Intramolecular isotope effects were determined for the N-demethylation of N-methyl-N-trideuteriomethylaniline catalyzed by two isozymes of cytochrome P-450 and several peroxidases in order to differentiate between deprotonation and hydrogen atom abstraction steps. Lactoperoxidase, hemoglobin, myoglobin, and two isozymes of horseradish peroxidase catalyzed the hydroperoxide-dependent N-demethylation at initial rates ranging from 20 to 1700 min⁻¹. These hemeproteins exhibited large and comparable intramolecular isotope effects (k_H/k_D = 8.6 to 10.1). In contrast, two isozymes of cytochrome P-450 as well as chloroperoxidase (v = 1.5 to 1700 min⁻¹) gave low isotope effects (k_H/k_D = 1.7 to 3.1) under identical conditions. Catalase exhibited an intermediate intramolecular isotope effect (k_H/k_D = 5.4). These results have been interpreted to indicate that most of the hemeproteins investigated catalyze N-demethylation reactions via the carbinolamine intermediate, while the reactions catalyzed by cytochrome P-450 and chloroperoxidase proceed via hydrogen atom abstraction.

Oxidative N-dealkylations of secondary and tertiary amine substrates are common reactions catalyzed by cytochrome P-450. These reactions are generally accepted to proceed via the insertion of an oxygen atom into the alpha-carbon-hydrogen bond of the N-alkyl group followed by amine elimination from the resulting carbinolamine (1). Oxygen 18 studies have demonstrated that the oxidant is the source of the oxygen atom in the carbinaldehyde (2, 3) and in the aldehyde product (4) of cytochrome P-450-catalyzed N-dealkylation reactions supported by various peroxo compounds or NADPH and molecular oxygen. However, the actual chemical steps involved in dealkylation reactions are still a matter of controversy.

Chloroperoxidase (5), horseradish peroxidase (5-7), and several other hemeproteins (5, 7) can also catalyze the hydroperoxide-dependent N-demethylation of many aromatic secondary and tertiary amines. The peroxidase-catalyzed N-demethylation of N,N-dimethylaniline has been characterized in detail (5, 6). This reaction has been shown to proceed by a ping-pong kinetic mechanism involving the oxidized enzyme intermediate compound 1 (6, 8). While this kinetic mechanism is distinctly different from the sequential mechanism implicated for the cytochrome P-450-catalyzed O-demethylation of p-nitroanisole (9), it is possible that cytochrome P-450 and peroxidases share a common chemical mechanism of substrate oxidation.

Several possible chemical mechanisms for the hemeprotein-catalyzed N-demethylation of an N,N-dimethylamine (I) are shown in Fig. 1. The amine can transfer an electron to the iron-oxo species of the hemeprotein to form an aminium cation radical (II) (pathway A). Although this step was originally postulated to be irreversible (7), it is shown as reversible in Fig. 1 since irreversible electron transfer would be inconsistent with the observed isotope effects on the V_m/K_m for N,N-dimethylaniline during peroxidase-catalyzed demethylation. The aminium radical can lose a proton to form the neutral radical (III) (pathway C), which can then transfer an electron (pathway E) to form the iminium cation (IV). Alternatively, the aminium radical (II) can transfer a hydrogen atom (pathway D) to form the iminium ion (IV) directly. The iminium ion would then react with water (pathway G) to form the carbinaldehyde (V). Another alternative is direct hydrogen atom abstraction from the amine (I) to form the radical (III) (pathway B), which can undergo electron transfer to form the iminium ion (IV) or recombine with an enzyme-bound oxygen moiety to form the carbinaldehyde (V) directly (pathway F), as has been suggested for cytochrome P-450-catalyzed N-demethylation reactions (10). The generally unstable carbinaldehyde (V) would decompose to yield the secondary amine (VI) and formaldehyde (VII) as the ultimate products.

One approach which can provide chemical information about N-demethylation reactions is the determination of isotope effects. Northrop (11, 12) has shown that the magnitudes of isotope effects on enzyme-catalyzed reactions are subject to unknown degrees of suppression from numerous pre-catalytic steps when they are determined by intermolecular competition (separate incubations of deuterated and nondeuter-
Isotope Effects in P-450 and Peroxidase N-Dealkylation Reactions

washed as previously described. With the exception of catalase, these preparations were at least 95% pure. Protein concentrations were determined using the method of Lowry et al. (22).

Crude horseradish peroxidase (Type 1, A$_{403}$/A$_{280}$ = 0.3), obtained from Sigma, was purified by a modification of the procedure of Shannon et al. (24) as previously described (25). The horseradish peroxidase B-C and A isozymes used for these studies had A$_{403}$/A$_{280}$ ratios greater than 3.2 and 3.6, respectively. Horseradish peroxidase concentrations were determined using the molar absorbance indices reported by Shannon et al. (24) for the various isozymes.

Catalase (beef liver), hemoglobin (bovine), myoglobin (whale), and lactoperoxidase (milk) were obtained from Sigma and used without further purification.

Materials—The substrate N-methyl-N,N-trideuteriomethylaniline was synthesized by lithium aluminium deuteride (Merck Sharp & Dohme Isotopes) reduction of the carbamate formed by reaction of N-methylalanine (Aldrich) and ethyl chloroformate in pyridine. The N-trideuteriomethylpentadeuteriophenylamine internal standard and N,N-trideuteriomethylaniline product were synthesized in an analogous fashion from 2,3,4,5,6-pentadeterioaniline (Aldrich) and aniline, respectively. The samples were usually stored as their oxalate salts. The chemical and isotopic purity for all the isotopically labeled substrate and products was greater than 98%.

N,N-Dimethylaniline was obtained from Aldrich and was redis- tillated before use. Ethyl hydroperoxide (10%) was obtained from Polysciences, Inc., hydrogen peroxide (30%) from Fischer, and cu- mene hydroperoxide from Matheson, Coleman, and Bell. NADPH was obtained from Sigma. All other materials were reagent grade and obtained from commercial sources. The hydroperoxide concentrations were determined by iodometric titration (26).

Incubation Conditions—Conditions where the rate of N-demethylation of N,N-dimethylaniline (0.5 mM) was linear with both time and enzyme concentration were established for all hemeproteins except catalase by measuring the amount of formaldehyde formed using a modification of the procedure of Nash (27) as previously described (5). The reaction catalyzed by catalase was not linear with time or protein concentration, and conditions were chosen such that detectable amounts of products were formed. However, it has previously been shown that initial rate conditions are not necessary for the accurate measurement of intramolecular isotope effects (13).

All of the incubation mixtures contained N-methyl-N,N-tri- deuteriomethylaniline (0.5 mM). The hemeprotein-catalyzed reactions were initiated by the addition of the hemeprotein, incubated at 25°C for the times indicated, and terminated by the addition of 0.45 ml of 60% trichloroacetic acid. The reactions catalyzed by the cytochrome P-450 isozymes (3.0 nmol) in potassium phosphate buffer (pH 7.4), were initiated by the addition of cumene hydroperoxide (1.0 mM) and incubated for 10 min. The following reactions in sodium potassium phosphate buffer (0.5 M), pH 6.0, contained ethyl hydroperoxide (1.6 mM) and were incubated for the times indicated: chloroperoxidase (0.83 µg; 10 min), horseradish peroxidase A (0.95 µg; 5 min), hemoglobin (20 µg; 7.5 min), myoglobin (20 µg; 7.5 min), lactoperoxidase (10 µg; 10 min), and catalase (300 µg; 10 min). The reactions containing either hydrogen peroxide (0.47 mM) and horseradish peroxidase B-C (0.34 µg) in sodium potassium phosphate buffer (0.4 M), pH 5.5, or ethyl hydroperoxide (0.32 mM) and horseradish peroxidase B-C (688 µg) in sodium potassium phosphate buffer (0.4 M), pH 7.4, were incubated for 5 min. After the addition of trichloroacetic acid, 100 nmol of N-trideuteriomethylpentadeuteriophenylamine were added as the internal standard and the samples were frozen and stored at -70°C until analysis.

Product Analysis—The frozen samples were thawed, 1.5 ml of 4 N NaOH were added to each sample, and the isotopically labeled N,N-dimethylaniline products were extracted into 2.0 ml of n-hexane. Following centrifugation to separate the phases, 1.5 ml of the n-hexane phases were transferred to conical test tubes, 50 µl of trifluoroacetic anhydride (Pierce Chemical Co.) were added, and the tubes were capped, vortexed, and stored overnight at 5°C. The sample volume was reduced to 100 µl under a stream of dry nitrogen, and aliquots (2 µl) were injected onto a glass column (5 feet x 2 mm inner diameter) packed with 3% OV17. Gas chromatography-mass spectrometry was carried out at a column temperature of 100°C using methane as both the carrier and chemical ionization gas on a Finnigan 3200 quadrupole mass spectrometer.
mass spectrometer. The N-trifluoroacetamide derivatives of N-methylaniline had a retention time of approximately 2.7 min. A Finnigan 4000 data system was used to integrate selected ion currents under the gas chromatographic peak due to the isotopically labeled derivatives of N-methylaniline. Ions were monitored at m/z 204, 207, and 212, which correspond to the MH+ ions of the trifluoroacetamide derivatives of N-methylaniline, N-trideuteriomethylaniline, and the internal standard N-trideuteriomethylpentadecanophenylamine, respectively.

Standard curves containing equimolar mixtures of N-methylaniline and N-trideuteriomethylaniline (25 to 500 nmol) along with the internal standard (100 nmol) were prepared and also carried through the analysis. The quantities of the two isotopically labeled N-methylaniline products formed during the incubations were calculated from their respective standard curves. The intramolecular isotope effects were calculated from the ratios of N-trideuteriomethylaniline to N-methylaniline.

RESULTS AND DISCUSSION

The intramolecular isotope effects observed for two isozymes of cytochrome P-450, four peroxidases, and several other hemeproteins during the N-demethylation of N-methyl-N-trideuteriomethylaniline are summarized in Table I. With the exception of chloroperoxidase and catalase, all of the isotope effects for the peroxidases, hemoglobin, and myoglobin were large (kH/kD > 5.5), while those for the cytochrome P-450-catalyzed N-demethylation were low (kH/kD < 3.1). Large and comparable isotope effects were observed during the N-demethylation reaction catalyzed by the B-C isozyme of horseradish peroxidase and chloroperoxidase in the presence of either ethyl hydroperoxide or hydrogen peroxide, suggesting that the isotope effects are independent of the identity of the oxidant employed. This conclusion is further substantiated by the small and comparable isotope effects observed during the cytochrome P-450-catalyzed N-demethylation reaction in the presence of cumene hydroperoxide or in a reconstituted system containing NADPH-cytochrome P-450 reductase, NADPH, and molecular oxygen.

The large isotope effects observed with horseradish peroxidase, lactoperoxidase, hemoglobin, and myoglobin (Table I) indicate that maximum carbon—hydrogen bond cleavage occurs in a symmetrical transition state during N-demethylation, consistent with a radical mechanism of bond cleavage. This conclusion is also consistent with the large intrinsic isotope effect observed for hydrogen atom abstraction from a-dideuteriobenzylamine by permanganate (kH/kD = 7.0) (19) and for the hydrogen atom abstraction mechanism in aliphatic (kH/kD = 5.3) and benzyl (kH/kD = 11) oxidations catalyzed by cytochrome P-450 (28, 29). These results provide persuasive evidence that the N-demethylation reactions catalyzed by these hemeproteins involve hydrogen atom abstraction. However, the data do not permit the distinction between hydrogen atom abstraction from an aminium cation radical to form an iminium cation (Fig 1, pathway D), as proposed by Griffin and Ting (7), and hydrogen atom abstraction from the tertiary amine substrate to form a radical (pathway B), as proposed by Shannon and Bruce (30) and by Kedderis and Hollenberg.1

In contrast to the large isotope effects observed for most of the hemeprotein-catalyzed demethylation reactions, the intramolecular isotope effects for the reaction catalyzed by two isozymes of cytochrome P-450 and chloroperoxidase were small (kH/kD < 3.1) (Table I). The low magnitude of the intramolecular isotope effects during the cytochrome P-450-catalyzed N-demethylation supported by cumene hydroperoxide or O2 and NADPH (kH/kD < 3.1) is similar to the small intramolecular isotope effects reported for the demethylation of several aliphatic (13, 31) and aromatic (32) tertiary amines in microsomes and reconstituted cytochrome P-450 systems. Although no intermolecular isotope effect was observed on the Vm/Km for N,N-dimethylaniline during its demethylation by cytochrome P-450 (32), the intramolecular competition technique used in this study has revealed the low magnitude of the intrinsic isotope effect on the demethylation of this substrate. The low magnitude of the intrinsic isotope effect has also been confirmed by the Northrop technique (11, 12) utilizing deuterium and tritium isotope effect measurements.2

The small intrinsic isotope effect exhibited by the two isozymes of cytochrome P-450 implicates a common deprotonation step following anilinium radical formation (pathways A and C). This conclusion is consistent with the low magnitude of the intrinsic isotope effects associated with deprotonation of the a-carbon of amine cation radicals reported for the oxidation of trimethylamine, benzyl-t-butylamine, and benzylamine by chlorine dioxide (kH/kD = 1.3 to 3.0) (14), the oxidation of trimethylamine by permanganate (kH/kD = 1.8) (15), the oxidation of N-methyldi-n-butylamine by alkaline potassium ferricyanide (kH/kD = 3.6) (16) and electrochemically (kH/kD = 1.7) (17), and the photochemical oxidation of N-methyl-N-trideuteriomethyl-t-butylamine (kH/kD = 2.2) (18). Shono et al. (33) have suggested that cytochrome P-450-catalyzed N-demethylation reactions proceed by initial electron transfer from nitrogen based on the similarity between the intramolecular isotope effects observed during the demethylation of N-methyl-N-trideuteriomethylamine by microsomes (kH/kD = 1.6) and by electrochemical oxidation (kH/kD = 1.9). Moreover, EPR evidence exists for one-electron oxidation of nitrogen by cytochrome P-450 (34).

Recombination of the carbon radical (111) and the nascent iron-bound hydroxyl radical would then lead to the carboxyl-lamine intermediate (V). This is substantiated by the dioxygen source demonstrated for the oxygen in a stable carboxylamine intermediate (2, 3) and aldehyde product (4). The latter, aldehyde oxygen, was trapped from exchange through rapid enzymatic reduction to the alcohol in situ. Moreover, the absence of isotope effects on Vm for N,N-dimethylaniline and N,N-di(trideuteriomethyl)amine (32) and for N,N-di-methylphenylene and N,N-di(trideuteriomethyl)phenyl-
mine (13) in reconstituted cytochrome P-450 systems is in agreement with the view that the rate-limiting step is electron transfer from nitrogen rather than cleavage of the α-carbon—hydrogen bond. Thus, three independent data provide compelling evidence for a mechanism involving deprotonation followed by radical recombination (depicted in Fig. 1 as pathways A, C, and F), while providing no support for the hydrogen atom abstraction–iminium ion mechanism (pathways A, D, and G) proposed by Griffin et al. (35) for the hydroperoxide-supported N-demethylation reactions catalyzed by cytochrome P-450. The results are also inconsistent with a hydrogen atom abstraction–radical recombination mechanism (10) (Fig. 1, pathways B and F), originally demonstrated by Groves et al. (28) for aliphatic carbon oxidation, since this mechanism is characterized by a large isotope effect \( k_{H}/k_{D} = 11.5 \). However, large isotope effects have been observed during cytochrome P-450-catalyzed benzyl oxidation (29) and O-demethylation reactions (36, 37), suggesting that these reactions proceed by a hydrogen atom abstraction mechanism.

The active sites of cytochrome P-450 and chloroperoxidase share several physical properties as evidenced by optical absorption (38), electron paramagnetic resonance (39), magnetic circular dichroism (40), and Mossbauer (41, 42), Raman (43), and extended x-ray absorption fine structure (44) spectroscopic studies. These common properties are thought to be due to thiolate ligation of the heme iron (44). The intramolecular isotope effect for the chloroperoxidase-catalyzed N-demethylation reaction is low \( k_{H}/k_{D} = 2.5 \), demonstrating that this enzyme is unique among the peroxidasinxexamined in catalyzing N-demethylation through a deprotonation step in common with cytochrome P-450. These data provide the first demonstrated similarity in reaction mechanism between chloroperoxidase and cytochrome P-450 and may suggest that the heme iron thiolate moiety, uniquely shared by these enzymes, directs the course of α-carbon—hydrogen elimination in this reaction.

In any enzyme-catalyzed reaction in which carbon—hydrogen bond cleavage is irreversible, the relationship between the intrinsic effect, \( k_{H}/k_{D} \), and the observed isotope effect on \( V_{m}/K_{m} \) is given by the equation:

\[
\frac{V_{m}^H}{V_{m}^D} = \frac{k_{H}/k_{D} + C_{i}}{1 + C_{i}}
\]

where \( C_{i} \) is the commitment to catalysis and represents the tendency of the enzyme-substrate complex to go forward through catalysis as opposed to its tendency to break down to free enzyme and substrate (11, 12). The intermolecular isotope effects of 1 to 3 on the \( V_{m}/K_{m} \) observed during the demethylation reaction catalyzed by horseradish peroxidase B-C (41) are much smaller than the intramolecular isotope effects \( k_{H}/k_{D} = 8.8 \) to 10.1, indicating that the intrinsic isotope effect is substantially suppressed in the observed \( V_{m}/K_{m} \) by a large commitment to catalysis. A large commitment to catalysis indicates that the enzyme-substrate complex is more prone to proceed through the isotopically sensitive catalytic step rather than to dissociate back to unreacted substrate and enzyme. Therefore, the masking of the intrinsic isotope effect, observed by intramolecular determination of \( V_{m}/K_{m} \), for horseradish peroxidase, suggests that the rate of bond cleavage is rapid, relative to the rate of substrate dissociation from the enzyme-substrate complex.

In contrast, the intramolecular isotope effect on the chloroperoxidase-catalyzed demethylation reaction \( k_{H}/k_{D} = 2.5 \) was essentially identical with the intramolecular isotope effect of 2.9 on the \( V_{m}/K_{m} \) for this reaction, demonstrating that the intrinsic isotope effect on carbon—hydrogen bond cleavage is fully expressed on \( V_{m}/K_{m} \). Thus, \( C_{i} \) must be negligible, indicating that the carbon—hydrogen bond cleavage rate must be slow relative to the rate of dissociation of the enzyme-substrate complex.

The mechanistic interpretation of the intermediate intramolecular isotope effect observed for the \( N \)-demethylation reaction catalyzed by catalase \( k_{H}/k_{D} = 5.4 \) (Table I) is presently unclear. The intermediate value of the isotope effect may arise from a less symmetrical transition state than that involved in the other reactions with larger isotope effects. Alternatively, a mixed mechanism, with the participation of both deprotonation (Fig. 1, pathway C) and hydrogen atom abstraction steps (pathways B and/or D), may occur in the demethylation reaction. In this regard, Sayo and Hosokawa have suggested that the active site of catalase for the \( N \)-demethylation of aminopyrine is different than that for the oxidation of methanol or the decomposition of hydrogen peroxide based on inhibition studies (45) and the effects of alkaline denaturation (46). They found that lyophilized or alkaline-denatured catalase could readily catalyze the \( N \)-demethylation of aminopyrine but not the decomposition of hydrogen peroxide (46). These observations suggest that the mechanism of \( N \)-demethylation by catalase may depend upon the orientation of the substrate in the active site.

The use of intramolecular isotope effects for estimating intrinsic isotope effects provides a unique means for investigating deprotonation and hydrogen atom abstraction steps in enzyme-catalyzed reactions. The present application of this technique to differentiate between these possible mechanisms for the \( N \)-demethylation reactions catalyzed by peroxidasex and cytochrome P-450 is the first comparative mechanistic study of a single reaction catalyzed by these enzymes. The results presented here provide the first direct evidence for the occurrence of deprotonation and hydrogen atom abstraction steps in hemeprotein-catalyzed demethylation reactions. Techniques similar to those used here may be of utility in investigating other enzyme-catalyzed reactions.

Acknowledgments.—We would like to acknowledge Dr. Yoshihito Watanabe for valuable discussions and Regina Wang and Lois Arntzen for helpful discussions and Regina Wang and Lois Arntzen for valuable discussions and valuable discussions and Regina Wang and Lois Arntzen for valuable discussions. We also wish to thank Rosa Casamano for the typing of this manuscript.

REFERENCES

Isotope Effects in P-450 and Peroxidase N-Dealkylation Reactions

34. Augusto, O., Belina, H. S., and Ortiz de Montellano, P. R. (1982) J. Biol. Chem. 257, 11288-11295
The use of intramolecular isotope effects to distinguish between deprotonation and hydrogen atom abstraction mechanisms in cytochrome P-450- and peroxidase-catalyzed N-demethylation reactions.

G T Miwa, J S Walsh, G L Kedderis and P F Hollenberg


Access the most updated version of this article at [http://www.jbc.org/content/258/23/14445](http://www.jbc.org/content/258/23/14445)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/258/23/14445.full.html#ref-list-1](http://www.jbc.org/content/258/23/14445.full.html#ref-list-1)