Hydroxylation of Benzene by Immobilized Cytochrome c in an Organic Solvent

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Recently we showed that cytochrome c (cyt.c) has some cytochrome P450 (P450)-like substrate oxidation activity, which was enhanced by immobilization on poly-γ-methyl-L-glutamate. We now report that immobilized cyt.c catalysed hydroxylation of benzene when benzene containing a small amount of water was used as the reaction solvent, whereas free cyt.c did not catalyse the reaction. The EPR spectrum of immobilized cyt.c showed that the immobilization had caused a change in the protein conformation, and this might have led cyt.c to acquire the new activity. The results of the reaction using [^{18}O]*m*-chloroperbenzoic acid (mCPBA) or [^{18}O]molecular dioxygen showed that 59% of the oxygen atom of the product, phenol, was derived from the oxidant, and 33% from molecular dioxygen. When [^{18}O]mCPBA was used as an oxidant, the value of deuterium isotope effect (k_H/k_D)was 1.08 when ^{18}O was incorporated into the product, and 1.48 when ^{18}O was not incorporated. These results indicate that immobilized cyt.c catalysed hydroxylation of benzene *via* two major pathways, of which one resembles that of P450.

Cytochrome P450 (P450) is a monooxygenase and catalyses a variety of oxidative reactions including hydroxylation of aliphatic and aromatic carbons, *N*- and *O*-dealkylation, *S*-oxidation, and epoxidation of olefins.[1] Recently we have reported that cytochrome c (cyt.c), one of the components of the electron transport system, catalysed several substrate oxidations, such as *N*-demethylation, *S*-oxidation, and olefin epoxidation, in the same manner as P450, *i.e.*, oxidation of substrates with incorporation of the oxygen atom of the reactive species into the product, and epoxidation with retention of the stereochemistry of the substrate.[2] We also reported that these P450-like activities of cyt.c were enhanced by covalent immobilization on poly-γ-methyl-L-glutamate (PMG). Generally, immobilization tends to increase the stability of enzymes *in vitro*, and to change the conformation of the protein partially when it was immobilized by covalent bonds onto the carrier. In our case, the conformational change of cyt.c caused by the immobilization appears to increase the P450-like reactivity, which is not an intrinsic function of cyt.c *in vivo*, that is, the enzyme function was induced by the immobilization.

In recent years several models which mimic the activity of P450 have been explored in attempts to understand better its catalytic cycle, as well as to develop new synthetic methods. Hydroxylation of aromatic substrates has been a focus of attention in studies with P450 model compounds, though investigation of this reaction is less advanced compared with other reactions. Some P450 chemical model systems are able to hydroxylate aromatic compounds, but the yields are only moderate.[3] In this work we investigated whether cyt.c and immobilized cyt.c could catalyse hydroxylation of aromatic carbon.

Recently, enzymatic reactions in organic solvents have been widely studied, and it has been found that many enzymes can work in water-immiscible organic solvents containing a little water. There are numerous advantages to conducting enzymatic conversions in organic solvents as opposed to water.[4] Initially, reactions catalysed by hydrolases were studied, including esterification, transesterification, acylation, amidation, and resolution of racemic alcohols, amines and carboxylic acids.[5] Later, oxidative reactions catalysed by oxidases, such as horseradish peroxidase,[6] alcohol dehydrogenase,[7] and polyphenol oxidase,[8] were also studied. The role of water in organic solvents has been studied too, and it has been assumed that water works as a molecular lubricant in proteins, due to its ability to form multiple hydrogen bonds, and activates enzymes by enhancing their conformational flexibility.[9]

In this report, we examined hydroxylation of benzene by cyt.c and immobilized cyt.c in benzene containing a small amount of aqueous buffer.

Results and Discussion

Benzene Hydroxylation Activity of Immobilized Cyt.c.— Hydroxylation of benzene was conducted in benzene containing 0–5% of 0.1 mol dm^{-3} phosphate buffer (pH 7.0). Hydrogen peroxide and mCPBA were used as oxidants. The activities of free and immobilized cyt.c are shown in Table 1. When free cyt.c was used as a catalyst, the amounts of phenol formed were at the same level as in the control system, that is, the cyt.c(−) system. When immobilized cyt.c was used, the amounts of phenol generated were much larger. The highest turnover number of immobilized cyt.c was 4.1 [oxidant; *m*-chloroperbenzoic acid (mCPBA), buffer content; 0.2%]. P450IIE1, one of the P450 isozymes, has been reported to catalyse hydroxylation of benzene more effectively than other P450 isozymes, and to be induced by ethanol, acetone, benzene, *etc.*[10] When the reaction was catalysed by acetone-treated rat hepatic microsomes in the NADPH*–O_2 system (in this system, P450 accepts two electrons from NADPH *via* P450 reductase and activates molecular dioxygen to form the reactive species, as *in vivo*), the turnover number of P450 was 6.4. So, it can be said that the activity of immobilized cyt.c was comparable to that of P450. When poly-L-glutamine-coated glass beads formed by converting methyl esters of PMG to amides, poly-L-glutamine-coated glass beads plus free cyt.c, and immobilized bovine serum albumin (BSA) were used in the place of immobilized cyt.c, the amounts of phenol formed were at the same level as those in the cyt.c(−) system. These results show that immobilized cyt.c catalysed hydroxylation of benzene, while free cyt.c did not. The amounts of benzoquinone and hydroquinone formed were very small (< 1 nmol), probably because the substrate, benzene, was in large excess over the catalyst.

Table 1 also shows the relation between the activity of immobilized cyt.c and the water content of the reaction mixtures. The reaction without water was examined with mCPBA as an oxidant. The activity of immobilized cyt.c in this

* NADPH = reduced nicotinamide adenine dinucleotide phosphate.

Table 1 Hydroxylation of benzene catalysed by immobilized cytochrome c

Oxidant	Buffer content[c] (%)	Catalyst (phenol formation/nmol) Free cyt.c	Immobilized cyt.c	Cyt.c(−)[d]	Polymer	Polymer + Free cyt.c	Immobilized BSA
H_2O_2[a]	0.25	0.3	61.8	0.8			
	0.5	1.8	56.7	5.7			
	1	2.0	65.1	4.0	3.1	3.4	4.0
	3	2.7	70.0	5.5			
	5	2.7	32.8	4.9			
mCPBA[a]	0	8.3	32.9	5.9			
	0.2	7.9	81.3	4.6			
	0.5	7.7	76.6	6.5			
	1	4.3	67.3	7.3			
	3	4.4	32.0	8.0			
	5	4.2	34.6	11.3			
H_2O_2[b]	100	1.2	0.9	0.7			

[a] Reaction mixtures contained free or immobilized cyt.c (20 nmol), oxidant (20 mmol dm^{-3}), and phosphate buffer (pH 7.0) (0.1 mol dm^{-3}; 0–5%) in benzene (total 1 cm^3), and were incubated for 20 min at 37 °C. [b] Reaction mixtures contained free or immobilized cyt.c (20 nmol), benzene (2 mol dm^{-3}), and hydrogen peroxide (1 mmol dm^{-3}) in phosphate buffer (pH 7.0) (0.1 mol dm^{-3}; total 1 cm^3), and were incubated for 20 min at 37 °C. [c] For example, 0.25% buffer content means 2.5 mm^3 of buffer and 997.5 mm^3 of benzene. 1% buffer content means 10 mm^3 of buffer and 990 mm^3 of benzene. [d] Cyt.c(−) system contained all the components of the reaction mixture except cyt.c.

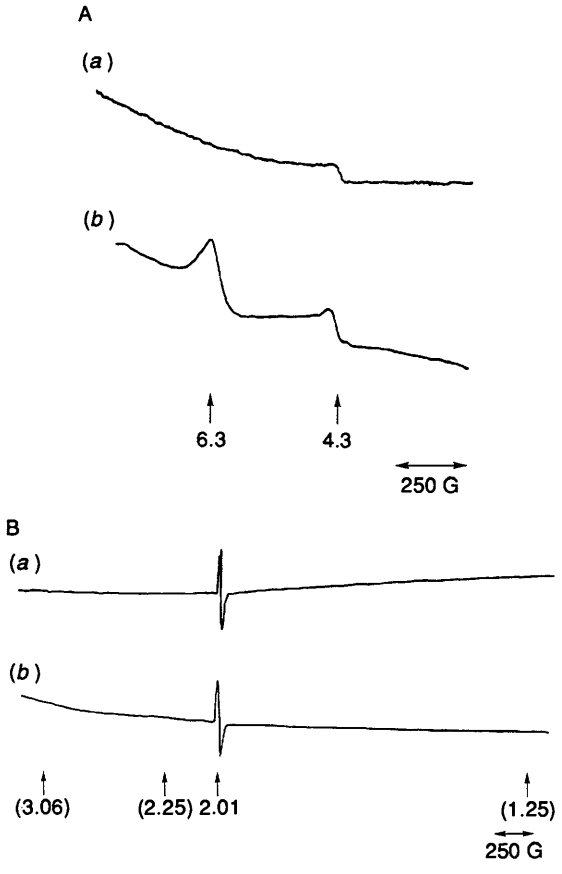

Fig. 1 EPR spectra of immobilized cyt.c and polymer-coated glass beads. (*a*) Glass beads coated with polymer azide (amplitude 125). (*b*) Immobilized cyt.c (amplitude 250). A. Spectra in the region of $g = 6$. B. Spectra in the region of $g = 2$. The scale of B is reduced to 69% of A.

case was about 40% of that in the reaction mixture containing 0.2% buffer. This result shows that a small amount of buffer was necessary for higher activity of immobilized cyt.c. Possibly the water added to benzene hydrated the immobilized cyt.c, increasing the flexibility of the cyt.c molecule for catalysis. On the other hand, in the systems using hydrogen peroxide and mCPBA as the oxidant, the activity of immobilized cyt.c decreased as the buffer content in the reaction mixtures was increased (more than 3%). This can be explained by supposing that excessive hydration of immobilized cyt.c results in the bound water molecules blocking access of the substrate to the heme pocket. In addition, the reaction was not catalysed by free or immobilized cyt.c when the buffer alone was used as the reaction solvent.

EPR Spectrum of Immobilized Cyt.c.—EPR spectra of glass beads coated with polymer azide and immobilized cyt.c are shown in Fig. 1. In the spectrum of the polymer-coated glass beads, there were two peaks, of which one ($g = 4.3$) was probably derived from iron contained by the glass beads, and the other ($g = 2.01$) was probably derived from some organic radicals on the activated polymer. In the spectrum of immobilized cyt.c, in addition to the two peaks which were seen in the spectrum of the polymer-coated glass beads, there was one clear peak ($g = 6.3$) due to high-spin heme iron. In native cyt.c, the axial ligands of heme iron comprise a nitrogen atom of a histidine residue and a sulfur atom of a methionine residue, so the heme iron is in low-spin form showing characteristic EPR signals ($g = 3.06, 2.25, 1.25$).[11] In the spectrum of immobilized cyt.c, however, there were no signals of low-spin heme iron, indicating that one of the two ligands was dissociated from the heme iron and the spin state was changed. It is known that the sixth ligand of iron, the sulfur atom of Met-80, is easily dissociated from the iron by lowering the pH,[12] or by reagents such as cyanide, azide or imidazole.[13] Therefore, the ligand dissociated from the iron is thought to be the sulfur atom of Met-80. From these results, it appears that the protein conformation of cyt.c was changed by immobilization, and the sulfur atom of Met-80 was dissociated from the heme iron, so presumably the heme groove was opened sufficiently for oxidants and/or substrates to approach the heme iron.

Mechanism of Benzene Hydroxylation Catalysed by Immobilized Cyt.c.—P450 incorporates an oxygen atom of the reactive species into the product during the reaction.[1] To investigate whether immobilized cyt.c incorporates oxygen of the reactive species into the product during hydroxylation of benzene, the reactions were carried out in the presence of [^{18}O]mCPBA, or [^{18}O]molecular dioxygen, or [^{18}O]water. The results show that 59% of the oxygen atom of phenol formed is derived from mCPBA, 33% is derived from molecular dioxygen, and none is derived from water (Table 2). When hydroxylation of benzene was catalysed by acetone-treated rat

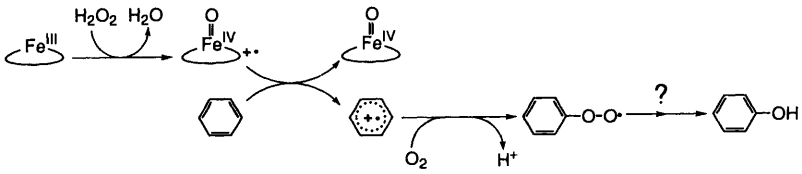

Fig. 2 Proposed mechanism of benzene hydroxylation catalysed by immobilized cyt.c with incorporation of the oxygen atom from molecular dioxygen into phenol

Table 2 ^{18}O Incorporation into phenol by immobilized cyt.c[a]

^{18}O Source	^{18}O Incorporation (%)
[^{18}O]mCPBA	59
$^{18}O_2$	33
$H_2{}^{18}O$	0

[a] Reaction mixtures contained immobilized cyt.c (20 nmol), mCPBA (20 mmol dm^{-3}), and phosphate buffer (pH 7.0) (50 mmol dm^{-3}; 2%) in benzene (total 1 cm^3), and were incubated for 20 min at 37 °C.

hepatic microsomes in the presence of [^{18}O]cumene hydroperoxide (CHP) or [^{18}O]mCPBA, almost 100% of the oxygen atom of phenol formed was ^{18}O (in this system, which is called the shunt pathway, P450 reacts directly with the oxidant to form the reactive species). For hydroxylation of aromatic carbon by P450, two mechanisms are proposed. In one mechanism, the oxygen atom of the reactive species is directly added to the aromatic ring to form an arene oxide intermediate, then the epoxide ring is opened and phenol is formed (direct addition mechanism).[14] In the other mechanism, one electron of the aromatic ring is abstracted by P450 reactive species, then the oxygen atom of this reactive species attacks the cation radical to form the product, phenol (one electron abstraction mechanism).[15]

On the basis of our results, two types of pathways may operate in benzene hydroxylation catalysed by immobilized cyt.c. In one type, mCPBA and heme iron of immobilized cyt.c react to form the iron-oxenoid type reactive species, and the oxygen atom of this reactive species is incorporated into phenol. This leads to the same result as that in the reaction catalysed by P450, though it is not clear which mechanism, direct addition or one electron abstraction, actually proceeded. In the other type, the oxygen atom of molecular dioxygen is incorporated into phenol. The mechanism is unclear, but one possible mechanism is shown in Fig. 2. Similarly to the one electron abstraction mechanism of P450, one electron of benzene is abstracted by the reactive species, and benzene cation radical is formed. Subsequently this radical reacts with molecular dioxygen accompanying the release of a proton to form phenylperoxy radical, then the O–O bond of the adduct is cleaved in some way. Thus, immobilized cyt.c is thought to catalyse hydroxylation of benzene partially in the same manner as P450.

In hydroxylation of an aromatic ring by P450, it is known that the phenomenon called NIH shift [migration of a hydrogen (deuterium) to the vicinal carbon] occurs. For example, in the hydroxylation of [4-^2H]toluene catalysed by rat liver microsomes, 54% of NIH shift was observed.[16] When hydroxylation of [4-^2H]toluene by immobilized cyt.c was carried out, 19.1% of the deuterium migrated and was retained in the product, *p*-cresol. This result showed the partial similarity between the reaction mechanism of immobilized cyt.c and that of P450, and supports our idea about the reaction mechanism.

From experiments using C_6H_6–C_6D_6 (1:1) mixture as a substrate and as a reaction solvent, the deuterium isotope effect (k_H/k_D) during the hydroxylation of benzene catalysed by cyt.c was determined. [^{18}O]mCPBA was used as the oxidant. When ^{18}O from the oxidant was incorporated into the product, k_H/k_D was 1.08, and when ^{18}O from the oxidant was not incorporated into the product, *i.e.*, the oxygen atom of molecular dioxygen was incorporated, k_H/k_D was 1.48. In the reaction catalysed by acetone-treated rat liver microsomes, k_H/k_D was 1.07. The fact that the k_H/k_D values in the reaction catalysed by immobilized cyt.c were both similar to that in the reaction by P450 is thought to support our proposed reaction mechanism described above.

Conclusions.—It has been shown that immobilized cyt.c. catalyses hydroxylation of benzene. This is very interesting, because P450 is the only known hemoprotein which catalyses this reaction. It is presumed that the immobilization induced a change of cyt.c. protein conformation, but it is not clear how this occurred or why the immobilized cyt.c was able to catalyse the reaction. These will be important targets for future study. Since P450 is involved in the metabolism of many xenobiotics having aromatic rings, the results of our work suggest that immobilized cyt.c may be useful in drug metabolism research.

Experimental

Materials.—PMG (Ajicoat F-4000) was kindly supplied by Ajinomoto Co., Inc. (Tokyo, Japan), as a 10% solution in ethylene dichloride. Silicone rubber adhesive (Tosu-seal 371) was purchased from Toshiba Silicone Co., Inc. (Tokyo, Japan). Porous glass beads (CPG-10), 150–180 μm in diameter, were purchased from CPG Inc. Cyt.c (from horse heart) and BSA were purchased from Sigma Chemical Co. [$^2H_6$]Benzene (99.95 atom %) was purchased from Merck. $H_2{}^{18}O$ (99.8 atom %) was purchased from Euriso-Top. $^{18}O_2$ gas (89 atom %) was purchased from ISOTEC. [^{18}O]mCPBA (78 atom %) was prepared from $K^{18}O_2$ and *m*-chlorobenzoyl chloride as previously described.[17] [4-^2H]Toluene (80 atom %) was prepared by forming a Grignard reagent from *p*-bromotoluene and magnesium, and hydrolysing it with D_2O. Other chemicals used were the purest available commercially.

Preparation of Immobilized Cyt.c, Immobilised BSA and Poly-L-glutamine-coated Glass Beads.—Immobilized cyt.c was prepared as reported.[2,18] Porous glass beads (1.0 g) were mixed with a solution of 10% PMG solution (1.0 g), silicone rubber adhesive (50 mg) and chloroform (5 cm^3). The organic solvent was removed under reduced pressure, and the remaining beads coated with PMG were dried at 80–90 °C *in vacuo* for 5–10 h.

The PMG-coated glass beads (1.0 g) were added to 40% hydrazine hydrate in ethanol (50 cm^3) and stirred gently at 70 °C for 2.5 h to convert methyl esters of PMG into hydrazides. The polymer hydrazide obtained was added to sodium nitrite (0.14 mol dm^{-3}) in hydrochloric acid (0.2 mol dm^{-3}; 120 cm^3) and stirred gently at 0 °C for 30 min to convert hydrazides into acyl azides. This activated polymer was mixed with cyt.c (10 mg) in phosphate buffer (pH 8.7) (0.1 mol dm^{-3}; 10 cm^3) and stirred gently to immobilize the protein by amide bond formation between carbonyl groups of the polymer and free amino groups of the protein. The contents of cyt.c immobilized on the polymer was calculated from the difference between the Soret band absorption of cyt.c solution before and after immobilization.

The immobilized cyt.c was dried intensively *in vacuo* before use.

Immobilized BSA was prepared according to the method described above but with BSA solution (1 mg cm^{-3}) instead of cyt.c solution. The contents of BSA immobilized was calculated from the difference between the BSA concentration of the solution before and after immobilization, determined by Lowry's method.[19]

Poly-L-glutamine-coated glass beads were prepared as follows. Methyl esters of the PMG-coated glass beads (0.4 g) were activated to acyl azides as described above, then the beads were added to aqueous ammonia (500 mmol dm^{-3}; 4 cm^3), and stirred gently for 2 h at 4 °C. This polymer amide was separated by filtration and washed thoroughly with phosphate buffer (pH 7.0) (0.1 mol dm^{-3}).

Hydroxylation of Benzene Catalysed by Cyt.c.—Hydroxylation of benzene catalysed by cyt.c was carried out as follows. Free cyt.c was placed in test tubes as an aqueous solution, then the water was removed by argon flushing, and the residue was dried *in vacuo* before the reaction. Reaction mixtures contained free or immobilized cyt.c (20 nmol) and phosphate buffer (pH 7.0) (0.1 mol dm^{-3}; 0–5%) in benzene (1 cm^3). They were placed in test tubes, incubated on a dry bath at 37 °C, and stirred with a magnetic stirrer. After preincubation for 3 min, the reaction was initiated by adding an oxidant (final 20 mmol dm^{-3}), then the mixtures were incubated for 20 min at 37 °C. When hydrogen peroxide was used as the oxidant, the reaction was stopped by cooling the mixtures with ice immediately. When mCPBA was used as an oxidant, the reaction was stopped by adding triphenylphosphine (150 mmol dm^{-3}; 200 mm^3) to reduce excess of mCPBA. After removal of cyt.c and the buffer by centrifugation, the organic phases were dried (sodium sulfate), and concentrated, and then the phenol formed was trimethylsilylated by mixing with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and anhydrous pyridine, and storage for 10 min at room temperature. Excess of BSTFA and pyridine were removed by argon flushing, and the residue was dissolved in a small amount of ethyl acetate and then analysed by GC or GC–MS (Shimadzu QP2000A). The formation of benzoquinone and hydroquinone (trimethylsilylated) was also analysed by GC–MS.

In the case of poly-L-glutamine-coated glass beads or immobilized BSA as the control, the same weight of the polymer-coated glass beads or immobilized BSA as that of immobilized cyt.c was used (20 mg/one reaction; 20 mg of immobilized BSA corresponded to about 5 nmol of BSA).

When the reaction was carried out in buffer, reaction mixtures contained free or immobilized cyt.c (20 nmol), benzene (2 mmol dm^{-3}) (10 mm^3 of acetonitrile was used as a co-solvent), and hydrogen peroxide (1 mmol dm^{-3}) in phosphate buffer (pH 7.0) (0.1 mol dm^{-3}; 1 cm^3). The mixtures were incubated for 20 min at 37 °C, and phenol was extracted with ethyl acetate (2 cm^3 × 2). The extracts were dried (sodium sulfate) and concentrated, and then phenol was trimethylsilylated as described above.

To investigate the incorporation of the oxygen atom of molecular dioxygen into phenol, the reaction was carried out under an atmosphere of $^{18}O_2$–argon according to the method previously described.[17] The reaction mixture without mCPBA was placed in a two-necked flask, and mCPBA was separately placed in a glass vessel connected to the flask. The system was also connected by a glass tube to a vial containing $^{18}O_2$ gas (100 cm^3), a balloon containing argon gas, and a vacuum pump. The mixture was evacuated 10 times, and the atmosphere was replaced with argon gas each time. After the last evacuation, the seal to the $^{18}O_2$ gas reservoir was broken and the gas was allowed to distribute through the system, followed by introduction of argon gas to equalize the pressure. The reaction was subsequently initiated by addition and mixing of mCPBA.

When the percentage of NIH shift was to be determined, reaction mixtures contained immobilized cyt.c (20 nmol), hydrogen peroxide (40 mmol dm^{-3}), and phosphate buffer (pH 7.0) (0.1 mol dm^{-3}; 2%) in [4-^{2}H]toluene (0.5 cm^3), and were incubated for 20 min at 37 °C.

When the deuterium isotope effect (k_H/k_D) was to be determined, reaction mixtures contained immobilized cyt.c (20 nmol), [^{18}O]mCPBA (20 mmol dm^{-3}), and phosphate buffer (pH 7.0) (0.1 mol dm^{-3}; 1%) in C_6H_6–C_6D_6 (1:1; 1 cm^3) mixture, and were incubated for 20 min at 37 °C.

Hydroxylation of Benzene Catalysed by Rat Liver Microsomes.—Induction of P450 was carried out according to the method previously reported.[10] Male Wistar rats (6 weeks) were given i.g. (intragastric) injections of 25% aqeuous acetone (5 cm^3 acetone kg^{-1}) for 2 days. Then hepatic microsomes were prepared as previously described.[20]

When the reaction was carried out in the NADPH–O_2 system, the reaction mixtures contained microsomes (4 mg protein), glucose-6-phosphate (4 mmol dm^{-3}), 5 units of glucose-6-phosphate dehydrogenase, magnesium chloride (4 mmol dm^{-3}), potassium chloride (60 mmol dm^{-3}), C_6H_6 (5 mmol dm^{-3}), or C_6H_6 (2.5 mmol dm^{-3}) and C_6D_6 (2.5 mmol dm^{-3}) (10 mm^3 of acetonitrile was used as a co-solvent) in phosphate buffer (pH 7.4) (0.1 mol dm^{-3}; 2.5 cm^3). After preincubation for 3 min at 37 °C with a water incubator, the reaction was initiated by adding $NADP^+$ (final 0.4 mmol dm^{-3}). The mixtures were incubated for 20 min at 37 °C, and phenol was extracted with methylene dichloride (4 cm^3 × 1). The extracts were dried (sodium sulfate), and concentrated, then phenol was trimethylsilylated.

When the reaction was carried out *via* the shunt pathway using mCPBA or CHP as the oxidant, the reaction mixtures contained microsomes (4 mg protein), magnesium chloride (4 mmol dm^{-3}), potassium chloride (60 mmol dm^{-3}), and benzene (5 mmol dm^{-3}) (10 mm^3 of acetonitrile was used as a co-solvent) in phosphate buffer (pH 7.4) (0.1 mol dm^{-3}; 2.5 cm^3). After preincubation for 3 min at 37 °C, the reaction was initiated by adding the oxidant (as 10 mm^3 of acetonitrile solution, final 2 mmol dm^{-3}). The mixtures were incubated for 20 min at 37 °C.

Measurement of EPR Spectra.—Immobilized cyt.c, glass beads coated with polymer azide were placed in quartz tubes. Molecular oxygen in the tubes was removed by argon flushing for 1 h, and the samples were frozen in liquid nitrogen. The EPR spectra were measured at 77 K by using a JEOL JES-FE3XG spectrometer.

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