[Chem. Pharm. Bull. 28(7)2077—2082(1980)]

Electron Spin Resonance Studies on the Cumene Hydroperoxide-Supported Oxidation of Aminopyrine by Catalase[1]

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(Received January 24, 1980)

Cumene hydroperoxide-supported N-demethylation of aminopyrine catalyzed by catalase has been investigated. The transient free radical of aminopyrine was detected by electron spin resonance at room temperature. 4-Diethylaminoantipyrine was also oxidized to the corresponding free radical. Although another free radical was detected in the absence of aminopyrine in the catalase-cumene hydroperoxide system, this radical is considered not to be the major oxidant of aminopyrine in the catalase-aminopyrine-cumene hydroperoxide system, because its concentration was too low. Cumene hydroperoxide previously added to the catalase solution greatly inhibited the oxidation of aminopyrine, whereas it did not inhibit the catalatic reaction. In contrast, sodium azide significantly inhibited the latter reaction and only slightly inhibited the former reaction. Methanol was not oxidized appreciably in our system. The present study suggests that the active site of catalase for the cumene hydroperoxide-supported N-demethylation of aminopyrine is different from that for the catalatic reaction.

Keywords——catalase; cumene hydroperoxide; aminopyrine; 4-diethylaminoantipyrine; aminopyrine free radical; 4-diethylaminoantipyrine free radical; electron spin resonance; N-demethylation of aminopyrine; cumene hydroperoxide-supported oxidation; inhibition of catalase

Catalase was shown by kadlubar *et al.*[3] to catalyze the oxidative N-demethylation of aminopyrine in the presence of several organic hydroperoxides which also supported the cytochrome P-450-catalyzed reaction. However, this catalytic activity was not well characterized. Recently, in the horseradish peroxidase (HRP)-hydrogen peroxide system, the transient free radical of aminopyrine was detected by electron spin resonance (ESR) at room temperature.[4] On the other hand. the aminopyrine free radical was not detected in the metmyoglobin-cumene hydroperoxide system, although the methyl radical was detected in the reaction mixture by means of a spin-trapping technique.[5] In order to elucidate the mechanism of oxidative N-demethylation of substrates catalyzed by various hemoproteins, we have now studied the cumene hydroperoxide-supported oxidation of aminopyrine catalyzed by catalase.

Experimental

Materials——Catalase (from bovine liver, C-40) was used as supplied by Sigma. The concentration of catalase was determined from the absorbance at 405 nm. An extinction coefficient of $\varepsilon = 340\,cm^{-1}\,mM^{-1}$ was used.[6] Catalatic activity of this preparaion was 11100 Sigma units per mg protein. One Sigma unit will decompose one μmol of H_2O_2 per minute at pH 7.0 at 25°, while the H_2O_2 concentration falls from 10.3 to 9.2 μmol per ml of reaction mixture. The rate of disappearance of H_2O_2 was followed by observing the rates of decrease in absorbance at 240 nm with a Hitachi 340 spectrophotometer. Aminopyrine was obtained from Aldrich Chemicals and purified by recrystallization from ligroin. Cumene hydroperoxide

1) Preliminary report: H. Sayo and M. Hosokawa, *Chem. Pharm. Bull.*, **28**, 683 (1980).
2) Location: *Ikawadani-cho, Tarumi-ku, Kobe 673, Japan.*
3) E.F. Kadlubar, K.C. Morton, and D.M. Ziegler, *Biochem. Biophys. Res. Commun.*, **54**, 1255 (1973).
4) B.W. Griffin, *FEBS Lett.*, **74**, 139 (1977); B.W. Griffin and P.L. Ting, *Biochemistry*, **17**, 2206 (1978).
5) B.W. Griffin and P.L. Ting, *FEBS Lett.*, **89**, 196 (1978).
6) M.L. Kremer, *Biochem. Biophys. Acta*, **198**, 199 (1970).

was obtained from Nakarai Chemicals and purified as its sodium salt.[7] A stock solution of the salt dissolved in a buffer was determined by iodometric titration.[8] 4-Diethylaminoantipyrine was prepared as described previously.[9] Phenyl N-*tert*-butylnitrone was obtained from Eastman Organic Chemicals. The buffer solution used in this study was 0.1 M NaH_2PO_4–Na_2HPO_4 (pH 7.4). Water was purified by the use of a Millipore MILLI-R/Q system. In some experiments, dissolved oxygen was removed by passing nitrogen through the solution. All other chemicals used were of reagent grade.

Methods——ESR spectra were recorded on a JEOL JES-FE 1X spectrometer, equipped with 100 kHz field modulation, at room temperature (25°±1°). After initiating the reaction by addition of cumene hydroperoxide, the mixture was transferred to a flat aqueous solution cell (JES-LC-11) and the ESR signal was scanned 1 min after mixing. The radical concentration was determined by double integration of the overmodulated ESR signal using a JEOL EC-100 computer system. An aqueous solution of 4-hydroxy-2,2,6,6-tetramethyl-1-oxylpiperidine was employed as an $S=1/2$ intensity standard. The g value was measured by comparison with that of aqueous peroxylamine disulfonate ($g=2.0055$). For kinetic experiments, the signal was overmodulated and the maximal ESR signal amplitude was recorded at a fixed magnetic field as a function of time.

Formaldehyde was assayed by the Nash procedure,[10] after the reaction had been quenched with 10% trichloroacetic acid and the solution centrifuged to remove precipitated protein. For determination of 2-phenyl-2-propanol (cumenol), the reaction mixture (1.5 ml) was made acidic by the addition of 0.1 ml of 10% HCl and extracted with 3 ml of ether. Five μl of the ether layer was injected into a JEOL JGC-20KFP gas-liquid chromatograph equipped with a flame ionization detector and a glass column (2 m×2 mm) containing 3% Silicone OV-17 on Chromosorb W (80—100 mesh). The column temperature was 100° and the carrier gas was N_2. The amount of cumenol was estimated by comparison with standards.

Results

Aminopyrine Free Radical

When cumene hydroperoxide was added to a buffered solution (pH 7.4) containing catalase and aminopyrine, the mixture turned blue-violet and an ESR signal with a g value of 2.0034 was observed at room temperature (Fig. 1A). This ESR signal was not observed if aminopyrine was omitted from the reaction mixture. The ESR signal intensity was not affected by the presence of oxygen. Fenton's reagent (ferrous ion/hydrogen peroxide in aqueous solution) generated an identical free radical species from aminopyrine.[4] Therefore, the radical was identified as the aminopyrine free radical. No ESR signal was observed if catalase was omitted from the reaction mixture (Fig. 1 B). When aminopyrine was replaced by 4-diethylaminoantipyrine, an ESR signal with a g value of 2.0035 was observed (Fig. 1 C). The same ESR signal was observed for the reaction of 4-diethylaminoantipyrine with Fenton's reagent.

Fig. 2 shows the time dependences of the concentration of the aminopyrine free radical and of the production of formaldehyde and cumenol. Acetophenone was not detected in the reaction mixture. The concentrations of cumenol and formaldehyde determined 2 hours after initiating the reaction (at that time the reaction is considered to be completed) were 1.3 and 0.67 mM, respectively. This indicates that cumene hydroperoxide was almost quantitatively reduced to cumenol and that the concentration of formaldehyde formed was nearly half that of cumenol.

The effect of varying the cumene hydroperoxide concentration on the aminopyrine free radical concentration at 2 min after initiating the reaction is shown in Fig. 3. The radical concentration approached a limiting value with increase in cumene hydroperoxide concentration. In order to investigate the possibility of inhibition by higher concentrations of cumene hydroperoxide, the remaining catalytic activity of catalase which had been used once for the oxidation of aminopyrine with various concentrations of the hydroperoxide was examined. Fig. 4 shows the time dependence of the ESR signal intensities in the second reaction, in which

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8) P.D. Bartlett and R. Altshul, *J. Am. Chem. Soc.*, **67**, 816 (1945).
9) H. Sayo and M. Masui, *Chem. Pharm. Bull.*, **24**, 2137 (1976).
10) T. Nash, *Biochem. J.*, **55**, 416 (1953).

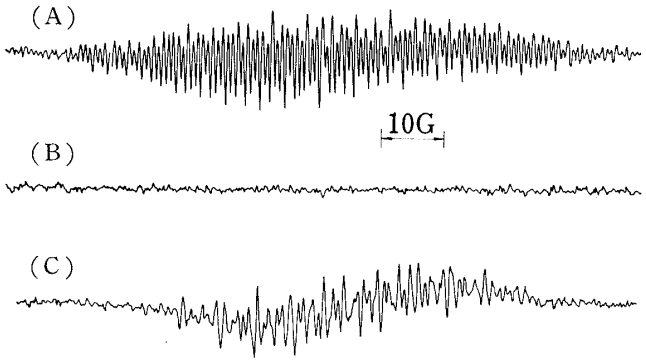

Fig. 1. ESR Spectra of the Aminopyrine Free Radical (A) and the 4-Diethylaminoantipyrine Free Radical (C)

(A): The reaction mixture contained 1.4 μM catalase, 6 mM aminopyrine, and 1.5 mM cumene hydroperoxide in 0.1 M sodium phosphate buffer, pH 7.4. (B): Catalase was omitted from A. (C): Aminopyrine was replaced by 4-diethylamino-antipyrine in A. All ESR spectra were recorded at room temperature (25°) with the following instrumental settings: power, 1mW; modulation amplitude, 0.5 G; scan rate, 25 G/min; time constant, 0.3 sec; gain 1.6×1000.

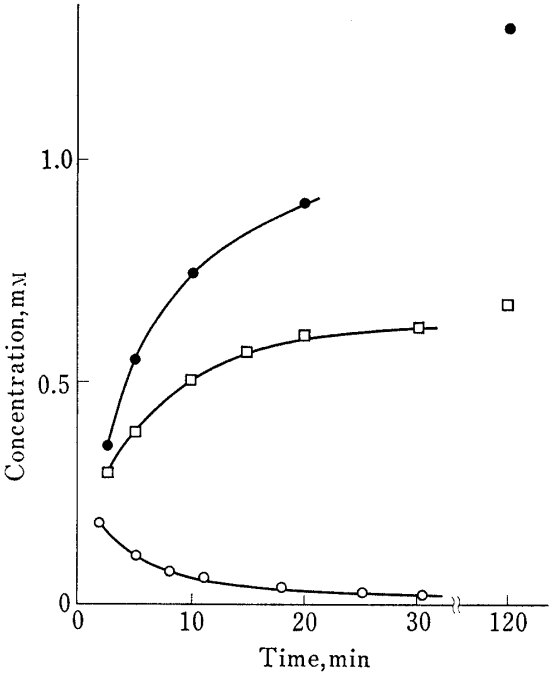

Fig. 2. Time Dependences of Aminopyrine Free Radical Concentration and Productions of Formaldehyde and 2-Phenyl-2-propanol

The reaction mixture contained 1.4 μM catalase, 6 mM aminopyrine, and 1.5 mM cumene hydroperoxide in 0.1 M phosphate buffer (pH 7.4), at 25°. After initiating the reaction by adding cumene hydroperoxide, the concentrations of the three substances were monitored as a function of time, as described in the text. ○, Aminopyrine free radical; □, Formaldehyde; ●, 2-Phenyl-2-propanol (cumenol).

the catalase used in the first reaction was used again. The concentrations of cumene hydroperoxide added in the second reactions were all 0.75 mM. It can be seen in Fig. 4 that the use of higher concentrations of cumene hydroperoxide in the first reaction resulted in impairment of the catalytic activity of catalase. This was confirmed by the experimental findings that in experiment 4 d) the amounts of formaldehyde and cumenol formed in the second reaction at 5 min after initiating the reaction were 4% and 8% of the control values, respectively.

As shown in Table I, when cumene hydroperoxide was previously added to the catalase solution, allowed to stand for 5 min and then mixed with the aminopyrine solution, the rate of the reaction and the concentration of the radical were very low (Run 2). This indicates that cumene hydroperoxide in the absence of aminopyrine impairs the N-demethylase activity of catalase. However, as shown in Table II, cumene hydroperoxide did not inhibit the catalase-catalyzed decomposition of hydrogen peroxide (catalatic reaction). In contrast, sodium azide significantly inhibited the latter reaction (Table II) and only slightly inhibited the N-demethylation (Table I, Runs 3 and 4).

Methanol was not oxidized appreciably under the conditions used for aminopyrine (Run 5). Although Rahimtula *et al.* have reported that ethanol, 1-propanol, 1-butanol, and 1-pentanol were oxidized in the catalase-cumene hydroperoxide system,[11] the concentrations of alcohols added in their study were much higher than that of methanol used in the present study.

11) A.D. Rahimtula and P.J. O'Brien, *Eur. J. Biochem.*, **77**, 201 (1977).

ESR Spectrum obtained from the Catalase-Cumene Hydroperoxide System in the Absence of Aminopyrine

As shown in Fig. 5, when cumene hydroperoxide was added to a buffered solution containing catalase, a broad singlet with a g value of 2.004 and a peak-to-peak width of 7.9 G was observed at room temperature. However, its intensity was very weak and a distinct spectrum

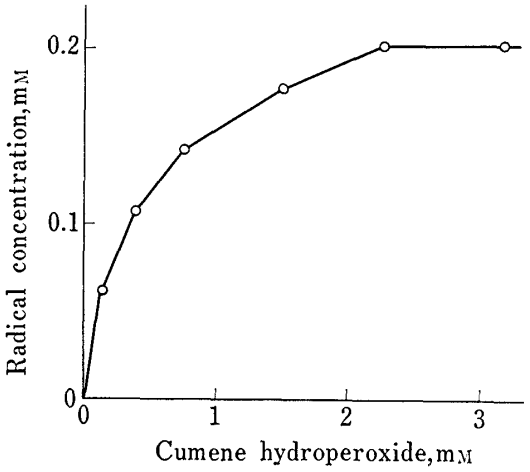

Fig. 3. Dependence of Aminopyrine Free Radical Concentration on Cumene Hydroperoxide Concentration

The radical concentrations were determined 2 min after initiating the reaction. The mixture contained 1.4 μM catalase, 6 mM aminopyrine, and various levels of cumene hydroperoxide as indicated in 0.1 M sodium phosphate buffer (pH 7.4), at 25°.

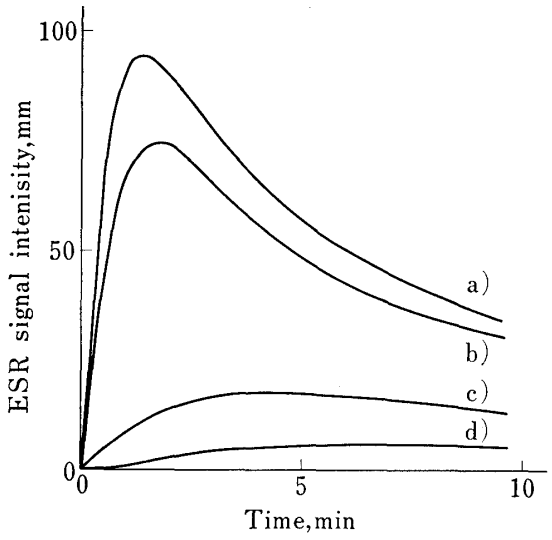

Fig. 4. Effect of Cumene Hydroperoxide Concentration in the First Reaction on the Time Dependence of Aminopyrine Free Radical Concentration in the Second Reaction

The reaction mixture in the first reaction contained 1.4 μM catalase, 6 mM aminopyrine, and cumene hydroperoxide b) 1.5 mM, c) 3 mM, d) 4.5 mM. a) is a control experiment without the first reaction. The reaction mixture in the second reaction contained 0.7 μM catalase, 3 mM aminopyrine, and 0.75 mM cumene hydroperoxide. Instrumental settings: power, 5 mW; modulation amplitude, 10 G; time constant, 1 sec; gain 1×1000.

Table I. Effects of Added Compounds and the Order of Their Addition on the Rate of the Reaction and the Concentration of the Aminopyrine Free Radical in the Catalase-catalyzed System at 25°

Run	Order of addition to the catalase solution[a] 1	2	3	Rate of HCHO formation[b]	Rate of cumenol formation[b]	Aminopyrine free radical,[d] mM
1	Aminopyrine	Cumene hydroperoxide	—	56 (100)[c]	88 (100)[c]	0.18 (100)[c]
2	Cumene hydroperoxide	allowed to stand for 5 min	Aminopyrine	5.4 (9.6)	10 (12)	0.004 (2.2)
3	NaN_3 (0.4 mM)	Aminopyrine	Cumene hydroperoxide	48 (86)	75 (85)	0.14 (78)
4	NaN_3 (2.0 mM)	Aminopyrine	Cumene hydroperoxide	40 (71)	61 (69)	0.10 (56)
5	Methanol	Cumene hydroperoxide	—	Negligible	Negligible	—

a) The final concentrations of various agents were as follows; catalase 1.4 μM, aminopyrine 6 mM, cumene hydroperoxide 1.5 mM, NaN_3 0.4 or 2.0 mM, and methanol 6 mM.
b) HCHO or cumenol mol/min/mol of catalase.
c) Numbers in parentheses are the values relative to run 1.
d) The concentration was determined 2 min after initiating the reaction.
e) All experiments were carried out in 0.1 M sodium phosphate buffer, pH 7.4.

Table II. Effects of Added Compounds on the Catalase-catalyzed Decomposition of H_2O_2

Addition to the catalase solution (1.4 μM)[a]	Activity[b]	% of control
Control	11100	100
+NaN_3 (0.4 mM)[a]	5200	47
+NaN_3 (2.0 mM)[a]	800	7.2
+Cumene hydroperoxide (1.5 mM)[a]	11100	100

a) The concentration in the mixture of catalase and additive. The mixture was allowed to stand for 5 min and then diluted with 0.05 M phosphate buffer (pH 7.0) for assay.
b) Sigma units per mg protein.

was obtained only after more than four accumulations. The presence of oxygen had no effect on the intensity of the signal, and no hyperfine structure was obtained from the deoxygenated mixture with smaller modulation amplitudes. No ESR signal was observed if catalase or cumene hydroperoxide was omitted.

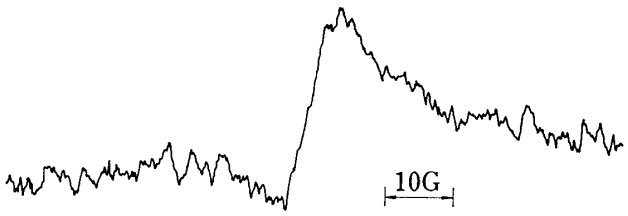

Fig. 5. ESR Spectrum of the Free Radical Generated by the Catalase–Cumene Hydroperoxide System in the Absence of Aminopyrine

The reaction mixture contained 5.6 μM catalase and 0.35 mM cumene hydroperoxide in 0.1 M sodium phosphate buffer, pH 7.4. The ESR spectrum was recorded at room temperature (25°) with the following instrumental settings: power, 20 mW; modulation amplitude, 5G; scan rate 50 G/min; time constant, 1 sec; gain, 5×1000; accumulation, nine times.

Spin-trapping of the Free Radical produced in the Catalase-Cumene Hydroperoxide System

The ESR spectrum when cumene hydroperoxide was added to a deoxygenated buffer solution (pH 7.4) containing catalase and phenyl N-*tert*-butylnitrone showed a triplet of doublets (A_N=16.5 G, A_H=3.6 G, g=2.0055), and its intensity increased with time. The concentration of the trapped radical 30 min after initiating the reaction was about 2 μM. The hyperfine splitting constants of the radical did not coincide with those of the hydroxyl radical[12] or methyl radical,[13] and the radical could not be identified. In the presence of oxygen this signal was very weak. Although the same spectrum was obtained in the absence of catalase, its intensity was much weaker than that in the presence of catalase.

Discussion

The present study leads us to conclude that aminopyrine is oxidized to the aminopyrine free radical by the catalase-cumene hydroperoxide system in a one-electron transfer step, and that the radical formed decomposes to formaldehyde and unidentified compounds. Since N-demethylation is not the sole process in the decomposition of the aminopyrine free radical,[14] and the expected products of the N-demethylation, 4-methylaminoantipyrine and 4-aminoantipyrine, are more easily oxidized than aminopyrine,[4,14] the stoichiometry of N-demethylation of aminopyrine cannot be determined from the concentrations of formaldehyde and cumenol alone.

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13) E.G. Janzen and B.J. Blackburn, *J. Am. Chem. Soc.*, **91**, 4481 (1969); E.G. Janzen and J. I-Ping, *J. Magn. Resonance*, **9**, 510 (1973).
14) H. Sayo and M. Masui, *J. Chem. Soc. Perkin II*, **1973**, 1640.

The next problem is to identify the oxidant of aminopyrine in the catalase-cumene hydroperoxide system. Although the methyl radical was claimed to be an oxidant in the metmyoglobin-cumene hydroperoxide system,[5] neither methyl radical or acetophenone, the latter of which is formed along with the former upon decomposition of the cumyloxy radical, was detected by the spin-trapping technique or by gas chromatography in our system.

Earlier ESR measurements on aqueous solutions containing catalase and ethyl hydroperoxide did not give a free radical signal.[15] In the present study, an unidentified free radical, which is presumably the cumyloxy or cumene peroxy radical, was detected in the catalase-cumene hydroperoxide system in the absence of aminopyrine by direct measurement and by spin-trapping at room temperature. However, the concentration of the radical was too low for it to be a significant oxidant of aminopyrine. Moreover, the presence of oxygen significantly reduced the concentration of the trapped radical, whereas it did not reduce that of the aminopyrine free radical in the catalase-aminopyrine-cumene hydroperoxide system.

A free radical was detected in the HRP–H_2O_2 system, but it was claimed that the radical does not participate to a major extent in the peroxidative mechanism, because its concentration is too low.[16] Thus, we also suggest that the major oxidant of aminopyrine in our system is not a free radical, but a higher oxidation state of catalase.

Another problem is the active site of catalase for the oxidation of aminopyrine. Although cumene hydroperoxide impaired the N-demethylase activity of catalase in the absence of aminopyrine, the catalase thus impaired showed normal catalatic activity. In contrast, sodium azide significantly inhibited the latter activity and only slightly inhibited the former activity. Moreover, methanol, which is an effective reductant of compound I of catalase, was not oxidized appreciably in the catalase-cumene hydroperoxide system.

These experimental results strongly suggest that the active site of catalase for the cumene hydroperoxide-supported N-demethylation of aminopyrine is different from that for the catalatic reaction. Further studies are necessary, however.

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16) Y. Morita and H.S. Mason, *J. Biol. Chem.*, **240**, 2654 (1965); R. Aasa, T. Vanngard, and H.B. Dunford, *Biochem. Biophys. Acta*, **391**, 259 (1975).