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PRELIMINARY COMMUNICATIONS

AROMATIC METHYL MIGRATION BY IRON-THIOL AND HEMIN-THIOL SYSTEMS

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The intramolecular migration and retention of aromatic ring substituents (NIH shift), which occur during enzymic hydroxylation by monooxygenases, provide a criterion for evaluating chemical model systems capable of introducing the hydroxyl group into aromatic rings. Udenfriend, Fenton, and Hamilton systems do not show the NIH shift (1), whereas the occurrences of NIH shift have been established with peroxytrifluoroacetic acid (2) and with photolysis of pyridine-N-oxide (3).

We have investigated 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 monooxygenases (4).

Ullrich et al. described (5) that the hydroxylation occurring in a system such as ferrous ion-oxygen-thiosalicylic acid did not exhibit the migration of substituents. This communication deals with the positive evidence of methyl migration during hydroxylation of p-toluidine as a substrate with the same model system used in the hydroxylation of aniline.

The reactions were carried out by the same procedure previously reported (4). Identification of products, 4-hydroxy-m-toluidine [I], 3-hydroxy-p-toluidine [II], 2-hydroxy-p-toluidine [III], and p-aminophenol was established by TLC and by LC in which two kinds of mobile phases were used. Quantitation was achieved in I, II, and III with LC by the modification of Brodie's method (6).

The methyl migration during hydroxylation by model systems used appeared to be maximum with reaction time for 2 hours and to have an optimal pH of about 4. Throughout this study, therefore, the reactions were carried out at pH 4 and for 2 hours at 40°.

As shown in Table I, in the system containing cysteine or cysteine methyl ester the reactions were observed only in the presence of hemin, whereas in the system containg thiosalicylic acid or β-mercaptopropionic acid and dithiol such as 2,3-dimercaptopropanol the reactions were found to be promoted by ferrous ion rather than hemin. It is apparent from above results that there occurred the

Table I. Methyl Migration and Hydroxylation of p-Toluidine by Model Systems.

Thiol Compound	Fe(II) I (μg)	Fe(II) II (μg)	Fe(II) III (μg)	Fe(II) I/III Ratio	Hemin I (μg)	Hemin II (μg)	Hemin III (μg)	Hemin I/III Ratio
Cysteine	0	0	0	-	51±4	26	280±31	0.183±0.034
Cysteine Methyl Ester	0	0	0	-	42±2	0	93±19	0.458±0.059*
o-Aminobenzenethiol	0	0	0	-	0	122±22	143±19	0
β-Mercaptopropionic Acid	17± 4	0	198±39	0.089±0.022*	0	t	43	0
Thiosalicylic Acid	53± 8	25	573±19	0.093±0.013*	32	0	150	0.20
2,3-Dimercaptopropanol	77±10	t	220±46	0.380±0.160	0	0	0	-
Thiophenol	0	0	180	0	78	0	t	-

The reaction vessels contained thiol compound 10^{-1}M, ferrous ion or hemin 10^{-3}M, p-toluidine 10^{-1}M, and sodium hydroxide in 80% acetone. Total volume was 10 ml.
* Significantly different from the value obtained with hemin-cysteine system ($P<0.01$). Values are means ± S.D. of four experiments.

Table II. Effect of Concentration of Thiol on Methyl Migration and Hydroxylation in Model Systems.

System	Conc. of Thiol (Thiol/Iron)	I μg (yield %)	II μg	III μg (yield %)	I/III Ratio
Thiosalicylic Acid - Fe(II)	50	35 ±11	t	432 ±107	0.080 ±0.007
	100	53 ± 8 (0.04)	31 ± 3	573 ± 19 (0.47)	0.093 ±0.014
	300	137 ± 8 (0.11)	t	860 ± 39 (0.70)	0.160 ±0.015*
	500	163 ±10 (0.13)	t	823 ± 84 (0.66)	0.199 ±0.010*
Cysteine - Hemin	50	10	10	193	0.052
	100	51 ± 4 (0.04)	26	280 ± 31 (0.23)	0.183 ±0.034*
	300	60 (0.05)	26	720 (0.58)	0.083
	500	30 (0.02)	14	340 (0.28)	0.088

Conditions are the same as indicated in Table I.
* Significantly different from the value obtained when the molar quantity of thiosalicylic acid to ferrous ion is 100 ($P<0.01$). Values are means ± S.D. of four experiments. Others are means of two experiments.

intramolecular migration of aromatic methyl group during hydroxylation in iron-thiol and hemin-thiol model systems. It is noteworthy that the change in the product ratio of I to III (I/III ratio) depends on the systems used.

The molar quantity of thiol compound to iron component influenced on the methyl migration and hydroxylations (Table II). In the system of iron-thiosalicylic acid, the methyl migration was found to increase with the concentration of thiol, whereas in the system of hemin-cysteine, maximum methyl migration and hydroxylations were observed when the molar quantity of cysteine to hemin was

Table III. Interaction of Hemin and Cysteine

System	λ_{max} (nm)			
Hemin-OH	370		590sh	
Hemin-Pyridine	403	525	555sh	
Hemin-Cysteine	380			640sh
Hemin-Pyridine-Cysteine (15s-30m)	413	535	565sh	
Hemin-Pyridine-Cysteine (30m-)	413	523	555	
Low-spin P-450 (Fe^{3+})*	415	535	565	
Reduced P-450 (Fe^{2+})*	415		555	

The solution contained hemin chloride 5×10^{-5}M, pyridine 1.24M, and cysteine 8.25M in pH 7.5 phosphate buffer.
* C. R. E. Jefcoate and J. L. Gaylor, Biochemistry, 8, 3464 (1969).

300. Despite the highest yield of methyl migration and hydroxylations was achieved with the system of iron-thiosalicylic acid, the maximum migration of methyl group in the products was found to be within smaller extent (0.13%) compared with the migration of 1.2% obtained with microsomal aniline hydroxylation (7). The ratio of I to III in the products appeared to be almost linear with the concentration of thiosalicylic acid.

It is clear that the interaction of the thiol group and ferrous ion or hemin plays an essential role in aromatic hydroxylation (4) and further in the migration of aromatic ring substituent.

Recently, Stern et al. (8) have reported mercaptide anion (RS^-) to be a ligand trans to CO in cytochrome P-450·CO complex by use of hemin and 2-mercaptoethanol. In connection with Stern's findings, we have observed similar spectra to low spin state of cytochrome P-450 in the hemin-pyridine-cysteine system within 30 minutes in pH 7.5 (Table III).

Thus we may say that iron-thiol and hemin-thiol systems are regarded as the best chemical model so far for studying the oxygen activation and structure of cytochrome P-450 monooxygenases.

Further investigations are in progress with a more detailed study with these systems to clarify the mechanism of these reactions and the structure of these systems and the results will be reported in the near future.

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