Chloroperoxidase-Catalyzed Oxidation of Aminopyrine

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Although in the absence of halide ion chloroperoxidase did not catalyze the ethylhydroperoxide (EHP)-supported oxidation of aminopyrine, in the presence of Br^- or Cl^-, chloroperoxidase did catalyze the oxidation of aminopyrine, generating the aminopyrine cation radical (AP^+). The initial rate of AP^+ formation was determined by monitoring the absorbance at 565 nm. The pH optimum of the reaction was centered around 5.0. The rate of AP^+ formation showed typical Michaelis–Menten saturation kinetics with respect to EHP, aminopyrine and Br^-. The rate of formation of bromine in the chloroperoxidase–EHP–Br^- system was also determined by measuring the change in absorbance at 267 nm. In the system containing 1 mM EHP and 0.2 M KBr at pH 5.0, the rate was 1.8 nmol of bromine/s/μg of chloroperoxidase, which was slower than that of AP^+ formation under the same conditions. The present results suggest that the formation of AP^+ is initiated by the halogenation of the *N,N*-dimethylamino group followed by the homolysis of the haloammonium cation, and that the most likely halogenating reagent is an enzyme-bound halogenating intermediate.

Keywords chloroperoxidase; ESR; aminopyrine; aminopyrine cation radical; bromine; ethylhydroperoxide

Chloroperoxidase (chloride : hydrogen peroxide oxidoreductase; EC 1.11.1.10) is a unique hemeprotein which has the physical properties of cytochrome P-450 and which exhibits several distinct catalytic activities. Its most unusual property is the ability to catalyze the halogenation of a large number of substrates in the presence of Cl^-, Br^- or I^-, and hydrogen peroxide.[1,2] Both an enzyme-bound halogenating intermediate[3-5] and a halogenium ion or hypohalous acid[6-8] have been proposed as the actual halogenating agent produced by chloroperoxidase. The enzyme also catalyzes the hydroperoxide-dependent *N*-demethylation of a variety of *N*-methylarylamines.[9] However, it has been reported that aminopyrine is not demethylated by chloroperoxidase and ethylhydroperoxide (EHP).[9]

On the other hand, *N*-demethylation of aminopyrine by peroxides is catalyzed by a number of hemeproteins such as horseradish peroxidase,[10] metmyoglobin,[10] liver microsomal cytochrome P-450,[11] catalase,[12] prostaglandin H synthase[13] and myeloperoxidase[14]; the peroxidase-mediated reactions involve the formation of the free radical intermediate as indicated by electron spin resonance (ESR) spectroscopy. In a previous paper,[15] we reported that chloroperoxidase catalyzes the EHP-supported oxidation of 1,4-diazabicyclo[2.2.2]octane (DABCO) in the presence of Cl^-, and that the radical cation of DABCO is formed as an intermediate in the oxidation. Since the voltammetric peak potential of aminopyrine[16] is less positive than that of DABCO,[17] the peroxide-supported oxidation of aminopyrine would appear to be catalyzed by chloroperoxidase, at least in the presence of Cl^-. In the present paper, we demonstrate that aminopyrine is in fact oxidized by the chloroperoxidase–EHP–Cl^- and the chloroperoxidase–EHP–Br^- systems, generating the aminopyrine cation radical, and that the most likely halogenating agent is an enzyme-bound intermediate.

Experimental

Materials and Methods 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. Aminopyrine was obtained from a commercial source and recrystallized from ligroin. EHP was prepared by the method of Rieche and Hitz.[18] The concentrations of stock EHP, NaOCl and Br_2 solutions were determined by iodometric titration. The buffer solutions used were 0.1 M sodium phosphate buffer (pH 3.0), 0.1 M sodium acetate buffer (pH 4.0—5.5) and 0.1 M sodium phosphate buffer (pH 6.0). In the experiment in which the concentration of KBr was varied, the total ionic strength was adjusted to 0.26 using potassium sulfate as an inert salt. Water was purified by the use of a Millipore MILLI-R/Q system. All other chemicals used were of reagent grade.

ESR spectra were recorded on a JEOL JES-FE 1X spectrometer, equipped with 100 kHz field modulation, at room temperature (25 ± 1 °C). After initiating the reaction by addition of an oxidant, the mixture was transferred to thin-walled capillaries of 1 mm inside diameter and the ESR signal was scanned immediately.

The initial rate of formation of the aminopyrine cation radical was determined at 25 °C by the use of a Union-Giken RA 601 stopped-flow spectrophotometer, equipped with a system 71 data processor. One reservoir was filled with the EHP solution and the other was filled with a mixture of the chloroperoxidase, aminopyrine and KCl/KBr solutions. The amount of the aminopyrine cation radical formed was determined from the absorbance of the solution at 565 nm using a molar absorption coefficient of $2.23 \times 10^3\ M^{-1}\ cm^{-1}$.[19]

The rate of formation of bromine in the chloroperoxidase–EHP–Br^- system was determined from the increase in absorbance at 267 nm using a molar absorption coefficient of $3.64 \times 10^4\ M^{-1}\ cm^{-1}$ and an equilibrium constant of $14.4\ M^{-1}$ for the formation of tribromide ion.[3]

Results

Identification of a Radical Produced by Chloroperoxidase-Catalyzed Oxidation of Aminopyrine in the Presence of Bromide or Chloride Ion Although in the absence of halide

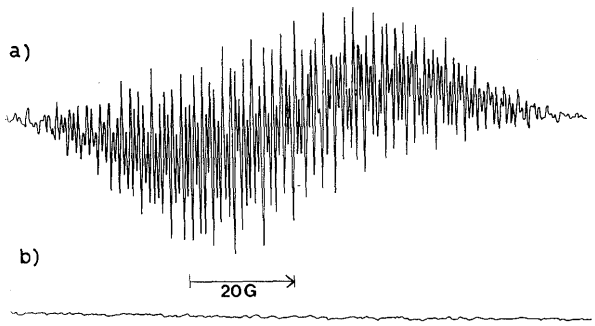

Fig. 1. ESR Spectra Obtained during the Oxidation of Aminopyrine by the Chloroperoxidase–EHP System in the Presence and Absence of KBr

(a) The reaction mixture contained chloroperoxidase (1.3 μg/ml), EHP (9 mM), aminopyrine (10 mM) and KBr (0.2 M) in 0.1 M sodium acetate buffer (pH 5.0). Spectrometer settings: power, 10 mW; modulation amplitude 0.8 G; scan rate, 25 G/min; time constant, 0.3 s; gain, 1 × 1000. (b) Identical to (a), except that KBr was omitted.

ions the EHP-supported oxidation of aminopyrine gave no ESR signal at pH 3—6, in the presence of Br^- or Cl^- at pH 5.0, it gave a fairly well resolved ESR spectrum, as shown in Fig. 1. The spectrum was identical to that previously obtained by the use of catalase and cumene hydroperoxide, and was assigned to the aminopyrine cation radical. The formation of the aminopyrine cation radical was also confirmed by its absorption maximum at 565 nm.[19] All control experiments were negative. The same ESR and visible absorption spectra were obtained on the oxidation of aminopyrine by bromine or hypochlorous acid.

Determination of Initial Velocity of Aminopyrine Cation Radical Formation Since the concentration of the aminopyrine cation radical can be determined very sensitively by spectrophotometry,[19] the increase in the absorbance of the reaction mixture at 565 nm as a function of time was measured by the use of a stopped-flow spectrophotometer. Typical examples of the curves are shown in Fig. 2. Although the aminopyrine cation radical decomposes fairly rapidly in aqueous buffer solutions, the initial increase in the absorbance can be used to obtain the initial rate of oxidation of aminopyrine.[20] For the studies reported here, the initial velocity was obtained from the difference in the absorbance between 2 and 4 s after initiating the reaction. Since the initial velocity of aminopyrine radical formation was linear with respect to the chloroperoxidase concentration between 0.4 and 2.2 μg/ml, the concentration of chloroperoxidase was set at 1.3 μg/ml throughout the study.

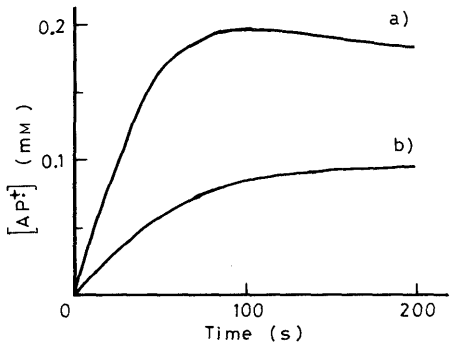

Fig. 2. Time Dependence of Aminopyrine Cation Radical Formation by the Chloroperoxidase–EHP–KBr/KCl System at pH 5.0

The reaction mixture contained chloroperoxidase (1.3 μg/ml), EHP (1 mM), aminopyrine (5 mM) and KBr or KCl (0.2 M) in 0.1 M sodium acetate buffer (pH 5.0). (a) In the presence of KBr; (b) KCl. $AP^{\cdot}$, aminopyrine cation radical.

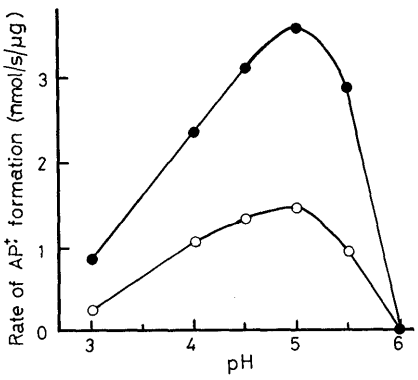

Fig. 3. Effect of pH on the Rate of Aminopyrine Cation Radical ($AP^{\cdot}$) Formation

The reaction mixture contained chloroperoxidase (1.3 μg/ml), EHP (1 mM), aminopyrine (5 mM) and KBr or KCl (0.2 M) in 0.1 M of the buffer described in Materials and Methods. ●, in the presence of KBr; ○, KCl.

Effects of pH and Substrate Concentration on the Rate of Aminopyrine Cation Radical Formation As shown in Fig. 3, chloroperoxidase exhibited a pH optimum centered around pH 5.0 for the EHP-dependent oxidation of aminopyrine in the presence of Br^-. The rate of the radical formation showed typical Michaelis–Menten saturation kinetics with respect to EHP, aminopyrine and KBr. The apparent K_m's for EHP, aminopyrine and KBr, calculated from the Lineweaver–Burk plots in Fig. 4, were 5.2, 0.9 and 23.0 mM, and V_{max}'s were 20.7, 3.4 and 3.9 nmol of the radical formed/s/μg of chloroperoxidase at [aminopyrine]= 5 mM and [KBr]=0.2 M, [EHP]=1 mM and [KBr]=0.2 M, and [EHP]=1 mM and [aminopyrine]=5 mM, respectively.

When potassium chloride was substituted for potassium bromide, a similar pH-profile (Fig. 3) and double reciprocal plots were obtained (Fig. 4A, B), although the initial rate of the radical formation is slower in the presence of chloride ion than that in the presence of bromide ion.

Enzymic Formation of Molecular Bromine from Bromide Ion The rate of formation of bromine in the chloroperoxidase–EHP–Br^- system was determined by measuring the change in absorbance at 267 nm without addition of aminopyrine. In the system containing 1.3 μg/ml chloroperoxidase, 1 mM EHP and 0.2 M KBr at pH 5.0, the concentration of bromine generated increased linearly with time up to 7.5 s and leveled off 20 s after initiating the reaction. The final concentration of total bromine formed was 0.031 mM, which corresponded to 3.1 mol% of EHP added. Since bromine is toxic to the enzyme, the initial rate was obtained from the slope between 2.5 and 7.5 s after initiating the reaction. The initial rate of bromine for mation increased linearly with increase in the concentration of chloroperoxidase between 0.4 and 2.2 μg/ml. The rate

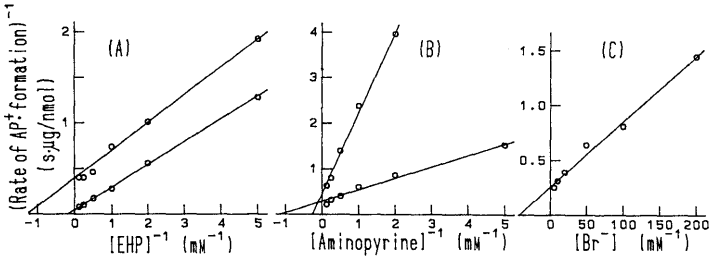

Fig. 4. Double-Reciprocal Plots of the Initial Rate of Aminopyrine Cation Radical Formation with (A) EHP, (B) Aminopyrine, or (C) Potassium Bromide as the Variable Substrate

The reaction mixture contained chloroperoxidase (1.3 μg/ml), and (A) aminopyrine (5 mM), KBr or KCl (0.2 M) and the indicated concentration of EHP; (B) EHP (1 mM), KBr or KCl (0.2 M) and the indicated concentration of aminopyrine; (C) EHP (1 mM), aminopyrine (5 mM) and the indicated concentration of KBr in 0.1 M sodium acetate buffer (pH 5.0). lower plots, in the presence of KBr; upper plots, KCl.

was 1.8 nmol of bromine/s/μg of chloroperoxidase, which was slower than that of the aminopyrine cation radical formation in the same system.

The Reaction of Aminopyrine with Bromine The reaction of aminopyrine (5 mM) with bromine (0.5 mM) in the presence of KBr (0.2 M) at pH 5.0 was too fast to observe in the stopped-flow apparatus. In a more dilute solution ([aminopyrine] = 0.5 mM, [Br_2] = 0.1 mM), a simple exponential trace was obtained. The aminopyrine cation radical formed in the reaction amounted to only 30 mol% of bromine added. The pseudo-first-order rate constant obtained was $47.2 \pm 1.2\,s^{-1}$ (mean $\pm$ S.D.). The rate constant, however, was not proportional to the concentration of aminopyrine ($69.1 \pm 2.1\,s^{-1}$ at 1.0 mM and $108.2 \pm 4.3\,s^{-1}$ at 2 mM).

Since the reaction of aminopyrine with bromine was extremely rapid, the decay in the absorbance after the maximum formation of the aminopyrine cation radical can be considered to represent the decay of the radical. Plots of $1/C$ against time, where C is the concentration of the radical, were linear up to 80% decay, characterizing the disappearance of the radical as a second-order process. The second-order decay constant, K_d, calculated from the slope was $108 \pm 4\,M^{-1}s^{-1}$ (mean $\pm$ S.D.) at pH 5.0. This compares to the k_d of $426 \pm 9\,M^{-1}s^{-1}$ at pH 7.8 obtained by Eling *et al.*[13]

Discussion

The results reported here are the first demonstration that chloroperoxidase can catalyze the EHP-supported oxidation of aminopyrine in the presence of Br^- or Cl^-, generating a free radical intermediate, although in the absence of added halide ion it cannot catalyze the reaction. Since the voltammetric peak potential of aminopyrine[16] is less positive than that of *N*,*N*-dimethylaniline,[21] the inability of chloroperoxidase to catalyze the oxidation of aminopyrine in the absence of halide ion indicates that the *N*,*N*-dimethylamino group of aminopyrine, because of steric restrictions, cannot donate electrons directly to the higher oxidation state of chloroperoxidase. Therefore, the oxidation of aminopyrine by the chloroperoxidase–EHP–Br^- system was initially considered to be caused by free bromine generated in the solution. However, careful analysis of the experimental results obtained here leads us to the conclusion that the oxidation of aminopyrine is initiated by the bromination of the *N*,*N*-dimethylamino group followed by the homolysis of the bromoammonium cation, and that the most likely halogenating reagent is not free bromine but an enzyme-bound halogenating intermediate. The bromine atom which is formed through homolysis of the bromoammonium cation may also react with aminopyrine to give the aminopyrine cation radical. The formation of the aminopyrine cation radical is proposed to occur as shown in Chart 1. The dependence of the rate of aminopyrine cation radical formation on the concentrations of aminopyrine, EHP and Br^- suggests that the first step in Chart 1 is rate-determining.

If free bromine is the actual halogenating reagent, the rate of the aminopyrine cation radical formation in the chloroperoxidase–EHP–Br^- system should be slower than that of bromine formation in the same system and independent of the aminopyrine concentration, since the rate of

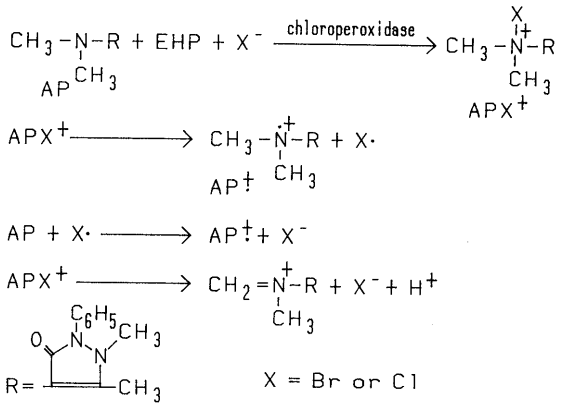

Chart 1

chemical reaction of aminopyrine with bromine is much faster than that of the enzymic formation of the aminopyrine cation radical and the radical formed amounts to only 30 mol% of bromine added. In the present study, however, completely conflicting experimental results were obtained, that is, (1) the rate of aminopyrine cation radical formation was faster than that of bromine formation in the same system; (2) the rate of the radical formation increased with increase in the concentration of aminopyrine. Therefore, we have reached this conclusion.

The reason why aminopyrine is halogenated by an enzyme-bound intermediate in spite of its steric bulkiness can be accounted for by a difference in the binding site of aminopyrine, that is, in the presence of halide ion aminopyrine is only required to approach the outer sphere of an enzyme-bound halogenating center, which must be remote from the heme iron.[3] Since the maximum formation of the aminopyrine cation radical in the chloroperoxidase–EHP–Br^- system amounted to only *ca.* 20 mol% of EHP added (Fig. 2), a fairly large amount of the bromoammonium cation is considered to undergo heterolysis.

The change in the rate of the aminopyrine cation radical formation in the pH range from 3.0 to 6.0 can be ascribed to a combined effect of the pH-dependence of halogenating activity of chloroperoxidase and the ionization of aminopyrine. The halogenating activity of the enzyme increases with decreasing pH and reaches a maximum at pH 2.75,[2] while the protonated form of aminopyrine, which cannot be halogenated, increases with decreasing pH below pH 5.0, since the pK_a of aminopyrine is around 5.0.[22] As a result of the combined effect, the pH optimum of the aminopyrine cation radical formation is apparently at 5.0.

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