Chloroperoxidase-Catalysed Oxidation of 4-Chloroaniline to 4-Chloronitrosobenzene

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The incubation of 4-chloroaniline with chloroperoxidase and H_2O_2 resulted in a rapid formation of 4-chloronitrosobenzene. This enzymic oxidation displayed a pH optimum at 4.4 with a K_m of $8.1 \times 10^{-4} \text{ M}$ and catalytic-centre activity of 312. The initial rate of the reaction was strongly affected by the presence of halide ions. 4-Chlorophenylhydroxylamine was even more rapidly converted into the nitroso compound. A reaction mechanism is proposed on the basis of currently accepted theory for the catalytic action of chloroperoxidase. A noteworthy aspect of this new reaction is the difference in the products previously reported for the action of classical peroxidases on anilines and the single nitroso product resulting from chloroperoxidase oxidation.

In recent years we have demonstrated a unique biochemical transformation of aromatic nitroso compounds into hydroxamic acids. The basis of these conversions is an adventitious reaction between the nitroso functional group and biochemical intermediates of thiamin-containing enzymes, including pyruvate decarboxylase (Corbett *et al.*, 1975) and transketolase (Corbett & Chipko, 1977). Most recently these biochemical pathways were shown to be operative in intact organisms (Corbett *et al.*, 1978). The potential environmental significance of these processes is not yet known, owing to our lack of knowledge on the occurrence of aromatic nitroso compounds in the environment. Subsequently we have begun an investigation of selected biological oxidation–reduction processes to determine if such aryl nitroso compounds might be significant, although temporary, metabolites of the extremely common aniline- and nitrobenzene-derived pollutants. We now report that chloroperoxidase (chloride–hydrogen peroxide oxidoreductase, EC 1.11.1.10) readily oxidizes the model compound 4-chloroaniline (I) to the corresponding nitroso metabolite (III) (Scheme 1).

Chloroperoxidase is a low-molecular-weight glycoprotein with properties very similar to other peroxidases, such as horseradish peroxidase. Like most other peroxidases, chloroperoxidase contains ferriprotophyrin IX as its prosthetic group and requires H_2O_2 for its enzymic actions (Morris & Hager, 1966). On the other hand, chloroperoxidase possesses some unique properties that set it apart from most peroxidatic enzymes. The most obvious of these properties is its ability to halogenate a

rather broad range of nucleophilic-acceptor substrates (Hager *et al.*, 1966; Thomas *et al.*, 1970). Another property that attracted our attention to this enzyme is its ionic, rather than radical, mechanism of substrate oxidation (Brown & Hager, 1967; Hollenberg *et al.*, 1974). We suspected that the probable products resulting from chloroperoxidase action on anilines might be considerably different from those resulting from horseradish peroxidase oxidation of anilines (Saunders *et al.*, 1964). Finally, chloroperoxidase might be typical of those halogenating enzymes that are very abundant in many aquatic marine organisms (Fenical, 1975; White & Hager, 1975). Thus the metabolism of anilines by chloroperoxidase might serve as a model for the metabolic disposition of aniline pollutants in the marine environment, in the same fashion that horseradish peroxidase is a model for metabolism in soils (Bordeleau & Bartha, 1972; Iwan *et al.*, 1976).

Scheme 1. Conversion of 4-chloroaniline and 4-chlorophenylhydroxylamine into 4-chloronitrosobenzene (I), 4-Chloroaniline; (II), 4-chlorophenylhydroxylamine; (III), 4-chloronitrosobenzene.

Materials and Methods

Chloroperoxidase (crude and purified grades), ox liver catalase, horseradish peroxidase (type II), monochlorodimedon and *N*-(1-naphthyl)ethylene-diamine dihydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Trisodium pentacyanoamine ferroate was obtained from Eastern Chemical Co. (Hauppauge, NY, U.S.A.), 4-chloroaniline (purity greater than 99%) from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and 4-chloronitrobenzene from Eastman Chemical Co. (Rochester, NY, U.S.A.). The method of Kaufman *et al.* (1973) was used for the synthesis of 4-chlorophenylhydroxylamine and 4-chloronitrosobenzene. T.l.c. was carried out on EM Silica Gel 60 F-254 precoated plates (Brinkmann Instruments, Westbury, NY, U.S.A.) with the solvents indicated.

The purities of the 4-chloroaniline and 4-chlorophenylhydroxylamine used in the incubations were established to be in excess of 99% by t.l.c. methods (Grady *et al.*, 1973) and elemental analyses. Reference 4-chloronitrosobenzene was purified by repeated recrystallization from aqueous ethanol until it gave a single spot on t.l.c. analysis that could be detected only with trisodium pentacyanoamine ferroate (Radomski *et al.*, 1973) and not by fluorescence quenching, since this compound has no significant u.v. absorption in the 254nm region.

Determination of chloroperoxidase halogenation activity

The enzymic activity of chloroperoxidase solutions was determined by the method of Morris & Hager (1966). This method is based on the decrease in A_{278} for a solution of monochlorodimedon (ϵ 12200 litre·mol⁻¹·cm⁻¹) as it is converted into dichlorodimedon (ϵ 120 litre·mol⁻¹·cm⁻¹) by the enzyme. One unit of chloroperoxidase activity is defined as that amount of enzyme that converts 1 μ mol of monochlorodimedon/min under the standard assay conditions. The specific activity of enzyme routinely employed in this study was about 600 units/mg, which is about 30% pure (Krejcarek *et al.*, 1976). Confirmation of the results obtained was carried out on an enzyme with a specific activity of 1400 units/mg (67% purity). Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The molarity of reagent H₂O₂ was determined by the thiosulphate oxidation method (Pietrzyk & Frank, 1974).

Incubation of 4-chloroaniline with chloroperoxidase

All incubations were carried out in 0.1 M-potassium phosphate buffer at 25°C, most often at pH 4.4. The final concentration of H₂O₂ was 2.0 mM. The concentration of 4-chloroaniline was varied from 0.125 to

2.0 mM. When KCl or KBr was included, a final concentration of 20 mM was used. Chloroperoxidase was most often added to give a final concentration of 1 μ g/ml, representing approx. 0.6 unit of chloroperoxidase activity/ml for the crude enzyme preparation and 1.4 units for the purer enzyme. The typical incubation was carried out on 2.0 ml volumes in 1 cm quartz cuvettes, and the reaction monitored by following the increase in A_{320} with a Beckman model 24 spectrophotometer. A solution of the buffer was prepared that contained all of the reactants, except enzyme, at twice the desired incubation concentration. The enzyme was prepared separately as a solution in the same buffer. The reaction was initiated by combining 1.0 ml of each of these two solutions in the cuvette. The reference cuvette was prepared by substituting 1.0 ml of buffer for the enzyme solution. The A_{320} change with time was recorded continuously with a strip chart recorder. Initial reaction velocities were determined from the slopes of the tracings within the first minute of the reaction. A quantitative measure of the total amount of 4-chloronitrosobenzene produced in each reaction was made from the absorption data on completion of the reaction, as evidenced by the levelling-off of the absorption tracing. A quantitative confirmation of the amount of 4-chloronitrosobenzene produced in each incubation was made by the colorimetric procedure described below. Control experiments were carried out by employing heat-inactivated enzyme and by excluding H₂O₂ from the incubations. Control incubations with 2 mM-Br₂ and 2 mM-NaOCl, but lacking chloroperoxidase, were also made.

Determination of the production of 4-chloronitrosobenzene

The conversion of 4-chloroaniline into 4-chloronitrosobenzene by chloroperoxidase and H₂O₂ was monitored by a determination of the increase in A_{320} . On completion of the incubation due either to consumption of one of the substrates or to enzyme inactivation, the u.v. spectrum of the incubation mixture was obtained. In all cases the u.v. spectrum in the region 260–350 nm was identical with that of authentic 4-chloronitrosobenzene in the same buffer [$\lambda_{\text{max.}}$ = 320 nm (ϵ 10300 litre·mol⁻¹·cm⁻¹); shoulder at 292 nm (ϵ 5900 litre·mol⁻¹·cm⁻¹)]. To confirm the results obtained by the u.v. method, a colorimetric procedure was developed similar to that reported by Kaufman *et al.* (1973). This method is a highly specific quantitative determination for aryl nitroso compounds. A portion (1.0 ml) of the incubation mixture was treated with 0.1 μ g of catalase in 10 μ l of phosphate buffer, pH 4.4. After 2 min the incubation mixture was treated with 0.5 ml of 5% (w/v) hydroxylamine hydrochloride solution, then 7 min later with

0.5ml of 5% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride. The resulting azo dye was quantitatively measured at 555nm by comparison with a standard curve prepared from pure 4-chloronitrosobenzene. The visible-absorption spectrum (400–650nm) of the incubation mixture was identical with that of the authentic azo dye. Final confirmation of 4-chloronitrosobenzene as the incubation product was made by carrying out the incubation in a 100ml volume with 0.5mm-4-chloroaniline, with all other conditions the same as described above. After 10min the scaled-up incubation mixture was extracted twice with 100ml of diethyl ether. The combined diethyl ether extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue was taken up in 0.5ml of methanol, from which 5 μ l portions were chromatographed on silica-gel thin-layer plates, along with authentic 4-chloronitrosobenzene. Detection was achieved by spraying the air-dried plates with 5% (w/v) trisodium pentacyanoamine ferroate (Radomski *et al.*, 1973). The R_F values of the incubation product and authentic 4-chloronitrosobenzene were identical in the three solvent systems investigated [R_F = 0.3 (hexane); R_F = 0.9 (chloroform); R_F = 0.7 [hexane/toluene (2:1, v/v)]].

Incubation of 4-chlorophenylhydroxylamine with chloroperoxidase

The action of chloroperoxidase on 4-chlorophenylhydroxylamine was followed spectrophotometrically (A_{320}) on incubation mixtures with the substrate at a concentration of 0.125 mM and H_2O_2 at 2.0 mM in potassium phosphate buffer, pH 4.4, containing 1 μ g of chloroperoxidase/ml. Simultaneous incubations were also conducted with 4-chloroaniline at the same concentration to compare the initial rates of reaction of the two substrates under identical conditions. 4-Chloroaniline and 4-chlorophenylhydroxylamine were also incubated with H_2O_2 in the absence of chloroperoxidase. A larger-scale incubation was carried out with 4-chlorophenylhydroxylamine under the above conditions, but in a total volume of 50ml. After 15min the incubation mixture was subjected to extraction with 80ml of diethyl ether, and the diethyl ether solution was evaporated *in vacuo* after drying over Na_2SO_4 . T.l.c. analysis of the residue indicated the presence of 4-chloronitrosobenzene and 4,4'-dichloroazoxybenzene, with the former comprising most of the product. Identification of 4,4'-dichloroazoxybenzene was achieved by comparative t.l.c. analysis with the authentic compound.

Incubation of 4-chloroaniline with horseradish peroxidase

A solution of 25 μ mol (0.5mM) of 4-chloroaniline and 100 μ mol (2mM) of H_2O_2 in 50ml

of 0.1M-potassium phosphate buffer, pH 4.5, was treated with 10 μ g of horseradish peroxidase dissolved in 100 μ l of buffer. Portions (1.0ml) were taken every minute for 20min and analysed for the presence of 4-chloronitrosobenzene by the colorimetric procedure described above. Analysis by the u.v. procedure at 320nm was not possible owing to the formation of highly coloured products that absorbed strongly at that wavelength.

Results

The action of chloroperoxidase on 4-chloroaniline (I) in the presence of H_2O_2 resulted in the rapid conversion into 4-chloronitrosobenzene (III) in high yield. This enzymic conversion gave only a single product (III), which was readily characterized by u.v. spectrophotometry and by colorimetric methods. The product was isolated by chromatographic methods and conclusively identified by direct comparison with authentic 4-chloronitrosobenzene.

The production of 4-chloronitrosobenzene did not occur in the absence of either chloroperoxidase or H_2O_2 . As expected, heat-inactivated enzyme also failed to produce the nitroso product. The incubation of either Br_2 or NaOCl with 4-chloroaniline under conditions paralleling the enzyme incubation did not result in the production of the nitroso compound. Elemental Br_2 appeared not to undergo any reaction with the substrate, as evidenced by the u.v. spectrum of the reaction mixture. On the other hand, NaOCl resulted in the production of unidentified and highly coloured oxidation products of 4-chloroaniline. Thus the ready conversion of 4-chloroaniline into 4-chloronitrosobenzene is not the result of a chemical reaction of enzyme-generated elemental halogen on the aniline substrate.

A careful investigation of the action of horseradish peroxidase and H_2O_2 on 4-chloroaniline failed to demonstrate the production of detectable amounts of the corresponding nitroso compound. Although Böttcher & Kiese (1960) reported that about 0.2% of 4-chloroaniline was converted into 4-chloronitrosobenzene by horseradish peroxidase, we were unable to detect any such conversion; rather we did observe the previously reported complex mixture of high-molecular-weight coloured products (Saunders *et al.*, 1964; Holland & Saunders, 1968). Thus the course of reaction of 4-chloroaniline with chloroperoxidase is considerably different from that which occurs with peroxidase.

Since the conversion of an aniline into the nitroso analogue is a four-electron oxidation, we suspected that the corresponding hydroxylamine might be an intermediate in the chloroperoxidase-catalysed conversion of 4-chloroaniline into the nitroso compound. Incubation of 4-chlorophenylhydroxylamine (II) with chloroperoxidase under the usual conditions

resulted in the initial rapid conversion into the nitroso compound. The initial rate of this oxidation was 3 times faster than the initial rate of oxidation of the aniline substrate under identical conditions, as shown in Fig. 1. In the absence of chloroperoxidase the initial rate of oxidation of the hydroxylamine to the nitroso compound by H_2O_2 was only about 0.06 that of the enzymic reaction. 4-Chloroaniline gave no detectable nitroso product. Thus the hydroxylamine is also a substrate for chloroperoxidase oxidation; however, it is not yet possible to conclude that 4-chlorophenylhydroxylamine is an actual intermediate in the enzymic oxidation of 4-chloroaniline to 4-chloronitrosobenzene, although this is an attractive and reasonable presumption.

The biphasic appearance of the interaction of 4-chlorophenylhydroxylamine with chloroperoxidase, as observed in Fig. 1, is most likely the result of a competing non-enzymic reaction. The 4-chloronitrosobenzene produced from 4-chlorophenylhydroxylamine can condense with the latter to produce 4,4'-dichloroazoxybenzene, which indeed was identified as a second component of this particular enzymic reaction. The failure to observe this

product during the enzymic oxidation of 4-chloroaniline is readily explained on the basis either that the concentration of the hydroxylamine is kept low by rapid conversion into the nitroso compound, as evidenced in Fig. 1, or that the hydroxylamine is not a discrete intermediate in the oxidation of 4-chloroaniline.

An investigation of the pH profile of this enzymic reaction showed an optimum at pH 4.4 for the initial velocity of the reaction, although appreciable activity was displayed throughout the pH range 3.0–7.0 (Fig. 2). A similar broad pH range has been reported for other non-halogenating oxidation reactions of chloroperoxidase (Thomas *et al.*, 1970).

Fig. 3 illustrates the noteworthy effect of halide anions on the oxidation of 4-chloroaniline by chloroperoxidase. Both bromide and chloride anions accelerated the initial rate of the oxidative reaction, but were not incorporated into the product. It was not possible to employ the u.v. analytical method to study the effect of iodide on this enzymic reaction because the production of I_3^- anions gave intense

Fig. 1. Comparative action of chloroperoxidase on 4-chloroaniline and 4-chlorophenylhydroxylamine. Incubation mixtures (a) and (b) contained in 2.0 ml of 0.1 M-potassium phosphate buffer, pH 4.4, 4 μmol of H_2O_2 , 1.2 units of chloroperoxidase and 0.25 μmol of 4-chloroaniline (a) or 0.25 μmol of 4-chlorophenylhydroxylamine (b). Incubation mixtures (c) and (d) contained in 2.0 ml of 0.1 M-potassium phosphate buffer, pH 4.4, 4 μmol of H_2O_2 and 0.25 μmol of 4-chlorophenylhydroxylamine (c) or 0.25 μmol of 4-chloroaniline (d).

Fig. 2. Effect of pH on the initial rate of oxidation of 4-chloroaniline by chloroperoxidase. Each incubation mixture contained 0.25 μmol of 4-chloroaniline, 4.0 μmol of H_2O_2 and 1.2 units of chloroperoxidase (sp. activity = 600) in 2.0 ml of 0.1 M-potassium phosphate buffer. The pH of the buffer was varied from 3.0 to 7.0. The initial rate of reaction was taken as A_{320} after 1.0 min.

Fig. 3. Effect of halide salts on the oxidation of 4-chloroaniline by chloroperoxidase

Each incubation mixture contained in 2.0 ml of 0.1 M-potassium phosphate buffer, pH 4.4, 4 μ mol of H_2O_2 , 1.3 units of chloroperoxidase, 0.50 μ mol of 4-chloroaniline and no added halide salt (a), 40 μ mol of KCl (b) or 40 μ mol of KBr (c).

background absorption at 320 nm. However, by employing the colorimetric analytical technique we found that iodide accelerated the reaction even more than did bromide. A halide ion-dependent oxidation of certain substrates such as thiourea and uric acid by chloroperoxidase has been previously reported (Thomas *et al.*, 1970; Silverstein & Hager, 1974), in addition to the halide ion-independent oxidation of more classical peroxidase substrates such as guaiacol and pyrogallol (Thomas *et al.*, 1970). The oxidation of 4-chloroaniline was strongly affected by halide ion, but was by no means dependent on the presence of halide. The initial rates of reaction were greater in the presence of chloride and bromide, but the enzyme inactivation evidenced by the abrupt levelling of the curves (Fig. 3) was of a much lower magnitude in the absence of halide ions. As a result the rate of oxidation of 4-chloroaniline remained linear for a greater length of time in the absence of halide ions, and the total conversion of the aniline into nitroso compound was much larger. The H_2O_2 -induced inactivation of chloroperoxidase has been previously observed (Shaw & Hager, 1961); and the relative effects of chloride and bromide on the present reaction are consistent with those reported for other

chloroperoxidase-catalysed reactions (Hager *et al.*, 1966; Brown, 1967). Because of the greater linearity in the rate of oxidation in the absence of halide ions, we concentrated all further studies on incubation mixtures not containing added halides.

A kinetic analysis of the enzymic oxidation of 4-chloroaniline in the absence of added halide was carried out by varying the concentration of the aniline while maintaining the concentration of H_2O_2 at a saturating amount. The concentration of H_2O_2 used is near the maximum allowable before rapid inactivation of chloroperoxidase results (Shaw & Hager, 1961). Fig. 4 illustrates the effect of the substrate concentration on the initial rate of 4-chloronitrosobenzene formation. Fig. 4 also illustrates the effect of enzyme concentration on the initial rates of reaction. As expected, there exists a direct linear relationship between reaction rate and enzyme concentration. A double-reciprocal plot of the data presented in Fig. 4 further establishes the Michaelis-Menten-type reaction kinetics. The apparent K_m was found to be 8.1×10^{-4} M for 4-chloroaniline. The turnover number of this substrate by chloroperoxidase was computed to be approx. 18700 min^{-1} (catalytic centre activity = 312), assuming a mol.wt. of 42000 (Morris & Hager, 1966) and specific activity of 2100 units/mg for the pure enzyme (Hollenberg & Hager, 1973). The reported turnover

Fig. 4. Effect of 4-chloroaniline concentration on the initial rate of oxidation of 4-chloroaniline by chloroperoxidase

Each incubation mixture contained in 2.0 ml of 0.1 M-potassium phosphate buffer, pH 4.4, 4.0 μ mol of H_2O_2 , the indicated amount of 4-chloroaniline and 1.16 units of chloroperoxidase (sp. activity = 1400) (a) or 0.49 unit of chloroperoxidase (sp. activity = 600) (b). Details on the initiation of the incubation mixtures are presented in the Materials and Methods section. v is the initial velocity of the reaction, and is expressed as A_{320} at $t = 1.0$ min. Each datum point is the average value of three separate incubations.

number of the chloroperoxidase-catalysed chlorination of monochlorodimedon is 67000 min^{-1} (catalytic-centre activity = 1117) (Hager *et al.*, 1966), whereas turnover numbers of halide-independent oxidation reactions of chloroperoxidase are much lower, such as 2300 min^{-1} for guaiacol (catalytic-centre activity = 38) (Thomas *et al.*, 1970).

The data presented in Fig. 4 were obtained from the crude (curve *b*) and purer (curve *a*) chloroperoxidase preparations. The direct linear relationship between the initial rates of 4-chloroaniline oxidation and the specific activities of the two enzyme preparations on the basis of monochlorodimedon chlorination is significant. This relationship is strong evidence that this oxidative process is a result of chloroperoxidase and not an enzymic contaminant of the commercially available chloroperoxidase, unless such an enzymic contaminant co-purifies in direct proportion to the chloroperoxidase enzyme. Further support for our contention that this oxidation of 4-chloroaniline is the result of chloroperoxidase, and not a trace contaminant, is evident from Fig. 3, which demonstrates the effects of halide ions on this oxidative process. These effects are in direct agreement with those previously reported for the halogenating action of chloroperoxidase (Thomas *et al.*, 1970).

We were surprised with the observation that the only product of this enzymic oxidation of 4-chloroaniline was 4-chloronitrosobenzene, especially since

Fig. 5. Effect of concentration of 4-chloroaniline on the production of 4-chloronitrosobenzene by chloroperoxidase

The amount of 4-chloronitrosobenzene produced in each incubation (x) was computed from A_{320} at $t = 10 \text{ min}$. The percentage conversion of 4-chloroaniline (○) into 4-chloronitrosobenzene was calculated from the former curve. Incubations were carried out by combining $4 \mu\text{mol}$ of H_2O_2 and 1.2 units of chloroperoxidase along with the indicated amounts of 4-chloroaniline in 2.0 ml of 0.1 M-potassium phosphate buffer, pH 4.4.

other peroxidases give complicated mixtures of oxidation products (Saunders *et al.*, 1964). Fig. 5 illustrates the high values obtained for percentage conversion of the aniline into nitroso compound. This conversion approaches 100% at low substrate concentrations. The lower yields for higher substrate concentrations are most likely a result of the consumption of most of the H_2O_2 during the reaction.

Discussion

Our discovery that chloroperoxidase catalyses the oxidation of arylamines to give aromatic nitroso compounds in high yield is quite significant for several reasons. First of all this particular enzymic oxidation has never been reported for chloroperoxidase, nor has it ever been considered more than a very minor process for other peroxidatic enzymes (Böttcher & Kiese, 1960). Some investigators have even presented evidence to suggest that nitroso-aromatics are not expected intermediates from the peroxidatic oxidation of anilines (Bordeleau *et al.*, 1972).

Even more significant is the fact that chloroperoxidase-catalysed production of nitroso compounds is the first reported enzymic redox process that gives nitroso-aromatics as the only products. Although nitroso-aromatics are often proposed as intermediates in enzymic reduction of nitro compounds to arylamines by nitroreductases, they are rarely detected in significant quantities (Mitchard, 1972). The best known biochemical route to nitroso-aromatics is the oxidation of arylhydroxylamines by oxyhaemoglobin, which is part of the cyclic redox process that leads to the production of ferrihaemoglobin (Kiese & Taeger, 1976). The production of nitroso-aromatics by the action of mammalian microsomal oxidases is apparently only a minor process. On the other hand, the oxidation of arylamines to arylhydroxylamines by cytochrome *P*-450-dependent oxidases is a well-known process, frequently invoked to explain the mechanism of toxicity and carcinogenesis of certain arylamines (Uehleke, 1973; Hlavica & Kehl, 1976). Further oxidation of arylhydroxylamines to nitroso compounds by cytochrome *P*-450 has not been clearly demonstrated. The traces of nitroso-aromatics detected in incubation mixtures of microsomes with arylamines are very likely produced by non-enzymic oxidation of the hydroxylamines (Kadlubar *et al.*, 1973).

The striking similarities between chloroperoxidase and cytochrome *P*-450 that others have noted are obviously insufficient to result in these two oxidative systems yielding the same metabolites from arylamines. Most notable among these similarities is the report that the Soret peak for the reduced chloroperoxidase-CO complex is at 443 nm, which corre-

Scheme 2. Conversion of native chloroperoxidase into reactive intermediates (IV), Native-chloroperoxidase; (V), Compound I; (VI), halogenating form of enzyme. P represents a protein ligand.

sponds closely to that for cytochrome *P*-450. On the other hand, horseradish peroxidase, catalase and lactoperoxidase display the reduced CO Soret peak at 420 nm (Hollenberg & Hager, 1973). Other studies of the haem moieties of chloroperoxidase and cytochrome *P*-450 have further demonstrated the remarkable similarities of these oxidative enzymes (Champion *et al.*, 1976; Makino *et al.*, 1976; Estabrook & Werringloer, 1977). Regardless of the close relationship in the active sites of these two enzymes, the oxidation of arylamines by the enzymes results in quite distinct products, with chloroperoxidase effecting an overall four-electron oxidation.

From a mechanistic viewpoint the greatest distinguishing factor between chloroperoxidase and other peroxidases is the fact that the former does not produce a detectable intermediate similar to Compound II of the peroxidases. This discovery, coupled with other studies on chloroperoxidase, indicates that chloroperoxidase oxidation proceeds by a two-electron transfer process from the substrate to Compound I, or at least by two nearly simultaneous one-electron transfers (Hager *et al.*, 1975). The tremendous difference between the aniline oxidation products resulting from chloroperoxidase and the other peroxidases can best be explained on the basis that the former generates ionic or nitrenium-ion reaction intermediates, whereas the peroxidases generate radical species.

Considerable evidence now suggests that the active site of chloroperoxidase Compound I is best represented as the tetravalent-iron porphyrin π -cation radical (V) (Scheme 2) (Hager *et al.*, 1975; DiNello *et al.*, 1975; Loew *et al.*, 1977). It has been proposed that the oxidative process occurs by electron transfer from a nucleophilic substrate to Compound I. In the case of halogenation reactions of chloroperoxidase, the donor is a halide ion, which in turn is converted into an enzyme-bound halonium ion (structure VI) and can then be transferred to the acceptor substrate. In a similar fashion we propose that aniline nitrogen electrons can be transferred to

Compound I to generate a nitrenium ion that is neutralized by covalent bond formation with hydroxide ion to produce the arylhydroxylamine. The arylhydroxylamine, also a good nucleophile, then reacts by a two-electron transfer to another molecule of Compound I to give the nitroso product. Further oxidation of the nitroso compound to give the nitro product does not occur, probably because the nitroso group is a very weak nucleophile. The increased rate of oxidation of 4-chloroaniline in the presence of halide ions might be explained on the basis of an intermediate formation of a haloamine by direct nucleophilic attack of the aniline or hydroxylamine on the enzyme-bound halonium species (VI). The conversion of the N-halogen bond to an N-O bond by hydrolysis is a reasonable expectation (Nojima *et al.*, 1976).

Since chlorinated anilines are common environmental residues that result from the application of numerous pesticides to soils, their ultimate fate in the environment necessitates careful scrutiny. It is doubtful that biotransformation pathways in soils will generate significant amounts of aromatic nitroso compounds, since peroxidase-type transformations predominate. However, the transport of anilines to coastal marine environments presents a new problem in view of our present results. If chloroperoxidase is typical of the halogenating enzymes present in numerous marine organisms, then the biotransformation of anilines through the intermediate formation of aryl nitroso compounds is a likely process.

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