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Arylamine Oxidations by Chloroperoxidase

MICHAEL D. CORBETT, DANIEL G. BADEN, AND BERNADETTE R. CHIPKO

Rosenstiel School of Marine and Atmospheric Science, The University of Miami, 4600 Rickenbacker Causeway, Miami, Florida 33149

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Chloroperoxidase was found to catalyze the peroxide oxidation of most of the anilines investigated to the corresponding nitroso compounds. Michaelis–Menten constants and maximal velocities were determined for each substrate at the optimal hydrogen peroxide concentration. The major factor controlling the rate of oxidation of the aniline substrates was found to be substituent size, with highly bulky substituents resulting in a marked decrease in the rate of oxidation. Turnover numbers for oxidizable substrates ranged from 67 200 min^{-1} for aniline down to 6800 min^{-1} for 4-isopropylaniline.

Our interest in the interaction of aromatic nitroso compounds with thiamine derivatives (*1*) and with thiamine-dependent enzymes (*2*, *3*) has prompted us to investigate potential biochemical reactions that might lead to the production of nitroso-aromatics. The thiamine-mediated conversion of nitrosoaromatics to aromatic hydroxamic acids (Fig. 1) requires a source of the nitroso compounds before any

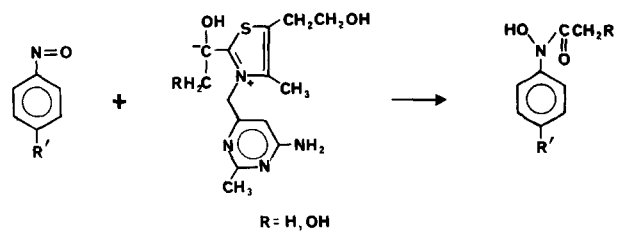

FIG. 1. The conversion of arylnitroso compounds to hydroxamic acids by thiamine-derived biochemical intermediates.

significance can be given to this process *in vivo*. Since the nitroso compounds are not primary xenobiotics, they can be present to participate in this novel reaction only if they are metabolites of the more common arylamine and nitroaromatic xenobiotics. Although nitrosoaromatics are rarely detected as urinary metabolites, it is well known that they appear in blood as intermediary metabolites of arylamines (*4*) and as nitro-aromatic metabolites resulting from the action of gastrointestinal bacteria (*5–7*).

Recently, we demonstrated that the conversion presented in Fig. 1 occurs in a micro-algal species. This reaction might be of considerable interest in environmental health, especially if appreciable amounts of nitrosoaromatics are conversion products of arylamines or nitroaromatics in the environment (*8*). We now report on the generality of a new enzymatic oxidation (*9*) that converts arylamines selectively to the corresponding

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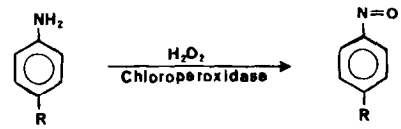

FIG. 2. Chloroperoxidase-catalyzed oxidation of arylamines to arylnitroso compounds.

nitroso compounds (Fig. 2). The enzyme that catalyzes this unique oxidation is chloroperoxidase, a peroxidatic enzyme isolated from the soil fungus, *Caldariomyces fumago* (*10*, *11*). In its natural role, chloroperoxidase is a halogenating enzyme that is thought to function in part by generating an enzyme-bound chloronium species from chloride ion. Some investigators consider this enzyme as being typical of uncharacterized, but widely distributed, halogenating enzymes in certain marine organisms, such as the Rhodophyceae (*12*, *13*). Thus, we decided to investigate the action of chloroperoxidase on selected aniline-derived xenobiotics as part of a preliminary study on biodegradative processes in the marine environment.

In previous work we found that chloroperoxidase rapidly converted 4-chloroaniline to 4-chloronitrosobenzene in the presence of H_2O_2 (Fig. 2), and that the conversion became quantitative at low substrate concentrations (*9*). The reaction is not dependent upon the presence of halide ion, although the reaction kinetics are greater when either bromide or chloride is present in large quantities. To determine the generality of this novel enzymatic oxidation, we investigated this same substrate along with 10 additional *para*-substituted anilines. The substituted anilines were selected in order to determine the electronic and steric effects of *para*-substituents upon the rate of the oxidation.

The kinetic data and results for each of the 11 substrates are summarized in Table 1. Two of the substrates were not oxidized by chloroperoxidase. The values for K_m and V_{max} were obtained at the optimal H_2O_2 concentration for aniline oxidation, which was found to be 4.5 m*M* and in the absence of added halide ions (Fig. 3). We observed in this study that after 1 to 3 min, depending upon the substrate and its concentation, the enzyme rapidly lost activity. Other investigators have reported that chloroperoxidase is

TABLE 1

RESULTS OF INCUBATION OF SUBSTITUTED ANILINES WITH HYDROGEN PEROXIDE AND CHLOROPEROXIDASE

Substrate	λ_{max} of product	$K_m(\times 10^{-3})$	V_{max} (μmol/min)	Turnover No. (TN) (min^{-1})	Relative TN	Percentage yield
Aniline	310	2.1	0.88	67 200	1.00	37
4-Fluoroaniline	315	5.0	0.82	62 600	0.93	46
4-Methylaniline	323	5.5	0.30	22 900	0.34	28
4-Chloroaniline	320	1.4	0.30	22 900	0.34	66
4-Bromoaniline	325	1.7	0.26	19 800	0.30	47
4-Ethylaniline	325	3.3	0.21	17 700	0.26	14
4-Iodoaniline	342	0.5	0.12	9200	0.13	56
3,4-Dichloroaniline	317	0.2	0.09	7600	0.11	34
4-Isopropylaniline	325	3.8	0.08	6800	0.10	7
2,4,6-Trimethylaniline	—	—	—	—	—	—
4-Aminophenylalanine	—	—	—	—	—	—

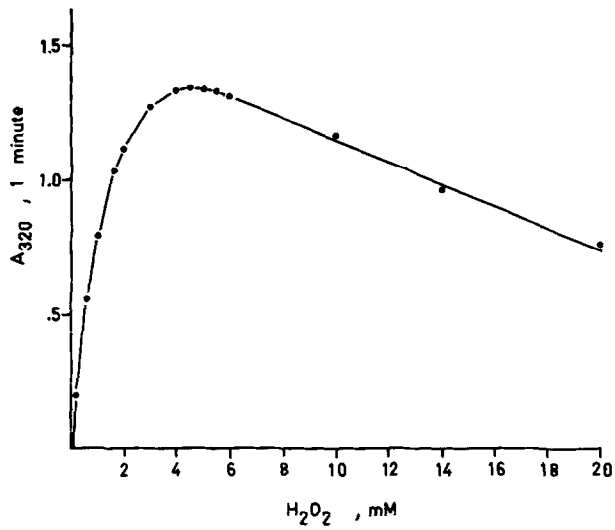

FIG. 3. Optimal concentration of peroxide for the chloroperoxidase-catalyzed oxidation of 4-chloroaniline.

subject to substrate inactivation by H_2O_2 when excessive concentrations are employed (*10*). It was this peroxide-induced enzyme inactivation that resulted in relatively low percentage conversions for some of the substrates (Table 1). Previously, when 0.1 m*M* 4-chloroaniline was oxidized by chloroperoxidase in the presence of 2 m*M* peroxide, a quantitative conversion to 4-chloronitrosobenzene was observed (*9*); however, under the conditions employed in the present study the percentage conversion decreased to 66%. A possible explanation for the observed higher percentage conversions of halogen-substituted anilines (Table 1) is that these substrates are more effective in inhibiting peroxide-induced enzyme inactivation. Although the kinetics of enzyme inactivation as a function of substrate were not investigated, we did observe in the present study a definite tendency for the halogen-substituted anilines to increase enzyme stability.

From our results it is apparent that substitution on the benzene ring resulted in a lower rate of oxidation of the substituted aniline to nitroso compound. Aniline itself was most rapidly oxidized, followed closely by the 4-fluoro analog. Introduction of either methyl or chloro groups at the 4-position caused a very large decrease in the oxidation rate. As substituent size was further increased through the halogen series, substantial decreases in the rates of oxidation were observed. Similarly, in the alkyl series, increasing substituent size from methyl to ethyl and isopropyl caused an extensive reduction in V_{max}.

The failure of 4-aminophenylalanine to undergo oxidation could be due to electrostatic repulsion by the amino acid functionality. 2,4,6-Trimethylaniline was included in this study with the expectation that it would not be oxidized by chloroperoxidase due to steric hindrance at the aniline nitrogen. This expectation proved to be justified. Inhibition studies with the two substrates that did not undergo oxidation established that these analogs are not appreciably bound to the enzyme active site, a conclusion based on the finding that these analogs had no effect upon the kinetics of oxidation of aniline when present in a 16-fold excess over aniline.

We were unable to establish any other correlation for either K_m or V_{max} with redox potential, basicity, or Hammett substituent constants. Substituent size is apparently the controlling factor as to whether a *para*-substituted aniline will serve as a substrate for chloroperoxidase.

Most significant is our demonstration of the fairly broad range of *para*-substituted anilines that are oxidized to the nitroso level by chloroperoxidase. This finding is in sharp contrast to the reported course of oxidation of these same substrates by horseradish peroxidase (*14, 15*). The latter peroxidatic enzyme converts anilines to complex mixtures of dimers, trimers, and higher condensation products, most likely by a free radical mechanism. Chloroperoxidase, on the other hand, gives a remarkably specific oxidation solely to the nitroso monomers in all cases studied.

Similar studies have been conducted with lactoperoxidase, a peroxidase considered to be intermediate in catalytic properties between horseradish peroxidase and chloroperoxidase (*16*). We could not detect any oxidation of 4-chloroaniline by lactoperoxidase. Studies with myeloperoxidase and related enzymes might prove to be quite interesting, since this enzyme is considered to be more similar to chloroperoxidase than is lactoperoxidase (*16, 17*). The possibility exists that highly specialized mammallian enzymes with oxidative properties similar to chloroperoxidase might act to convert arylamines to toxic, and possibly carcinogenic, nitroso compounds.

EXPERIMENTAL

Chloroperoxidase (purified grade), monochlorodimedon, *p*-aminophenylalanine, and 4-fluoroaniline were obtained from Sigma. Aniline, 4-bromoaniline, 4-iodoaniline, and 4-methylaniline were obtained from Eastman. 4-Ethylaniline, 4-isopropylaniline, 2,4,6-trimethylaniline, 4-chloroaniline, and 3, 4-dichloroaniline were obtained from Aldrich. Substrates were purified by distillation or recrystallization to obtain each in pure form as evidenced by tlc analysis. The corresponding nitroso compounds were prepared in a manner similar to a previously described method (*18*). Purification of each nitroso-aromatic was achieved by codistillation with aqueous ethanol, followed by recrystallization from ethanol. The purity of each nitroso compound was determined as described previously (*9*). Ultraviolet and visible spectrophotometric measurements were made on a Beckman Model 24 spectrophotometer.

The enzymatic activities of commercial chloroperoxidase preparations were determined by the standard method of Morris and Hager (*11*). Studies were conducted on chloroperoxidase that was of approximately 67% purity (1400 units/mg). Protein was determined according to Bradford (*19*) employing crystalline bovine serum albumin as the standard. Peroxide concentration was determined by the thiosulfate oxidation method (*20*).

Incubation of substrates with chloroperoxidase. Incubations were conducted in pH 4.4 KH_2PO_4 buffer (0.10 *M*) at 23°C. To obtain kinetic data, the H_2O_2 concentration in the incubation solution was 4.5 m*M*. This optimal H_2O_2 concentration was determined with 4-chloroaniline as the substrate at a concentration of 0.5 m*M* (Fig. 3). The substrate concentration for each of the substituted anilines (Table 1) was varied from 0.25 to 4 m*M*. Chloroperoxidase was added to give a final concentration of 1 μg/ml or

1.4 units/ml. Incubations were carried out on 2.0-ml volumes contained in 1-cm quartz cuvettes and the course of the reaction was monitored by following the increase in optical absorbance at the λ_{max} of the nitroso product (Table 1). The reaction was initiated by combining 1.0 ml of a solution of the buffer containing all of the reactants, except enzyme, at twice their final reaction concentration with 1.0 ml of the buffer containing enzyme. The reference cuvette was prepared by substituting 1.0 ml of buffer for the enzyme solution. Initial reaction velocities were determined from the slopes of the time tracings of absorbance changes. The resulting values were converted to micromoles per minute by use of the extinction coefficients of each authentic nitroso compound measured in the KH_2PO_4 buffer. Kinetic constants (Table 1) for each substrate were determined graphically and by the method of least squares (*21*). The identity of the final product was confirmed by comparison of uv absorption spectra, and by a colorimetric determination for the nitroso functional group (*9*). The values for percentage conversion (Table 1) were computed after 5-min incubation periods at 0.25 m*M* substrate concentrations.

Inhibition studies. For those substrates that failed to undergo oxidation by chloroperoxidase, a repetition of the incubations was made except that aniline was present at a concentration of 0.25 m*M*. The nonoxidized substrates were present at a final concentration of 4 m*M*. The rate of conversion of aniline to nitrosobenzene under such conditions was compared to the oxidation rate in the absence of the potential inhibitor.

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