Peroxidase-catalyzed N-Demethylation Reactions

SUBSTRATE DEUTERIUM ISOTOPE EFFECTS

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Deuterium isotope effects on the kinetic parameters for the hydroperoxide-supported N-demethylation of N,N-di(trideuteromethyl)aniline catalyzed by chloroperoxidase and horseradish peroxidase were determined using N,N-di(trideuteromethyl)aniline. The isotope effect on the $V_{\text{max}}$ for the chloroperoxidase-catalyzed demethylation reaction supported by ethyl hydroperoxide was $1.42 \pm 0.31$. The isotope effects on the $V_{\text{max}}$ for the horseradish peroxidase-catalyzed reaction supported by ethyl hydroperoxide and hydrogen peroxide were $1.99 \pm 0.39$ and $4.09 \pm 0.27$, respectively. Isotope effects ranging from 1.76 to 5.10 were observed on the $V_{\text{max}}/K_m$ for the hydroperoxide substrate (i.e. the second order rate constant for the reaction of the hydroperoxide with the peroxidase to form compound I) in both enzyme systems when the N-methyl groups of N,N-dimethylaniline were deuterated. These results are not predicted by the simple ping-pong kinetic model for peroxidase-catalyzed N-demethylation reactions. The data are most simply explained by a mechanism involving the transfer of deuterium (or hydrogen) from N,N-dimethylaniline to the enzyme during catalysis. The deuterium must subsequently be displaced from the enzyme by the hydroperoxide, causing the observed isotope effects.

Chloroperoxidase (chloride:hydroperoxide oxido-reductase, EC 1.11.1.10), horseradish peroxidase (donor:hydrogen peroxide oxido-reductase; EC 1.11.1.7), and several other hemeproteins can catalyze the hydroperoxide-dependent N-demethylation of a variety of N-methyl arylamines (1-5). Detailed studies of the N-demethylation reactions catalyzed by chloroperoxidase (4) and horseradish peroxidase (5) have established that these reactions proceed according to the following overall equation,

$$\text{ROOH} + R'R''N'\text{CH}_3 \rightarrow \text{ROH} + R'R''NH + \text{HCHO} \quad (1)$$

where ROOH is the hydroperoxide substrate and R'R''N'CH$_3$ is the N-methyl arylamine substrate (secondary or tertiary). The lack of inhibition of the chloroperoxidase- and horseradish peroxidase-catalyzed N-demethylation of DMA by reagents which react specifically with the superoxide anion, singlet oxygen, and the hydroxyl radical suggests that these activated oxygen species are not free intermediates in the reaction (4, 5). Moreover, the near identity of the $V_{\text{max}}/K_m$ for the hydroperoxide substrate in the horseradish peroxidase-catalyzed demethylation of DMA (5) with the reported rates of horseradish peroxidase compound I formation (6) suggests that this oxidized enzyme intermediate mediates the demethylation reaction. The results of a detailed steady state kinetic analysis of the chloroperoxidase-catalyzed demethylation of DMA indicate that the reaction proceeds by a ping-pong kinetic mechanism (7), as would be expected for a reaction mediated by peroxidase compound I. Initial velocity studies of the horseradish peroxidase-catalyzed demethylation of DMA are also consistent with a ping-pong kinetic mechanism (5). In this mechanism, the hydroperoxide substrate reacts with the native peroxidase to form the oxidized enzyme intermediate, compound I, with the concomitant release of the alcohol product. DMA then binds to compound I and is oxidized, resulting in the formation of N-methylaniline and formaldehyde and the regeneration of the native peroxidase. However, while these kinetic results describe the interactions of the substrates with the enzyme, they do not provide chemical detail about the interactions. Previous studies (4, 5) have demonstrated that the peroxidase-catalyzed N-demethylation of DMA does not proceed via N-oxidation of the amine moiety and support the involvement of a carbinolamine intermediate. The observation by EPR spectroscopy of free radicals during horseradish peroxidase-catalyzed oxidation of DMA has led to the suggestion that substrate free radicals are free intermediates in the demethylation reaction (8). However, these studies were done under conditions where the oxidation of DMA to $N,N',N''$-tetramethylbenzidine is known to occur (9), and a subsequent report (10) has suggested that these radicals are charge-transfer complexes between tetramethylbenzidine radicals and DMA. Although the identity of the intermediate species has not been conclusively determined, it is clear that the formation of formaldehyde from DMA involves the cleavage of a carbon-hydrogen bond at some point in the overall reaction.

For reactions in which a carbon-hydrogen bond is broken at some point during catalysis, an investigation of the effect of substituting a deuterium or tritium for the hydrogen atom that is released during catalysis can provide mechanistic information regarding both the chemical and kinetic details of the overall reaction. As pointed out by Northrop (11-13), the interpretation of an isotope effect on enzyme-catalyzed...

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$^1$The abbreviations used are: DMA, N,N-dimethylaniline; DMA-$^3$H$_6$, N,N-di(trideuteromethyl)aniline; EtOOH, ethyl hydroperoxide.

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Reactions as indicating the rate-limiting nature of bond cleavage may be oversimplified in many cases. Unlike simple chemical reactions where the intrinsic or actual magnitude of an apparent rate constant observed experimentally, enzymatic reactions are complex multistep processes where the apparent isotope effects observed are often of lesser magnitude than the intrinsic isotope effect on bond cleavage. The intrinsic isotope effect on bond cleavage in enzymatic reactions is often masked by complicating rate factors (11-13). Nevertheless, apparent isotope effects can provide mechanistic information about enzyme-catalyzed reactions.

Since the peroxidase-catalyzed demethylation of DMA involves the breaking of carbon-hydrogen bonds, we have investigated the effect of deuteration of the methyl groups of DMA on the kinetic parameters of the reaction as an additional diagnostic tool for studying the mechanism of the reaction. The results presented here indicate that the peroxidase-catalyzed N-demethylation of DMA proceeds by a mechanism where hydrogen (or deuterium) is transferred from DMA to the enzyme and must be displaced by the hydroperoxide substrate on the next turnover.

EXPERIMENTAL PROCEDURES

Enzyme Preparation—Chloroperoxidase was isolated and purified from Caldariomyces fumago as reported previously (14). The preparations used for these studies had specific activities of greater than 2000 units/mg of protein in the standard chlorination assay (14) and exhibited \\A_{254}/A_{366} ratios greater than 1.40, indicating that the enzyme preparations were at least 95% pure (14). Protein concentrations were determined using the method of Lowry et al. (15).

Crude horseradish peroxidase (type I, 0.3, obtained from Sigma, was purified by a modification of the procedure of Shannon et al. (16) as previously described (17). The horseradish peroxidase (B-C) isozyme was used for these studies and had an A_{400} A_{520} ratio greater than 3.2. Horseradish peroxidase concentrations were determined using the molar absorbance indices reported by Shannon et al. (16).

Materials—N,N-Dimethylaniline (distilled before use) and 2,4-pentanedione (gold label) were obtained from Aldrich. Ethyl hydroperoxide (10%) and ammonium acetate (ultrapure) were obtained from Polysciences Inc. Hydrogen peroxide (30%) was obtained from Fisher, and aniline (redistilled before use) was obtained from Eastman Kodak Co. The high performance liquid chromatography grade was purchased from Fisher. All other materials were reagent grade and obtained from commercial sources. The hydroperoxide concentrations were determined by iodometric titration (18).

The method of Pones and White (19) was used to synthesize N,N-di(trideuteromethyl)aniline. A saturated solution of sodamide in liquid nitrogen was placed in a thick walled glass tube and a 2-fold excess of trideuteromethyl iodide (Stohler Isotope Chemical Co., >89.5 atom % D) was added. The total reaction volume was 3 ml. The tube was sealed and heated overnight in an oven at 130 °C. After cooling, the contents of the tube were made basic and then distilled into 1 N HCl. The distillate was made basic and extracted twice with 20 ml of diethyl ether. The ether extracts were combined, dried over sodium sulfate, concentrated by a gentle stream of dry nitrogen, applied to a preparative TLC plate, and chromatographed in hexanes:ethyl acetate (7:2) with four solvent passes. The band corresponding to N,N-di(trideuteromethyl)aniline was scraped from the plate and the product was eluted from the silica with diethyl ether. The eluate was dried over sodium sulfate and the ether removed by a gentle stream of dry nitrogen. The product was >97% pure as judged by high performance liquid chromatography, and the extent of deuteration was >99.5 atom excess. The isotope effect on the amine substrate was varied were incubated for 5 min. A 1-ml aliquot of the terminated reaction mixture was incubated with 0.5 ml of the Nash reagent as previously described (4), and the absorbance of the resulting conjugate was read at 421 nm on a Gilford 2400-S UV-visible spectrophotometer. The horseradish peroxidase-catalyzed reaction was varied where the concentration of the amine substrate was varied were incubated for 5 min. A 1-ml aliquot of the terminated reaction mixture was incubated with 0.75 ml of Nash reagent as previously described (5) and the fluorescence of the resulting lutein derivative was read in an Amino-Bowman spectrophotofluorometer using an excitation wavelength of 410 nm and measuring the emission at 500 nm. Under the conditions used for these studies, the rate of formaldehyde formation was linear with time for the times indicated. Standard curves, in which solutions containing known amounts of formaldehyde were taken through the same procedure, were run with each experiment.

All experiments were done at least twice with each point carried out in duplicate. All lines were determined by linear regression of the data and had correlation coefficients greater than 0.990. The data presented are mean values plus or minus standard errors from at least two determinations of K_m and at least four determinations of V_max. The standard errors on the isotope effects were determined by the propagation of error of the measured values.

RESULTS

The initial rate of the chloroperoxidase-catalyzed demethylation exhibited normal Michaelis-Menten saturation kinetics when N,N-di(trideuteromethyl)aniline (DMA-^3H) was substituted for DMA. As shown in Fig. 1, the double reciprocal plots of the initial rates versus the substrate concentrations exhibited good linearity for both DMA and DMA-^3H with the V_max concentrations of the respective isoenzymes being kept constant (Fig. 1a) and for V_max when the concentration of the amine substrate was kept constant (Fig. 1b). The kinetic parameters for the horseradish peroxidase-catalyzed demethylation of DMA and DMA-^3H were determined as previously described (16) and for the hydroperoxide-supported analysis of the data and had correlation coefficients greater than 0.990. The data presented are mean values plus or minus standard errors from at least two determinations of K_m and at least four determinations of V_max. The kinetic parameters for the horseradish peroxidase-catalyzed demethylation of DMA and DMA-^3H were shown in Tables II and III with EtOOH and hydrogen peroxide as the respective oxidants. The isotope effect on the V_max for the EtOOH-supported demethylation was 1.99 ± 0.39, while the isotope effect on the V_max for the hydrogen peroxide-supported reaction was 4.09 ± 0.27. The differences in the magnitudes of the isotope effects on V_max with the two hydroperoxides are probably due to differences in the magnitudes of the rate factors suppressing the expression of the intrinsic isotope effect, those factors being smaller with hydrogen peroxide than with EtOOH. This difference is most likely a consequence of the almost 5-fold greater rate of horseradish peroxidase compound I formation with hydrogen peroxide relative to EtOOH (6). The isotope effects on the V_max/K_m for DMA in the horseradish peroxidase-catalyzed demethylation reaction were 3.00 ± 0.35 with hydrogen peroxide as the oxidant and 0.99 ± 0.20 with EtOOH as the oxidant. In the horseradish peroxidase-catalyzed demethylation reaction, isotope effects of 2.14 ± 0.43 and 5.10 ± 0.53 were observed on the V_max/K_m for EtOOH and hydrogen peroxide, respectively.
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Isotope effects on the kinetic parameters for chloroperoxidase-catalyzed demethylation of N,N-di-(trideuteromethyl)aniline

The reactions were run in sodium potassium phosphate buffer (0.5 M), pH 6.0, under the conditions described under "Experimental Procedures" and assayed for formaldehyde formation by the Nash assay.

| Substrate | V_max/K_m (nmol formaldehyde/min/µg) | V_max (nmol formaldehyde/min/µg) |
|-----------|-----------------------------------|----------------------------------|
| DMA       | 928.4 ± 145.6 919.9 ± 14.5 | 81.7 ± 12.8  |
| DMA-2H_6  | 311.3 ± 46.5 524 ± 14.0 | 57.6 ± 8.5  |
| Isotope effects | 2.99 ± 0.78 1.76 ± 0.52 | 1.42 ± 0.31  |

Isotope effects on the kinetic parameters for horseradish peroxidase-catalyzed demethylation of N,N-di-(trideuteromethyl)aniline supported by hydrogen peroxide

The reactions were run in sodium potassium phosphate buffer (0.4 M), pH 5.5, under the conditions described under "Experimental Procedures" and assayed for formaldehyde formation by the Nash assay.

| Substrate | V_max/K_m (nmol formaldehyde/min/µg) | V_max (nmol formaldehyde/min/µg) |
|-----------|-----------------------------------|----------------------------------|
| DMA       | 603.8 ± 28.0 28740.0 ± 1033.6 | 431.1 ± 15.5  |
| DMA-2H_6  | 201.5 ± 14.5 863.6 ± 315.5 | 105.4 ± 5.9  |
| Isotope effects | 3.00 ± 0.35 5.10 ± 0.53 | 4.09 ± 0.27  |

DISCUSSION

Steady state kinetic studies of the N-demethylation of DMA catalyzed by chloroperoxidase (7) and horseradish peroxidase (5) indicate that the reactions proceed by a ping-pong mechanism. This mechanism can be presented by the following model,
in demethylation. Each half-reaction is mediated by a distinct enzyme form, and the two enzyme forms (native peroxidase and peroxidase compound I) are not connected to each other by a reversible step. Thus, substitution of DMA-^2H_4 for DMA should only affect the latter half-reaction involving compound I and DMA. This conclusion would also be predicted from the definitions of the Vamax/Km parameters for each substrate, since the Vmax/Km values are the second order rate constants for each half-reaction in the ping-pong model. The definitions of the Vmax/Km values for each substrate, derived from Scheme 1 using the net rate constant method of Cleland (21), are

\[ V/K_m = \frac{k_i k_o [E]^T}{k_i + k_o} \]

where [E]^T represents the total enzyme concentration. It is usually assumed that only catalysis (k_i in Scheme 1) is affected by substrate deuteration, but even if substrate binding (k_i) and product release (k_o) were also affected, no isotope effect is predicted on the V/Km parameter on the basis of Scheme 1 (11, 12). For example, the oxidation of 1-deuterio-glucose catalyzed by glucose oxidase proceeds without an isotope effect on the Vmax/Km for oxygen (22), as expected for a ping-pong mechanism (11, 12). We are unaware of any reports in the literature where deuterium of one of the substrates in a reaction which proceeds by a ping-pong mechanism results in an isotope effect on the Vmax/Km for the unlabeled substrate.

In contrast to the predictions for a classical ping-pong mechanism (11, 12), isotope effects were observed on the Vmax/Km for the hydroperoxide substrate in the peroxidase-catalyzed demethylation reaction when DMA-^2H_4 was substituted for DMA (Tables I–III). Therefore, the simple ping-pong kinetic model shown in Scheme 1 must be modified to a model which is consistent with both the steady state kinetics and isotope effect data. The isotope effects are most simply explained by a mechanism in which deuterium is transferred from DMA-^2H_4 to the enzyme and must subsequently be displaced by the hydroperoxide substrate on the next turnover, resulting in either a primary or secondary isotope effect. The large magnitude of the isotope effect on the Vmax/Km for hydrogen peroxide (Table III) suggests a primary isotope effect on compound I formation. However, a mechanism involving bond cleavage of the transferred deuterium which is both chemically reasonable and consistent with current knowledge of compound I formation (23, 24) is not apparent to us.

One possible mechanism which would explain the results by a secondary isotope effect is shown in Fig. 2. The ferric peroxidase is shown with a hydroxide as an axial ligand. This is not necessarily a representation of the native resting state of the enzyme and, in fact, there is considerable controversy as to whether the ferric heme of horseradish peroxidase is penta- or hexacoordinate (25). In any case, the original state of the enzyme is not important beyond the first turnover, where all steady state data are obtained. The ferric enzyme is shown as the hydroxide complex in Fig. 2 to more clearly demonstrate a possible origin of the isotope effect on the Vmax/Km for the hydroperoxide when DMA-^2H_4 is the substrate. The hydroxide substrate (ROOH) binds (with k_i) to the enzyme to form an initial complex. Because the hydroperoxide itself is probably too weak a nucleophile to displace the iron-bound ligand (24), the hydroperoxide may be deprotonated (with k_h) by a basic active site residue (B) to produce the hydroperoxyl anion, which would be an excellent nucleophile because of the α effect of the adjacent oxygen atom (26). The hydroperoxyl anion then displaces (with k_o) the hydroxide to coordinate to the heme iron. The ferric heme of the enzyme is oxidized as the hydroperoxide oxygen-oxygen bond is broken, resulting in the formation (with k_h) of peroxidase compound I and the alkoxo anion. The alkoxo anion recombines with the proton on active site residue B to form the alcohol (ROH), which dissociates (with k_o) from compound I. Compound I then binds (with k_i) the DMA-^2H_4 (R=^2H^-N-CD_3) to form another initial complex and abstracts (with k_h) a deuterium atom (or hydrogen atom when DMA is the substrate) to form a protein-bound DMA radical and peroxidase compound II. The DMA radical then transfers (with k_o) an electron to compound II, reducing it to the ferric deuterioxide (or hydroxide) species and forming an iminium cation. The iminium cation can react (with k_o) with water in the active site or can be released from the enzyme to react with water in the medium, forming a carboxylamine. The carboxylamine formed from DMA would be very unstable and would rapidly decompose to form N-methylalanine and formaldehyde.

Thus, in the mechanism shown in Fig. 2, the isotope effect on the Vmax/Km for the hydroperoxide substrate with DMA-^2H_4 would arise from having to displace (with k_o) the deuterioxide on the next turnover as opposed to a hydroxide when DMA is the substrate. Since deuterioxide is about twice as good a nucleophile as hydroxide (27), it would be expected to be a worse leaving group than hydroxide in the displacement reaction and would result in isotope effects of approximately 2 on the Vmax/Km. Consistent with these considerations, the isotope effects on the Vmax/Km for EtOOH were 1.76 ± 0.52 in the chloroperoxidase-catalyzed demethylation reaction (Table I) and 2.14 ± 0.43 in the horseradish peroxidase-catalyzed reaction (Table II) when DMA-^2H_4 was substituted for DMA. The much larger isotope effect (5.16 ± 0.53) observed on the Vmax/Km for hydrogen peroxide in the horseradish peroxidase-catalyzed reaction (Table III) implies that either the model shown in Fig. 2 is not a complete description of the events taking place during peroxidase compound I formation or that deuterioxide may be an even better nucleophile relative to hydroxide when bound to the heme iron in the active site of horseradish peroxidase. The hydrophobic environment of the active site of horseradish peroxidase (28) may make the enzyme-bound deuteroxide a better nucleophile (26) than indicated by the data of Steffa and Thornton (27) for the free anion in aqueous solution.

The results of a recent intramolecular isotope effect study of the heme protein-catalyzed N-demethylation of N-methyl-N-trideuteriomethylaniline were interpreted as indicating that the horseradish peroxidase-catalyzed reaction proceeds via α-carbon hydrogen abstraction (k_h) in Fig. 2) while the chloroperoxidase-catalyzed reaction involves electron transfer from nitrogen followed by α-carbon deprotonation of the anilinium cation, radical (29). The electron transfer-deprotonation mechanism for chloroperoxidase-catalyzed N-demethylation is the stepwise equivalent of hydrogen atom abstraction (k_h in Fig. 2). The initial electron transfer step must be reversible in order to predict the observed isotope effect on the Vmax/Km for DMA in the chloroperoxidase-catalyzed reaction (Table II).

Based on their studies of the horseradish peroxidase-catalyzed N-demethylation of aminopyrine, Griffin and Ting (30) proposed a mechanism involving initial irreversible electron transfer from nitrogen followed by hydrogen atom abstraction to yield the iminium cation. When the expression for the Vmax/Km for DMA is derived (21) for this mechanism, it does not include the rate constant for the hydrogen atom abstrac-
tion step and thus predicts that there will be no isotope effect on the $V_{max}/K_m$ for DMA. Although an isotope effect was not observed on this parameter in the EtOOH-supported reaction catalyzed by horseradish peroxidase (Table II), isotope effects were observed for the hydrogen peroxide-supported reaction (Table III) and for the chloroperoxidase-catalyzed reaction (Table I). Therefore, the mechanism proposed by Griffin and Ting (30) is inconsistent with the observed isotope effects. Since the same compound I species is formed upon reaction of different hydroperoxides with horseradish peroxidase (51), the differences in the magnitudes of the isotope effects observed on the $V_{max}/K_m$ for DMA in the horseradish peroxidase-catalyzed reaction (Tables II and III) must be due to differences in the rate factors suppressing the intrinsic isotope effect (11-13), those factors being larger in the EtOOH-supported reaction.

The proposed mechanism for the oxidative demethylation of DMA (Fig. 2) is similar to the mechanism proposed by Shannon and Bruce (32) for the iodosobenzene-supported demethylation of DMA catalyzed by chlorotetraphenylporphinatoiron (III). An alternative mechanism involving the release of DMA radicals from the enzyme and their subsequent reaction with water to form a carbinolamine species can be excluded because it would be inconsistent with the 1:1 stoichiometry of hydroperoxide consumption and formaldehyde formation observed for the horseradish peroxidase-catalyzed demethylation reaction (5). A mechanism involving the recombinant of the enzyme-bound DMA radical with the oxygen in compound II, as has been suggested for the hydroperoxide-supported oxidations catalyzed by cytochrome P-450 (33), would be inconsistent with the isotope effects observed on the $V_{max}/K_m$ for the hydroperoxide when DMA-$^3$H$_2$ is the substrate (Tables I-III).

In the detailed model for peroxidase-catalyzed demethylation reactions (Fig. 2), the iminium cation formed during the oxidation of DMA can react with water in the active site (derived from either the oxidant via displacement of the hydroxide or the medium) or it can dissociate from the enzyme and react with water in the medium, forming an unstable carbinolamine of DMA which would rapidly decompose to N-methylamine and formaldehyde. Thus, the proposed mechanism would be consistent with either the oxidant or solvent water as the source of the oxygen atom in the formaldehyde formed in peroxidase-catalyzed demethylation reactions. We have recently demonstrated (34) that the oxidant was the source of the oxygen atom incorporated into the product of the rabbit liver cytochrome P-450$_{2M_d}$-catalyzed N-demethylation of N-methylcarbazole when the reaction was supported by peroxy compounds or carried out using a reconstituted enzyme system containing highly purified cytochrome P-450, NADPH-cytochrome P-450 reductase, NADPH, and molecular oxygen. Foster et al. (38) did not observe any isotope effect on the demethylation of 1-(o-carbamoylphenyl)-3,3-dimethyltriazene catalyzed by rat liver microsomes but did observe isotope effects of 1.85 with p-nitroanisole, 1.90 with p-methoxyacetanilide, and 2.10 with p-methoxyanisole. The interpretation of isotope effects on cytochrome P-450-catalyzed demethylation reactions in microsomal suspensions or reconstituted enzyme systems is complicated by the presence of a second enzyme system, NADPH-cytochrome P-450 reductase, for which cytochrome P-450 is a substrate. The presence of the reductase may further add to the suppression of the magnitude of the isotope effects on the overall reaction. The determination of isotope effects on cytochrome P-450-catalyzed demethylation reactions using highly purified enzyme preparations and hydroperoxides as co-substrates would eliminate any suppression of the isotope effects due to the reductase and might simplify their interpretation. Comparison of the isotope effects on hydroperoxide-supported demethylation reactions catalyzed by cytochrome P-450 with those reported here for the peroxidase-catalyzed demethylation of DMA might add to our understanding of the catalytic relationship between cytochrome P-450 and peroxidases.

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