FREE RADICAL INTERMEDIATE IN THE *N*-DEMETHYLATION OF AMINOPYRINE BY HORSERADISH PEROXIDASE–HYDROGEN PEROXIDE

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1. Introduction

The oxidative cleavage of alkyl groups on nitrogen, oxygen and sulfur to their corresponding aldehydes has been considered to be a characteristic mono-oxygenation reaction of liver microsomal cytochrome *P*-450, requiring NADPH and molecular oxygen [1]. Kadlubar et al. [2] first reported that cumene hydroperoxide and various other organic hydroperoxides could replace both NADPH and O_2 in certain cytochrome *P*-450-catalyzed *N*-demethylations. Other hydroperoxide-supported activities of liver microsomal cytochrome *P*-450, including *O*-dealkylation and aromatic hydrocarbon hydroxylase activities, analogous to the O_2/NADPH requiring reactions, have since been reported [3,4]. Since a peroxidase activity of liver microsomal cytochrome *P*-450 has been implicated by these findings, we have undertaken a study of the *N*-demethylase activity of other hemeproteins as possible models for the liver enzyme. The first report of aminopyrine *N*-demethylation by hydrogen peroxide catalyzed by horseradish peroxidase (HRP) was made by Gillette et al. [5] who did not further characterize this activity. Catalase was shown by Kadlubar et al. [2] to exhibit a significant aminopyrine *N*-demethylase activity in the presence of those organic peroxides which also supported the cytochrome *P*-450-catalyzed reaction.

Preliminary accounts of this work were presented at the VIIth International Conference on Magnetic Resonance in Biological Systems, September 19–24, 1976, St. Jovite, Quebec, Canada and at The American Chemical Society Southwest Regional Meeting, December 1–3, 1976, Fort Worth, Texas, USA.

This report presents preliminary results of a detailed study of the hydrogen-peroxide supported *N*-demethylation of aminopyrine catalyzed by HRP. It is shown that the turnover number for formaldehyde production in this system is much larger than any values which have been reported for the oxidation of this substrate by a variety of cytochrome *P*-450 systems. More significantly, we demonstrate for the first time an enzymatically-generated free radical species of aminopyrine which is strongly implicated by the experimental data as a reactive intermediate in the pathway of *N*-demethylation by the HRP-hydrogen peroxide system.

2. Materials and methods

HRP with an RZ value of approximately 3.0, was the Type VI, salt-free powder available from Sigma and was used as supplied. Aminopyrine (4-dimethylaminoantipyrine) was purchased from Aldrich. Other chemicals used were reagent grade. Formaldehyde was assayed by the Nash procedure [6] after quenching the reaction with 15% trichloroacetic acid.

All spectrophotometric measurements were made with a Beckman Model 25 ultraviolet – visible spectrophotometer. EPR spectra were recorded at room temperature with a Varian E-4 spectrometer. After initiating the reaction by addition of enzyme, the mixture was transferred to a calibrated capillary tube and the EPR signal scanned immediately. Both $CuSO_4$ and Fremy's salt (peroxylamine disulfonate) in aqueous solution were employed as $S = ½$ intensity standards, with excellent agreement between the two.

Concentrations of the aminopyrine free radical were determined quantitatively by computer double-integration of the overmodulated EPR signal recorded under non-saturating power. Although limitations on the number of data points collected prevented integration of the resolved aminopyrine radical signal, it was experimentally verified with Fremy's salt that the computed area is independent of modulation amplitude, as required by theory.

3. Results and discussion

Table 1 compares the turnover number for aminopyrine *N*-demethylation by horseradish peroxidase determined under optimal conditions with various values reported for cytochrome *P*-450. As can be seen from the data, HRP is a far better catalyst of this reaction than is cytochrome *P*-450. A transient free radical derived from aminopyrine (fig.1A) has been detected by EPR in the HRP–hydrogen peroxide system at room temperature. A search for other systems which could give rise to this aminopyrine free radical revealed that Fenton's reagent (ferrous ion/hydrogen peroxide in aqueous acid solution) generates identically the same free radical species from aminopyrine (fig.1B). Since this system is known to produce hydroxyl radicals [7] mannitol, a trap for hydroxyl radicals was added, and found to significantly inhibit the signal intensity of the radical. It should be noted that the only non-zero control experiment for the EPR spectra shown in fig.1 was the control for Fenton's reagent with iron omitted; a weak signal, identical to those shown, was detected due to the slow decomposition of hydrogen peroxide in acid solution. As shown in table 2, mannitol had no effect on the quasi-steady state radical concentration which could be detected in the HRP–hydrogen peroxide system or on the rate of *N*-demethylation by this system. Also shown in table 2 is the effect of KF and NaN_3 on the HRP-catalyzed reaction; both radical concentration and the rate of formaldehyde production are unaffected by KF and significantly inhibited by NaN_3.

The maximum signal intensity of the radical which could be measured in the HRP–hydrogen peroxide system as a function of pH is compared in fig.2 with the pH dependence of *N*-demethylation of aminopyrine in this system. Although the EPR signal intensity more closely resembles a steady state value rather than a rate, the correlation of the radical concentration with the rate of *N*-demethylation is quite good. At the pH optimum for the EPR signal intensity, 0.1 M Tris–HCl, pH 8.0, (fig.2) the radical concentration attains a value of approximately 2 mM, as determined by double integration of the signal; this

Table 1
Turnover numbers for aminopyrine *N*-demethylation

Oxidant	Hemeprotein	Source	Turnover number (μmoles $H_2C{=}O$/min/μmol heme)
O_2, NADPH	Cytochrome *P*-450	Rat liver[a] [11]	7.9
		Pig liver[b] [2]	5.0
		Rabbit liver[c] [12]	4.3
		Rabbit lung[c] [12]	8.3
Cumene hydroperoxide	Cytochrome *P*-450	Pig liver[b] [2]	8.3
Hydrogen peroxide	Peroxidase	Horseradish[d]	2.5×10^4

[a] Phenobarbital-treated animals; assay carried out in 50 mM Tris–HCl buffer, pH 7.5, at 30°C
[b] Phenobarbital-treated animals; assay carried out in 0.1 M potassium phosphate buffer, pH 7.5, at 37°C. Data converted to heme basis using a value of 0.7 nmol cytochrome *P*-450/mg protein (R. A. Prough, personal communication)
[c] Control animals; experimental conditions of assay unspecified
[d] Assay conditions; 0.1 M potassium phosphate buffer, pH 7.0, 37°C

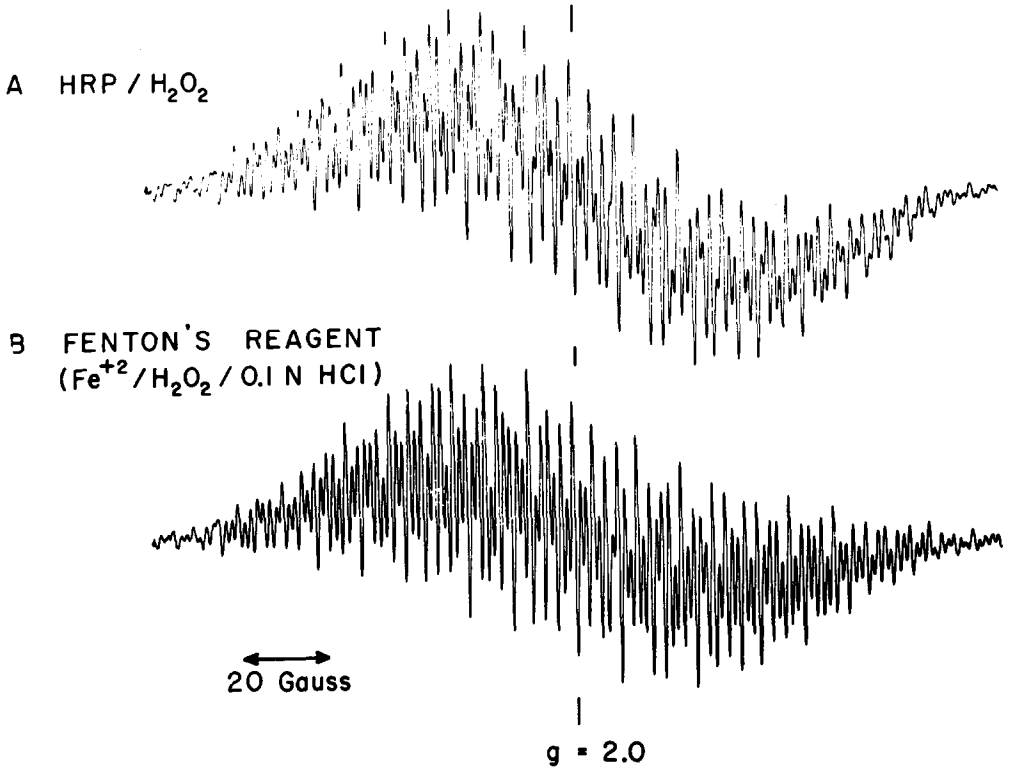

Fig.1. EPR spectrum of aminopyrine free radical. (A). The reaction mixture contained 10 mM aminopyrine, 10 mM hydrogen peroxide and 1.9 μM HRP in 0.1 M potassium acetate buffer, pH 5.8. (B). The reaction mixture contained 30 mM aminopyrine, 30 mM hydrogen peroxide and 0.5 mM ferrous ammonium sulfate in 0.1 N HCl. Both spectra were recorded at room temperature with instrument settings of: 10 mW power, 0.82 G modulation amplitude, 0.3 s time constant, 25 G/min scan rate, gain, 3.2×10^3 for A and 4.0×10^3 for B.

Table 2
Effects of added compounds on the EPR signal intensity of the aminopyrine free radical and on the rate of aminopyrine *N*-demethylation in the HRP-hydrogen peroxide system

Experiment[a]	EPR S.I. (Units)[b]	% of control	Rate of $H_2C{=}O$ prod.[b]	% of control
Control	78	100	1.04×10^4	100
Control + Mannitol	80	102	1.20×10^4	115
Control + KF	78	100	1.15×10^4	110
Control + NaN_3	18	23	5.56×10^2	5

[a] The control experiment contained aminopyrine, hydrogen peroxide and HRP. Mannitol, KF and NaN_3 were each added to a final concentration of 0.1 M. All experiments were carried out in 0.1 M potassium phosphate buffer, pH 7.0. Other conditions for the EPR experiments are described in the legend of fig.1. For assay of *N*-demethylase activity, reaction mixtures contained 12 mM aminopyrine, 0.43 mM hydrogen peroxide and 4.5 nM HRP, incubated at 37°C.

[b] EPR signal intensity has arbitrary units; rates of formaldehyde production are expressed as μmoles formaldehyde/min/μmol HRP.

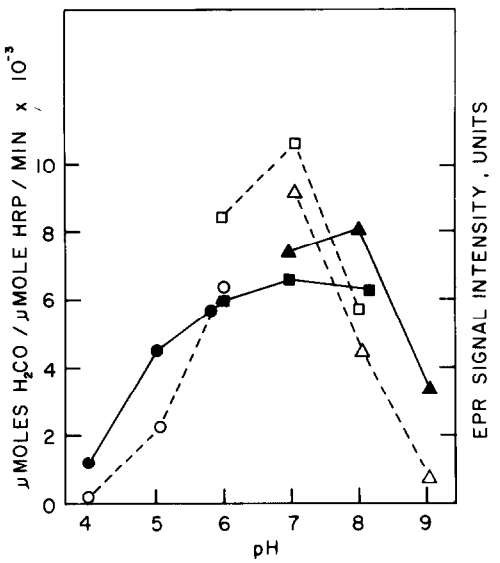

Fig.2. pH-Dependence of EPR signal intensity of aminopyrine free radical and rate of *N*-demethylation of aminopyrine in the HRP–hydrogen peroxide system. Open symbols: rate of *N*-demethylation in systems containing 6 mM aminopyrine, 0.2 mM hydrogen peroxide and 9 nM HRP in 0.1 M of the specified buffer at 37°C. Closed symbols: EPR signal intensity of aminopyrine free radical in 0.1 M of the specified buffer, recorded under the experimental conditions described in the legend of fig.1. (○,●) K^+-acetate, (□,■) K^+-phosphate and (△,▲) Tris–HCl buffers.

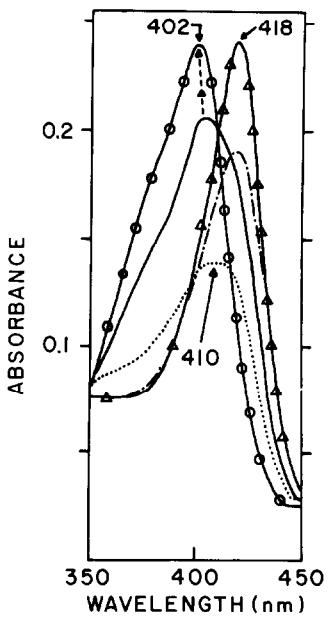

Fig.3. Effect of aminopyrine on the absorbance spectra of the higher oxidation states of HRP. The absorbance spectrum of 2.4 μM HRP in 0.1 M potassium phosphate pH 7.0 at room temperature was recorded (—○—). Then 12 μM hydrogen peroxide was added, forming Compound I (· · · · ·). Additions of aminopyrine to Compound I were made as follows: (– · – · –) 0.65 μM aminopyrine, (—△—) 2.1 μM aminopyrine, forming Compound II completely, (——) 4.2 μM aminopyrine, regenerating ferric HRP.

represents a significant fraction (20%) of the total aminopyrine concentration in the reaction mixture.

Preliminary experiments have shown that aminopyrine, at stoichiometric concentrations, is an excellent electron donor to both Compounds I and II of HRP (fig.3). It is well-established that these conversions represent one-electron transfers which produce free radicals from the electron donor substrates of HRP [8,9]. These spectrophotometric data suggest that the aminopyrine free radical detected in this system is a one-electron oxidized species which arises by the same mechanism. The observation of the same radical in Fenton's reagent and its inhibition by mannitol are also consistent with a one-electron oxidation of aminopyrine by hydroxyl radicals. Furthermore, it has been reported that anodic oxidation of aminopyrine in acetonitrile involves an initial one-electron transfer which produces a free radical having an EPR signal very similar to that reported here [10]. These different methods of generating the same free radical species support the interpretation that it is a one-electron oxidized radical cation of aminopyrine.

The experimental data which implicate the aminopyrine free radical as a reactive intermediate in the HRP-catalyzed *N*-demethylation of this substrate are summarized:

(a) It is a transient species, decaying to undetectable concentrations in a short period of time, depending on the experimental conditions.

(b) The measured radical concentrations represent a significant fraction of the total aminopyrine concentration.

(c) A good correlation of the pH-dependence of both the radical concentration and the rate of aminopyrine *N*-demethylation is observed.

(d) The production of both the radical species and

formaldehyde is inhibited considerably by NaN_3, but neither is affected by mannitol or KF.

Additional experiments are in progress to establish the mechanism of *N*-demethylation by the HRP-hydrogen peroxide system and to test the validity of this system as a model for cytochrome *P*-450-catalyzed *N*-demethylations.

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