, 1 *M* HCl–1 *M* $CH_3COONa$; pH 4.0–6.0, 1 *M* $CH_3COOH$–1 *M* $CH_3COONa$, pH 7.0, 0.5 *M* Tris–0.5 *M* HCl.

tion was carried out under the respective conditions for each thiol compound (Table I).

The yield of hydroxylation with the system containing hemin and cysteine was higher than that containing hemin and cysteine esters. On the contrary, the yield with the system containing thioglycolate esters was better than that containing thioglycolic acid. Although no significant influence of the alkyl chain length in esters on the hydroxylation was observed, a slight decrease in the system containing octadecyl ester chain was noticed. Concentration dependency of thiolester on the oxidation yield and the product ratio were clearly observed; higher concentration of a thiolester in the system induced an increase in the yield of hydroxylation and a promotion of *o*-hydroxylation over *p*-hydroxylation.

Aniline hydroxylation was considerably stimulated by addition of a reducing agent or a second thiol compound, *e.g.* ascorbate and cysteine, whereas glutathione and dithionite were ineffective. No *m*-hydroxylation to aniline was observed in any of the systems applied in the present study.

### *Acetanilide Hydroxylation by Model Systems*

Acetanilide, which belongs to a modified type II substrate to cytochrome P-450 [22], was hydroxylated to form *p*-, *m*- and *o*-acetaminophenol isomers with several model complexes (Table II). From the study on pH dependency of acetaniline hydroxylation, optimal pH ranges were found to be at 4–5 under conditions similar to aniline hydroxylation (Fig. 3).

TABLE II. Hydroxylation of Acetanilide by Various Model Systems.

| System[a] | pH | Acetone (%) | Total Yield (μmol) | Conversion (%/2 hr) | Product Ratio *p*-AAP:*m*-AAP:*o*-AAP |
|---|---|---|---|---|---|
| Hm–Cys | 4.0 | 50 | 24.09 ± 0.94 | 2.41 | 53.3:5.8:40.9 |
| –CysE | 4.0 | 50 | 31.89 ± 0.44 | 3.10 | 55.8:4.4:39.9 |
| –TGE | 4.0 | 50 | 11.74 ± 0.54 | 1.17 | 57.8:3.7:38.5 |
| –TGB | 4.0 | 50 | 10.80 ± 0.22 | 1.08 | 54.9:4.5:40.6 |
| –TGO | 4.0 | 50 | 10.09 ± 0.31 | 1.01 | 55.2:4.2:40.7 |
| –TGEH | 4.0 | 50 | 20.43 ± 0.04 | 2.04 | 46.7:7.3:46.0 |
| –TGOD | 4.0 | 50 | 12.49 ± 0.49 | 1.25 | 56.0:4.1:39.9 |
| Hm–TGEH–Cys | 4.5 | 50 | 33.16 ± 0.43 | 3.32 | 57.9:5.5:36.7 |
| Hm–TGEH–Asc | 4.0 | 50 | 41.44 ± 2.40 | 4.14 | 46.4:12.1:41.6 |
| Fe(II)–Cys | 5.0 | 50 | 22.63 ± 1.09 | 2.26 | 57.6:5.4:37.0 |
| Cu(II) | 4.5 | 50 | – | – | |
| Asc | 4.5 | 50 | 1.38 | 0.17 | 44.9:16.7:38.4 |
| $H_2O_2$ | 4.5 | 50 | 0.18 | 0.22 | 100:0 :0 |
| Cu(II)–Asc | 4.5 | 50 | 14.53 | 1.83 | 37.9:22.9:39.2 |
| $-H_2O_2$ | 4.5 | 50 | 1.68 | 0.21 | 48.8:13.1:38.1 |
| $-Asc-H_2O_2$ | 4.5 | 50 | 3.76 | 0.47 | 45.5:7.7:46.8 |
| $Asc-H_2O_2$ | 4.5 | 50 | 4.86 | 0.61 | 42.0:6.2:51.8 |
| Cu(II)–Asc | 3.0 | 50 | 2.12 ± 0.09 | 0.27 | 34.4:34.4:31.1 |
| | 3.5 | 50 | 2.03 ± 0.08 | 0.25 | 35.5:33.5:31.0 |
| | 4.0 | 50 | 20.09 ± 0.20 | 2.51 | 33.8:23.2:43.1 |
| | 4.5 | 50 | 10.75 ± 0.50 | 1.34 | 39.0:19.4:41.6 |
| | 5.0 | 50 | 10.25 ± 0.22 | 1.28 | 41.4:16.3:42.3 |

[a]The concentration of components and conditions were as for Fig. 3. In the systems containing Cu(II) ion, the concentrations were 50 m*M* Asc, 1 m*M* Cu(II), 25 m*M* $H_2O_2$ and 80 m*M* acetanilide. Data are means ± S.D. for 2–4 experiments.

Under these conditions, both hemin–thioglycolate ester and hemin–cysteine complexes induced relatively higher hydroxylation to acetanilide than to aniline.

Corresponding well to the observation in aniline hydroxylation, the addition of a reducing agent such as cysteine or ascorbate was found to induce higher hydroxylation activity than without the reducing agents. Acetanilide hydroxylation activity was also observed when Fe(II) was used in place of hemin and the product ratio (*p*-AAP:*m*-AAP:*o*-AAP) gave almost identical values to those obtained using hemin containing systems.

The Cu(II)–ascorbate–$H_2O_2$ system, which induced high aniline hydroxylation activity [23], catalyzed acetanilide hydroxylation, but acetanilide was hydroxylated best by a Cu(II)–ascorbate system. The maximum hydroxylation activity was found at pH 4.0 in 50% acetone. It is noteworthy (Table II) that at low pH the ratio of three hydroxylated products was unaltered but at pH 4.5 or 5.0 *m*-hydroxylation decreased. A considerable difference in the product ratio was shown between hemin–thiol complexes and Cu(II)-containing systems.

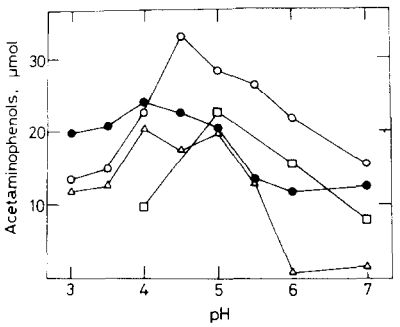


Fig. 3. pH-Dependency on acetanilide hydroxylation by various types of model system. The reaction mixture contained 1 m*M* hemin or Fe(II), 100 m*M* cysteine or TGEH or both and 100 m*M* acetanilide in 10 ml of buffer–acetone solvent (50% acetone). Conditions were as for Fig. 2. –○–, hemin–cysteine–TGEH system; –●–, hemin–cysteine system; –△–, hemin–TGEH system; –□–, Fe(II)–cysteine system.

### *Acetanilide Hydroxylation by Rat Liver Microsomes*

Acetanilide hydroxylation with rat liver microsomal fractions using three types of NADPH generating system was studied to compare with the results

TABLE III. Hydroxylation of Acetanilide by Rat Liver 105,000 × g Microsomes

| System[a] | Microsomes (mg protein/ml incub.mix.) | P-450 Content (nmol/ml incub.mix.) | Rate of AAP Formation (nmol/mg prot./30 min) | Product Ratio *p*-AAP:*m*-AAP:*o*-AAP |
|---|---|---|---|---|
| 105,000 × g Microsomes | | | | |
| System (A) | 2 | 1.95 | 38.1 | 50.0:2.6:47.4 |
| System (B) | 2 | 1.95 | 40.5 | 69.6:13.0:17.4 |
| System (C) | 5 | 4.86 | 22.6 | 64.6:8.0:27.4 |

[a]Conditions are described in experimental section. Data are means for 2–3 experiments.

obtained in the model systems (Table III). With the microsomal fractions, the three isomeric metabolites were successfully detected, and no other side products were found. Although the product ratio was found to be changed depending on the system, a similar tendency among the products, *p*-AAP > *o*-AAP > *m*-AAP, was observed. The product ratio in the reactions with microsomal systems was akin to that with the hemin– (or Fe(II)–) thiol compound systems, suggesting an involvement of a similar process for hydroxylation in both the liver and the model systems. However, the product ratio in the Cu(II)–ascorbate system was found to be different from that in microsomal systems.

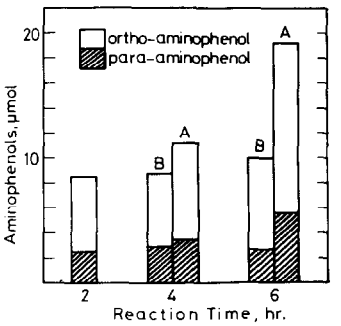


Fig. 4. Enhancement of aniline hydroxylation upon addition of cysteine in hemin–cysteine system. The reaction mixture contained 1 m*M* hemin, 100 m*M* cysteine and 100 m*M* aniline in 10 ml of buffer–acetone solvent (50% acetone) of pH 4.5. In the experiments for reaction time of 4 and 6 hr, the reactions were compared with two sets of the system; in (A) cysteine (100 m*M*) was added every two hr but in (B) cysteine was not added at 4 and 6 hr.

## Discussion

In aniline hydroxylation with the model systems, neither high activity nor high selectivity to *para*-hydroxylation were observed by comparison with those with liver post-mitochondrial fraction (Table I). Judging from both the recent observation that high aniline hydroxylation activity was achieved by the Cu(II)–ascorbate–$H_2O_2$ system, in which the hydroxyl radical is an active oxygen species [23], and the new evidence that aniline hydroxylation was mediated by hydroxyl radicals generated in cytochrome P-450- or hemoglobin-catalyzed Habar–Weiss reaction between $O_2^-$ and $H_2O_2$ [24], aniline may not be an appropriate substrate for studying hydroxylation with model systems. Therefore, acetanilide, known as a modified type II substrate, was chosen as a probe for evaluating model systems of cytochrome P-450 monooxygenases.

In acetanilide hydroxylation with the hemin–thiol model complexes, high hydroxylation activity and reaction selectivity associated with the product ratio were achieved (Tables II and III).

In separate experiments it was clearly shown that aniline hydroxylation activity was enhanced upon addition of cysteine every 2 hours during a total of 6 hours of reaction time with the hemin–cysteine system (Fig. 4). The results indicated that a requirement of thiol compound was necessary in order to obtain a high yield of hydroxylation, because thiol groups were oxidized during the oxidation of added substrate. Thus, the high yield of hydroxylation with simultaneous shortening of reaction time was achieved simply by addition of reducing agent or another type of thiol compound that has a different proton dissociation constant [25], as is evident from Tables I and II for aniline and acetanilide hydroxylation, respectively. The requirement of a second reducing agent for the stimulation of hydroxylation indicated a necessity for the co-existence of an electron-donating compound in the model systems. Therefore, the reaction profile of the systems mimics that of NADPH-dependent cytochrome P-450 monooxygenases.

The similarity in the reaction pattern associated with the product ratio in acetanilide hydroxylation was observed in liver microsomal and hemin (or Fe(II))–thiol model systems. Since aniline hydroxylation with the model system does not involve ·OH, $O_2^-$, $^1O_2$ or $H_2O_2$ but has a potent active oxygen intermediate in equilibrium among various multivalent iron complexes retaining the thiolate–heme linkage [14], a similar hydroxylation mechanism may be involved in the case of acetanilide by the model

systems. The estimation of the reaction mechanism in the system is important in order to evaluate the efficiency of the model system. Work on this subject is continuing, and the results will be reported.

Recently we have found that a system consisting of hemin and thiolester (thioglycolate esters or cysteine esters) induces aromatic intramolecular methyl migration (methyl NIH shift) during hydroxylation of *p*-methylanisole, whereas a system containing hemin or Fe(II) ion and thiolcarboxylate (thioglycolic acid or cysteine) does not induce methyl NIH shift [26]. Furthermore, we have reported that the hemin–thiolester complex produces a characteristic hyperporphyrin spectrum with hemin–dithiolate coordination in the presence of alkali in acetone solvent [27] and that a hydroxyl group such as methanol can coordinate to the *trans* position of the penta-coordinate hemin–thiolate complex by a ligand-exchange reaction of hyperporphyrin complex with methanol. This suggests a possible coordination model of cytochrome P-450 in the ferric low-spin state [28, 29].

Based on the results presented previously and herein, it was concluded that the complex consisting of hemin and thioglycolate ester with the addition of another thiol compound or reducing agent, which simulates both the oxidation activity to substrate and the optical and EPR properties of cytochrome P-450, is a good model of this enzyme.

## Acknowledgements

This research was supported in part by a Grant-in Aid for Scientific Research from the Ministry of Education, Science and Culture, and a Grant-in-Aid for New Drug Development from the Ministry of Health and Welfare of Japan.

## References

1 R. Sato and T. Omura, 'Cytochrome P-450', Kodansha, Tokyo and Academic Press, New York (1978).
2 T. Omura and R. Sato, *J. Biol. Chem., 235,* 1375 (1962).
3 Y. Hashimoto, T. Yamano and H. S. Mason, *J. Biol. Chem., 237,* 3843 (1962).
4 L. K. Hanson, W. A. Eaton, S. G. Sliger, I. C. Gunsalus, M. Gouterman and E. R. Connell, *J. Am. Chem. Soc., 98,* 2672 (1976).
5 E. Bayer, H. A. O. Hill, A. Röder and R. J. P. Williams, *Chem. Commun.,* 109 (1969).
6 J. Peisach and W. E. Blumberg, *Proc. Natl. Acad. Sci. U.S.A., 67,* 172 (1970).
7 S. C. Tang, S. Koch, G. C. Papaefthymiou, S. Foner, R. B. Frankel, J. A. Iber and R. H. Holm, *J. Am. Chem. Soc., 98,* 2414 (1976).
8 J. O. Stern and J. Peisach, *J. Biol. Chem., 249,* 7495 (1974).
9 J. P. Collman and T. N. Sorell, *J. Am. Chem. Soc., 97,* 4133 (1975).
10 H. Sakurai and S. Ogawa, *Biochem. Pharmacol., 24,* 1257 (1975) and *Chem. Pharm. Bull., 27,* 2171 (1979).
11 H. Sakurai and M. Kito, *Biochem. Pharmacol., 24,* 1647 (1975) and *Chem. Pharm. Bull., 25,* 2330 (1977).
12 H. Sakurai and M. Kito, *Biochem. Pharmacol., 25,* 2133 (1976).
13 H. Sakurai, S. Shimomura, K. Fukuzawa and K. Ishizu, *Chem. Pharm. Bull.,* 26, 1348 (1978).
14 H. Sakurai, *Chem. Pharm. Bull., 28,* 3437 (1980).
15 H. Sakurai, S. Shimomura and K. Ishizu, *Biochem. Biophys. Res. Commun., 101,* 1102 (1981).
16 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem., 193,* 265 (1951).
17 T. Omura and R. Sato, *J. Biol. Chem., 239,* 2370 (1964).
18 H. Sakurai and S. Ogawa, *J. Chromatogr. Sci., 14,* 499 (1976).
19 T. Kamataki, M. Kitada, K. Chiba, H. Kitagawa, Y. Imai and R. Sato, *Biochem. Pharmacol., 29,* 1141 (1980).
20 B. Mellström and C. von Bahr, *Drug. Metab. Disposit., 9,* 565 (1981).
21 H. Shigematsu, S. Yamano and H. Yoshimura, *Chem. Pharm. Bull., 25,* 825 (1977).
22 J. B. Schenkman, H. Remmer and R. W. Estabrook, *Mol. Pharmacol., 3,* 113 (1967).
23 H. Sakurai *et al.*, in preparation.
24 M. Ingelman-Sundberg and G. Ekström, *Biochem. Biophys. Res. Commun., 106,* 625 (1982).
25 J. P. Danehy and K. N. Parameswaran, *J. Chem. Eng. Data, 13,* 386 (1968).
26 H. Sakurai, E. Hatayama, K. Fujitani and H. Kato, *Biochem. Biophys. Res. Commun., 108,* 1649 (1982).
27 H. Sakurai and T. Yoshimura, *Inorg. Chim. Acta, 56,* L49 (1981).
28 H. Sakurai and T. Yoshimura, *Inorg. Chim. Acta, 66,* L25 (1982).
29 H. Sakurai and T. Yoshimura, in 'Microsomes, Drug Oxidations and Drug Toxicity', R. Sato and R. Kato, Eds., Japan Scientific Soc. Press and Wiley–Interscience, New York (1982) p. 113.