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Peroxidase-Catalyzed N-Demethylation Reactions: Deuterium Solvent Isotope Effects†

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ABSTRACT: The effect of D_2O on the kinetic parameters for the hydroperoxide-supported N-demethylation of *N,N*-dimethylaniline catalyzed by chloroperoxidase and horseradish peroxidase was investigated in order to assess the roles of exchangeable hydrogens in the demethylation reaction. The initial rate of the chloroperoxidase-catalyzed N-demethylation of *N,N*-dimethylaniline supported by ethyl hydroperoxide exhibited a pL optimum (where L denotes H or D) of 4.5 in both H_2O and D_2O. The solvent isotope effect on the initial rate of the chloroperoxidase-catalyzed demethylation reaction was independent of pL, suggesting that the solvent isotope effect is not due to a change in the pK of a rate-controlling ionization in D_2O. The solvent isotope effect on the V_{max} for the chloroperoxidase-catalyzed demethylation reaction was 3.66 ± 0.62. In contrast, the solvent isotope effect on the V_{max} for the horseradish peroxidase catalyzed demethylation reaction was approximately 1.5 with either ethyl hydroperoxide or hydrogen peroxide as the oxidant, indicating that the exchange of hydrogens in the enzyme and hydroperoxide for deuterium in D_2O has little effect on the rate of the demethylation reaction. The solvent isotope effect on the V_{max}/K_M for ethyl hydroperoxide in the chloroperoxidase-catalyzed demethylation reaction was 8.82 ± 1.57, indicating that the rate of chloroperoxidase compound I formation is substantially decreased in D_2O. This isotope effect is suggested to arise from deuterium exchange of the hydroperoxide hydrogen and of active-site residues involved in compound I formation. A solvent isotope effect of 2.96 ± 0.57 was observed on the V_{max}/K_M for *N,N*-dimethylaniline in the chloroperoxidase-catalyzed reaction. This isotope effect is suggested to arise from deuterium exchange of the α-carbon hydrogens of the anilinium cation radical intermediate in the chloroperoxidase-catalyzed demethylation reaction.

Chloroperoxidase (chloride:hydrogen peroxide oxidoreductase; EC 1.11.1.10) and horseradish peroxidase (donor:hydrogen peroxide oxidoreductase; EC 1.11.1.7) catalyze the hydroperoxide-supported N-demethylation of a variety of *N*-methylarylamine compounds (Kedderis et al., 1980; Kedderis & Hollenberg, 1983a). The results of a steady-state kinetic analysis of the chloroperoxidase-catalyzed N-demethylation of *N,N*-dimethylaniline (DMA)[1] supported by ethyl hydroperoxide (EtOOH) were consistent with a ping-pong Bi-Bi kinetic mechanism for the reaction (Kedderis & Hollenberg, 1983b). Initial velocity studies of the horseradish peroxidase catalyzed demethylation of DMA were also consistent with a ping-pong mechanism (Kedderis & Hollenberg, 1983a). In this mechanism, the hydroperoxide reacts with the native peroxidase to form the oxidized enzyme intermediate

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[1] Abbreviations: DMA, *N,N*-dimethylaniline; EtOOH, ethyl hydroperoxide.

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compound I and the corresponding alcohol. Compound I then binds DMA and oxidizes it, resulting in the formation of formaldehyde and *N*-methylaniline and regeneration of the native peroxidase. We have previously reported the kinetic isotope effects on the demethylation of DMA catalyzed by chloroperoxidase and horseradish peroxidase when the *N*-methyl groups of DMA were deuterated (Kedderis & Hollenberg, 1984a). The observation of significant isotope effects on the V_{max}/K_M for the hydroperoxide substrate with *N*,*N*-bis(trideuteriomethyl)aniline suggested that the peroxidase-catalyzed demethylation reaction proceeds by a mechanism in which hydrogen (or deuterium) is transferred from DMA to the enzyme and must subsequently be displaced by the hydroperoxide on the next turnover. In order to elucidate the roles of exchangeable hydrogens in the peroxidase-catalyzed demethylation of DMA, we have investigated the solvent isotope effects on the kinetic parameters of the reaction. The large solvent isotope effects observed for the chloroperoxidase-catalyzed demethylation reaction are interpreted to arise from deuterium exchange of the hydroperoxide hydrogen and of active-site residues involved in compound I formation, as well as from deuterium exchange of the anilinium cation radical intermediate in the demethylation of DMA.

Experimental Procedures

Enzyme Preparation. Chloroperoxidase was isolated and purified from *Caldariomyces fumago* as reported previously (Hollenberg & Hager, 1978). The preparations used for these studies had specific activities greater than 2000 units/mg of protein in the standard chlorination assay and exhibited A_{403}/A_{280} ratios greater than 1.40, indicating that the enzyme preparations were at least 95% pure. Protein concentrations were determined according to the method of Lowry et al. (1951).

Crude horseradish peroxidase (type I, A_{403}/A_{280} = 0.3), obtained from Sigma Chemical Co., was purified by a modification of the procedure of Shannon et al. (1966) as previously described (Hollenberg et al., 1974). The horseradish peroxidase (B,C) isozyme was used for these studies and had an A_{403}/A_{280} ratio greater than 3.2. Horseradish peroxidase concentrations were determined by using the molar absorbance indexes reported by Shannon et al. (1966).

Materials. *N*,*N*-Dimethylaniline (redistilled before use) and 2,4-pentanedione (gold label) were obtained from Aldrich Chemical Co. D_2O, KOD, and D_3PO_4 were obtained from Sigma Chemical Co. Ethyl hydroperoxide (10%) and ammonium acetate (ultrapure) were obtained from Polysciences Inc. Hydrogen peroxide (30%) was obtained from Fisher Scientific Co. All other materials were reagent grade and obtained from commerical sources. The hydroperoxide concentrations were determined by iodometric titration (Silbert & Swern, 1958).

Preparation of Buffers. Phosphate buffers were prepared by dissolving the appropriate amount of sodium phosphate (monobasic) in glass-distilled water and titrating with concentrated potassium hydroxide solution. Phosphate buffers below pH 4.5 were prepared by diluting the appropriate amount of phosphoric acid in glass-distilled water and titrating with concentrated potassium hydroxide solution. All pH measurements were made on a Corning Model 110 digital pH meter equipped with a Sensorex combination electrode. Deuterium oxide buffers were prepared by dissolving the appropriate amount of sodium phosphate (monobasic) in D_2O (>99.8 atom % D) and titrating with KOD (40% solution in D_2O, 98 atom % D). For buffers below pD 4.5, D_3PO_4 (85% solution in D_2O, 99 atom % D) was diluted in D_2O and titrated with KOD. The pD of the buffers was determined by using the equation $pD = pH_{obsd} + 0.4$ (Glasoe & Long, 1960), where pH_{obsd} is the pH of the buffer as measured with a standard pH electrode. The pL (where L denotes H or D) of the buffers was not affected by addition of the enzymes or substrates.

Assay for N-Demethylation. The *N*-demethylase activities of chloroperoxidase and horseradish peroxidase were assayed by measuring the amount of formaldehyde formed by using a modification of the procedure of Nash (1953) as previously described (Kedderis et al., 1980). In the pL range from 4.5 to 7.0, *N*,*N*-dimethylaniline was added to the 3-mL reaction mixtures in less than 15 μL of acetone. In the pL range from 3.0 to 4.5, *N*,*N*-dimethylaniline was added to the reaction mixtures in phosphate buffer titrated to the appropriate pL with KOH or KOD. The reactions were initiated by the addition of the peroxidase, incubated at 25 °C for the times indicated, and terminated by the addition of 0.75 mL of 60% trichloroacetic acid. The horseradish peroxidase catalyzed reactions where the concentration of *N*,*N*-dimethylaniline was varied were incubated for 5 min. The chloroperoxidase-catalyzed reactions were incubated for 10 min at pL 3.5–6.0 and for 5 min at pL 3.0, 6.5, and 7.0. In preliminary studies, the time course for product formation was determined at each pL, and the incubation times selected for further studies at each pL value were within the linear portion of product formation vs. time, and therefore, the results are initial rates. A 1-mL aliquot of the terminated reaction mixture was incubated with 0.5 mL of the Nash reagent as previously described (Kedderis et al., 1980), and the absorbance of the resulting conjugate was read at 421 nm on a Gilford 2400-S UV–visible spectrophotometer. The horseradish peroxidase catalyzed reactions where the concentrations of the hydroperoxide substrates were varied were incubated for 3 min, and formaldehyde formation was quantitated by using the fluorescent assay as previously described (Kedderis & Hollenberg, 1983a). Formaldehyde formation from the chloroperoxidase-catalyzed reactions in D_2O was also quantitated by the fluorescent assay. For the fluorescent assay, the reactions were terminated by the addition of 0.45 mL of 60% trichloroacetic acid. A 1.5-mL aliquot of the terminated reaction mixture was incubated with 0.75 mL of the Nash reagent as previously described (Kedderis & Hollenberg, 1983a), and the fluorescence of the resulting lutidine derivative was read in an Aminco-Bowman spectrophotofluorometer using an excitation wavelength of 410 nm, and the emission at 500 nm was measured. 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 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 standard errors on the isotope effects were determined by propagation of error of the measured values.

Results

Solvent Isotope Effects on Chloroperoxidase-Catalyzed N-Demethylation. The substitution of D_2O for H_2O in the chloroperoxidase-catalyzed N-demethylation of DMA results in a marked inhibition of the reaction, giving a solvent isotope effect of 3.6 (Kedderis et al., 1980). To investigate the possibility that the solvent isotope effect was due to a shift in the p*K* of a rate-controlling ionization in D_2O, the effect of pL (where L denotes H or D) on the solvent isotope effect was

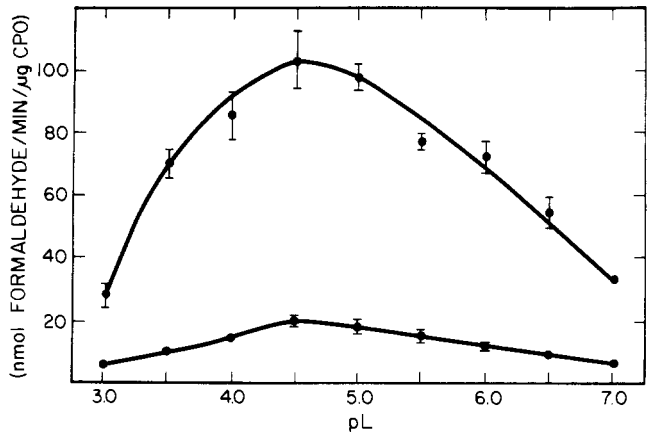

FIGURE 1: Initial rate of the demethylation reaction at saturating substrate concentrations as a function of pL. The reaction mixtures were adjusted to the indicated pH (upper curve) or pD (lower curve) values. The reactions were run in sodium potassium phosphate buffer (0.5 M), pL as indicated, in a final volume of 3 mL. The concentrations of *N*,*N*-dimethylaniline were 48.30 mM at pL 3.0, 29.15 mM at pL 3.5, 13.43 mM at pL 4.0, 5.27 mM at pL 4.5, 1.58 mM at pL 5.0, 1.05 mM at pL 5.5, 0.53 mM at pL 6.0, and 0.13 mM at pL 6.5 and 7.0. The concentrations of ethyl hydroperoxide were 3.22 mM at pL 3.0 and 5.5, 5.36 mM at pL 3.5–5.0, 2.14 mM at pL 6.0 and 6.5, and 1.61 mM at pL 7.0. The concentrations of chloroperoxidase were 0.895 μg at pL 3.0, 0.29 μg at pL 3.5–6.5, and 0.45 μg at pL 7.0. The incubation conditions and formaldehyde determinations were the same as described under Experimental Procedures. The data are presented as the mean values plus or minus standard errors from four determinations.

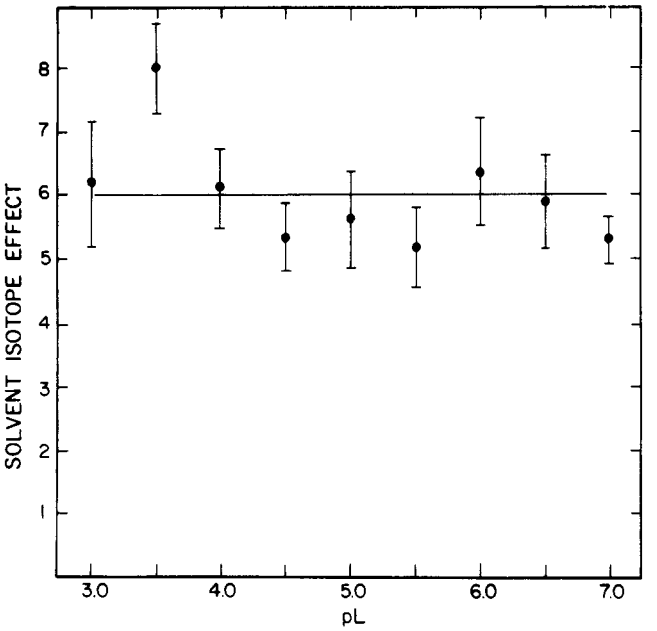

FIGURE 2: Solvent isotope effect on the initial rate of the demethylation reaction as a function of pL. The solvent isotope effect at a given pL was calculated from v_{H_2O}/v_{D_2O} by using the data in Figure 1.

studied throughout the pL range of 3.0–7.0. The substrate concentrations used were previously determined to be saturating in H_2O (Kedderis & Hollenberg, 1984b). As shown in Figure 1, the initial rate of the demethylation reaction exhibited a pL optimum at 4.5 in both H_2O and D_2O. When the solvent isotope effects on the initial rates were determined (v_{H_2O}/v_{D_2O}) and plotted as a function of pL (Figure 2), they were found to be essentially independent of pL, suggesting that the solvent isotope effect on the demethylation reaction was due to an effect on the rate of catalysis rather than to the shifting of the pK of a rate-controlling ionization in D_2O. Throughout the entire pL range, the solvent isotope effect on the initial velocity of the demethylation reaction was approximately 6.0 under these conditions. At pL 3.5, the solvent isotope effect appeared to be somewhat larger (7.98), but application of a Student's t test to the data suggested that this difference was not statistically significant ($p > 0.05$).

Table I: Solvent Isotope Effects on Chloroperoxidase-Catalyzed Demethylation of *N*,*N*-Dimethylaniline[a]

condn	$V_{max}/K_{M\,DMA}$ [nmol of HCHO min^{-1} (μg of CP)$^{-1}$ mM^{-1}]	$V_{max}/K_{M\,EtOOH}$ [nmol of HCHO min^{-1} (μg of CP)$^{-1}$ mM^{-1}]	V_{max} [nmol of HCHO min^{-1} (μg of CP)$^{-1}$]
H_2O	928.4 ± 145.6	91.9 ± 14.5	81.7 ± 12.8
D_2O	314.1 ± 21.1	10.4 ± 1.1	22.3 ± 1.5
isotope effect	2.96 ± 0.57	8.82 ± 1.57	3.66 ± 0.62

[a] The reactions were run in sodium potassium phosphate buffer (0.5 M), pL 6.0, under the conditions described under Experimental Procedures and assayed for formaldehyde formation by the Nash assay. CP designates chloroperoxidase.

Table II: Solvent Isotope Effects on the Kinetic Parameters for Horseradish Peroxidase Catalyzed Demethylation Supported by Ethyl Hydroperoxide[a]

condn	$V_{max}/K_{M\,DMA}$ [nmol of HCHO min^{-1} (μg of HRP)$^{-1}$ mM^{-1}]	$V_{max}/K_{M\,EtOOH}$ [nmol of HCHO min^{-1} (μg of HRP)$^{-1}$ mM^{-1}]	V_{max} [nmol of HCHO min^{-1} (μg of HRP)$^{-1}$]
H_2O	453.4 ± 71.6	7653.5 ± 1207.9	154.6 ± 24.4
D_2O	628.3 ± 51.3	5112.7 ± 417.1	104.3 ± 8.5
isotope effect	0.72 ± 0.13	1.50 ± 0.38	1.48 ± 0.26

[a] The reactions were run in sodium potassium phosphate buffer (0.4 M), pL 6.0, under the conditions described under Experimental Procedures and assayed for formaldehyde formation by the Nash assay. HRP designates horseradish peroxidase.

The exchange of hydrogen for deuterium in D_2O can occur on chloroperoxidase, and the hydroperoxide hydrogen of EtOOH is also freely exchangeable. The only readily exchangeable hydrogens on DMA are those at the ortho and para ring positions (Tice et al., 1963), which would not be expected to be involved in the demethylation reaction. The kinetic parameters for the demethylation reaction were determined in D_2O at pD 6.0 to investigate the origin(s) of the solvent isotope effect. As shown in Figure 3, normal Michaelis–Menten saturation kinetics were observed in D_2O. The kinetic parameters and solvent isotope effects for the chloroperoxidase-catalyzed demethylation reaction are shown in Table I.

Solvent Isotope Effects on Horseradish Peroxidase Catalyzed N-Demethylation. The substitution of D_2O for H_2O in the horseradish peroxidase catalyzed N-demethylation of DMA supported by either EtOOH or hydrogen peroxide was previously found to produce solvent isotope effects of less than 2 on the initial rate of the reaction (Kedderis & Hollenberg, 1983a). The kinetic parameters and solvent isotope effects for the horseradish peroxidase catalyzed demethylation of DMA are shown in Tables II and III with EtOOH and hydrogen peroxide as the respective oxidants. Normal Michaelis–Menten saturation kinetics wee observed in D_2O in all cases (data not shown).

Discussion

Although D_2O usually shifts the pK of a monoprotic acid about 0.4 unit higher (Klinman, 1978), the initial velocity curves for the chloroperoxidase-catalyzed N-demethylation of DMA in H_2O and D_2O both exhibited optima at pL 4.5 (Figure 1), and the calculated solvent isotope effects were

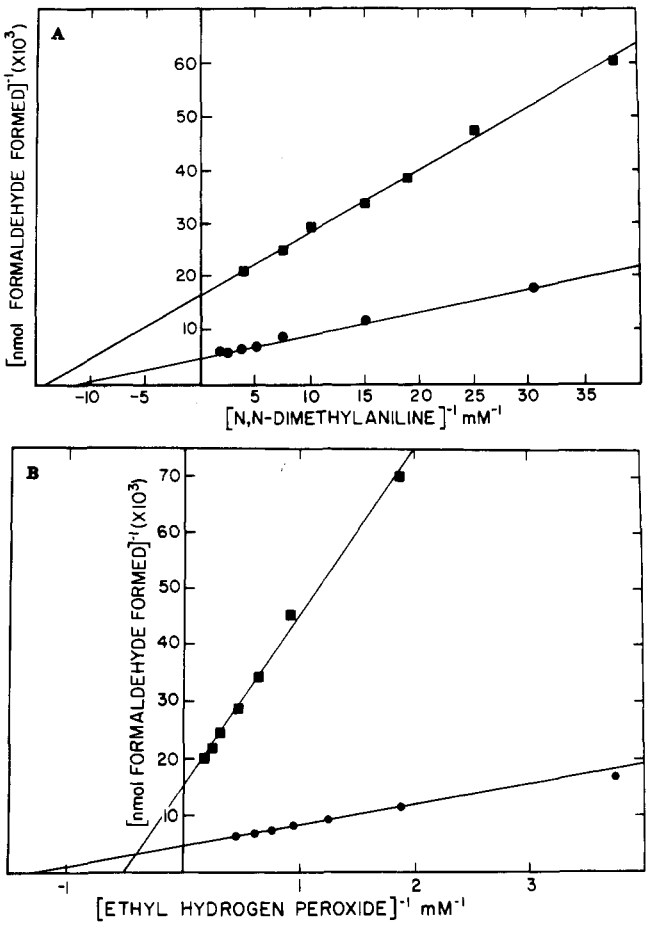

FIGURE 3: Double-reciprocal plots of the initial rate of the demethylation of DMA by chloroperoxidase in H_2O and D_2O. The 3-mL reaction mixtures contained 0.5 M sodium potassium phosphate buffer, pL 6.0, and 0.29 μg of chloroperoxidase. (A) The concentrations of DMA were varied as indicated in the presence of 1.61 mM EtOOH in H_2O (●) or 5.36 mM EtOOH in D_2O (■). (B) The concentrations of EtOOH were varied as indicated in the presence of 1.05 mM DMA in H_2O (●) or 0.26 mM DMA in D_2O (■). The reactions were initiated by addition of the enzyme and incubated for 10 min at 25 °C. Formaldehyde formation was determined by using the Nash assay in H_2O and the fluorescent assay in D_2O as described under Experimental Procedures.

Table III: Solvent Isotope Effects on the Kinetic Parameters for Horseradish Peroxidase Catalyzed Demethylation Supported by Hydrogen Peroxide[a]

condn	$V_{max}/K_{M\,DMA}$ [nmol of HCHO min^{-1} (μg of HRP)$^{-1}$ mM^{-1}]	$V_{max}/K_{M\,H_2O_2}$ [nmol of HCHO min^{-1} (μg of HRP)$^{-1}$ mM^{-1}]	V_{max} [nmol of HCHO min^{-1} (μg of HRP)$^{-1}$]
H_2O	603.8 ± 28.0	28740.0 ± 1033.6	431.1 ± 15.5
D_2O	445.1 ± 28.2	24211.4 ± 1545.5	297.8 ± 19.0
isotope effect	1.36 ± 0.11	1.19 ± 0.22	1.45 ± 0.11

[a] The reactions were run in sodium potassium phosphate buffer (0.4 M), pL 5.5, under the conditions described under Experimental Procedures and assayed for formaldehyde formation by the Nash assay. HRP designates horseradish peroxidase.

found to be independent of pL within experimental error (Figure 2), suggesting that the solvent isotope effects were not due to a shift in the pK of a rate-controlling ionization. An analysis of the effect of pH on the kinetic parameters for the chloroperoxidase-catalyzed demethylation of DMA indicated that the rate of the reaction is controlled by two ionizations on the enzyme with pK's of approximately 3.1 and 6.8 (Kedderis & Hollenberg, 1984b). Although it appears that

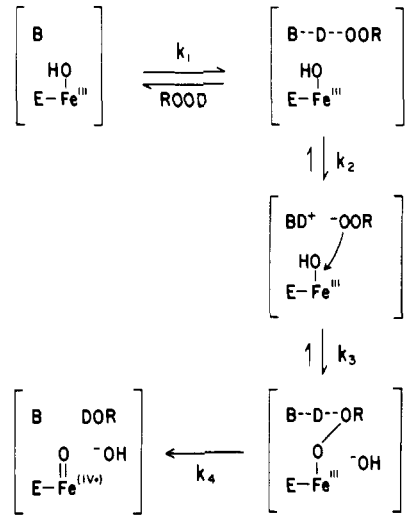

FIGURE 4: Mechanism of peroxidase compound I formation in D_2O. Abbreviations: E, enzyme; ROOD, hydroperoxide substrate; B, basic active-site residue.

there was no significant shift in the pK's of the two rate-controlling ionizations for the chloroperoxidase-catalyzed demethylation reaction in D_2O, a rigorous analysis of the pD dependence of the steady-state kinetic parameters was not performed. Such an analysis might reveal a pK shift which was not detectable in the initial velocity curves, particularly if the shift were small (<0.4 unit) and expressed in the V_{max}/K_M for the two substrates rather than in the V_{max}.

Large solvent isotope effects were observed for the chloroperoxidase-catalyzed demethylation reaction (Table I). The solvent isotope effect of 8.82 on the V_{max}/K_M for EtOOH indicates that the rate of formation of chloroperoxidase compound I is substantially decreased in D_2O. This large isotope effect is most likely a consequence of deuterium exchange of the hydroperoxide hydrogen, as illustrated in Figure 4. The mechanism shown in Figure 4 is consistent with current knowledge of peroxidase compound I formation (Jones & Dunford, 1977; Dunford & Araiso, 1979). Compound I formation is proposed to occur by the following steps: (a) binding of the hydroperoxide to the native peroxidase (with k_1); (b) active-site ionization of the hydroperoxide (with k_2) to form the more nucleophilic hydroperoxy anion (Dunford & Araiso, 1979); (c) nucleophilic attack of the iron by the hydroperoxy anion (with k_3); (d) oxidation of the iron and oxygen–oxygen bond scission (with k_4) to form compound I and the corresponding alcohol. Deuterium exchange of the hydroperoxide hydrogen would result in isotope effects on the active-site ionization (k_2) and oxidation (k_4) steps. It is also possible that active-site residues which assist in compound I formation by hydrogen bonding of the hydroperoxide may exchange hydrogen for deuterium and contribute to the observed isotope effect on the V_{max}/K_M for EtOOH. These conclusions are supported by the observation of a solvent isotope effect of 12.65 on the initial rate of oxygen evolution from EtOOH catalyzed by chloroperoxidase under comparable conditions.[2] This reaction involves the reduction of a second molecule of EtOOH by chloroperoxidase compound I (Thomas et al., 1970).

In contrast to chloroperoxidase, the small magnitude of the solvent isotope effects on the kinetic parameters for the horseradish peroxidase catalyzed demethylation reaction (Tables II and III) indicates that deuterium exchange of hydrogens in the enzyme and hydroperoxide substrate has little effect on

[2] G. L. Kedderis and P. F. Hollenberg, unpublished observations.

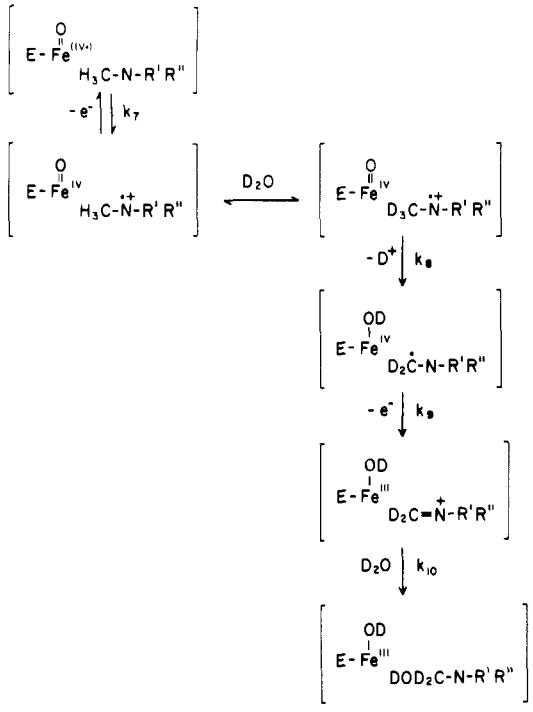

FIGURE 5: Mechanism of oxidative N-demethylation of DMA by chloroperoxidase compound I. Abbreviations: E, enzyme; R″R′–N–CH_3, *N*-methylarylamine substrate.

the rate of the demethylation reaction. The small solvent isotope effects are consistent with the studies of Dunford et al. (1978) on the effect of D_2O on the rate of horseradish peroxidase compound I formation and on the rate of substrate oxidation by compounds I and II. They observed a solvent isotope effect of approximately 1.6 on compound I formation over a pL range of 4–10 and interpreted the effect as arising from deuterium exchange on hydrogen peroxide. Dunford et al. (1978) have also investigated the effect of D_2O on the rates of oxidation of ferrocyanide, *p*-aminobenzoic acid (Hubbard et al., 1975), *p*-cresol, and iodide (Critchlow & Dunford, 1972) by horseradish peroxidase compounds I and II. They found that only reducing substrates which possessed an exchangeable hydrogen at the locus of oxidation exhibited a decreased rate of oxidation in D_2O.

A solvent isotope effect of 2.96 was observed on the V_{max}/K_M for DMA in the chloroperoxidase-catalyzed demethylation reaction (Table I). This effect was not anticipated since the hydrogen atoms on the *N*-methyl groups of DMA are not acidic enough to exchange with the solvent (Tice et al., 1963). A possible explanation for the solvent isotope effect is suggested by the results of a recent intramolecular isotope effect study of the chloroperoxidase-catalyzed demethylation of DMA (Miwa et al., 1983), which suggest that α-carbon–hydrogen-bond cleavage proceeds via an electron-transfer–deprotonation mechanism as shown in Figure 5. In this mechanism, DMA reversibly transfers an electron (with k_7) to chloroperoxidase compound I to form an anilinium cation radical, followed by a slow deprotonation (with k_8) of the cation radical to form a neutral DMA radical. The DMA radical transfers an electron (with k_9) to chloroperoxidase compound II, forming an iminium cation which can react (with k_{10}) with water to form the unstable carbinolamine of DMA (Kedderis & Hollenberg, 1984a). Because the α-hydrogens of aminium cation radicals are more acidic than those of the amine itself (Masui & Sayo, 1971; Audeh & Lindsay Smith, 1971), they could exchange with D_2O to produce the observed solvent isotope effect on the deprotonation step (k_8 in Figure 5). This explanation is supported by the virtual identity of the magnitudes of the solvent isotope effect on the V_{max}/K_M for DMA (2.96) and the intermolecular isotope effect (2.99) produced by *N*,*N*-bis(trideuteriomethyl)aniline (Kedderis & Hollenberg, 1984a). This result would be predicted by an electron-transfer–deprotonation mechanism of carbon–hydrogen-bond cleavage since deprotonation would be the common isotope-sensitive step in D_2O and with deuterated DMA. The mechanism shown in Figure 5 also predicts that the *N*-methylaniline formed during the demethylation of DMA in D_2O will contain some deuterium in the *N*-methyl group. Experiments are currently in progress to test this hypothesis using NMR spectroscopy.

The ferric deuteroxide species shown in Figure 5 was first proposed to explain the observation of significant isotope effects on the V_{max}/K_M for the hydroperoxide substrate in the N-demethylation of *N*,*N*-bis(trideuteriomethyl)aniline catalyzed by chloroperoxidase and horseradish peroxidase (Kedderis & Hollenberg, 1984a). The oxidation of *N*,*N*-bis(trideuteriomethyl)aniline was proposed to result in the formation of a ferric deuteroxide species by a mechanism similar to that shown in Figure 5. The isotope effects on the V_{max}/K_m for the hydroperoxide substrate were suggested to arise from the displacement of a deuteroxide during compound I formation as opposed to a hydroxide when DMA is the substrate (Kedderis & Hollenberg, 1984a). The postulated exchange of the α-carbon hydrogens of the DMA cation radical for deuterium in D_2O during chloroperoxidase catalysis would result in the formation of a ferric deuteroxide species (Figure 5), which would also contribute to the solvent isotope effect on the V_{max}/K_M for EtOOH (Table I) via the nucleophilic displacement step (k_3 in Figure 4) in the formation of compound I.

In contrast to the electron-transfer–deprotonation mechanism for chloroperoxidase, the N-demethylation of DMA by horseradish peroxidase has been suggested to proceed by initial hydrogen atom abstraction (Miwa et al., 1983; Kedderis & Hollenberg, 1984a), which would directly produce the neutral DMA radical without an anilinium cation radical intermediate. Consistent with this mechanism, significant solvent isotope effects were not observed on the V_{max}/K_M for DMA in the horseradish peroxidase catalyzed demethylation reaction (Tables II and III). The events following the formation of the DMA radical would be the same as those shown in Figure 5 (k_9 and k_{10}), resulting in the formation of a ferric hydroxide species. Significant deuterium exchange of the ferric hydroxide species in D_2O would contribute to the solvent isotope effect on the V_{max}/K_M for the hydroperoxide substrate. However, the solvent isotope effects on this parameter (Tables II and III) are smaller than the isotope effects which were observed with *N*,*N*-bis(trideuteriomethyl)aniline (Kedderis & Hollenberg, 1984a), suggesting that the ferric hydroxide species does not exchange with the solvent during the steady state of the horseradish peroxidase catalyzed demethylation reaction.

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Fluorine NMR Studies on Stereochemical Aspects of Reactions Catalyzed by Transcarboxylase, Pyruvate Kinase, and Enzyme I†

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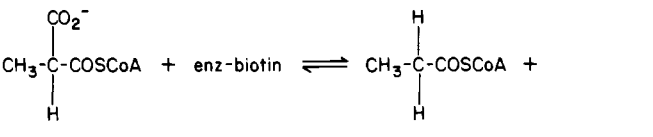
ABSTRACT: The stereochemistry of the transcarboxylase-catalyzed carboxylation of 3-fluoropyruvate has been studied by using fluorine NMR of unpurified reaction mixtures. When the product 3-fluorooxaloacetate was trapped by using malate dehydrogenase, only the 2*R*,3*R* diastereomer of 3-fluoromalate was formed. The fluoromethyl group of fluoropyruvate does not take up deuterium label from the solvent during the reaction. These results confirm and extend those obtained previously by Walsh and co-workers [Goldstein, J. A., Cheung, Y. F., Marletta, M. A., & Walsh, C. (1978) *Biochemistry 17*, 5567–5575] showing that transcarboxylase is specific for one of the two prochiral hydrogens in fluoropyruvate. Transcarboxylase, coupled to malate dehydrogenase, has been used to analyze samples of chiral fluoropyruvate obtained by dephosphorylation of (*Z*)-fluorophosphoenolpyruvate in D_2O in the presence of either pyruvate kinase or enzyme I from the *Escherichia coli* sugar transport systems. Analysis of the fluoromalate produced showed that fluoroenolpyruvate is deuterated from opposite faces by these two enzymes: enzyme I protonates (deuterates) fluoroenolpyruvate exclusively from the 2-*re* face and pyruvate kinase does so mainly from the 2-*si* face. Fluoropyruvate is carboxylated by transcarboxylase with absolute retention of configuration.

Transcarboxylase (methylmalonyl-CoA:pyruvate carboxyltransferase, EC 2.1.3.1) is a biotin-containing multimeric enzyme that has been purified from *Propionibacterium shermanii* (Wood et al., 1969). The transfer of CO_2 from methylmalonyl coenzyme A (methylmalonyl-CoA) to pyruvate involves two half-reactions 1 and 2, which take place on different subunits of the enzyme. Biotin, which is linked to the ε-amino group of a lysyl residue, carries the CO_2 from one subunit to another. The reaction sequence in eq 1 and 2 has been supported by results from kinetic studies (Northrop & Wood, 1969a,b) and by the isolation and subsequent utilization



(1)

$$CH_3\text{–}CO\text{–}CO_2^- + \text{enz–biotin–}CO_2^- \rightleftharpoons {}^-O_2C\text{–}CH_2\text{–}CO\text{–}CO_2^- + \text{enz–biotin} \quad (2)$$

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