Hydroxylation of *Para*-Chlorotoluene by Model Complexes of Cytochrome P-450

HIROMU SAKURAI*, ETSUJI HATAYAMA

Faculty of Pharmaceutical Sciences, University of Tokushima, Sho-machi 1, Tokushima 770, Japan

and KAZUYOSHI FUJITANI

Kyoto College of Pharmacy, Misasagi, Yamashina-ku, Kyoto 607, Japan

Received August 31, 1983

Hydroxylation of p-*chlorotoluene with hemin–thiol complexes, Fenton's system and Udenfriend's system was studied and the complexes assessed as models of cytochrome P-450 monooxygenases. Five species of possible hydroxylation products of* p-*chlorotoluene, namely,* p-*chlorobenzyl alcohol, 2-chloro-5-methylphenol,* p-*chlorobenzaldehyde, 4-chloro-2-methylphenol and 5-chloro-2-methylphenol, were studied using high performance liquid chromatography. The oxidation reactions were characterized by the yields of hydroxylation products and the product ratio. The system consisting of hemin and cysteine ethyl ester as well as Udenfriend's system gave relatively high hydroxylation yields and the former only induced a methyl migration during hydroxylation (methyl NIH shift). However, neither Fenton's nor Udenfriend's systems induced a methyl NIH shift. The hemin–thiol complex is thus concluded to be a good chemical model of cytochrome P-450 monooxygenases.*

Introduction

In the preceding papers [1–6] on hydroxylation studies of aniline, *para*-toluidine and acetanilide, we demonstrated that hemin–thiol complexes operated like cytochrome P-450 monooxygenases. In further investigations into the ability of hemin–thiol complexes to serve as models of the enzymes, we used another type of substrate [7], classified as a type I substrate to the enzymes [8]. Chlorobenzene has been utilized as a suitable substrate to study hydroxylation by cytochrome P-450 and its model systems [9, 10]. However, the hydroxylation pattern of *para(p)*-chlorobenzene derivatives is not yet known in detail. This led us to examine the hydroxylation of the chlorobenzene derivative. We report here our results on hemin–thiol activity in hydroxylation of *p*-chlorotoluene and our finding that the complex induced aromatic methyl migration during hydroxylation (methyl NIH shift), though the value was small compared with the reported values of the NIH shift using [*p*-^{2}H]-chlorobenzene [10].

*Author to whom correspondence should be addressed.

Experimental

Materials and Methods

Hemin (Type I, bovine) was obtained from Sigma Chemical Co. Oxidation products of *p*-chlorotoluene were purchased or prepared by synthesis in our laboratories. The hydroxylation system consisted of hemin 1 mM, thiol compound 100 mM, *p*-chlorotoluene 100 mM, buffer solution (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) and acetone. Hydroxylation of *p*-chlorotoluene with model systems was carried out as reported previously [1]. The reaction products were determined by high performance liquid chromatography using a Hitachi 635 S connected to an Oyo Bunko Kiki Uvilog 5III UV-detector operated at 228 nm. Separation of the five hydroxylation products and *p*-chlorotoluene was carried out on a Chemco Nucleosil $5C_{18}$ column (ϕ 4.0 mm × 150 mm) and its pre-column (ϕ 4.6 mm × 30 mm) with a mixture of H_2O–CH_3CN (62:38) as mobile phase at a flow rate of 0.5 ml min^{-1} at room temperature. Samples of 10 μl were injected.

Results and Discussion

The optimal conditions for detecting five possible hydroxylation products of *p*-chlorotoluene (Fig. 1) were examined; they were simultaneously separated using the mobile phase of H_2O–CH_3CN (62:38) on a Nucleosil $5C_{18}$ column. The time required for separation was within 30 min (Fig. 2). The amounts and peak-heights or areas of the five compounds were linearly correlated over the ranges studied (*p*-chlorobenzyl alcohol [I], 0–7 nmol; 2-chloro-5-methyl-

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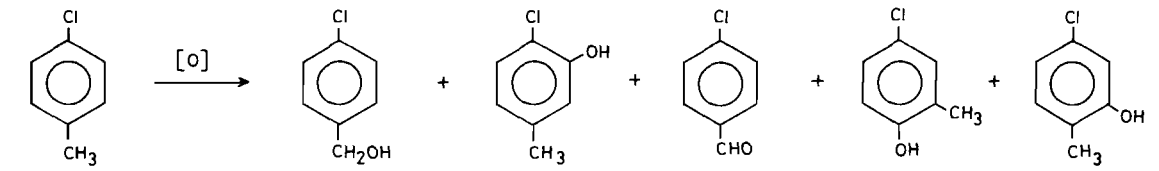

Fig. 1. Hydroxylation products of *p*-chlorotoluene by model systems of cytochrome P-450.

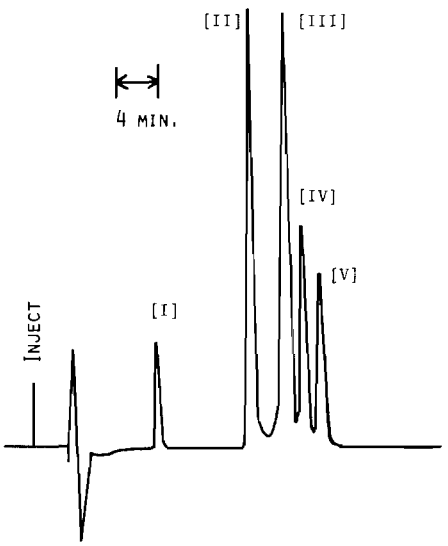

Fig. 2. Chromatogram for separation of hydroxylation products of *p*-chlorotoluene. [I]: *p*-chlorobenzyl alcohol; [II]: 2-chloro-5-methylphenol; [III]: *p*-chlorobenzaldehyde; [IV]: 4-chloro-2-methylphenol; [V]: 5-chloro-2-methylphenol. Separation conditions are described in the Experimental.

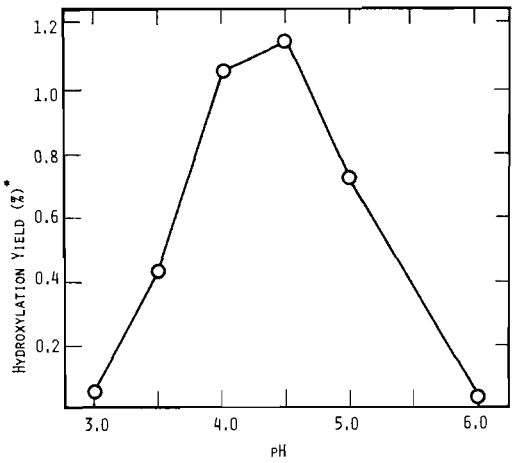

Fig. 3. pH-Dependency on hydroxylation of *p*-chlorotoluene by the hemin–cysteine ethyl ester system. Reaction conditions are as follows; hemin 1 mM, CysE 100 mM, substrate 100 mM, solvent 50% acetone, reaction time 2 hours and reaction temperature 40 °C. Points represent means of at least two experiments. *The total hydroxylation yield is based on the initial amount of substrate.

phenol [II], 0–8.2 nmol; *p*-chlorobenzaldehyde [III], 0–2.8 nmol; 4-chloro-2-methylphenol [IV], 0–9.9 nmol; 5-chloro-2-methylphenol [V], 0–5.4 nmol). Average recoveries [1] of the compounds were 100%.

Under these experimental conditions, it was confirmed that *p*-chlorotoluene was hydroxylated by the hemin–thiol model systems, the main products being [I]–[V]. The other oxidation products which were detected on the chromatogram depended on the various types of model system tested, but structures of these products were not investigated further.

For the system consisting of hemin and cysteine ethyl ester (CysE), the assay conditions were tested and a pH range of 4.0–4.5 in 50% acetone was found to be optimal (Fig. 3). Under these conditions after a reaction time of 2 hours, hydroxylation of *p*-chlorotoluene by various types of model system was studied and the results are summarized in Table I. The characteristic features deduced from the Table are that the system containing thiol esters such as CysE and TGE showed higher hydroxylation yields than those with the system containing corresponding thiol carboxylates such as Cys and TG, and these four systems induced aromatic methyl migration during hydroxylation (methyl NIH shift) [11], which is one of the unusual characteristics of cytochrome P-450 monooxygenases and can be used in evaluating chemical models for their capacity [12]. However, the system containing thioglycolate of higher alcohol such as octyl ester showed a low hydroxylation yield and induced no methyl NIH shift. The order of hydroxylation yield in hemin–thiol complexes examined was aliphatic oxidation to aldehyde [III] ≈ aromatic hydroxylation [V] > aliphatic oxidation to benzyl alcohol [I] ≈ aromatic hydroxylation [II] > methyl NIH shift [IV]. Further, the selectivity of the hydroxylation to *ortho*-position [V] (25–40%) rather than to *meta*-position [II] (10–23%) of the methyl group in *p*-chlorotoluene was found to be retained when it was compared with the result of

TABLE I. Hydroxylation of *p*-Chlorotoluene by Various Model Systems[a].

System[b]	pH	Conversion[c] (%/2 hrs)	Product Ratio (%)[d]				
			[I]	[II]	[III]	[IV]	[V]
Hemin–Cys	4.0	0.56 ± 0.02	5	10	56	1	28
Hemin–CysE	4.0	1.05 ± 0.07	6	23	35	3	33
Hemin–TG	4.0	0.20 ± 0.03	26	11	23	1	39
Hemin–TGE	4.0	0.41 ± 0.04	5	16	44	trace	35
Hemin–TGO	4.0	0.25 ± 0.01	10	13	53		25
Hemin–CysE–Asc	4.5	0.42	15	19	27		40
Fenton[e]	4.7	0.03 ± 0.02	69	7	10		14
Udenfriend[f]	7.5	1.39 ± 0.09	7	7	42		44

[a] Abbreviations: cysteine, Cys; cysteine ethyl ester, CysE; thioglycolic acid, TG; thioglycolic acid ethyl ester, TGE; thioglycolic acid octyl ester; TGO, ascorbic acid, Asc; ethylenediamine tetraacetic acid, EDTA. [b] Concentration of components and reaction conditions were as for Fig. 3. [c] The total hydroxylation yield is based on the initial amount of substrate. Data are means ± S.D. for 3–4 experiments. [d] Numbering of the products is as for Fig. 1. [e] $FeSO_4$ 5 mM, H_2O_2 50 mM and *p*-chlorotoluene 100 mM in 50% acetone–0.5 *M* acetate buffer, pH 4.7, under nitrogen atmosphere. [f] $FeSO_4$ 5 mM, EDTA 20 mM, Asc 100 mM and *p*-chlorotoluene in 50% acetone–0.5 *M* phosphate buffer, pH 7.5, under air.

toluene-hydroxylation by liver microsomes and model systems [13]. Enhancement of hydroxylation yield was not seen after addition of a second reducing agent such as ascorbate and sodium dithionate, whereas enhancement was observed in the acetanilide hydroxylation by the same hemin–thiol complexes [6]. No alteration was observed upon addition of non-ionic detergent such as Brij 58 (data are not given in the Table).

A relatively high hydroxylation yield comparable to that obtained with the hemin–CysE complex was obtained with the Udenfriend's system [14], whereas using the Fenton's system [15] a distinctly different hydroxylation pattern was seen (Table I); the yield of hydroxylation was very low and hydroxylation to benzyl alcohol was abundant.

It is known that Fenton's system is a well authenticated source of the hydroxyl radical, ·OH [16], whereas the active oxygen species of Udenfriend's system is not likely to involve ·OH, but metal-chelated oxygen species [16, 17]. In the hemin–Cys reaction, we previously suggested that the active oxygen intermediate contains hemin–thiolate bonding rather than a hydroxyl radical, superoxide anion, singlet oxygen or hydrogen peroxide species [18]. Judging from the results on *p*-chlorotoluene hydroxylation presented here and the data cited above, it is indicated that the active oxygen species in *p*-chlorotoluene hydroxylation by the hemin–thiol model complexes involves a metal-chelation site, probably thiol (or thiolate)–heme–oxygen. However, occurrence of the methyl NIH shift in the hemin–thiol systems shows that its reaction mechanism is different from that in the Udenfriend's system, which lacks the methyl NIH shift.

It was recently reported that an oxy-radical such as the hydroxyl radical induced the NIH shift during the non-enzymatic hydroxylation of [*p*-^{2}H]-chlorobenzene in aprotic solvents [10]. The evaluation of the model system which may involve either radical generation or oxygen species including metal-chelation is thought to be indispensable for understanding the mechanism of cytochrome P-450 dependent monooxygenases, and is being investigated further.

Acknowledgements

This research was supported in part by a Grant-in-Aid from the Research Foundation for Pharmaceutical Sciences, 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.

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