18O Studies of the Peroxidase-catalyzed Oxidation of N-Methylcarbazole

MECHANISMS OF CARBINOLAMINE AND CARBOXALDEHYDE FORMATION

(Received for publication, June 12, 1986)

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Cloroperoxidase, horseradish peroxidase, hemoglobin, myoglobin, lactoperoxidase, and microperoxidase catalyzed the ethyl hydroperoxide-dependent oxidation of N-methylcarbazole to N-(hydroxymethyl)carbazole and N-formylcarbazole as major products. Mass spectral analysis of the N-(hydroxymethyl)carbazole formed during the peroxidase-catalyzed N-demethylation of N-methylcarbazole in \(^{18}O\)-enriched medium indicated partial incorporation (7.5–25.9\%) of solvent water oxygen into the carbinolamine intermediate in all systems investigated, suggesting that the peroxidase active site is partially accessible to solvent water during N-demethylation. In contrast, solvent water oxygen was not incorporated into the N-formylcarbazole formed during the peroxidase-catalyzed oxidation of N-methylcarbazole. N-(Hydroxymethyl)carbazole was not further metabolized by the peroxidases in the presence of ethyl hydroperoxide, indicating that it is not an intermediate in N-formylocarbazole formation. The horseradish peroxidase-catalyzed formation of N-formylcarbazole was decreased by 77\% when the hydroperoxide-supported reactions were carried out in a nitrogen atmosphere, while the formation of N-(hydroxymethyl)carbazole was decreased by 46\%. When the horseradish peroxidase-catalyzed reactions were carried out in a \(^{18}O\)-enriched atmosphere, \(^{18}O\) incorporation into N-(hydroxymethyl)carbazole was 64.4\% of the total oxygen, while 81.8\% of the oxygen incorporated into N-formylcarbazole came from \(^{18}O\). These results suggest that there are two different mechanisms for the formation of N-(hydroxymethyl)carbazole, both involving the initial oxidation of N-methylcarbazole to a neutral carbon-centered radical. The radical can be further oxidized in the enzyme active site to an iminium cation, which reacts with water derived from either the oxidant or the medium to form the carbinolamine. Alternatively, the substrate radical can react with molecular oxygen to form a hydroperoxy radical, which decomposes to form the carboxaldehyde and carbinolamine.

Peroxidases and several other heme proteins can catalyze the hydroperoxide-dependent N-demethylation of secondary and tertiary amines (1–5). Detailed studies of the N-demethylation of N,N-dimethylaniline catalyzed by chloroperoxidase (chloride/hydrogen-peroxide oxidoreductase; EC 1.11.1.10) and horseradish peroxidase (donor:hydrogen-peroxide oxidoreductase; EC 1.11.1.7) have established that these reactions are described by the following equation (4, 5):

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

where ROOH is the hydroperoxide substrate and R'\text{R}''\text{N}-\text{CH}_3 is the N-methylarylamine substrate. Steady-state kinetic studies have demonstrated that the demethylation of N,N-dimethylaniline catalyzed by chloroperoxidase and horseradish peroxidase proceeds by a ping-pong kinetic mechanism (5, 6). In this kinetic mechanism, the hydroperoxide reacts with the native peroxidase to form the oxidized enzyme intermediate, compound I, and the corresponding alcohol. The N-methylarylamine substrate then binds to compound I and is oxidized, resulting in the formation of the desmethylarylamine and formaldehyde with the regeneration of the native peroxidase. While these studies establish the kinetic mechanism of peroxidase-catalyzed N-demethylation reactions, they do not provide information regarding the chemistry of substrate oxidation.

Previous studies have demonstrated that the peroxidase-catalyzed N-demethylation of N,N-dimethylaniline does not proceed via an N-oxide intermediate (4, 5), supporting the involvement of a carbinolamine precursor of formaldehyde. Studies of the kinetic deuterium isotope effects associated with peroxidase-catalyzed N-demethylation reactions have revealed information about the chemistry of carbon-hydrogen bond cleavage. Large intramolecular isotope effects (\(k_D/k_H > 8.5\)) were observed during the N-demethylation of N-methyl,N-trideuteriomethylaniline catalyzed by horseradish peroxidase, lactoperoxidase, hemoglobin, and myoglobin, indicating that the reaction involves initial hydrogen atom abstraction from the N-methyl group (7). In contrast, the small intramolecular isotope effects (\(k_D/k_H < 3.1\)) observed with chloroperoxidase and purified isozymes of cytochrome P-450 indicated that the N-demethylation reactions catalyzed by these heme proteins proceed via an initial electron transfer from nitrogen followed by deprotonation of the cation radical intermediate (7). Additionally, significant kinetic deuterium isotope effects were observed on the \(V_{\text{ox}}/K_m\) for the hydroperoxide substrate (the second order rate constant for peroxy-
idase compound I formation) during the N-demethylation of N,N-di(trideuteriomethyl)aniline catalyzed by horseradish peroxidase and chloroperoxidase (8). These results were interpreted to indicate that N-demethylation proceeds by a mechanism which were hydrogen (or deuterium) is transferred from the N-methylaminoalcohol substrate to the peroxidase, resulting in the formation of a ferric-hydroxide (or deuterioxide) enzyme species and an iminium cation. The hydroxide (or deuterioxide) must then be displaced from the enzyme by the hydroperoxide on the next turnover, causing an isotope effect on peroxidase Compound I formation (8).

The identification of the source of the oxygen atom incorporated into the product of peroxidase-catalyzed N-demethylation reactions has not been possible because of the rapid exchange of the carbonyl oxygen of formaldehyde with solvent water oxygen (9). The iminium cation formed during N-demethylation could react with water in the enzyme active site (derived from either the oxidant via displacement of the hydroxide or from the medium) or it could dissociate from the enzyme and react with water in the medium, forming a generally unstable carbinolamine which would decompose to yield the desmethylamine and formaldehyde. One way to determine the source of the oxygen atom in the product of peroxidase-catalyzed N-demethylation reactions is to use an N-methylcarboline substrate such as N-methylcarbazole (NMC), which yields a stable isolatable carbinolamine intermediate (10–12). 15O studies of the cytochrome P-450-catalyzed N-demethylation of N-methylcarbazole have demonstrated that the oxidant is the source of the oxygen atom in the carbinolamine intermediate formed by hepatic microsomes supported by NADPH and molecular oxygen (13) and by purified cytochrome P-450 supported by hydroperoxides or in a reconstituted monoxygenase system supported by NADPH and molecular oxygen (14).

In this study, we have investigated the peroxidase-catalyzed oxidation of NMC in H218O-enriched medium and under an 18O atmosphere. Quantitation of 18O into the metabolites N-(hydroxymethyl)carbazole (NFC) and N-formylcarbazole (NFC) suggests that there are two different mechanisms of carbinolamine formation.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparation—**Chloroperoxidase was isolated and purified from Caldariomyces fumago as reported previously (15). The preparations used for these studies had specific activities of greater than 2000 units/mg of protein in the standard clorhination assay (15) and exhibited A440/A390 ratios greater than 1.40, indicating that the enzyme preparations were at least 95% pure (15). Protein concentrations were determined using the method of Lowry et al. (16).

Crude horseradish peroxidase (Type I, A405/A390 = 0.3), obtained from Sigma, was purified by a modification of the procedure of Shannon et al. (17) as previously described (18). The horseradish peroxidase (B-C) isozyme used for these studies and had an A405/A390 ratio greater than 3.2. Horseradish peroxidase concentrations were determined using the molar absorbance indices reported by Shannon et al. (17).

Cytochrome P-450,2 and NADPH-cytochrome P-450 reductase were purified from liver microsomes of phenobarbitol-pretreated rabbits as previously described (14). Catalase (bovine liver), hemoglobin (bovine), myoglobin (whale), lactoperoxidase (bovine milk), and microperoxidase (equine heart cytochrome c heme-undecapeptide) were obtained from Sigma and used without further purification.

**Chemicals—**NMC and NHMC (carbazole-9-methanol) were synthesized by the oxidation of NHMC with periodide (1,1,1-triaethylpentyl)-1,1-dihydro-1,2-benzodioxol-3(1H)-one; Aldrich) using the procedure described by Dass and Martin (19). The products were separated using silica gel column chromatography, and NFC was purified by preparative thin layer chromatography. The purified product, recrystallized from ethyl acetate, yielded a single spot with the expected structure (Fig. 2) and exhibited an infrared absorbance band at 1695 cm⁻¹ (aromatic carbonyl stretch); m.p. (lit.) 99–100 °C (20, 101 °C).

Ethyl hydroperoxide (10%) was obtained from Polysciences, Inc. H218O was obtained in 97% enrichment from MSD Isotopes. O2 (99% purity) was obtained from US Services, Inc. All other materials were reagent grade and obtained from commercial sources.

**Incubation Conditions—**All of the mixtures contained NMC (1 mM) and ethyl hydroperoxide (2 mM) in a final volume of 1.0 ml. The mixtures containing chloroperoxidase (200 μg) were prepared in phosphate buffer (0.1 M, pH 5.0) or buffer containing 15% H218O. The mixtures containing the following heme proteins were prepared in potassium phosphate buffer (0.5 M), pH 6.0, or buffer containing 19.5% H218O: horseradish peroxidase (50 μg), lactoperoxidase (50 μg), hemoglobin (150 μg), myoglobin (50 μg), microperoxidase (100 μg), and catalase (100 μg). The mixtures were preincubated at 25°C for 5 min, and the mixtures were initiated by the addition of ethyl hydroperoxide. All incubations were carried out for 20 min in duplicate. The reactions were extracted twice with 3 ml of ethyl acetate containing 1% diethylamine, and the extracts were processed for chromatographic analysis as previously described (12).

Some incubations containing horseradish peroxidase, as described above, were carried out in atmospheres containing nitrogen or 18O2. The incubations were done on a vacuum train using a manifold which allowed 3 samples to be degassed and then incubated under 18O2 at the same time. Incubations containing cytochrome P-450,2 and NADPH-cytochrome P-450 reductase as previously described (14) were run with each experiment as a positive control. 18O enrichment of the atmosphere was determined from the cytochrome P-450-dependent incorporation of 18O2 into the NHMC formed during the N-demethylation of NMC (13, 14).

**High Pressure Liquid Chromatography—**The dried samples were dissolved in 0.2 ml of hexane/isopropanol (50:1), and aliquots (10 μl) were injected onto a Varian 5000 liquid chromatograph equipped with a CDS-1111 microprocessor and a Whatman Partisil 10 column (4.6 × 250 mm). The metabolites of NMC were separated using hexane/isopropanol (50:1) as the mobile phase at a flow rate of 1.7 ml/min.

In some experiments, α-naphthoflavone (7.5 nmol) was added to the incubation mixtures as the internal standard prior to ethyl acetate extraction. The formation of NFC and NHMC was quantitated by the peak area ratio method using standard curves generated by taking known amounts of each metabolite through the procedure described above.

**Gas Chromatography-Mass Spectroscopy—**The dried samples were reconstituted in 50 μl of ethyl acetate and allowed to react with N,O-bis(trimethylsilyl)trifluoroacetamide (Supelco, Inc.), evaporated to dryness under a stream of nitrogen, and redissolved in 20 μl of ethyl acetate. Aliquots (1 μl) of the reconstituted extract were injected onto a Finnigan 3225 gas chromatograph-mass spectrometer equipped with a 30-m fused silica capillary column coated with SE-54 (J & W Scientific) and chromatographed as previously described (14). NFC and the trimethylsilyl ether of NHMC had retention times of 9.8 and 10.3 min, respectively. Ionization was by electron impact with the filament operated at 70 eV. Ion current was integrated at m/z 271 (M + 2) and m/z 269 (M + 1) for the gas chromatographic peak due to NFC. (See Fig. 2 for the complete mass spectrum of NFC.) The ratio (M + 2)/(M + 1) was calculated, and the difference between that in 18O-containing media and that in 16O-containing media was divided by the fractional enrichment of 18O in the media. This result was multiplied by 100 to yield the percent 18O incorporated into each metabolite.

**RESULTS**

Horseradish peroxidase, lactoperoxidase, hemoglobin, myoglobin, and microperoxidase catalyzed the ethyl hydroperoxide-dependent oxidation of NMC to two major metabolites as shown by HPLC analysis of the reaction mixture (Fig. 2). Control experiments demonstrated that metabolite formation...
was dependent upon the presence of both the hydroperoxide and peroxidase. Under the experimental conditions used, the oxidation of NMC catalyzed by chloroperoxidase did not result in sufficient amounts of products to be detected by HPLC. However, the chloroperoxidase-catalyzed formation of both metabolites was readily detectable by gas chromatographic-mass spectral analysis (data not shown). Catalase did not oxidize NMC to detectable products.

The metabolite peak in Fig. 1 with a retention time of 19.0 min had the same retention time on HPLC and gas chromatography as authentic NHMC, and its mass spectrum was identical to the synthesized standard (14). The other major metabolite peak had a retention time (4.6 min) which did not correspond to any of the previously characterized microsomal metabolites of NMC (11, 12). The mass spectrum of this metabolite (Fig. 2) had a parent ion at m/z 195 and exhibited a prominent loss of 28 mass units (CO), suggesting a carboxaldehyde structure. These data are consistent with NFC as the metabolite. When authentic NFC was synthesized, its retention times (HPLC and gas chromatography) and mass spectrum were identical to that of this metabolite (Fig. 2).

Previous studies have established that the carbamidamine of NMC, NHMC, does not exchange oxygen with the aqueous medium at pH 7.4 (13) or 7.7 (14). In order to verify that NHMC did not exchange oxygen with the aqueous medium under the conditions used for this study, NHMC (1 mM) was incubated in potassium phosphate buffer (0.5 M), pH 6.0, and in phosphate buffer containing 19.5% H$_2^{18}$O using the conditions described under "Experimental Procedures." Mass spectral analysis indicated that the ratio of m/z 271:269 was 6.06 ± 0.31% (mean ± S.E.) for NHMC incubated in phosphate buffer and 5.83 ± 0.13% for NHMC incubated in H$_2^{18}$O-enriched phosphate buffer. These results are in reasonable agreement with the calculated m/z 271:269 ratio of 5.10% for C$_{16}$H$_{18}$NOSi (21) and indicate that NHMC does not exchange oxygen with the aqueous medium.

Experiments were carried out to determine the source of the oxygen atom in the products of the hydroperoxide-supported oxidation of NMC catalyzed by several peroxidases. As shown in Table I, $^{18}$O incorporation from the medium into the NHMC formed during the N-demethylation of NMC was observed for all of the hemeproteins investigated, ranging from 7.5 to 25.9%. In contrast, no $^{18}$O incorporation from the
medium into the NFC formed during the peroxidase-catalyzed oxidation of NMC was observed. The ratio of m/z 197:195 for the NFC formed in H\textsubscript{2}O\textsuperscript{18} phosphate buffer was 1.66 ± 0.09% (mean ± S.E.). In H\textsubscript{2}O\textsuperscript{18}-enriched phosphate buffer, the ratio of m/z 197:195 was 1.74 ± 0.07%. These results are in reasonable agreement with the calculated m/z 197:195 ratio of 1.22% for C\textsubscript{6}H\textsubscript{5}NO (21). Further experiments demonstrated that NHMC was not oxidized to NFC by any of the hemeproteins examined in the presence or absence of ethyl hydroperoxide.

When the hydroperoxide-supported oxidation of NMC by horseradish peroxidase was carried out under anaerobic conditions, the formation of NFC was decreased by 77% while the formation of NHMC was decreased by 46% (Table II). When the horseradish peroxidase-catalyzed oxidation of NMC was carried out in an atmosphere of \textsuperscript{18}O\textsubscript{2}, \textsuperscript{18}O was incorporated into both NFC (79.5-84.1%) and NHMC (62.1-66.7%).

DISCUSSION

The data presented here demonstrate that a variety of peroxidases oxidize NMC to yield NHMC and NFC as the major products. Thus, the peroxidases can catalyze the hydroperoxide-dependent N-demethylation of NMC via the formation of the carbinolamine intermediate NHMC. When the peroxidase-catalyzed oxidation of NMC was carried out in \textsuperscript{18}O\textsubscript{2}-enriched medium, incorporation of solvent water oxygen into the NHMC formed ranged from 7.5 to 25.9%, depending upon the identity of the hemeprotein. These results suggest that the peroxidase active site is partially accessible to solvent water during N-demethylation. In contrast to carbinolamine formation, solvent water oxygen was not incorporated into the carboxaldehyde metabolite NFC. Moreover, NHMC was not further metabolized by any of the peroxidases. These results indicate that NHMC is not an intermediate in NFC formation. When the horseradish peroxidase-catalyzed oxidation of NMC was carried out under a nitrogen atmosphere, the formation of NFC and NHMC was decreased by 77 and 46%, respectively. When the horseradish peroxidase-catalyzed oxidation was carried out under a \textsuperscript{18}O\textsubscript{2} atmosphere, \textsuperscript{18}O incorporation into NFC and NHMC was 81.8 and 64.4%, respectively. These results suggest that there are two different mechanisms of carbinolamine formation, involving molecular oxygen and one involving water.

These data are best interpreted in terms of the mechanisms shown in Fig. 3. Peroxidase compound I oxidizes NMC via either hydrogen atom abstraction or electron transfer-deprotonation (7), resulting in the formation of a neutral substrate radical. The decomposition of the iminium cation from the enzyme active site (3), because mechanism predicts 100% incorporation of solvent water oxygen into the carbinolamine intermediate.

The neutral substrate radical shown in Fig. 3 could also react with molecular oxygen to form a hydroperoxy radical, which can abstract a hydrogen atom to form a substrate-derived hydroperoxide. The hydroperoxide could then decompose to yield the carboxaldehyde NFC. The decomposition of organic hydroperoxides to yield aldehydes is well established (22). This mechanism of NFC formation is similar to the mechanism of the cobalt (III)-catalyzed oxidation of aromatic compounds (23). It is also similar to the proposed mechanism of the oxidative decarboxylation of indole-3-acetic acid by horseradish peroxidase (24).

An alternative mechanism of carbinolamine formation involving recombination of the substrate radical with the oxygen moiety of compound II, as has been suggested for cytochrome P-450-catalyzed N-demethylation (13, 14), would be inconsistent with the isotope effects observed on the V\textsubscript{max}/K\textsubscript{m} for the hydroperoxide substrate during the N-demethylation of N,N-di(trideuteriomethyl)aniline (8). The data are also inconsistent with a proposed mechanism involving the release of the iminium cation from the enzyme active site (3), because that mechanism predicts 100% incorporation of solvent water oxygen into the carbinolamine intermediate.

The neutral substrate radical shown in Fig. 3 could also react with molecular oxygen to form a hydroperoxy radical, which can abstract a hydrogen atom to form a substrate-derived hydroperoxide. The hydroperoxide could then decompose to yield the carboxaldehyde NFC. The decomposition of organic hydroperoxides to yield aldehydes is well established (22). This mechanism of NFC formation is similar to the mechanism of the cobalt (III)-catalyzed oxidation of aromatic compounds (23). It is also similar to the proposed mechanism of the oxidative decarboxylation of indole-3-acetic acid by horseradish peroxidase (24). Although attempts to observe NMC-derived free radicals by room temperature EPR were unsuccessful, \textsuperscript{2} these species would be expected to have very short solution lifetimes.

In addition to decomposition, the NMC-derived hydroperoxide shown in Fig. 3 can be reduced by peroxidase to yield NHMC. Thus, for horseradish peroxidase-catalyzed NHMC oxidation, 18\textsuperscript{O} Studies of Peroxidase-catalyzed N-Demethylation

| Table II |

| Effect of anaerobic conditions on the horseradish peroxidase-catalyzed oxidation of NMC |
| Netmole |
| Atmosphere | NHMC formed | NFC formed |
| nmol | nmol |
| Air | 19.7 | 12.1 |
| Nitrogen | 10.6 (64%) | 2.8 (23%) |

\textsuperscript{2} R. N. Pandey, P. F. Hollesberg, and B. M. Hoffman, unpublished observations.
formation, approximately 64% of the carbinolamine oxygen is derived from molecular oxygen, while the remainder is from water derived from either the solvent (17%) or presumably from the oxidant (19%). For horseradish peroxidase-catalyzed NFC formation, approximately 82% of the carboxaldehyde oxygen is derived from molecular oxygen; none is from solvent water, and the remaining 18% is presumably derived from the oxidant. This could result from reaction of the substrate free radical or iminium ion with ethyl hydroperoxide (present at 2 mM) to form an unstable peroxide, which could decompose to NFC and ethanol. Alternatively, the peroxide could decompose to NHMC and acetaldehyde, providing another pathway for incorporation of oxygen from the oxidant into the carbinolamine metabolite.

The data presented here demonstrate the involvement of molecular oxygen in approximately 60% of the horseradish peroxidase-catalyzed N-demethylation of NMC. Molecular oxygen has also been shown to be involved in the horseradish peroxidase-catalyzed N-demethylation of N,N-dimethyl-p-toluidine (25). In contrast to these systems, anaerobic conditions had no effect on the horseradish peroxidase-catalyzed N-demethylation of N,N-dimethylaniline, suggesting that molecular oxygen is not involved in the demethylation of this substrate. The apparent substrate-dependence of the involvement of molecular oxygen in peroxidase-catalyzed demethylation reactions may arise from differences in the rates of escape of nascent substrate radicals from the peroxidase active site and from differences in the reactivity of the radicals with molecular oxygen.

This is the first report of the peroxidase-catalyzed oxidation of an N-methylarylamine to the corresponding N-formyl derivative. The formation of N-formyl metabolites of chlorotoluuron (26), aminopyrine (27, 28), N-methylbenzamide (29), and methadone (30) has been observed in vivo and in vitro with hepatic subcellular fractions. In the case of N-methylbenzamide, N-formylbenzamide was shown to be formed via oxidation of N-hydroxymethylbenzamide by hepatic alcohol dehydrogenase (29). This is in contrast to the peroxidase-catalyzed formation of NFC, where NHMC is not an intermediate. We have also observed the formation of NFC during the metabolism of NMC by highly purified hepatic cytochrome P-450. Studies of the mechanism of formation of this unusual metabolite by cytochrome P-450 are currently in progress.

Acknowledgments—We thank Dr. Lowell P. Hager for supplying the chloroperoxidase and for his encouragement. We also thank Dr. Duward Shriner for his help with the experiments using 18O2.

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