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Aromatic Methyl Group Migration and Hydroxylation of *p*-Toluidine by Iron-Thiol and Hemin-Thiol Systems as a Model of Cytochrome P-450

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The aromatic methyl group migration and hydroxylation of *p*-toluidine by several Fe(II)-thiol compound and hemin–thiol compound systems were investigated as a model of cytochrome P-450 of liver microsomes. In these systems, 4-hydroxy-*m*-toluidine, 3-hydroxy-*p*-toluidine and 2-hydroxy-*p*-toluidine were formed as the main products of the reaction.

The yield of methyl group migration and hydroxylation were dependent on the type of thiol compounds, chemical form of iron (Fe(II) or hemin), pH of the reaction mixture, reaction periods as well as on the concentration of the substrates or the thiol compounds. These results on the methyl group migration of *p*-toluidine suggest that the Fe(II)-thiol and hemin–thiol systems can be regarded as a good chemical model for the study of the function and structure of cytochrome P–450.

Keywords——aromatic methyl group migration; hydroxylation; *p*-toluidine; model system; thiol compound; hemin; cytochrome P–450; NIH shift

The cytochrome P-450 monooxygenases are membrane-bound mixed function oxidases which mediate the hydroxylation of a wide variety of substrates including steroides, aromatic compounds, hydrocarbons and barbiturates.[2] The intramolecular migration and retention of aromatic ring substituents (NIH shift), which occur during enzymatic hydroxylation by the monooxygenases, provide a criterion for evaluating chemical model systems capable of introducing the hydroxy group into aromatic rings. In the hydroxylation, a mechanism involving the enzyme-catalyzed formation of arene oxide, followed by the spontaneous rearrangement of this unstable intermediate to a phenol, has been proposed.[3]

Udenfriend, Fenton and Hamilton systems, which are well-known model systems of the hydroxylation, do not show the NIH shift,[4] whereas the occurrence of NIH shift has been established with peroxytrifluoroacetic acid[5] and with photolysis of pyridine-N-oxide.[6]

In the previous papers, we reported the regioselective hydroxylation of aniline observed in biological systems by several ferrous ion-thiol and hemin-thiol complexes as a possible model system closer to cytochrome P-450.[7] In this connection, Ullrich, *et al.* described that the hydroxylation occurring in a system such as ferrous ion-oxygen-thiosalicylic acid did not exhibit the migration of substituents.[8] However, in a preliminary report, we showed the positive evidence of methyl group migration during hydroxylation of *p*-toluidine in the

1) Location: *a*) *Sho-machi, Tokushima 770, Japan*; *b*) *Yamashina-ku, Kyoto 607, Japan.*
2) J.E. Tomaszewski, D.M. Jerina and J.W. Daly, *Annu. Rep. Med. Chem.*, **9**, 290 (1974).
3) D.M. Jerina, J.W. Daly and B. Witkop, *J. Am. Chem. Soc.*, **90**, 6523 (1968).
4) D.M. Jerina, *Chem. Tech.*, **1973**, 120.
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7) *a*) H. Sakurai and S. Ogawa, *Biochem. Pharmacol.*, **24**, 1257 (1975); *b*) H. Sakurai and S. Ogawa, *Chem. Pharm. Bull.* (Tokyo), "submitted."
8) V. Ullrich and Hj. Staudinger, in Concepts in Biochemical Pharmacology, Part 2. Handbook of Experimental Pharmacology, Vol. XVIII, Springer–Verlag, 1971, p. 251.

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carefully designed model system.[9] This paper represents the clear evidence that non-enzymatic hydroxylation of aromatic ring was really accompanied by intramolecular migration of methyl group with several ferrous ion-thiol and hemin-thiol compound systems.

Materials and Methods

Thiol compounds; cysteine, cysteine methyl ester, β-mercaptopropionic acid, *o*-aminobenzenethiol, thiosalicylic acid, 2,3-dimercaptopropanol and thiophenol and *p*-toluidine were purchased from Nakarai Chemicals, Kyoto or Wako Pure Chemical Industries, Osaka. Hemin chloride (Fe(III)-protoporphyrin IX chloride) of bovine crystalline, type I, was obtained from Sigma, St. Louis. 4-Hydroxy-*m*-toluidine (I) (Eastman Kodak Co.), and 3-hydroxy-*p*-toluidine (II), 2-hydroxy-*p*-toluidine (III) (Aldrich Chemical Company, Inc.), *p*-toluidine and *p*-aminophenol were purified by sublimation. Acetone, ether and chloroform were purified by distillation. Thiol compounds were dissolved in acetone, water or 0.1 M sodium hydroxide solution immediately before use.

A typical reaction mixture contained the following reagents; 10^{-1}M thiol compound, 10^{-3}M ferrous sulfate heptahydrate (Fe(II)) or hemin chloride and 10^{-1} M *p*-toluidine and pH of the solution was adjusted to 4.0 and 6.0 with sodium hydroxide. The reaction mixture was adjusted to 10 ml with acetone (80%).

The reaction mixtures were incubated at 40° for various periods of time with vigorous shaking in the atmosphere of air, and the reaction was stopped by addition of 2 N hydrochloric acid. The reaction–products were identified by thin–layer chromatography (TLC) and liquid chromatography (LC). Quantitative measurement was also carried on LC according to the method previously reported.[10]

Results

Identification of Products

When substrate *p*-toluidine is hydroxylated by liver microsomes or non–enzymatic hydroxylating systems, the possible products of the reactions are 4-hydroxy-*m*-toludine (I), 3-hydroxy-*p*-toluidine (II), 2-hydroxy-*p*-toluidine (III), *p*-aminophenol, *p*-aminobenzylalcohol and N-hydroxy-*p*-toluidine as shown in Chart 1. With the model system consisting of thiol

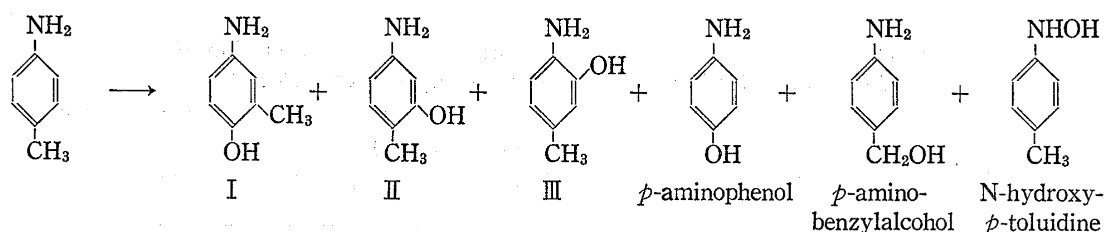

Chart 1. Possible Reaction Products of Hydroxylation of *p*-Toluidine by the Chemical Model of Cytochrome P–450

compound, oxygen and Fe(II) or hemin, compounds (I), (II), (III) and *p*-aminophenol were identified as the products by TLC and LC.[10] Accurate determination of the yield of *p*-aminophenol, however, was quite difficult owing to overlapping of the peak of the product with those of unidentified products.

Hydroxylation of *p*-Toluidine by Model Systems

The results of the methyl group migration and hydroxylation of *p*-toluidine with various Fe(II)–thiol and hemin-thiol model systems during the incubation for two hours at pH 4 or 6 are summarized in Table I. The methyl group migration accompanied with hydroxylation was observed in the systems consisting of hemin and thiol compounds which possessed only an amino group or those with both amino and carboxyl groups, while, in the presence of thiol compounds with a carboxyl group or dithiol compounds such as 2,3-dimercaptopropanol,

9) H. Sakurai and M. Kito, *Biochem. Pharmacol.*, 24, 1647 (1975).
10) H. Sakurai and M. Kito, *Talanta*, 23, 842 (1976).

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Table I. Methyl Group Migration and Hydroxylation of *p*-Toluidine by the Chemical Model of Cytochrome P–450

Coordinating group	Thiol compound	pH	Reaction products (μg) Fe(II)			Hemin		
			I	II	III	I	II	III
SH-NH_2-COOH	Cysteine	4	0	0	0	51± 4	26± 5	280±31
		6	27± 6	t	123±38	48± 7	t	127± 4
SH-NH_2	Cysteine	4	0	0	0	42± 2	0	93±19
	Methyl ester	6	0	0	0	35± 5	t	80±16
	o-Aminoben-	4	0	0	0	0	122±22	143±19
	zenethiol	6	29± 8	36±19	43±19			
SH-COOH	*β*-Mercapto-	4	17± 4	0	198±39	0	t	43
	propionic acid	6	32± 2	0	180±23			
	Thiosalicylic	4	53± 8	31± 3	573±19	32	0	150
	acid	6	30± 2	12± 9	310±46			
SH-SH	2,3-Dimercapto-	4	77±10	t	220±46	0	0	0
	propanol	6	53± 7	0	t			
SH	Thiophenol	4	0	0	180	78	0	0

The reaction vessels contained thiol compound 10^{-1} M, Fe(II) or hemin 10^{-3} M, *p*-toluidine 10^{-1} M and sodium hydroxide in 80% acetone. Total volume was 10 ml. Uncertainties are the standard deviations of four experiments. Others are means of two experiments. Trace amount of product is shown by the symbol of t.

the reactions were found to be promoted by ferrous ion rather than by hemin. Since high yields of methyl group migration and hydroxylation were observed in the Fe(II)–thiosalicylic acid system as well as in the hemin–cysteine system, the reaction profiles with these two systems were compared in further detail.

a) Effect of pH on Hydroxylations——Table II shows the results of the methyl group migration and hydroxylation of *p*-toluidine at various pH values of the reaction mixture during

Table II. Effect of pH of Reaction Mixture on Hydroxylation of *p*-Toluidine by the Chemical Model of Cytochrome P–450

System	pH	Reaction products (μg) I	II	III	I/III
Thiosalicylic	2	0	15± 5	333±123	0
Acid-Fe (II)	3	0	13± 3	285± 35	0
	4	53± 8	31± 3	573± 19	0.093±0.013
	6	30± 2	12± 9	310± 46	0.098±0.018
Cysteine–	2	0	0	30	0
hemin	3	0	0	30	0
	4	51± 4	26± 5	280± 31	0.183±0.034
	6	48± 7	t	127± 4	0.373±0.057
	7	61± 4	t	120± 8	0.515±0.063

The reaction mixtures contained same concentrations of reagents as described in Table I, and were incubated for two hours. Values are means±S.D. of four experiments. Others are means of two experiments.

two hours. The maximum reaction rate was observed at pH 4 in both Fe(II)-thiosalicylic acid and hemin-cysteine systems. Also, the change of the ratio of product [I] to [III] depended on the system used. The ratio was about 0.09 in the system of Fe(II)-thiosalicylic acid, but 0.18—0.52 (pH 4—7) accounted for the system of hemin-cysteine. Methyl group migration in the hemin-cysteine system is favourable than in the Fe(II)-thiosalicylic acid system.

b) Effect of Reaction Periods on Hydroxylations——The results of methyl group migration and hydroxylation of *p*-toluidine at various reaction periods at pH 4 are shown in Table III.

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TABLE III. Effect of Reaction Periods on Hydroxylation of *p*-Toluidine by the Chemical Model of Cytochrome P-450

System	Periods (hr)	Reaction products (μg) I	II	III	I/III
Thiosalicylic acid-Fe (II)	0.5	22	25	330	0.067
	1.5	40	50	610	0.066
	2.0[a]	53	31	573	0.093
	4.0	20	25	560	0.036
Cysteine-hemin	1.0	0	6	0	—
	2.0[a]	51	26	280	0.182
	3.0	22	16	400	0.055
	4.0	16	10	200	0.073

The reaction mixtures contained same concentrations of reagents as described in Table I, and were incubated at pH 4. Values are means of two or four experiments (*a*) four experiments).

The maximum yield of the reaction was obtained with the incubation of 1.5—2 hours and 2—3 hours in the system of Fe(II)-thiosalicylic acid and hemin-cysteine, respectively. The yield of the products were rather decreased with exessive increase of the incubation period in both systems, presumably due to degradation or *trans*-formation of these compounds to other products unidentified at present.

c) Effect of the Concentration of Substrates on Hydroxylation with Fe(II)-Thiosalicylic Acid——The yield of hydroxylation at various concentrations of *p*-toluidine in the system of Fe(II)-thiosalicylic acid at pH 4 during two hours was decreased as the concentration ratio of *p*-toluidine to Fe(II) increased (Fig. 1). At low concentration of *p*-toluidine, it was difficult to determine the yields of products due to its low amount. From these results, the concentration of *p*-toluidine was fixed 100 times larger than that of Fe(II) in the later experiments unless otherwise mentioned.

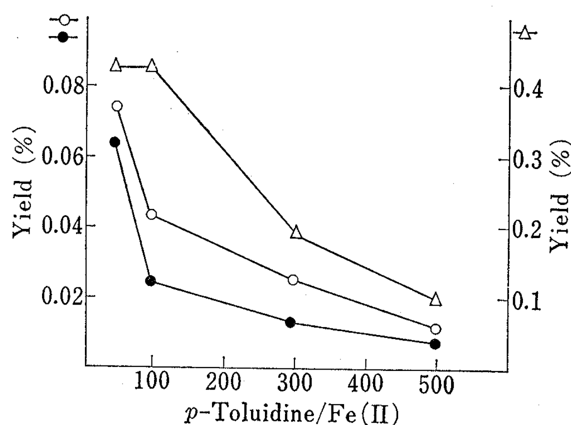

Fig. 1. Effect of Concentration of *p*-Toluidine on the Yield of its Hydroxylation by Fe(II)-Thiosalicylic Acid

The reaction mixtures contained same concentrations of reagents as described in Table I and were incubated for two hours at pH 4.
—○—, product (I); —●—, product (II); —△—, product (III). (Chemical structure of these products is shown in Chart 1.)

d) Effect of the Concentration of Thiol Compounds on Hydroxylation——The methyl group migration and hydroxylation of *p*-toluidine at various concentrations of thiol compound was investigated in the system of Fe(II)-thiosalicylic acid and hemin-cysteine at pH 4 during two hours (Table IV). From this result, it is obvious that the molar quantity of thiol compound to iron component influenced remarkably on the methyl group migration and hydroxylation. In the system of Fe(II)-thiosalicylic acid, the methyl group migration was found to increase with the concentration of thiol compound and the maximum rate was not found in the conditions applied, whereas in the system of hemin-cysteine, the maximum methyl group migration and hydroxylation were observed when the molar quantity of cysteine to hemin was 300. The ratio of (I) to (III) in the products almost linearly increased with the concentration of thiosalicylic acid, but it was somewhat deviated for the hemin-cysteine system.

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TABLE IV. Effect of Concentration of Thiol Compound on Methyl Group Migration and Hydroxylation of *p*-Toluidine by the Chemical Model of Cytochrome P–450

System	Conc. of thiol (thiol/iron)	Reaction products (μg)			I/III
		I	II	III	
Thiosalicylic Acid-Fe (II)	10	t	t	120	—
	50	35±11	t	432±107	0.080±0.007
	100	53± 8	31± 3	573± 19	0.093±0.014
	300	137±11	t	860± 39	0.160±0.015[a]
	500	163±10	t	823± 84	0.199±0.010[a]
Cysteine-hemin	50	10	10	193	0.052
	100	51± 4	26± 5	280± 31	0.183±0.034[a]
	300	60	26	720	0.083
	500	30	14	340	0.088

The reaction mixtures contained the same concentrations of reagents as described in Table I, and were incubated for two hours at pH 4.
a) Significantly different from the value obtained when the molar quantity of thiosalicylic acid to ferrous ion is 100 ($p<0.01$). Uncertainties are the standard deviations of four experiments. Others are means of two experiments.

TABLE V. Effect of Inorganic Sulfur on the Migration of the Methyl Group of *p*-Toluidine by the Chemical Model of Cytochrome P-450

System	Reaction products (μg)	
	I	III
Hemin-Na_2S	0	0
Hemin-cysteine	48±7	127± 4
Hemin-Na_2S-cysteine	37±2	0
Fe(II)-Na_2S	0	0
Fe(II)-cysteine	27±6	123±38
Fe(II)-Na_2S-cysteine	40±3	98±21

The reaction mixtures contained thiol compound 10^{-1}M, Fe (II) or hemin 10^{-3}M, *p*-toluidine 10^{-1}M, Na_2S 10^{-3}M and sodium hydroxide and were incubated for two hours at pH 6 in 80% acetone. Total volume was 10 ml. Values are means±S.D. of four experiments.

e) **Effect of Inorganic Sulfur to Model Systems**——Besides, the effect of inorganic sulfur was investigated at pH 6 (Table V). The hydroxylations were not observed in the systems containing inorganic sulfur and Fe(II) or hemin. It seems that the addition of inorganic sulfur decreased the extent of methyl group migration in the system of hemin-cysteine, whereas inorganic sulfur slightly increased the methyl group migration in the systems containing cysteine and Fe(II).

Discussion

The enzymatic hydroxylation of aromatic substrates has been demonstrated to be accompanied by migration of the heavy isotope from the site of hydroxylation to an adjacent position in the aromatic ring. The substrates are generally classified into two groups, owing to the extent and direction of the migration; class I—substrates containing a substituent capable of undergoing ionization to form a neutral 2,5-cyclohexadienoid intermediate, and class II—substrates containing either electron-donating or electron-withdrawing substituents.[11] Based on this classification, aniline and *p*-toluidine fall under the substrate of class I. In the present

11) J. Daly, D. Jerina and B. Witkop, *Arch. Biochem. Biophys.*, **128**, 517 (1968).

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report the hydroxylation of *p*-toluidine which belongs to class I as aniline was investigated in order to make sure the criterion for chemical model systems described in the Introductoin.[5]

It is apparent from Table I—IV that there occurred intramolecular migration of methyl group of *p*-toluidine during hydroxylation in Fe(II)–thiol and hemin-thiol model systems. When Fe(III) or Cu(II) was used instead of Fe(II) or hemin, the methyl group migration and hydroxylation were not observed. Furthermore, the Udenfriend system failed to show the methyl group migration with *p*-toluidine, although the product (III) was formed, when the same concentration ratio of reagent and substrate as the hemin–cysteine system was used.

These results strongly indicate that the interaction of a thiol group and a ferrous state of iron plays an essential role both in aromatic hydroxylation[7] and in the migration of aromatic ring substituent. We have already shown that the ferric iron in hemin is reduced easily to ferrous state in the presence of thiol compound.[12] However, excess of thiol compounds to Fe(II) or hemin inhibited the methyl group migration and hydroxylation (Table IV), presumably the excessive thiol compound prevents access of *p*-toluidine to the heme moiety.

Despite the fact that the highest yield of methyl group migration and hydroxylation was achieved with the system of Fe(II)–thiosalicylic acid, the maximum migration amount of methyl group (0.13%) was found to be only 1/10 compared with the reported data obtained with microsomal aniline hydroxylation.[11]

From these results, the system consisting of hemin and biologically important thiol compounds is an excellent model for cytochrome P-450 in both chemical structure and function. Intensive study on this model system would provide a clue for clarifying the migration mechanism in cytochrome P-450.

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