The peroxide-supported N-demethylation catalyzed by chloroperoxidase, a heme protein isolated from Caldaromyces fumago, have been investigated as models for cytochrome P-450-catalyzed N-dealkylations. The turnover number for the ethyl hydrogen peroxide-supported dealkylation of N,N-dimethylaniline by chloroperoxidase (1476) was much greater than that for cytochrome P-450-catalyzed dealkylations. The dealkylations of N,N-dimethylaniline by chloroperoxidase yielded N,N-dimethylaniline and formaldehyde in equimolar amounts with no other products detectable by high-pressure liquid chromatography analysis of the reaction mixture. Ethyl hydrogen peroxide could be replaced by other hydroperoxides, peroxides, or peracids. Chloride ions stimulated the reaction at low pH. The dealkylation reaction exhibited normal Michaelis-Menten saturation kinetics with respect to N,N-dimethylaniline ($K_m = 0.88$ mm) and ethyl hydrogen peroxide ($K_m = 0.8$ mm) at low substrate concentrations. However, substrate inhibition occurred at higher concentrations of N,N-dimethylaniline.

The chloroperoxidase-catalyzed demethylations were inhibited by inhibitors of cytochrome P-450 such as azide or n-propyl gallate, but not by metyrapone, SKF-525A, or piperoxyn butoxide. Although tiron and DL-epinephrine, trapping agents for the superoxide anion, inhibited the demethylation reactions, superoxide dismutase had no effect. There was no significant inhibition by a-phenyl-t-butyl-nitrone or 5,5-dimethylpyrrolinone-N-oxide, which react with free radicals. Diphenylfurane and DL-histidine, which react with singlet oxygen, did not inhibit the reaction. Substitution of D$_2$O for H$_2$O resulted in a marked inhibition with a solvent isotope effect ($V_H/V_O$) of 3.6. Chloroperoxidase did not catalyze the demethylation of N,N-dimethylaniline-N-oxide, indicating that the reaction does not proceed via an N-oxide intermediate.

Chloroperoxidase (chloride:hydrogen-peroxide oxidoreductase; EC 1.11.1.10), a heme protein isolated from the mold Caldaromyces fumago, catalyzes the biological halogenation reactions involved in the biosynthesis of caldariomycin (2,2-dichloro-1,3-cyclopentenedione) (1). The purified enzyme is a monomer having a molecular weight of approximately 42,000 and containing 1 mol of ferric protoporphyrin IX/mol of enzyme. Chloroperoxidase catalyzes three different types of reactions, all of which use hydrogen peroxide, hydroperoxides, or peracids as oxidants (1, 2). In the presence of a suitable halogen donor (Cl$^-$, Br$^-$, or I$^-$, but not F$^-$), the enzyme catalyzes the peroxidative formation of a carbon-halogen bond with a suitable halogen acceptor as shown in Equation 1. Suitable halogen acceptors (AH) include most nucleophiles with an activated position available for attack by an electrophilic halogen. $\beta$-Keto acids and $\beta$-diketones are extremely good acceptors due to the presence of an enolizable proton. Chloroperoxidase also catalyzes the peroxidative oxidation of typical peroxidase substrates such as o-dianisidine, guaiacol, or pyrogallol (represented by BH$_2$ in Equation 2). In addition, chloroperoxidase can function as a catalase and catalyze the decomposition of hydrogen peroxide to give molecular oxygen (Equation 3).

$$\text{AH} + X^- + H_2O_2 + H^+ \rightarrow AX + 2H_2O \quad (1)$$
$$\text{BH}_2 + H_2O_2 \rightarrow B + 2H_2O \quad (2)$$
$$2H_2O_2 \rightarrow 2H_2O + O_2 \quad (3)$$

Chloroperoxidase is quite similar to many other protoheme peroxidases such as horseradish peroxidase, Japanese radish peroxidase, and cytochrome c peroxidase in many of its physical and catalytic properties (1-5). All of the peroxidases are monomeric hemeproteins with molecular weights ranging from 35,000 to 50,000 and exhibit similar optical spectra for the native (high spin ferric) and reduced (high spin ferrous) forms as well as their complexes with cyanide and azide. Although chloroperoxidase is similar to the plant and animal peroxidases in many of its catalytic functions, it exhibits one striking catalytic difference in its ability to catalyze chlorination reactions at low pH. Therefore, it has been suggested that chloroperoxidase differs significantly from other peroxidases in the vicinity of the active site (3). Hager and co-workers (3-10) have drawn attention to the surprising number of unusual physicochemical properties which chloroperoxidase shares with the bacterial and microsomal cytochrome P-450s, hemeproteins which exhibit catalytic properties which appear to be different from the peroxidases. Similar to cytochrome P-450, chloroperoxidase forms a reduced $+CO$ complex having the maximum of the Soret band at an unusually long wavelength, $\lambda_{max} = 443$ nm (4). In addition, the UV and visible absorption spectra for the oxidized and reduced forms of chloroperoxidase and the complexes of the ferric enzyme with various ligands are similar to those of cytochrome P-450$_{fem}$ (4). Mossbauer spectroscopy of the ferric enzyme, its complexes with various ligands, and the ferrous enzyme showed remarkable similarities to P-450$_{fem}$ (3) and suggested that...
chloroperoxidase and cytochrome P-450, have very similar, if not identical, local environments around the heme iron (5). Magnetic circular dichroism spectroscopy of chloroperoxidase (7) in the oxidized high spin and reduced +CO states also demonstrates marked similarities between the two proteins. The EPR spectrum of the high spin ferric form of chloroperoxidase (10) shows a deviation from axial symmetry at the heme iron which is much greater than that observed with most hemeproteins and which is similar in magnitude to that exhibited by cytochrome P-450. Analysis of the EPR spectrum of the low spin form of chloroperoxidase indicates that the two proteins are quite similar and have axial ligands with similar electronic properties (10). The striking similarities in the physicochemical properties of chloroperoxidase and cytochrome P-450 led us to suggest (3, 4) that the two proteins may provide similar environments for the heme active site and that these environments are quite different from those provided by other hemeproteins.

Based on the strong physical evidence which indicated the similarities between the active sites of chloroperoxidase and cytochrome P-450, we have investigated the ability of chloroperoxidase to catalyze some of the reactions catalyzed by cytochrome P-450. One of the characteristic reactions catalyzed by the liver microsomal cytochrome P-450 dependent mixed function oxidases is the NADPH and molecular oxygen-supported cleavage of alkylic groups attached to nitrogen, sulfur, or oxygen atoms. We report here the results of a detailed study of the N-demethylation reactions catalyzed by chloroperoxidase. Based on the results of this study, the peroxide-supported demethylation of N,N-dimethylaniline by chloroperoxidase would appear to be a satisfactory model system for investigating the reaction mechanism of the cytochrome P-450-catalyzed dealkylation reactions.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation**—Chloroperoxidase was isolated and purified from *Caldariorumycses fumago* as reported previously (1). The preparations used for these studies had specific activities greater than 2000 units/mg of protein in the standard chlorination assay and exhibited A403/A250 ratios greater than 1.40, indicating that the enzyme preparations were at least 95% pure. Protein concentrations were determined using the method of Lowry et al. (11).

Crude horseradish peroxidase (Type I, A403/A250 = 0.3), obtained from Sigma Chemical Co., was purified by a modification of the procedure of Shannon et al. (12) as previously described (13). The isozymes used for these studies were horseradish peroxidase (A fraction) and horseradish peroxidase (B-C fraction). Horseradish peroxidase concentrations were determined using the molar absorbance indices reported by Shannon et al. (12) for the various isozymes.

**Materials**—N,N-Dimethylaniline, N-methylaniline, aminoprine, metyrapone, 2,4-pentanedione (gold label), benzoyl peroxide, m-chloroanobenzoic acid, α-phenyl-β-t-butyl nitroene, and 5,5′-dimethyl-1-pyrroline-N-oxide were obtained from Aldrich Chemical Co. The epinephrine, n-propyl gallate, thiourea, and D_2O (>98.8 atom %) were obtained from Sigma Chemical Co. Catalase (beef liver), hemoglobin (bovine), myoglobin (whale), lactoperoxidase (milk), microperoxidase (horse heart), and cytochrome c (horse heart) were obtained from Sigma and used without further purification. Superoxide dismutase was obtained from Miles Laboratories.

Ethyl hydrogen peroxide and ammonium acetate (ultrapure) were obtained from Polysciences, Inc. Hydrogen peroxide (30%) was obtained from Fisher Scientific Co. The succinyl peroxide and t-butyl hydroperoxide were obtained from ICN Pharmaceuticals, Inc., peracetic acid from Praxair and Bauer, Inc., and cumene hydrogen peroxide from Matheson, Coleman, and Bell. All other materials were reagent grade and obtained from commercial sources. The peroxide concentrations were determined by iodometric titration (14). The method of Craig and Purushothaman (15) was used for the synthesis of N,N-dimethylaniline dihydrochloride from N,N-dimethylaniline and m-chloroperbenzoic acid. The N,N-dimethylaniline-N-oxide was recrystallized twice from ethyl acetate (melting point, 152-154°C; literature, 152-153°C).

**Enzyme Assays**—The N-demethylase activity of chloroperoxidase and the other hemeproteins was assayed by measuring the amount of formaldehyde formed using a modification of the procedure of Nash (16). Unless specified otherwise, the reaction mixtures contained sodium/potassium phosphate buffer (0.5 M), pH 6.0, N,N-dimethylaniline (0.53 mM), and ethyl hydrogen peroxide (2.1 mM) in a final volume of 3 ml. The reaction was initiated by the addition of the enzyme, incubated at 25°C for 15 min, and terminated by the addition of 0.75 ml of 60% trichloroacetic acid. The incubation mixtures were extracted twice with ethyl acetate (5 ml) to remove the violet color formed during the course of the reaction at higher protein concentrations. To 1 ml of the extracted aqueous phase, 0.5 ml of Nash reagent (30 g of ammonium acetate and 0.4 ml of 2.4-pentandione per 50-ml volume) was added and the solutions were mixed and incubated at 25°C for 45 min. The absorbance of the resulting conjugate was read at 421 nm on a Gilford 2400-S UV-visible spectrophotometer. All experiments were carried out in duplicate or triplicate and included controls in which the peroxidase was omitted. The data presented are average values.

**Spectral Measurements**—Room temperature optical spectra were recorded on an Aminco DW-2 UV-visible recording spectrophotometer using cells with pathlengths of 1 cm and protein concentrations of 0.9-6.0 mg/ml.

**Preparation of D_2O Buffers**—Sodium/potassium phosphate buffer (0.5 M), pH 6.0, was prepared by dissolving the appropriate amount of sodium phosphate (monobasic) in D_2O and titrating with concentrated potassium hydroxide in D_2O. The pH of the buffer was determined by using the equation pD = pH + 0.4 (17). The D_2O buffer was diluted with aqueous 0.5 M sodium/potassium phosphate buffer, pH 6.0, to a mole fraction of D_2O equal to 0.5 such that the final pH was 6.0, where pD represents the negative log of the summed hydrogen and deuterium ion concentrations. The pL was determined by using the equation pL = pH + 0.076n^2 + 0.3314n (18), where pH is the pH of the mixture as measured by a standard pH electrode and n is the mole fraction of D_2O in the mixture.

**RESULTS**

**Demethylation of N,N-Dimethylaniline by Chloroperoxidase**—The time course for the chloroperoxidase-catalyzed formation of N-methylaniline and formaldehyde from N,N-dimethylaniline with ethyl hydrogen peroxide as the oxidant is shown in Fig. 1. Under the conditions used for these studies, the rates of formation of both products were linear for at least 10 min at 25°C. In general, the ratio of formaldehyde formed to N-methylaniline formed was slightly less than 1 (Fig. 1), suggesting that essentially equimolar amounts of N-methylaniline and formaldehyde were formed by chloroperoxidase, as in the cytochrome P-450-catalyzed dealkylation reaction. The fact that the amount of formaldehyde measured at each time point was slightly less than the amount of N-methylaniline measured may reflect further oxidative metabolism of formaldehyde by chloroperoxidase. Since chloroperoxidase exhibited a high catalatic activity under the conditions used for this assay, the stoichiometry with respect to ethyl hydroperoxide was not determined. HPLC analysis of the reaction products (Fig. 2) indicated that N-methylaniline was the major product formed (>95% of the reaction products detected) during the course of the chloroperoxidase-catalyzed metabolism of N,N-dimethylaniline.

As shown in Fig. 3, the initial rate of formation of formaldehyde was linear with respect to the concentration of chloroperoxidase in the reaction mixture up to a final protein concentration of 1.8 μg.

**Demethylation Activity as a Function of pH**—As shown in Fig. 4, chloroperoxidase exhibited a relatively broad pH optimum centered around pH 6.0 for the ethyl hydrogen peroxide-dependent demethylation of N,N-dimethylaniline. This profile is similar to the pH profile for the evolution of oxygen from hydrogen peroxide except that the pH optimum is cen-

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1 The abbreviation used is: HPLC, high pressure liquid chromatography.
The reaction mixtures contained sodium/potassium phosphate buffer (0.10 ml) and formaldehyde from N,N-dimethylaniline by chloroperoxidase. The reactions were initiated by the addition of ethyl hydrogen peroxide, incubated at 25°C for the times indicated, and terminated by the addition of 0.10 ml of 6.2 N HCl. N-Ethylaniline (82.6 nmol) was added as the internal standard and the reaction mixture was made basic with 5 N NaOH (0.30 ml) and extracted three times with 2.5 ml of ethyl acetate. The ethyl acetate extracts were combined, isopropanol (0.20 ml) was added, and the extracts were reduced to approximately 0.10 ml. An aliquot of the concentrated extract was analyzed by HPLC on a Glencor HPLC using a Partisil 10/25 column (4.1 x 250 mm) eluted with hexanexethyl acetate (7.5:1) with a flow rate of 2.2 ml/min, and the eluate was monitored at 280 nm. N-Methylaniline was determined from a standard curve generated by processing reaction mixtures with known concentrations of N-methylaniline, but without ethyl hydrogen peroxide added, through the identical procedure. The amount of formaldehyde in the aqueous phase was determined using the Nash reaction as described under "Experimental Procedures." All points represent the average of duplicates for a single experiment. The ratio of formaldehyde to N-methylaniline for each time point is plotted at the top of the figure.

Fig. 2. HPLC elution profile of the metabolites formed upon demethylation of N,N-dimethylaniline by chloroperoxidase. A, the reaction mixture was the same as that described in the legend to Fig. 1. The reaction mixture was incubated at 25°C for 9 min, and the reaction was then terminated, extracted with ethyl acetate, and analyzed by HPLC as described in the legend to Fig. 1. B, a standard mixture consisting of N,N-dimethylaniline ($R_t = 2.0$ min), N-ethylaniline ($R_t = 2.6$ min), N-methylaniline ($R_t = 3.5$ min), and aniline ($R_t = 9.7$ min) was separated using the conditions given in the legend to Fig. 1.

The stimulation by halide anions is thought to be due to specific binding of the halide anion to the acidic form of the enzyme in the vicinity of the heme active site.
The oxygenating agents were tested at the concentrations indicated using the assay procedure described under "Experimental Procedures." The chloroperoxidase added was 1.8 μg.

| Oxygenating agent                  | Concentration (mM) | Turnover number (nmol formaldehyde formed/min/nmol heme) |
|-----------------------------------|--------------------|----------------------------------------------------------|
| Hydrogen peroxide                 | 0.3                | 79                                                       |
| Ethyl hydrogen peroxide           | 1.6                | 1096                                                     |
| Benzoyl peroxide                  | 2.0                | 55                                                       |
| Succinyl peroxide                 | 2.1                | 32                                                       |
| Peracetic acid                    | 2.0                | 38                                                       |
| t-Butyl hydroperoxide             | 2.1                | 41                                                       |
| m-Chloroperbenzoic acid          | 1.9                | 8                                                        |
| Cumene hydroperoxide              | 2.1                | 32                                                       |
| t-Amyl hydroperoxide              | 1.3                | 25                                                       |

* More chloroperoxidase (9 μg) was used in order to get sufficient product formation.

The results shown in Fig. 4 indicate that the binding of chloride to chloroperoxidase stimulates the ethyl hydrogen peroxide-supported demethylation reaction in a manner similar to that observed for oxygen evolution.

**Ability of Chloroperoxidase to Use Other Oxygenating Agents**—Chloroperoxidase was able to use several different peroxides and hydroperoxides (hydrogen peroxide, ethyl hydroperoxide, benzoyl peroxide, succinyl peroxide, t-butyl hydroperoxide, cumene hydroperoxide, and t-amyl hydroperoxide) as well as organic peracids (peracetic acid and m-chloroperbenzoic acid) to support the demethylation of N,N-dimethylaniline (Table I). Under the assay conditions used, ethyl hydrogen peroxide was the most effective of the oxidants in terms of the velocity of the demethylation reaction. However, the rates in Table I are initial velocities determined under conditions which were not optimized for each of the oxidants and therefore cannot be considered as appropriate measures of the relative ability of the oxidants to support chloroperoxidase-catalyzed demethylation reactions.

Although sodium chloride supports a number of chloroperoxidase-catalyzed chlorination and peroxidation reactions (13), it did not support the formation of formaldehyde as determined by the Nash assay. However, since chlorite reacts directly with formaldehyde oxidizing it to formic acid (19), which could not be detected by the Nash assay, we also measured the chlorite-dependent formation of N-methylanthranilic acid from N,N-dimethylaniline using the HPLC assay described in Fig. 1. The HPLC analysis results indicated that essentially no N-methylaniline or aniline was formed when the reaction was supported by chlorite. However, several new peaks were observed during HPLC analysis of the chlorite reaction mixture which probably represent ring-chlorinated reaction products.  

**Demethylation of Various Substrates by Chloroperoxidase**—The turnover numbers for several secondary and tertiary N-methyl amines given in Table II indicate that N,N-dimethylaniline, N,N-dimethylaniline, N-methyl-p-chloroanilnine, and N-methyl-p-nitroanilnine were good substrates for demethylation by chloroperoxidase. Since the assay conditions were not optimized for each individual substrate, the activities shown do not represent maximum velocities and therefore may not reflect the ability of the compounds to serve as substrates. Compounds which were not substrates for demethylation by chloroperoxidase included N,N-dimethylamipyrindine, benzphetamine, aminopyrine, and p-nitroanisole.

**Demethylation of N,N-Dimethylaniline by Other Hemeproteins**—Hemeproteins other than cytochrome P-450 can catalyze the N-demethylation of various substrates (20–22). Kudlub and co-workers (21) have reported that catalase could use organic peroxides, but not hydrogen peroxide, for the demethylation of N,N-dimethylaniline, aminopyrine, and benzphetamine. As shown in Table III, several hemeproteins were able to catalyze the demethylation of N,N-dimethylaniline when hydrogen peroxide, ethyl hydrogen peroxide, or cumene hydroperoxide were present to serve as the oxidant. The N,N-dimethylaniline and peroxide concentrations used for these studies were based on the optimum conditions determined for demethylation by chloroperoxidase at pH 6.0 (experiments not shown). However, except for chloroperoxidase...
**N-Demethylation by Chloroperoxidase**

**Fig. 5.** Initial velocity pattern for \( N,N \)-dimethylaniline demethylation with \( N,N \)-dimethylaniline as the variable substrate. The reaction mixtures contained sodium/potassium phosphate buffer (0.5 M), pH 6.0, ethyl hydrogen peroxide (2.8 mM), 0.29 \( \mu \)g of chloroperoxidase, and \( N,N \)-dimethylaniline as indicated in a final volume of 3.0 ml. The reaction was initiated by addition of the enzyme and incubated at 25°C for 10 min. The amount of formaldehyde formed was assayed by the Nash reaction as described under "Experimental Procedures." The inset is a double reciprocal plot of the data.

Although the two horseradish peroxidase isozyme preparations (B-C and A) were significantly more active than most of the other hemoproteins, neither preparation exhibited a catalytic activity comparable to that observed with chloroperoxidase when ethyl hydrogen peroxide was the oxidant. The horseradish peroxidase isozymes and lactoperoxidase exhibited much greater catalytic activity (at least 10 times greater) with hydrogen peroxide or ethyl hydrogen peroxide than with cumene hydroperoxide. Since the assay conditions were not optimized for each protein, the activities shown should be taken as approximate values and may not reflect accurately the ability of these proteins to carry out demethylation. The demethylation activities for the horseradish peroxidase isozymes and chloroperoxidase were significantly greater than those reported by Kadlubar et al. (21) for the demethylation of \( N,N \)-dimethylaniline by pig liver microsomes using NADPH and \( O_{2} \) (3.4 nmol of formaldehyde/min/mg of protein) or cumene hydroperoxide (35 nmol of formaldehyde/min/mg of protein). They were also greater than that reported by Nordblom et al. (23) for the cumene hydroperoxide-supported demethylation of \( N,N \)-dimethylaniline by highly purified rabbit liver cytochrome P-450 (19 nmol of formaldehyde/min/mmol of cytochrome P-450).

**Effect of Substrate Concentration on the Rate of the Demethylation Reaction—**The rate of formaldehyde formation exhibited typical Michaelis-Menten saturation kinetics with respect to \( N,N \)-dimethylaniline up to a final concentration of approximately 0.5 mM (Fig. 5). At higher substrate concentrations (not shown here), significant inhibition of the reaction by the amine substrate was observed. The apparent \( K_{m} \) for \( N,N \)-dimethylaniline, calculated from the Lineweaver-Burk plot in Fig. 5, was 0.08 mM, and the \( V_{max} \) for the demethylation reaction supported by ethyl hydrogen peroxide was 71.8 nmol of formaldehyde formed/min/\( \mu \)g of chloroperoxidase.

Chloroperoxidase exhibited normal Michaelis-Menten saturation kinetics with respect to ethyl hydrogen peroxide (Fig. 6). The apparent \( K_{m} \) for ethyl hydrogen peroxide calculated from the Lineweaver-Burk plot in Fig. 6 was 0.8 mM, and the estimated \( V_{max} \) was 78.4 nmol of formaldehyde formed/min/\( \mu \)g of chloroperoxidase. When similar studies were attempted with hydrogen peroxide, marked substrate inhibition by hydrogen peroxide was observed (experiments not shown). The marked inhibition by higher levels of hydrogen peroxide appeared to be due to a competition for Compound I between the peroxide and \( N,N \)-dimethylaniline with the reaction of the peroxide with Compound I resulting in oxygen evolution.

**Fig. 6.** Initial velocity pattern for \( N,N \)-dimethylaniline demethylation with ethyl hydrogen peroxide as the variable substrate. The reaction mixtures contained sodium/potassium phosphate buffer (0.5 M), pH 6.0, \( N,N \)-dimethylaniline (1.05 mM), 0.29 \( \mu \)g of chloroperoxidase, and ethyl hydrogen peroxide as indicated in a final volume of 3.0 ml. The incubation conditions and formaldehyde determinations were the same as described in the legend to Fig. 5. The inset is a double reciprocal plot of the data.

**TABLE IV**

Inhibition of \( N,N \)-dimethylaniline demethylation catalyzed by chloroperoxidase

| Inhibitor          | Concentration | Relative velocity % |
|--------------------|---------------|---------------------|
| Control            |               | 100                 |
| SKF-525A           | 0.5           | 97                  |
| Piperonyl butoxide | 0.5           | 96                  |
| \( n \)-Propyl gallate | 0.5       | 92                  |
| Metyrapone         | 0.5           | 95                  |
| Indole             | 0.5           | 76                  |
| Sodium cyanide     | 0.1           | 98                  |
| Sodium azide       | 0.1           | 4                   |

**Effect of Reagents Which React with Active Oxygen on**

\(^3\) D. A. Long and P. F. Hollenberg, unpublished observations.
**Demethylation**—Epinephrine and tiron, reagents which react with the superoxide anion (24, 25) caused a marked inhibition of the chloroperoxidase-catalyzed demethylation (Table V). When superoxide dismutase was added to the reaction mixture, there was a marked increase (approximately 30%) in the amount of formaldehyde formed. Subsequent studies demonstrated that superoxide dismutase catalyzed the dealkylation of N,N-dimethylaniline in the presence of hydrogen peroxide or ethyl hydrogen peroxide. Whether this activity represents a peroxidase impurity in the superoxide dismutase (26, 27) or an enzyme activity of the dismutase is not known. However, when the demethylation due to superoxide dismutase was taken into account, there was no inhibition of the chloroperoxidase-catalyzed demethylation by the superoxide dismutase. Under the conditions of the demethylation assay in the presence of N,N-dimethylaniline, chloroperoxidase catalyzed the rapid oxidation of epinephrine to adrenochrome, as measured by an increase in the absorbance of the reaction mixture at 475 nm (28). The chloroperoxidase-catalyzed oxidation of epinephrine was stimulated by superoxide dismutase, and it was subsequently demonstrated that superoxide dismutase could catalyze this reaction in the presence of hydrogen peroxide or ethyl hydrogen peroxide.

Azide, histidine, and diphenylfuran have been reported to be effective quenching agents for singlet oxygen (27, 29). The inhibition of demethylation by relatively high concentrations of histidine or diphenylfuran was not great enough to suggest the involvement of singlet oxygen as an intermediate in the chloroperoxidase-catalyzed demethylation. Although sodium azide inhibited the demethylation reaction, this inhibition was probably due to binding of the azide as an axial ligand of the heme iron of chloroperoxidase rather than the trapping of the singlet oxygen. Binding of the azide anion to the heme iron would interfere with substrate binding (peroxide and/or amine) to the enzyme active site, thereby causing the observed inhibition.

Thiourea, ethanol, and ascorbic acid, three reagents which react with free radicals, and are used as scavengers for the hydroxyl radical (30), all caused marked inhibition of demethylation. However, all three compounds are substrates for peroxidation by chloroperoxidase (2) and the inhibition probably reflects a competition with N,N-dimethylaniline for the enzyme active site rather than trapping of a free radical. This is supported by the observation that mannitol, another hydroxyl radical scavenger (30), did not inhibit the reaction. Reactive free radicals which are too short-lived for direct observation by EPR can be trapped using nitroxides which react with the transient radical to produce a more stable radical addition product, or “spin adduct” which can then be identified using EPR techniques (31, 32). If the chloroperoxidase-catalyzed demethylation proceeded via the formation of a free radical intermediate, we would expect that the reaction would be markedly inhibited by spin trapping agents such as α-phenyl-2-butyl-nitronene (33-36), 5,5-dimethylpyrroline-N-oxide (33, 34, 37), or nitromethane (38, 39). Although all three compounds caused inhibition (Table VI), the magnitude of the inhibition by α-phenyl-2-butyl-nitronene and 5,5-dimethylpyrroline-N-oxide at concentrations of 75 mM was relatively small, suggesting that free radical formation probably does not play a major role in the demethylation reaction. Preliminary experiments indicate the formation of an EPR-detectable spin adduct following addition of spin trapping reagents to the complete reaction mixture (enzyme, N,N-dimethylaniline, and ethyl hydrogen peroxide). The identification of this free radical adduct and an investigation into the role of this free radical in the demethylation reaction are in progress.

**Effect of D2O on the demethylation reaction**

Since the lifetime of singlet oxygen in D2O is approximately 10 times longer than in H2O (40), we would expect a marked increase in the rate of demethylation in D2O if singlet oxygen were an intermediate in the reaction. However, when D2O was used for the reaction in place of H2O, there was a marked inhibition of demethylation (Table VII), suggesting that singlet oxygen does not participate as an intermediate in demethylation. The primary deuterium solvent isotope effect \( V_{H_2O}/V_{D_2O} \) (saturated) was 3.6.

**Role of an N-Oxide Intermediate in Demethylation**—One possible mechanism for the oxidative N-demethylation of N,N-dimethylaniline involves N-oxidation of the amine to form N,N-dimethylaniline-N-oxide, which could then be decomposed to form formaldehyde and N-methylaniline. If N,N-dimethylaniline-N-oxide were an intermediate in the reaction, then chloroperoxidase should catalyze the peroxide-dependent metabolism of the N-oxide to formaldehyde and N-meth-
ylaniline at a rate equal to or greater than that observed with \(N,N\text{-dimethylaniline}\). When \(N,N\text{-dimethylaniline-N-oxide}\) was substituted for \(N,N\text{-dimethylaniline}\) in the standard assay for \(N\text{-demethylation}\), described under "Experimental Procedures," there was no formation of formaldehyde, indicating that the \(N\)-oxide is not an intermediate in the dealkylation reaction catalyzed by chloroperoxidase.

**DISCUSSION**

Chloroperoxidase can catalyze the peroxide-dependent dealkylation of several \(N\text{-methyl arylamines}\) in a manner similar to that described for the peroxide-supported demethylation of \(N,N\text{-dimethylaniline}\) and benzphetamine by highly purified rabbit liver microsomal cytochrome P-450. For both the chloroperoxidase and the cytochrome P-450-catalyzed dealkylations, the reaction can be written in the following manner:

\[
ROOH + R'R''-N-\text{CH}_3 \rightarrow R-\text{OH} + R'R''-\text{NH} + \text{HCHO}
\]

where ROOH is the peroxide and R'R''-N-CH₃ is the \(N\)-methyl arylamine substrate (secondary or tertiary). The stoichiometry for the formation of \(N\)-methylaniline and formaldehyde from \(N,N\text{-dimethylaniline}\) is essentially 1:1. This is the first report of catalytic similarities between cytochrome P-450 and chloroperoxidase.

During the initial phase of the dealkylation reaction, \(N\)-methylaniline and formaldehyde are the only reaction products detectable. However, at longer time periods or when higher protein concentrations are used, the incubation mixture becomes slightly purple. The identity of the product or products responsible for this purple color and the mechanism by which they are formed is not known, but the purple color may be due to the formation of small amounts of stable free radicals similar to those formed during the peroxide-supported dealkylation of \(N,N\text{-dimethylaniline}\) by metmyoglobin or horseradish peroxidase (41). Room temperature EPR experiments indicate the formation of stable free radicals under conditions where the formation of the purple color is observed.

Chloroperoxidase exhibited normal Michaelis-Menten saturation kinetics with respect to both \(N,N\text{-dimethylaniline}\) (\(K_m\) of 0.08 mM) and ethyl hydrogen peroxide (\(K_m\) of 0.8 mM). Although many oxidants were able to support the demethylation reaction (Table I), the turnover rate was dependent on the identity of the oxidant and varied by more than 200-fold. For the alkyl hydroperoxides, the activity sequence was ethyl > t-butyl > t-amyl, suggesting the importance of the steric properties of the hydroperoxides. The turnover numbers for the arylamine substrates were also highly dependent on the identity of the substrate (Table II). Although the results do not permit a detailed analysis of the structure-activity relationship, it is apparent that substitution on the benzene ring usually resulted in a significant decrease in the dealkylation rate, possibly due to steric restrictions at the arylamine binding site. This might also explain the failure of benzphetamine and aminopyrine to serve as substrates.

In addition to chloroperoxidase, the horseradish peroxidase preparations (B-C and A) exhibited catalytic activities for the demethylation of \(N,N\text{-dimethylaniline}\). A similar report of catalytic activities for the reaction had very similar, if not identical, local environments around the heme sites (the heme and its environment) of chloroperoxidase and cytochrome P-450, has been provided by the results of studies on chloroperoxidase using magnetic circular dichroism spectroscopy (7), resonance Raman scattering (9), and EPR spectroscopy (10). The existence of such strong physical evidence for the structural similarities of the active sites (the heme and its environment) of chloroperoxidase and cytochrome P-450 suggested that these proteins might exhibit similar catalytic properties.

Several laboratories have demonstrated that microsomal preparations are able to use organic hydroperoxides for the dealkylation or hydroxylation of various substrates of cytochrome P-450 including \(N,N\text{-dimethylaniline}\), ethoxy coumarin, cyclohexane, coumarin, and steroids (21, 42–44). Coon and co-workers (23) have demonstrated that highly purified rabbit liver microsomal cytochrome P-450 can use organic hydroperoxides in place of NADPH and O₂ for the metabolism of a variety of substrates. Purified cytochrome P-450, has been shown to catalyze the hydroxylation of camphor when oxidants such as hydrogen peroxide, t-butyl hydroperoxide, cumene hydroperoxide, or peracetic acid are used in place of NADPH and molecular oxygen (45). The ability of the P-450-type cytochromes to use organic hydroperoxides for hydroxylation and demethylation reactions suggests that the peroxides interact with cytochrome P-450 to generate an activated oxygen intermediate which is essentially identical to the activated oxygen intermediate which functions as the hydroxylating agent in the normal reaction cycle. Since the peroxide-supported reactions do not require oxygen and are not inhibited by carbon monoxide, it has been suggested that the enzyme is not reduced to the ferrous form in this process but forms an activated oxygen species analogous to Compound I of peroxidases and catalases (45).

Horseradish peroxidase Compound I is a spectrophotometrically identifiable intermediate (46) which is formed rapidly upon reaction of the peroxidase with peroxides, peracids, or other oxidants and which retains both of the oxidizing equivalents of the oxidant (47, 48). Chloroperoxidase also reacts with peroxides to form a spectrally observable intermediate which is essentially identical to the activated oxygen intermediate which functions as the hydroxylating agent in the normal reaction cycle. Since the peroxide-supported reactions do not require oxygen and are not inhibited by carbon monoxide, it has been suggested that the enzyme is not reduced to the ferrous form in this process but forms an activated oxygen species analogous to Compound I of peroxidases and catalases (45).

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Studies on the isotopic compositions of oxygen evolved from \(^{18}\text{O}\)-labeled peroxo acids by chloroperoxidase indicate that the reaction proceeds by a scrambling mechanism in which the oxygen atoms in the evolved oxygen are derived from different peroxo acid molecules (49, 50).

Similar studies on the disproportionation of hydrogen peroxide by chloroperoxidase demonstrated that oxygen evolution was not accompanied by oxygen scrambling (49, 50). Therefore, we suggested that
oxygen evolution from hydrogen peroxide and peroxo acids occurs via the formation of a three-oxygen intermediate which can decompose through either a scrambling or retention mechanism depending on the identity of the R group on the hydroperoxide, and we postulated that the active intermediate in chloroperoxidase-catalyzed reactions is a ferryl type of structure containing a single oxygen atom derived from the hydroperoxide that can behave as an electrophilic OH· species in a manner similar to the cytochrome P-450 hydroxylating intermediate.

Support for the involvement of a Compound I-type intermediate in the peroxide-supported reactions catalyzed by cytochrome P-450 comes from the observation of Gunsalus and co-workers (45) that the exposure of substrate-free cytochrome P-450, peroxides, to peracetic acid results in the formation of a spectral intermediate having a Soret spectrum similar to that of horseradish peroxidase Compound I. The addition of camphor to P-450 carboxyl at this point causes a decrease in the absorbance at 440 nm concomitant with the formation of hydroxylated substrate, and there is a shift in the position of the Soret peak from 418 to 391 nm, which is characteristic for the substrate-bound form of the ferric enzyme.

The ability of microsomal cytochrome P-450 to use peroxides to support hydroxylations and dealkylations has resulted in the suggestion that, in the presence of NADPH and O2, cytochrome P-450 functions in the manner of a peroxidase in which the peroxide is generated at the active site of the enzyme (23, 42-44). Coon and co-workers (23) have suggested that the peroxide is generated at the active site of the heme protein by 2-electron reduction of the oxygen bound to the heme iron to form O22-, the dianion of hydrogen peroxide, which then undergoes protonation to give H2O2 and subsequently eliminates the hydroxyl anion to give an [FeO]- complex. Finally, the “activated” oxygen in this complex is then inserted into a favorably positioned carbon-hydrogen bond of the enzyme-bound substrate to yield a hydroxylated product with the regeneration of the native ferric cytochrome P-450 at the same time.

The experimental results presented here demonstrate the ability of chloroperoxidase to catalyze dealkylation reactions in a manner similar to cytochrome P-450 and suggest that the active oxygen species in the chloroperoxidase-catalyzed demethylation of N,N-dimethylaniline may be similar or even identical to that in the cytochrome P-450-catalyzed dealkylations. Several different hydroxylating intermediates including the hydroxy radical, the superoxide anion, singlet oxygen, and an oxenoid species have been suggested as the active hydroxylating agent in the oxygenation reactions catalyzed by cytochrome P-450. However, there has been no unequivocal demonstration of the involvement or lack of involvement of any of these species in the hydroxylation reactions. The results of the inhibition studies using trapping agents for various activated oxygen species (Tables V and VI) do not conclusively prove either the involvement or lack of involvement of any of these species as intermediates in the reaction; however, they do prove that singlet oxygen, the hydroxy radical, substrate radicals, or superoxide anions are not formed during the course of the reaction as intermediates which are released free into the incubation medium. This does not preclude the involvement of one of these reactive oxygen species while bound to the heme protein.

The deuterium solvent isotope effect for the chloroperoxidase-catalyzed demethylation reaction (V/D0/V0 = 3.6) may be due to any one of several different effects of D2O on the protein or the substrates. With respect to the protein, the solvent isotope effects may be related to changes in the conformation of the protein or changes in the ionization behavior of the amino acid residues which are critical for catalysis. Groups which could be affected include those responsible for Compound I formation, substrate binding, or assisting in the demethylation reaction. With respect to the effect of D2O on the substrates, the peroxide proton on ethyl hydrogen peroxide would readily exchange with deuterium in the solvent. A third possible explanation of the solvent isotope effect is a possible shift in the pK of the ionizing residue(s) on the enzyme responsible for the demethylation activity or a shift in the pK of the amine substrate. The origin(s) of the deuterium solvent isotope effect is currently under investigation.

The N-oxidation of N,N-dimethylaniline occurs concomitantly with N-demethylation (51). Since liver microsomal preparations rapidly metabolize N,N-dimethylaniline-N-oxide to give stoichiometric formation of formaldehyde and N-methylaniline (52), it has been suggested that the pathway for liver microsomal N-dealkylation involved the formation of a tertiary amine N-oxide as an intermediate. Subsequently, two pathways for tertiary amine demethylation were shown to be operative in liver microsomal preparations (59), a C-oxidation pathway and an N-oxidation pathway with subsequent demethylation of the N-oxide. In addition, the oxidation of N,N-dimethylaniline to the N-oxide is catalyzed by a reconstituted rabbit liver microsomal enzyme system containing highly purified cytochrome P-448 (54). When N,N-dimethylaniline-N-oxide was incubated with chloroperoxidase, there was no formaldehyde formed in the presence or absence of peroxide, indicating that the chloroperoxidase-catalyzed demethylation of N,N-dimethylaniline does not proceed via the formation of N,N-dimethylaniline-N-oxide as an intermediate. If N,N-dimethylaniline-N-oxide were formed by chloroperoxidase, it is not further demethylated by the enzyme.

The catalytic properties of heme-proteins which contain the same heme group differ greatly from protein to protein. This has previously been attributed to the strong influence of the protein structure, in particular the axial ligands and the amino acid residues in the heme crevice, on the reactivity of the heme iron. Cytochrome P-450, horseradish peroxidase, hemoglobin, and chloroperoxidase all contain protoporphyrin IX as their prosthetic group, and yet their catalytic functions apparently are quite different. However, the results presented here, which demonstrate the ability of chloroperoxidase and other hemeproteins to catalyze N-dealkylations, as well as studies demonstrating the ability of the P-450-type cytochromes to use peroxides for the hydroxylation and dealkylation reactions, indicate that these hemeproteins may not differ as much in catalytic activity as previously presumed and that the major differences in physiological function may be related to the location of the enzymes with respect to peroxide generation, substrate specificity, and the specificity of the reductase for the various hemeproteins. These possibilities are currently under investigation.

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