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ANILINE IS HYDROXYLATED BY THE CYTOCHROME P-450-DEPENDENT HYDROXYL RADICAL-MEDIATED OXYGENATION MECHANISM

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Hydroxylation of aniline, catalyzed by rabbit liver microsomal cytochromes P-450 in reconstituted systems, was inhibited by catalase, superoxide dismutase, catechol, mannitol, hydroquinone, dimethylsulfoxide and benzoate, whereas the cytochrome P-450-catalyzed O-demethylation of paranitroanisole, measured under the same conditions, was unaffected by these agents. A similar inhibition profile of the hydroxylation reaction was observed in reconstituted systems where cytochrome P-450 had been replaced by hemoglobin. The results indicate that aniline hydroxylation is mediated by hydroxyl radicals generated in an iron-catalyzed Haber-Weiss reaction between O_2^- and H_2O_2 and may explain some of the special properties of this reaction previously described.

INTRODUCTION

Among different types of cytochrome P-450-catalyzed hydroxylation reactions, the parahydroxylation of aniline is known to exhibit special properties. Firstly, the substrate aniline, induces a type II spectral shift in the P-450 difference spectrum; a shift produced by compounds binding directly to the heme moiety of P-450. Secondly, the hydroxylation reaction is not specific for cytochrome P-450. Significant rates of paraaminophenol formation are obtained using e.g. any of the hemoproteins cytochrome b_5, hemoglobin, myoglobin, cytochrome c, indoleamine dioxygenase or tryptophan dioxygenase instead of cytochrome P-450 (1,2). In these instances the hemoprotein used is reduced by NADPH or NADH via NADPH-cytochrome P-450 reductase (2) or added flavin (1). The discovery of hemoglobin acting as a hydroxylase (3,4) led to the finding of an aniline hydroxylase system in

Abbreviations used are: DMSO, dimethylsulfoxide; SOD, superoxide dismutase; P-450 LM, liver microsomal cytochrome P-450; P-450 LM_2, P-450 LM_{3b}, P-450 LM_{3c}, P-450 LM_4, P-450 LM_6, forms of cytochrome P-450 designated according to their electrophoretic mobilities.

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erythrocytes (5,6) with hemoglobin as the terminal component. The mechanism of this hydroxylation reaction has hitherto remained obscure.

Recently, a new type of cytochrome P-450-dependent oxygenation mechanism was identified (7). Using ethanol as substrate, it was found that hydroxyl radicals, generated by an iron-catalyzed Haber-Weiss reaction between superoxide anions and hydrogen peroxide, constituted the active oxygenating species, yielding upon interaction with ethanol, acetaldehyde as product. Other systems than cytochrome P-450 generating superoxide anion as e.g. dihydroxyfumarate in water, also catalyzed the ethanol oxidation (7) by the same reaction mechanism. It may thus be suggested that any system producing O_2^- should in the presence of trace amounts of iron be capable of producing hydroxyl radicals participating in the oxidation of ethanol or other compounds interacting with hydroxyl radicals. We considered aniline to be a candidate for this oxygenation mechanism, since the unspecificity of the parahydroxylation reaction, with regard to the hemoprotein participating in the reaction, would then be better understood.

MATERIALS AND METHODS

Microsomal phospholipids were extracted from liver microsomes obtained from phenobarbital-treated rabbits according to Bligh and Dyer (8). Dilauroylphosphatidylcholine, catalase (11 500 units/mg), D-mannitol, catechol, superoxide dismutase (specific activity, 2410 units/mg), hydroquinone, hemoglobin (type I), sodium benzoate, methional and α-keto γ-methiolbutyric acid were purchased from Sigma. Dihydroxyfumaric acid was obtained from Merck. Electrophoretically homogeneous preparations of cytochrome P-450 LM_2 and NADPH-cytochrome P-450 reductase were prepared from liver microsomes of phenobarbital-treated rabbits as previously described (9). The cytochrome P-450 LM_2-preparation used had a specific content of 15.6 nmol/mg and the specific content of flavin in the NADPH-cytochrome P-450 reductase preparation used was 21.4 nmol/mg, when flavin was determined according to Iyanagi & Mason (10). Unilamellar phospholipid vesicles containing cytochrome P-450 LM_2 and NADPH-cytochrome P-450 reductase were prepared by the cholate gel filtration technique (9). Usually, 10 mg of microsomal phospholipids in chloroform solution was taken to absolute dryness under a stream of nitrogen. The residue was suspended in 1 ml of 10 mM tris-HCl, pH 7.4, containing 50 mM NaCl and 0.2 mM EDTA and 20 mg cholate was added. The solution was sonicated for 1-2 min and subsequently, 10 nmol of cytochrome P-450, 4 nmol of NADPH-cytochrome P-450 reductase were added. After 30 min, the solution was chromatographed on a precoated Sephadex G-50 column (1 x 15 cm), equilibrated in the tris

buffer and the vesicles were isolated from the void volume. Incubations with aniline were performed with vesicles corresponding to 0.3 nmol P-450 in 50 mM potassium phosphate buffer, pH 7.4, containing 0.25 mM aniline and 1 μCi of [14C]-aniline (New England Nuclear). Preincubation was performed for 3 min at 37°C and the incubations were started by the addition of 10 μl 10 mM NADPH and terminated 5 min later by the addition of 1 ml 2 M NaOH. Hundered nmol of paraaminophenol was added as carrier and after extraction with ethylacetate (11), aliquots of the water phase were subjected to liquid scintillation counting in an Intertechnique SL 30 scintillator using aquoluma plus as scintillator liquid. Incubations were also conducted in a similar way using a reconstituted system based on dilauroylphosphatidylcholine. Fifty μg of this phospholipid in 50 μl of water was incubated at 22°C with 0.3 nmol of P-450 and 0.12 nmol of NADPH-cytochrome P-450 reductase for 3 min before addition of buffer, substrate and cofactor. From incubation mixtures containing hemoglobin, paraaminophenol was quantitated as described by Mieyal et al.(4).

RESULTS

Membrane vesicles containing cytochrome P-450 LM_2, NADPH-cytochrome P-450 reductase and microsomal phospholipids at a molar ratio of about 2.5:1:800 were prepared by the cholate gel filtration technique. The vesicles were incubated with aniline or paranitroanisole in the presence of NADPH and increasing amounts of catalase. As shown in Fig. 1, catalase nearly completely inhibited the cytochrome P-450-dependent aniline hydroxylation, but did not affect the rate of paranitrophenol production. Similar results were also reached when incubations were performed in the reconstituted system based on dilauroylphosphatidylcholine (Fig. 1) The results thus indicate that hydrogen peroxide participates in the cytochrome P-450 LM_2-dependent hydroxylation of aniline.

Catechol, known as an efficient scavenger of hydroxyl radicals, effectively inhibited the cytochrome P-450 LM_2-dependent hydroxylation of aniline in the vesicular system and in the dilauroylphosphatidylcholine system (Fig. 2). The half-maximal effect was seen at a catechol concentration of 0.15 mM. In contrast, the cytochrome P-450 LM_2-catalyzed O-demethylation of paranitroanisole was unaffected by catechol (Fig. 2).

Other hydroxyl radical scavengers as hydroquinone, dimethylsulfoxide, mannitol and benzoate were also tested as inhibitors of aniline

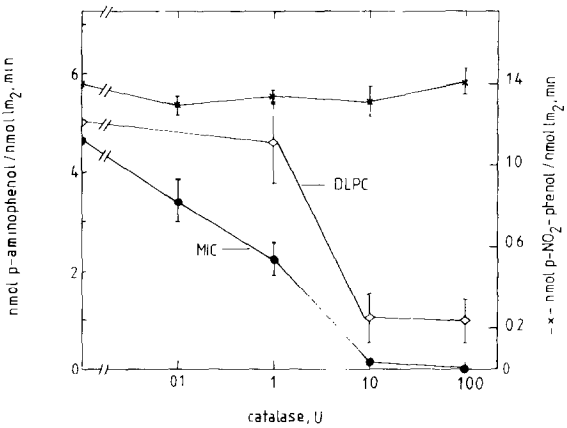

Fig. 1. Effect of catalase on cytochrome P-450 LM -dependent hydroxylation of aniline and O-demethylation of paranitroanisole. Incubations with aniline were performed in reconstituted vesicles prepared from microsomal phospholipids (MIC, ●---●)or in the dilauroyl-phosphatidylcholine system (◇---◇) at 37 C as outlined under "Methods". Incubations were performed for 5 min using a substrate concentration of 0.25 mM. Incubations with paranitroanisole (x---x) were carried out in the vesicular system as described previously (13). Each point represents the mean of three different experiments +- S.E.

hydroxylation. As is evident from Table I, all of these scavengers efficiently inhibited this reaction, whereas O-demethylation of paranitroanisole was unaffected.

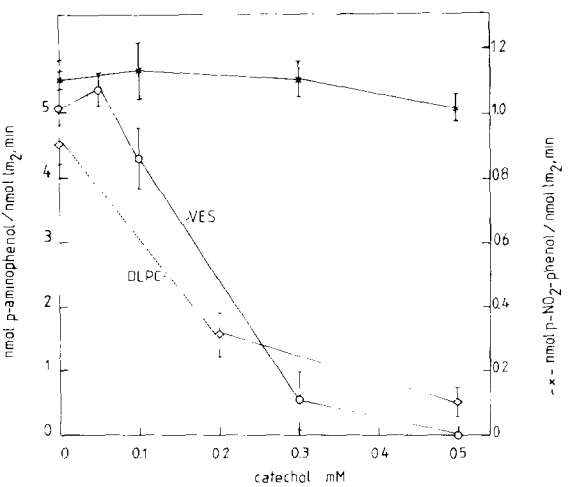

Fig. 2. Effect of catechol on cytochrome P-450 LM -dependent hydroxylation of aniline and O-demethylation of paranitroanisole. Aniline hydroxylation was detected in vesicles (O---O) and in the dilauroylphosphotidylcholine system (◇---◇) whereas paranitroanisole O-demethylation was registered in the vesicular system (x---x). Conditions were as described in legend to Fig. 1. Each point represents the mean of three different experiments +- S.E.

Table I. Effect of hydroxyl radical scavengers and superoxide dismutase on cytochrome P-450 LM_2-dependent hydroxylation of aniline and O-demethylation of paranitroanisole in reconstituted membrane vesicles.a/

Addition	product formed, nmol/nmol LM_2, min paraaminophenol	% inhib.	paranitrophenol
none	5.5	-	1.24
hydroquinone, 50 µM	1.2	78	1.20
- " - , 200 µM	0.1	98	1.15
DMSO, 10 mM	2.9	47	1.25
-"- , 50 mM	2.7	51	1.30
Mannitol, 10 mM	5.0	9	1.21
-"- , 700 mM	2.1	62	1.23
Benzoate, 100 mM	3.7	33	1.17
-"- , 500 mM	0.3	95	1.05
SOD, 0.5 µg/ml	5.4	2	1.18
-"-, 2 µg/ml	5.0	9	1.35
-"-, 10 µg/ml	3.6	35	1.30
-"-, 50 µg/ml	3.0	46	1.22

a/ Incubations were performed as outlined in legend to Fig. 1. The values represent the mean of two different sets of experiments.

Decreasing the steady-state level of superoxide anions by adding increasing amounts of superoxide dismutase to the vesicular system, selectively inhibited the P-450 LM_2-dependent metabolism of aniline but not the O-demethylation of paranitroanisole (Table I). The half-maximal effect was seen at a concentration similar to that observed when examining the P-450 LM_2-dependent ethanol oxidation (7).

When cytochrome P-450 LM_2 in the vesicles was replaced by a crude cytochrome P-450-preparation containing cytochromes P-450 LM_{3b}, P-450 LM_{3c}, P-450 LM_4 and P-450 LM_6, catechol and catalase inhibited the NADPH-dependent aniline hydroxylation in a similar manner. Complete inhibition was obtained at a catechol concentration of 0.5 mM whereas catalase at 10 U/ml gave 75 % inhibition and 100 U/ml 80 % inhibition; in the latter case 1.5 nmol of product was formed per min and nmol of P-450.

Aniline hydroxylation was also accomplished in a system containing dihydroxyfumarate. This compound is known to autooxidize spontaneously in water solution, thereby generating superoxide anions (12). Paraaminophenol was formed at 0.19, 0.47 and 1.1 nmol/min in 50 mM potassium phosphate buffer, pH 7.4, at 22°C when the concentration of dihydroxyfumarate was 5, 10 and 20 mM, respectively.

In separate experiments cytochrome P-450 was replaced by hemoglobin in the reconstituted systems. NADPH-dependent paraaminophenol formation from aniline was detected in a reaction mixture composed of 1 mM NADPH, 0.3 μM NADPH-cytochrome P-450 reductase and 1 μM hemoglobin. The phenol was thus formed at a rate of 0.23 nmol/nmol,min at 38°C in 20 mM potassium phosphate buffer, pH 6.8; upon addition of catalase (100 U), superoxide dismutase (10 μg), the hydroxyl radical scavengers methional (1 mM) or α-keto γ-metiolbutyric acid (1 mM), the reaction was inhibited by 80, 40, 78 and 100 %, respectively.

DISCUSSION

The results presented indicate that hydrogen peroxide, superoxide anions and hydroxyl radicals participate in the cytochrome P-450 and hemoglobin-dependent hydroxylation of aniline. It may thus be suggested that the conceivable mechanism for aniline hydroxylation in these systems is similar to that observed using ethanol as substrate (7). In this case, autooxidation of the oxycytochrome P-450 complex results in the liberation of superoxide anions. Hydrogen peroxide generated by dismutation of the superoxide anions react in an iron-catalyzed Haber-Weiss reaction

$$H_2O_2 + O_2^- \xrightarrow{Fe^{3+}} \cdot OH + OH^- + O_2$$

yielding ·OH which in turn react with the substrate. As a consequence acetaldehyde is formed.

Hydroxyl radicals are known to react with the benzene ring yielding primarily a hydroxycyclohexadienyl radical (14), which in the presence of oxygen is converted to phenol (15,16). Likewise, phenol reacts with

hydroxyl radicals (17) and as a result, catechol and hydroquinone are formed. Aniline would be expected to react in a similar way.

In conclusion, the results presented indicate that the unspecificity observed concerning the type of hemoprotein catalyzing the aniline hydroxylation is explained by the capacity of the respective hemoprotein to, upon reduction, produce activated oxygen species in the form of superoxide anions. It seems plausible that this type of indirect oxygenation mechanism is relevant also for the cytochrome P-450-dependent metabolism of a lot of other aromatic compounds, such as benzene and other benzene derivatives structurally related to aniline, e.g. acetaminophen and phenacetin.

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