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Hydroxylation of Benzene by Horseradish Peroxidase and Immobilized Horseradish Peroxidase in an Organic Solvent

Reiko Akasaka, Tadahiko Mashino, and Masaaki Hirobe*

Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan

Abstract: Horseradish peroxidase (HRP) catalyzed hydroxylation of benzene with an oxidant when benzene was used as the reaction solvent. HRP immobilized on poly(γ-methyl-L-glutamate) also catalyzed the reaction with higher activity than that of free HRP. The results of the reaction using [^{18}O]hydrogen peroxide show that free and immobilized HRP incorporated the oxygen atom of the reactive species into the product, indicating that both forms of HRP can catalyze hydroxylation of benzene in an analogous manner to that of cytochrome P450.

Cytochrome P450 (P450) is a monooxygenase and catalyzes a variety of oxidative reactions including hydroxylation of aliphatic and aromatic carbons, *N*- and *O*-dealkylations, *S*-oxidation, and epoxidation of olefins[1]. P450 is the only known hemoprotein which catalyzes the hydroxylation of aromatic hydrocarbons and consequently the differences between P450 and other hemoproteins have been extensively investigated, including the nature of the fifth ligand of the heme iron, characteristics of the reactive species, and amino acid sequence of the distal site of the heme[2]. And further, several chemical 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. Some P450 chemical model systems are able to hydroxylate aromatic hydrocarbons, but the yields are only moderate[3].

We have studied the reactivity of cytochrome c (cyt.c), one of the components of the electron transport system, and found that cyt.c catalyzes some substrate oxidation reactions in the same manner as P450, and further, that these activities are increased by covalent immobilization of cyt.c on poly(γ-methyl-L-glutamate) (PMG)[4]. We have also reported that immobilized cyt.c catalyzed hydroxylation of benzene when benzene was used as the reaction solvent, and we found that this activity was higher in the presence of a small amount of water[5]. Recently it has been found that many enzymes can work in water-immiscible organic solvents containing a small amount of water[6]. It has been proposed that water works as a molecular lubricant in proteins, due to its ability to form multiple hydrogen bonds, and activates enzymes in organic solvents by enhancing their conformational flexibility[7].

Horseradish peroxidase (HRP) is known to catalyze some P450-catalyzed reactions, such as *N*-demethylation and *S*-oxidation in buffer (that is, under usual conditions for enzymatic reactions), but it does not catalyze hydroxylation of aromatic hydrocarbons except phenolic compounds in buffer[8]. In this communication, we report that HRP catalyzed hydroxylation of benzene when benzene was used as the reaction solvent, and that HRP immobilized on PMG also catalyzed the reaction with higher activity than that of free HRP.

Immobilized HRP was prepared according to the method described previously[4]; 45% of the HRP used was immobilized, and the enzyme content was 85 nmol/g dry support. [^{18}O]hydrogen peroxide was prepared by disproportionation of $K^{18}O_2$ (78 atom %)[4]. $^{18}O_2$ gas (89 atom %) was purchased from ISOTEC. $H_2{}^{18}O$ (99.8 atom %) was purchased from EURISO-TOP.

When benzene was used as the reaction solvent, reaction mixtures contained 3 nmol of free or immobilized HRP, 20 mM hydrogen peroxide or *m*-chloroperoxybenzoic acid (mCPBA), and 0~5%(v/v) of 0.1 M phosphate buffer (pH 7.0) in 1 ml of benzene. The reaction under $^{18}O_2$/Ar mixture was carried out as described previously[5]. When buffer was used as the reaction solvent, reaction mixtures contained 3 nmol of free or immobilized HRP, 2 mM benzene (20 µl of acetonitrile was used as a co-solvent), and 1 mM hydrogen peroxide in 1 ml of 0.1 M phosphate buffer (pH 7.0). The condition of the reaction in buffer is according to general methods to determine native activities of HRP. The mixtures were incubated for 20 min at 37°C (the reaction was over within this reaction time). The product, phenol, was trimethylsilylated and detected by GC-MS.

Table 1. Hydroxylation of Benzene Catalyzed by Free and Immobilized HRP.

run	oxidant	buffer content (%)	phenol formation (nmol)		
			free HRP	immobilized HRP	HRP(-)
1	H_2O_2[a]	1	29.1	40.9	4.0
2		3	17.9	27.2	4.1
3		5	19.7	21.4	4.8
4	mCPBA[a]	0	6.2	38.0	8.6
5		0.2	6.6	90.6	8.0
6		0.5	8.2	75.8	7.6
7		1	8.3	69.9	6.1
8		3	8.0	53.8	7.7
9		5	10.6	37.4	8.5
10	H_2O_2[b]	100	0.1	0.1	0.1

Reaction solvent: a) benzene, b) buffer. The amounts of free or immobilized HRP used in each reaction were 3 nmol.

Phenol formation in the reaction is shown in Table 1. Free HRP catalyzed hydroxylation of benzene when hydrogen peroxide was used as the oxidant, and the activity of the protein was 9.7 nmol phenol formed / nmol HRP (run 1 in Table 1). But when mCPBA was used, the amounts of phenol formed were at the same level as in the control, HRP(-) system. In contrast, immobilized HRP catalyzed the reaction with either hydrogen peroxide or mCPBA as the oxidant, with the activity of 13.6 or 30.2 nmol phenol formed / nmol HRP, respectively (runs 1 and 5 in Table 1). In hydroxylation of benzene catalyzed by P450 in buffer, i.e., under a normal condition, the activity of P450 was 6.4 nmol phenol formed / nmol P450[5]. Thus, the activity of free HRP was similar to that of P450, and was increased by immobilization. The amounts of benzoquinone and hydroquinone, the products of further oxidation of phenol, formed in the reaction were very small. In addition,

the reaction was not catalyzed by free or immobilized HRP when only the buffer was used as the reaction solvent.

We previously found that free cyt.c did not catalyze hydroxylation of benzene, but free HRP did catalyze this reaction. The difference of activity between these two hemoproteins probably derives from their native characteristics, i.e., cyt.c is a member of the electron transport system, and its heme iron is coordinated by a sulfur atom of a methionine residue as the sixth ligand, in addition to the fifth ligand, an imidazole of a histidine residue[9], whereas HRP is a peroxidase, which reacts with hydrogen peroxide to form the reactive species, and oxidizes substrates such as amines and phenols[2a].

The activity of HRP was enhanced by immobilization, particularly when mCPBA was used as the oxidant. It is thought that the heme of free HRP is well covered by the apoprotein and the heme cavity is small[10]. Therefore, we propose that covalent immobilization of HRP on PMG caused some conformational change of the apoprotein and increased the accessibility of heme to substrate and oxidant, leading to the higher reactivity of immobilized HRP as compared with free HRP.

Table 1 also shows the relation between the activity of immobilized HRP and the buffer content of the reaction mixtures (The same relation was reported before for the reactions catalyzed by immobilized cyt.c[5]). The activity of immobilized HRP without buffer was about 40% of that with 0.2% buffer. On the other hand, the activity of immobilized HRP decreased as the buffer content in the reaction mixture was increased. These results show that a small amount of buffer is necessary for higher activity of immobilized HRP, while too much buffer presumably results in excessive binding of water molecules to the protein, blocking access of the substrate, benzene, to the heme pocket.

P450 incorporates an oxygen atom of the reactive species into the product during the reaction[1]. To investigate whether free HRP and immobilized HRP incorporate oxygen of the reactive species into the product during hydroxylation of benzene, the reactions were carried out in the presence of [^{18}O]hydrogen peroxide, or [^{18}O]molecular dioxygen, or [^{18}O]water. The results are shown in Table 2. The ^{18}O content of the product was corrected based on the original ^{18}O content of each ^{18}O source. In the case of free HRP, 95% of the oxygen in phenol formed was derived from the oxidant, that is, from the reactive species. Very little was derived from molecular dioxygen or water. In the case of immobilized HRP, 61% of the oxygen in phenol formed was derived from the oxidant, 20% from molecular dioxygen, and 1% from water. The result for immobilized HRP resembled that obtained for immobilized cyt.c reported before[5]. Thus, free HRP was shown to catalyze the reaction in the same manner as P450, whereas immobilized HRP seems to operate via two mechanisms. In one, the oxygen atom of the reactive species is incorporated into the product, phenol, as in the case of P450, and in the other, the oxygen atom of molecular dioxygen is incorporated.

Table 2. ^{18}O Incorporation into Phenol by Free and Immobilized HRP.

^{18}O source	^{18}O incorporation (%)	
	free HRP	immobilized HRP
$H_2{}^{18}O_2$	95	61
$^{18}O_2$	2	20
$H_2{}^{18}O$	1	1

We have shown here that free HRP catalyzes the hydroxylation of benzene when benzene was used as the reaction solvent, and its immobilization on PMG enhanced this reactivity. The very high content of the substrate in this reaction system may cause free HRP, which is essentially a peroxidase, in contrast to cyt.c, to have an activity which it does not show in a buffer system. The difference of reactivity between P450 and HRP in buffer was suggested to be mainly a consequence of the difference between their reactive species[2b]. But we have shown here that the reactive species of HRP does have a potential to hydroxylate benzene like P450 in our reaction system.

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