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Mechanism of N-Demethylation of Aminopyrine by Catalase-Organic Hydroperoxide Systems

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Cumene hydroperoxide (CHP)- and ethyl hydroperoxide (EHP)-supported oxidations of aminopyrine catalyzed by catalase were investigated by electron spin resonance (ESR) studies. The oxidations were also monitored through the measurement of formaldehyde. The EHP-supported oxidation of aminopyrine went to completion within 5 min and was inhibited by sodium azide. When EHP was added to catalase prior to the addition of aminopyrine, the ESR signal intensity of the aminopyrine free radical and the concentration of formaldehyde did not increase to appreciable values. This retardation of the oxidation of aminopyrine was considerably relieved by previously adding sodium azide to catalase. The rate of EHP-supported oxidation of methanol was comparable to that of aminopyrine. CHP previously added to catalase inhibited the EHP-supported oxidation of aminopyrine, whereas it did not inhibit that of methanol. The present results suggest that the active site of catalase for the EHP-supported oxidation of aminopyrine is different from that for the oxidation of methanol.

Keywords——catalase; cumene hydroperoxide; ethyl hydroperoxide; aminopyrine; aminopyrine free radical; N-demethylation of aminopyrine; cumene hydroperoxide-supported oxidation; ethyl hydroperoxide-supported oxidation; electron spin resonance; sodium azide

In the previous paper we described the cumene hydroperoxide (CHP)- supported oxidation of aminopyrine by catalase.[2)] The transient free radical of aminopyrine was detected by electron spin resonance(ESR) spectroscopy at room temperature. Although another free radical was detected in the catalase-CHP system in the absence of aminopyrine, this radical was considered not to be the major oxidant of the system, because its concentration was too low. CHP which had been added to the catalase solution prior to addition of aminopyrine impaired the N-demethylase activity of catalase, whereas 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 was not oxidized appreciably in the catalase-CHP system. These experimental results strongly suggested that the active site of catalase for the CHP-supported N-demethylation of aminopyrine is different from that for the catalatic reaction.

Marklund studied the peroxidatic activity of catalase towards phenolic compounds and suggested that the active sites for the different classes of substrates are not necessarily identical.[3)] This suggestion is similar to ours.[2)]

The present study was undertaken to elucidate the mechanism of N-demethylation of aminopyrine catalyzed by catalase. Ethyl hydroperoxide (EHP) was used in place of CHP, and the results were compared with those for CHP. The inhibitory effects of the hydroperoxides and sodium azide were also investigated.

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}

1) Location: *Ikawadani-cho, Tarumi-ku, Kobe, 673, Japan.*
2) H. Sayo and M. Hosokawa, *Chem. Pharm. Bull.*, **28**, 2077 (1980).
3) S. Marklund, *Biochim. Biophys. Acta*, **321**, 90 (1973).

was used.[4] The catalatic activity of this preparation determined as described previously[2] was 14000 Sigma units per mg protein. Aminopyrine and cumene hydroperoxide were obtained and purified as described previously.[2] Ethyl hydroperoxide was prepared by the method of Rieche.[5] The concentrations of hydroperoxides were determined by iodometric titration.[6] The buffer solution used in this study was 0.1 M NaH_2PO_4-Na_2HPO_4 (pH 7.4). Water was purified by means of a Millipore MILLI-R/Q system. 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 a hydroperoxide or aminopyrine, the mixture was transferred to a flat aqueous solution cell (JES-LC-11) and the ESR signal was scanned 1 min after mixing. 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,[7] after the reaction had been quenched with 10% trichloroacetic acid and the solution centrifuged to remove precipitated protein. Visible absorption spectra were recorded on a Hitachi 340 spectrometer at 25°.

Results

Time Dependence of Generation of the Aminopyrine Free Radical

When EHP was added to a buffered solution of pH 7.4 containing catalase and aminopyrine, the mixture turned blue-violet and the transient free radical of aminopyrine was detected by ESR. Fig. 1 shows the time dependence of ESR signal intensities under various

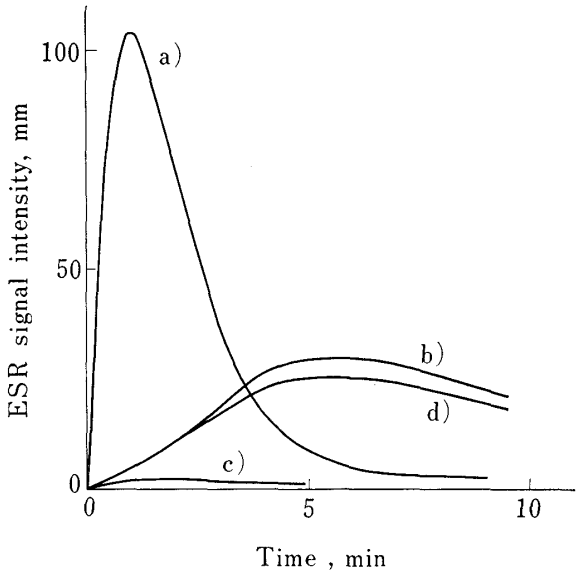

Fig. 1. Time Dependence of the ESR Signal Intensity of Aminopyrine Free Radical in the Ethyl Hydroperoxide-supported Oxidation

The reaction mixture contained 0.7 μM catalase, 3 mM aminopyrine, and 0.75 mM EHP. a) The three compounds were mixed in that order. b) Sodium azide (2 mM) was previously mixed with the catalase in a). c) EHP was added to the catalase solution, and the mixture was allowed to stand for 150 sec then mixed with aminopyrine. d) Sodium azide (2 mM) was previously mixed with the catalase in c). Instrumental settings: power, 5 mW; modulation amplitude, 10 G, time constant, 1 sec; gain, 1×1000. All experiments were carried out in 0.1 M sodium phosphate buffer (pH 7.4) at 25°.

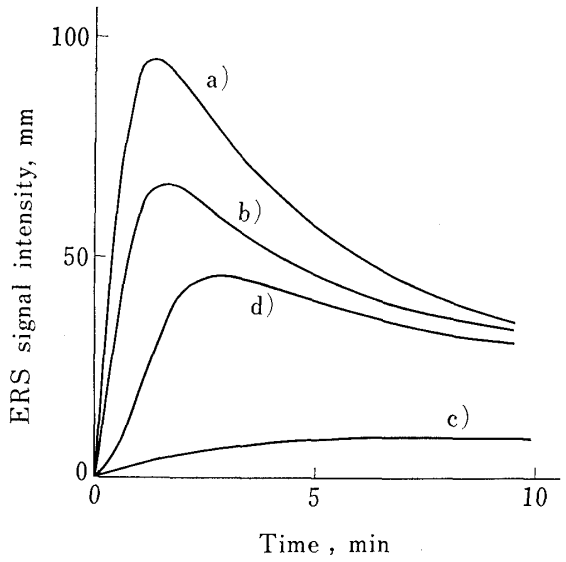

Fig. 2. Time Dependence of the ESR Signal Intensity of Aminopyrine Free Radical in the Cumene Hydroperoxide-supported Oxidation

The reaction mixture contained 0.7 μM catalase, 3 mM aminopyrine, and 0.75 mM CHP. a) The three compounds were mixed in that order. b) Sodium azide (2 mM) was previously mixed with the catalase in a). c) CHP was added to the catalase solution, and the mixture was allowed to stand for 150 sec then mixed with aminopyrine. d) Sodium azide (2 mM) was previously mixed with the catalase in c). Instrumental settings were the same as in Fig. 1.

4) M.L. Kremer, *Biochim. Biophys. Acta*, **198**, 199 (1970).
5) A. Rieche, *Chem. Ber.*, **62**, 218 (1929); *idem*, *ibid.*, **62**, 2458 (1929).
6) P.D. Bartlett and R. Altschul, *J. Am. Chem. Soc.*, **67**, 816 (1945).
7) T. Nash, *Biochem. J.*, **55**, 416 (1953).

experimental conditions. As shown in Fig. 1a), the ESR signal decayed at a faster rate than in the CHP-supported system (*cf.* Fig. 2).

When sodium azide was previously added to the catalase solution and then aminopyrine and EHP were added in that order, the rates of appearance and disappearance of the ESR signal and the maximal ESR signal intensity were greatly decreased (Fig. 1b). When EHP was added to catalase prior to addition of aminopyrine and the mixture was allowed to stand for 150 sec and then mixed with aminopyrine, the ESR signal intensity did not increase to an appreciable value (Fig. 1c). On the other hand, when EHP was added to catalase solution previously mixed with sodium azide and the mixture was allowed to stand for 150 sec and then mixed with aminopyrine, depression of ESR signal intensity was less marked (Fig. 1d).

Similar experiments were carried out with CHP in place of EHP. As shown in Fig. 2, sodium azide inhibited the oxidation of aminopyrine to a smaller extent in the CHP-supported system than in the EHP-supported one.

Table I. Effects of Added Compounds and of the Order of their Additions on the Formation of Formaldehyde in the Catalase-Organic Hydroperoxide System

Run	Order of additions of the catalase solution[a]				HCHO concentration,[b]			
					CHP-supported		EHP-supported	
	1	2	3	4	5 min[c]	2 hr[c]	5 min[c]	2 hr[c]
1	Aminopyrine	Hydroperoxide	——	——	100	169 (100)[d]	69 (100)[d]	70 (100)[d]
2	NaN_3	Aminopyrine	Hydroperoxide	——	58	155 (92)	21 (30)	61 (87)
3	Hydroperoxide	Allowed to stand for 30 sec	Aminopyrine	——	37	123 (73)	24 (35)	29 (41)
4	NaN_3	Hydroperoxide	Allowed to stand for 30 sec	Aminopyrine	33	132 (78)	22 (32)	50 (71)
5	Hydroperoxide	Allowed to stand for 150 sec	Aminopyrine	——	10	35 (21)	4 (6)	6 (9)
6	NaN_3	Hydroperoxide	Allowed to stand for 150 sec	Aminopyrine	29	128 (76)	14 (20)	26 (37)

a) The final concentrations of various agents were as follows; catalase 0.7 μM, aminopyrine 3 mM, CHP or EHP 0.75 mM, NaN_3 2 mM.
b) The concentration of HCHO is expressed relative to the value (0.24 mM) determined at 5 min after initiating the reaction by adding CHP taken as 100.
c) Reaction time after initiating the reaction by adding the last reactant.
d) Numbers in parentheses are the values relative to run 1.
e) All experiments were carried out in 0.1 M sodium phosphate buffer (pH 7.4) at 25°.

Concentration of Formaldehyde produced by N-Demethylation of Aminopyrine

Table I shows the effects of added compounds and of the order of their additions on the concentration of formaldehyde produced by the EHP- and CHP-supported oxidation of aminopyrine. It is apparent from run 1 that the EHP-supported N-demethylation goes essentially to completion within 5 min. This result is in accord with the time dependence of the ESR signal intensity. When sodium azide was previously added to the catalase solution and then aminopyrine and EHP or CHP were added in that order, the concentrations of formaldehyde at 5 min after initiating the reactions were considerably depressed. However, the concentrations of formaldehyde at 2 hr after initiating the reactions were only slightly smaller than those without addition of sodium azide (run 2).

When CHP was previously added to the catalase solution and the mixture was allowed to stand for 30 sec before adding aminopyrine, the formation of formaldehyde was suppressed significantly in the initial stage (run 3). However, the concentration of formaldehyde at 2 hr after initiating the reaction amounted to 73% of that in run 1. In contrast, when EHP was added in place of CHP, the concentration of formaldehyde at 2 hr after initiating the reaction amounted to only 41% of that in run 1 (run 3).

When the standing time in run 3 was increased to 150 sec, the depression of N-demethylation by the hydroperoxides became considerable in both reactions (run 5). However, if sodium azide was added to the catalase solution prior to addition of CHP, the concentration of formaldehyde in the CHP-supported oxidation amounted to 76% of that in run 1 for a period of 2 hr (run 6).

Organic Hydroperoxide-supported Oxidation of Methanol

In the previous paper, it was reported that the rate of the CHP-supported oxidation of methanol was very small.[2] Actually, the concentration of formaldehyde determined at 5 min after initiating the reaction was only 4—9% of that in the oxidation of aminopyrine, as shown in Table II. The CHP-supported oxidation of aminopyrine went to completion within 2 hr,[2] whereas that of methanol required 4 hr for completion. Furthermore, the limiting concentration of formaldehyde was 20% of that in the oxidation of aminopyrine and only 9% of the concentration of CHP. The concentration of formaldehyde determined at 2 hr after initiating the CHP-supported oxidation of the mixture of aminopyrine and methanol (run 3) was slightly smaller than that in run 1 and also smaller than the sum of those in run 4 and in run 5.

Table II. Organic Hydroperoxide-supported Oxidation of Aminopyrine and Methanol by Catalase[a]

Run	Substrate (concentration)	HCHO concentration,[b] μM			
		CHP-supported		EHP-supported	
		5 min	2 hr	5 min	2 hr
1	Aminopyrine (6 mM)	435	680	228	217
2	Methanol (6 mM)	39	106	187	183
3	Aminopyrine (3 mM) + Methanol (3 mM)	353	663	264	252
4	Aminopyrine (3 mM)	321	623	189	179
5	Methanol (3 mM)	18	61	126	122

a) The final concentration of catalase was 1.4 μM. CHP or EHP was finally added to the mixture to give a concentration of 1.5 mM.
b) The concentration was determined at 5 min and 2 hr after initiating the reaction by adding CHP or EHP.
c) All experiments were carried out in 0.1 M sodium phosphate buffer (pH 7.4) at 25°.

On the other hand, the EHP-supported oxidation of methanol took place at a rate comparable to that of aminopyrine. Therefore, both oxidations went to completion within 5 min. The concentration of formaldehyde determined at 5 min after initiating the EHP-supported oxidation of the mixture of aminopyrine and methanol (run 3) was larger than that in run 1 or run 2 and smaller than the sum of those in run 4 and run 5.

When sodium azide (4 mM) was previously added to catalase, the EHP-supported oxidation of methanol was significantly inhibited, and the concentration of formaldehyde formed at 5 min after initiating the reaction was 1.6% of that without addition of sodium azide. On the other hand, when CHP (0.75 mM) was previously added to catalase and the solution was allowed to stand for 10 min, the EHP-supported oxidation of methanol was not depressed at all, whereas that of aminopyrine was depressed significantly (the concentration of formaldehyde was 18% of the control).

Discussion

The experimental findings that the EHP-supported N-demethylation of aminopyrine went essentially to completion within 5 min and that the limiting concentration of formaldehyde was about 41% of that in the CHP-supported N-demethylation can be interpreted in

terms of the decomposition of EHP by catalase. Although CHP was not decomposed appreciably by catalase in the absence of aminopyrine, it is well established that EHP reacts with catalase fairly rapidly to form acetaldehyde without addition of reductants.[8] Since EHP is consumed for both the EHP-supported oxidation of aminopyrine and the decomposition of EHP itself, the concentration of EHP in the reaction mixture decreases rapidly, and the oxidation comes to an end within several minutes.

The inhibition of the N-demethylase activity by sodium azide in both the CHP- and EHP-supported systems is considered to be reversible, since the limiting concentration of formaldehyde amounted to 87—92% of that without addition of sodium azide even though the rate of the N-demethylation was considerably deperssed in the initial stage.

On the other hand, when CHP was previously added to catalase and the solution was allowed to stand for 150 sec, the rate of the N-demethylation of aminopyrine was significantly depressed and only a small portion of the N-demethylase activity was recovered in a period of 2 hr. Consequently, the concentration of formaldehyde at 2 hr after initiating the reaction amounted to only 21% of the control. This inactivation of the N-demethylase activity of catalase by CHP was considerably relieved by adding sodium azide to catalase.

The following explanations may be offered for these findings: (a) when CHP binds to catalase already carrying aminopyrine, CHP abstracts an electron from the lone-pair electrons of aminopyrine with the aid of catalase; (b) when CHP binds to catalase not carrying aminopyrine, CHP abstracts an electron from catalase itself and inactivates its N-demethylase activity; (c) sodium azide binds to the same site of catalase as CHP and is gradually replaced by CHP during the reaction, which causes reversible inhibition of the N-demethylase activity of catalase; (d) when CHP is added to catalase already carrying sodium azide, the sodium azide protects catalase against inactivation by CHP presumably by interfering with the binding between catalase and CHP.

The experimental findings that CHP previously added to catalase inhibited the EHP-supported oxidation of aminopyrine, whereas it did not inhibit that of methanol, and that the concentration of formaldehyde formed by the EHP-supported oxidation of the mixture of aminopyrine (3 mM) and methanol (3 mM) was larger than that formed by the oxidation of aminopyrine (6 mM) alone and also larger than that formed by the oxidation of methanol (6 mM) alone appear to suggest that the active site of catalase for the EHP-supported oxidation of aminopyrine is different from that for the oxidation of methanol.

Although the present study has provided some information on the mechanism of organic hydroperoxide-supported oxidation of aminopyrine, many problems remain. For example, aminopyrine is oxidized through a one-electron transfer, whereas the hydroperoxides are reduced through a two-electron transfer. In addition, the relationship between the CHP-supported oxidation and catalase compound I must be investigated. Further studies are in progress.

8) G.R. Schonbaum and R. Chance, "The Enzymes," 3rd ed., Vol. 13C, Academic Press, Inc., New York, 1976, p. 363.