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Studies on the Ethylhydroperoxide-Supported Oxidation of 1,4-Diazabicyclo[2.2.2]octane by Chloroperoxidase

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The chloroperoxidase-catalyzed oxidation of 1,4-diazabicyclo[2.2.2]octane (DABCO) by H_2O_2 or ethylhydroperoxide (EHP) in the presence of Cl^- was investigated at pH 2.75, 5.0, and 7.0, and the results were compared with those of the chemical oxidation by HOCl. The oxidation of DABCO in the chloroperoxidase-EHP-Cl^- system at pH 5.0 gave a fairly well-resolved electron spin resonance spectrum, which was identified as that of the radical cation of DABCO. The detection of the radical cation suggests that the first step of the chloroperoxidase-catalyzed oxidation of DABCO is the formation of the DABCO chloroammonium cation followed by the homolysis of the cation.

Keywords——chloroperoxidase; ESR; ethylhydroperoxide; 1,4-diazabicyclo[2.2.2]octane; DABCO cation radical; ethylhydroperoxide-supported oxidation; hypochlorous acid; triethylenediamine

Chloroperoxidase (EC 1.11.1.10),[1] horseradish peroxidase (EC 1.11.1.7),[1-3] catalase (EC 1.11.1.6)[4] and several other hemeproteins[1,3] can catalyze the hydroperoxide-dependent *N*-dealkylation of many aromatic secondary and tertiary amines. However, substrates for the dealkylation so far reported are confined to aromatic amines, and no aliphatic amine has been found to be oxidized by these hemeproteins at a significant rate.

Chloroperoxidase can utilize chloride ion as a donor for enzymatic halogenation reactions. Free HOCl or Cl_2 was suggested to be the actual halogenating agent produced by chloroperoxidase.[5-9] Since HOCl is a strong oxidizing agent which can oxidize aliphatic secondary[10] and tertiary amines,[11-14] chloroperoxidase is considered to have the capability of catalyzing oxidative fragmentation of the amines. Therefore, hydrogen peroxide- and ethylhydroperoxide-supported oxidations of 1,4-diazabicyclo[2.2.2]octane (DABCO) catalyzed by chloroperoxidase were investigated in some detail. DABCO was chosen as the model substrate because the mechanism of the oxidation of DABCO by HOCl has been extensively studied by Rosenblatt *et al.*[14-16] The oxidation of *N*,*N*′-dimethylpiperazine (DMP) and *N*,*N*-dimethylcyclohexylamine (DMCH) was also studied for comparison.

Experimental

Materials——Chloroperoxidase (from *Caldariomyces fumago*) was obtained from Sigma. The enzyme preparation had an RZ value of 0.8 and a specific activity of 1040 units/mg. Monochlorodimedone (MCD) was prepared by the method of Hager *et al.*[17] DABCO was obtained from a commercial source and recrystallized from acetone. DMP and DMCH were obtained from a commercial source and distilled before use. *N*,*N*′-Dichloropiperazine (DCP) was prepared by the method of Dennis *et al.*[14] Ethylhydroperoxide (EHP) was prepared by the method of Rieche.[18] The concentrations of stock H_2O_2, EHP and NaOCl solutions were determined by iodometric titration.[19] The buffer solutions used were 0.2 M sodium phosphate buffer (pH 2.75), 0.2 M sodium acetate buffer (pH 5.0) and 0.2 M sodium phosphate buffer (pH 7.0). The buffer solutions were deoxygenated by flushing with N_2 gas before use.

Methods——The rate of conversion of MCD to dichlorodimedone was measured in terms of the decrease of absorbance at 278 nm.[20] Formaldehyde was assayed as described previously.[21] DCP was determined by the use of high-performance liquid chromatography (HPLC). Piperazine was determined by the method of Chen and Farquharson.[22] Electron spin resonance (ESR) spectra were recorded on a JEOL JES-FE-1X spectrometer, equipped with an EC-LS-11 flat cell and a Union Giken MX-7 mixing device. The DABCO radical cation was generated by the continuous flow method at room temperature (24±1 °C).

Results

At pH 5.0, the oxidation of DABCO by the chloroperoxidase-EHP-Cl^- system gave a fairly well-resolved ESR spectrum, as shown in Fig. 1 (lower panel). The spectrum was identical to that obtained by the oxidation of DABCO with HOCl (Fig. 1, upper panel) and was assigned to the radical cation of DABCO. The hyperfine splitting constants of the spectrum ($A_1=16.9$ G(2N), $A_2=7.3$ G(12H)) agreed with those previously reported for the DABCO radical cation generated electrochemically.[23] No ESR spectrum was observed on the oxidation of DABCO by the following systems: chloroperoxidase-EHP-Cl^- at pH 2.75 and 7.0, chloroperoxidase-H_2O_2-Cl^- at pH 2.75, 5.0 and 7.0.

Formaldehyde produced during the oxidation of DABCO by the chemical oxidation with HOCl and by the enzymatic oxidation in the chloroperoxidase-(EHP or H_2O_2)-Cl^- system was determined at pH 2.75, 5.0 and 7.0. The oxidations of DMP, a monocyclic analogue of DABCO, and DMCH were also investigated for comparison. The results are listed in Table I. The oxidations of DABCO and DMP by HOCl were completed in 30 s and the amount of formaldehyde formed was nearly constant between pH 2.75 and 7.0. On the other hand, the

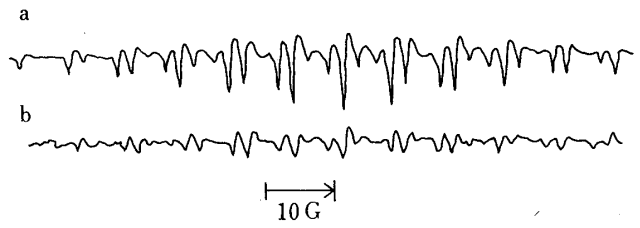


Fig. 1. ESR Spectra Obtained during the Oxidation of DABCO by HOCl and the Chloroperoxidase-EHP-Cl^- System

a) The reaction mixture contained NaOCl (1.2 mM) and DABCO (1.6 mM) in 0.2 M acetate buffer (pH 5.0). Spectrometer settings: power, 10 mW; modulation amplitude, 0.5 G; scan rate, 12.5 G/min; time constant, 3 s; gain, 4×1000. b) The reaction mixture contained chloroperoxidase (100 nM), EHP (25 mM), DABCO (20 mM) and KCl (400 mM) in 0.2 M acetate buffer (pH 5.0). Spectrometer settings: power, 5 mW; modulation amplitude, 1.0 G; scan rate, 6.25 G/min; time constant, 3 s; gain, 6.3×1000.

TABLE I. Amount of Formaldehyde Produced by the Chemical and Chloroperoxidase-Catalyzed Oxidations of Aliphatic Tertiary Amines

	pH	HOCl			CPO–EHP–Cl^-			CPO–H_2O_2–Cl^-		
		0.5	1.5	5.0	5	15	30	5	15	30 (min)
DABCO	2.75	86.8±0.6	88.0±0.3	88.0±0.3	4.0±0.1	4.6±0.2	5.0±0.3	0	0	0
	5.0	88.6±0.4	89.5±0.3	89.9±0.3	28.1±1.3	46.7±1.8	57.4±1.2	3.1±0.1	3.7±0.2	3.5±0.1
	7.0	80.0±0.3	80.0±0.4	77.8±0.3	1.1±0.1	1.5±0.2	1.8±0.1	0	0	0
DMP	2.75	99.6±0.4	98.3±0.7	99.6±0.6	1.4±0.2	2.3±0.2	2.5±0.1	1.9±0.2	2.3±0.2	1.8±0.1
	5.0	89.9±1.0	89.9±0.6	91.3±0.5	38.0±0.4	77.8±0.8	92.3±0.5	9.4±0.2	12.3±0.4	14.0±0.3
	7.0	88.5±0.5	89.2±0.6	89.2±0.4	3.0±0.1	7.5±0.2	8.4±0.4	2.1±0.2	3.0±0.1	4.2±0.4
DMCH	2.75	1.9±0.2	2.2±0.3	2.5±0.2	0	0	0	0	0	0
	5.0	15.5±0.4	53.8±0.8	57.0±1.0	6.6±0.2	8.4±0.2	7.9±0.3	2.6±0.1	2.6±0.1	2.6±0.1
	7.0	56.3±0.7	57.7±0.4	56.4±0.3	0	0	0	0	0	0

Numbers are mol% of formaldehyde formed relative to HOCl or EHP added; means±standard deviation of four experiments. The reaction mixture contained 333 μM oxidant, 1.3 mM amine, 200 mM KCl, and for the enzyme-catalyzed reaction, 25 nM chloroperoxidase. All experiments were carried out at 25 °C. CPO, chloroperoxidase.

oxidation of DMCH by HOCl at pH 5.0 was not completed in 30 s, and at pH 2.75 the amount of formaldehyde produced was very small even 5 min after initiating the reaction.

As regards the oxidation of the amines by the chloroperoxidase-EHP-Cl^- system, the rate of oxidation was much slower than that by HOCl and the pH optimum lies at pH 5.0. The amount of formaldehyde produced leveled off 30 min after initiating the reaction. The largest amount of formaldehyde was formed on the oxidation of DMP at pH 5.0, while the oxidation of DMCH did not produce an appreciable amount of formaldehyde at pH 2.75—7.0.

Other products of the oxidation of DABCO, piperazine and DCP, were determined by the use of HPLC. The oxidation of DABCO by HOCl at pH 5.0, 5 min after initiating the reaction, gave 1 and 24 mol% of piperazine and DCP relative to HOCl added. On the other hand, 15 and 8.7 mol% of piperazine and DCP relative to EHP added were obtained by the oxidation in the chloroperoxidase-EHP-Cl^- system at pH 5.0, 30 min after initiating the reaction.

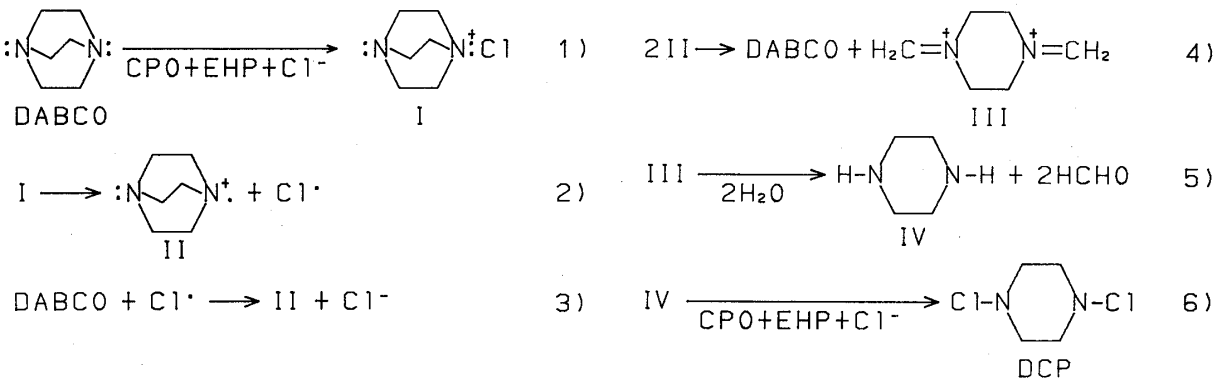
Although the chlorination activity of chloroperoxidase with H_2O_2 as the oxidant has been extensively studied by Hager *et al.*,[20,24] that with EHP as the oxidant has not been reported. Therefore, the chlorination activity of chloroperoxidase was measured at pH 2.75, 5.0 and 7.0 with EHP as the oxidant and compared with that with H_2O_2 as the oxidant. The values of the activity were 378 (pH 2.75), 49 (pH 5.0), and 0.6 (pH 7.0). Although at pH 2.75 the activity with EHP as the oxidant was much smaller than that with H_2O_2 as the oxidant, both activities were almost the same at pH 5.0.

Discussion

We have now established that chloroperoxidase also catalyzes the EHP-supported oxidation of DABCO in the presence of chloride ion. The radical cation of DABCO was identified as an intermediate in the oxidation. This is the first example of an enzyme-mediated reaction in which aliphatic amines are oxidized at a significant rate and their radical cations are detectable by ESR.

Rosenblatt *et al.* proposed the following mechanism for the reaction of HOCl with DABCO[15]: the first step of the reaction is the formation of the DABCO chloroammonium cation followed by the homolysis of the cation, and the end products were formaldehyde and DCP. Because hypochlorous acid is a two-electron oxidant, the DABCO cation radical was considered to arise from the homolytic cleavage of the nitrogen-chloride bond in the DABCO chloroammonium cation and the subsequent one-electron oxidation of DABCO by the chlorine atom.

The detection of the DABCO radical cation in the chloroperoxidase-EHP-Cl^- system



CPO = chloroperoxidase

Chart 1

suggests that, apart from the formation of free HOCl in this system, a similar mechanism is operating for the appearance of the radical cation. The mechanism shown in Chart 1 is proposed for the chloroperoxidase-catalyzed oxidation of DABCO by EHP in the presence of Cl^-. Since EHP is present in a limited amount in the reaction mixture and reaction 6 competes with reaction 1 for EHP, the end products will be formaldehyde, piperazine and DCP. From Chart 1, 1 mol of piperazine or DCP is formed together with 2 mol of formaldehyde. Therefore, the sum of piperazine and DCP formed should be equal to half the amount of formaldehyde produced. However, the sum obtained at pH 5.0, 30 min after initiating the reaction, amounts to only 41 mol% of formaldehyde produced. The rest of the formaldehyde is considered to be produced by the slow decomposition of DCP.[16]

Several recent papers have addressed the mechanism of chlorination of organic substrates by peroxide and Cl^- catalyzed by chloroperoxidase. A theme common to all of the papers is a role for HOCl, whether free in solution or enzyme-bound. An enzyme-bound chlorinating intermediate was proposed on the basis of evidence such as enzyme–substrate specificity, difference between chemical and enzymatic halogenation products, and steady-state kinetic results.[25-28] On the other hand, product analysis studies led to the conclusion that free HOCl is the most likely chlorinating reagent and that the reaction does not occur at the enzyme active site.[5-8,29,30]

Although our finding that the DABCO radical cation, formaldehyde, piperazine and DCP were detected in the chloroperoxidase-catalyzed oxidation of DABCO by EHP in the presence of Cl^- is to be expected for the reaction between HOCl and DABCO, the experimental fact that the amounts of formaldehyde produced by the oxidation of DABCO and DMP at pH 2.75 in the chloroperoxidase-EHP-Cl^- system were much less than those produced by the oxidation with HOCl does not support the role of free HOCl as the key intermediate in the enzyme-catalyzed pathway. Since DABCO and DMP are diacidic bases and the pK_a values for the proton ionization were reported to be 8.82 and 2.95, and 8.23 and 4.18,[31] large portions of DABCO and DMP exist in the diprotonated form at pH 2.75, whereas at pH 5.0 one of their amino groups is in the free form. Therefore, the oxidation of DABCO and DMP in the chloroperoxidase-EHP-Cl^- system may involve the binding of the nonprotonated amino group to the enzyme. Further studies are needed on this.

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