SPIN TRAPPING EVIDENCE FOR FREE RADICAL OXIDANTS OF AMINOPYRINE IN THE METMYOGLOBIN–CUMENE HYDROPEROXIDE SYSTEM

Brenda Walker GRIFFIN and Patrick L. TING
Biochemistry Department, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235, USA

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1. Introduction

The liver microsomal hemeprotein cytochrome *P*-450 exhibits both monooxygenase activity [1], requiring O_2 and NADPH, and peroxidase activity [2,3], requiring only added hydroperoxides, toward electron donor substrates. Several cumene hydroperoxide-supported activities of a purified preparation of liver microsomal cytochrome *P*-450 were described in [4]. Based on the stoichiometry of product formation from *N,N*-dimethylaniline, it was proposed [4] that both the monooxygenase and peroxidase reaction pathways involve a common higher oxidation state of the hemeprotein, structurally and functionally equivalent to compound I of horseradish peroxidase. However, the proposal that this species of cytochrome *P*-450 inserts an 'active' oxygen atom directly into substrates is inconsistent with the well-established function of compound I as an electron abstraction oxidant [5]. An alternative mechanism, namely, that the oxidant(s) could be free radical species arising from the hydroperoxide, was not eliminated by the study cited [4]. For example, the reaction stoichiometry was not conclusively established because the total amount of cumene hydroperoxide consumed or other possible products arising from the hydroperoxide were not reported. Moreover, substantial destruction of cytochrome *P*-450 heme by hydroperoxide was observed, which was inhibited by the addition of substrates. This result is consistent with a free radical process that results in oxidation of the heme group in the absence of competing electron donors.

It was recently shown that aminopyrine, a typical substrate of cytochrome *P*-450, undergoes *N*-demethylation and concomitant oxidation to a free radical species by a variety of heme catalyst–hydroperoxide systems [6]. Studies of the horseradish peroxidase-catalyzed reaction have implicated the free radical as an intermediate in the reaction pathway [7,8]. In this system, compounds I and II of horseradish peroxidase are the one-electron oxidants of aminopyrine [7]. However, the aminopyrine free radical can also be formed electrochemically [9] and by oxidation with Fenton's reagent [7]. The present study was undertaken to determine the identity of the oxidant of aminopyrine in hemeprotein–cumene hydroperoxide systems. Electron paramagnetic resonance (EPR) spin trapping techniques used in conjunction with high performance liquid chromatography have clearly established a role for free radicals derived from cumene hydroperoxide in the metmyoglobin-catalyzed oxidation of aminopyrine.

Abbreviations: NADPH, nicotinamide adenine dinucleotide phosphate (reduced form); EPR, electron paramagnetic resonance; MNP, 2-methyl-2-nitrosopropane

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2. Materials and methods

Oxymyoglobin was purified from fresh beef heart [10] and then oxidized by $K_3Fe(CN)_6$, which was

removed by chromatography on a Sephadex G-25 column. Aminopyrine and 2-methyl-2-nitrosopropane (MNP) were supplied by Aldrich. MCB cumene hydroperoxide (technical) was purified as its sodium salt [11]. Stock solutions of the salt dissolved in H_2O were determined by iodometric titration [12]. Acetone-d_6 was purchased from Merck, Sharp and Dohme. All other reagents were the highest quality commercially available.

EPR spectra were recorded at room temperature with a Varian E-4 spectrometer. The components of the reaction mixture were mixed in a test tube, and then immediately transferred to a calibrated quartz capillary tube for scanning the EPR spectrum. Relative EPR signal intensities were measured as the average of the amplitudes of the largest low- and high-field components of a given nitroxide EPR singal. For measurement of reaction products, a Waters Associates high performance liquid chromatograph with μ Bondapak C_{18} column was employed. An aliquot of the reaction mixture was quenched with an equal volume of methanol and chromatographed under isocratic conditions with the solvent system tetrahydrofuran (10%):H_2O (55%):methanol (45%). Detection of acetophenone and cumenol was at 254 nm and 210 nm, respectively; amounts were determined by measurement of the areas of chromatographic peaks and comparison with standard curves.

3. Results and discussion

Nitroso compounds can trap reactive free radicals ($R^{\cdot}$) by the following addition reaction which produces a stable nitroxide free radical product [13]:

$$(CH_3)_3C{-}N{=}O + R^{\cdot} \longrightarrow (CH_3)_3C{-}\underset{\displaystyle R}{\underset{|}{N}}{-}O^{\cdot} \quad (1)$$

High concentrations of the spin trap are required to insure trapping sufficient amounts of $R^{\cdot}$ for EPR detection of the product. However, the limited solubility in aqueous solutions of the more common spin traps has limited their application in biological systems [14,15]. One recently published study utilized a spin trap to probe the formation of free radicals during lipid peroxidation by rat liver microsomes [15]. In that study the observation of trapped radicals in microsomes required not only O_2 and NADPH but the addition of mM concentrations of Fe^{2+}, and phenyl-*t*-butyl nitrone was used as the spin trap. We have investigated a simpler system consisting of a purified hemeprotein (metmyoglobin) and an organic hydroperoxide (cumene hydroperoxide), without added metal ions, and have employed the nitroso compound shown in eq. (1), 2-methyl-2-nitrosopropane (MNP). The advantage of nitroso spin traps is that protons of the trapped radical generally produce a characteristic splitting of the nitroxide EPR signal, which allows positive identification of the trapped radicals.

The EPR spectrum recorded when cumene hydroperoxide was added to a buffered solution containing metmyoglobin and MNP (dissolved in acetone) is shown in fig.1. Two distinct nitroxide signals are evident: the characteristic 3-line nitroxide signal (seen also in the controls) is attributable to the trapped $(CH_3)_3{-}C^{\cdot}$ radical formed by light-induced decomposition of the spin trap [13]; the second nitroxide signal was observed only when both the hemeprotein and cumene hydroperoxide were present. The hyperfine splitting pattern and constants of this latter signal (fig.1) identify an MNP-trapped methyl ($CH_3^{\cdot}$) radical, which has been previously detected in organic solvent systems [16].

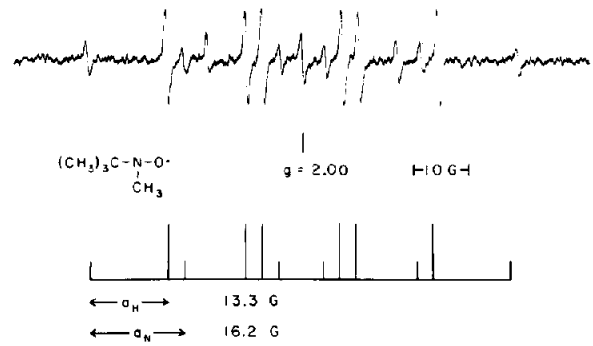


Fig.1. EPR signals arising from spin trapping reactions of MNP in the presence of metmyoglobin and cumene hydroperoxide. The reaction mixture contained 24 μM metmyoglobin, 30 mM cumene hydroperoxide, 50 mM MNP and 10% acetone-d_6 in 0.1 M Tris–HCl buffer, pH 9.0. Instrument settings: 20 mW microwave power; 9.160 GHz; 0.82 G modulation amplitude; 0.3 s time constant; 25 G/min scan rate; 6.2×10^3 gain.

Although it was necessary to employ a solvent for MNP, acetone at these concentrations did not inhibit the rather low rate of aminopyrine *N*-demethylation (8.1/min at 37°C [6]). The EPR signal characteristic of the MNP-trapped methyl radical was also detected when other solvents, including acetone-d_6 and various alcohols, were used and, thus, does not depend on the solvent. However, the alcohols gave rise to additional nitroxide signals, identified as trapped solvent radicals [16], consistent with the findings in [15]. Acetone-d_6 was employed in all experiments because its high chemical and isotopic purity eliminated the possibility that any observed proton splittings could arise from trapped solvent radicals.

The pH dependence of the EPR signal amplitude of the MNP-trapped methyl radical is shown in fig.2; the signal was maximal at the highest pH examined, pH 9.0. Since this effect could reflect the pH stability of the nitroxide radical itself, the nitroxide signal resulting from the self-trapping reaction of MNP was used as a control: it had a constant amplitude over the pH range 5.0–9.0. Thus, the pH dependence depicted in fig.2 does not appear to be an effect of pH on the stability of the nitroxide product. Although the same EPR signal was detected in several buffers, in Tris–HCl the intensity of this signal increased as a function of time up to several hours. Although we detected no EPR signals attributable to MNP-trapped Tris radicals, as has been reported [15], this effect of Tris remains unexplained.

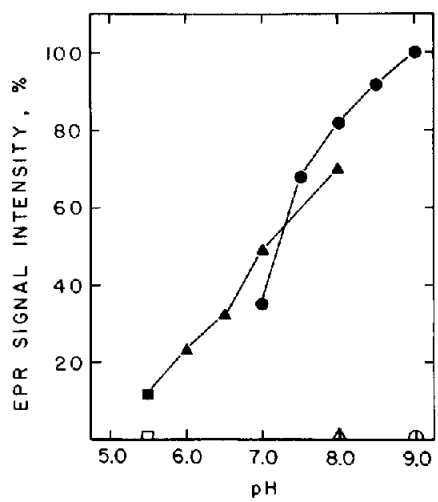


Fig.2. pH dependence of MNP-trapped methyl radicals in the metmyoglobin–cumene hydroperoxide system. The reaction mixtures contained 25 μM metmyoglobin, 20 mM cumene hydroperoxide, 50 mM MNP, and 10% acetone-d_6 in one of the following buffers (0.1 M): (•) Tris–HCl; (▲) potassium phosphate; (■) potassium acetate. Open symbols represent the appropriate control experiments with the heme protein omitted.

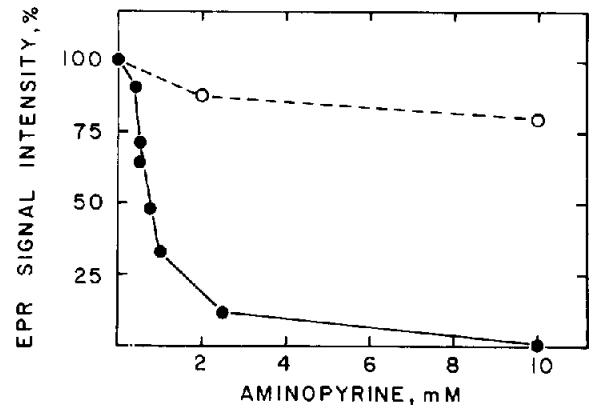
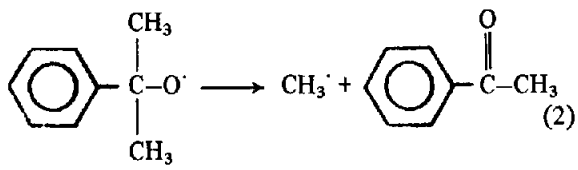


Fig.3. Effect of aminopyrine on the relative EPR signal amplitude of MNP-trapped methyl radicals in the metmyoglobin–cumene hydroperoxide system. The reaction mixtures (•) contained 25 μM metmyoglobin, 20 mM cumene hydroperoxide, 50 mM MNP, 10% acetone-d_6 and the indicated concentration of aminopyrine in 0.1 M Tris–HCl buffer pH 9.0. (o) Control experiment: effect of aminopyrine on the relative EPR signal amplitude of MNP-'self-trapped' radicals in the presence of cumene hydroperoxide alone or metmyoglobin and H_2O_2, also at pH 9.0.

Increasing concentrations of aminopyrine caused the MNP-trapped methyl radical EPR signal to disappear, as shown in fig.3. The aminopyrine free radical, presumably formed in this system, is optimally stable below pH 6.0 and, thus, was not detected at the pH of these experiments. Several explanations of the effect of aminopyrine are possible, including a direct reaction of aminopyrine or the aminopyrine free radical with the nitroxide. By use of the MNP-'self-trapped' radical as a control, it was established that the amplitude of this signal, measured in the presence of cumene hydroperoxide or metmyoglobin–H_2O_2 (known to generate the aminopyrine free radical [8]), decreased no more than 20% at aminopyrine concentrations which completely inhibited the appearance of the MNP-trapped methyl radicals (fig.3). From this result, it is concluded that

the principal effect of aminopyrine is on the generation or trapping reactions of the methyl radical.

Purely chemical studies have established the origin of methyl radicals formed during the decomposition of cumene hydroperoxide [17]:



(2)

One electron reduction of cumene hydroperoxide produces the hydroxide anion and the cumyloxy radical, which may undergo decomposition as indicated in eq. (2). Reduction of either cumyloxy or methyl radicals could explain the observed effect of aminopyrine. Although the EPR results provided indirect evidence for the existence of cumyloxy radicals in this system, conclusive proof was obtained by measuring acetophenone production (cf. eq. (2)). In the absence of aminopyrine (table 1), measurable amounts of acetophenone were formed. Aminopyrine at low concentrations caused increased production of both acetophenone and cumenol (table 1), and this effect was more marked at pH 7.0 than pH 9.0. Thus, while the results of table 1 indicate that aminopyrine stimulated the production of methyl radicals equivalent to acetophenone (cf. eq. (2)), the EPR signal amplitude of the MNP-trapped methyl radical actually decreased in the presence of aminopyrine (fig.3). These results strongly suggest that aminopyrine functions as an electron donor to the methyl radical, which would result in formation of the aminopyrine free radical species. If aminopyrine was an effective reductant of the cumyloxy radical, then the amount of acetophenone should have remained constant or decreased in the presence of the substrate. The involvement of hydroxyl radicals was eliminated by the finding that high concentrations of mannitol did not inhibit the observed EPR signals [7].

Table 1
Effect of aminopyrine on the products of cumene hydroperoxide decomposition stimulated by metmyoglobin

pH	Aminopyrine added (mM)	Cumenol formed (mM)	Acetophenone formed (mM)
7.0	0	0.71	0.38
	0.44	1.42	0.82
9.0	0	0.68	0.44
	0.44	1.04	0.58

Experimental conditions: 22 μM metmyoglobin, 20 mM cumene hydroperoxide, and stated concentration of aminopyrine in 0.1 M buffer (Tris–HCl, pH 9.0 or potassium phosphate, pH 7.0) at 22°C. The reaction products were assayed 5 min. after the reaction was initiated, as described in section 2

Although these experiments have not eliminated a possible catalytic role for a higher oxidation state of metmyoglobin [18] in the *N*-demethylation of aminopyrine by cumene hydroperoxide, they have clearly demonstrated the existence of free radicals derived from the hydroperoxide which are capable of oxidizing aminopyrine. Similar studies of cytochrome *P*-450-dependent oxidations supported by cumene hydroperoxide are in progress.

Acknowledgements

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