Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 4295–4299, April 1997
Biochemistry

Microperoxidase/H_2O_2-mediated alkoxylating dehalogenation of halophenol derivatives in alcoholic media

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Communicated by Vincent Massey, University of Michigan School of Medicine, Ann Arbor, MI, February 18, 1997 (received for review November 15, 1996)

ABSTRACT **The results of this study report the H_2O_2-driven microperoxidase-8 (MP8)-catalyzed dehalogenation of halophenols such as 4-fluorophenol, 4-chlorophenol, 4-bromophenol, and 2-fluorophenol in alcoholic solvents. In methanol, the conversion of the para-halophenols and 2-fluorophenol to, respectively, 4-methoxyphenol and 2-methoxyphenol, as the major dehalogenated products is observed. In ethanol, 4-ethoxyphenol is the principal dehalogenated product formed from 4-fluorophenol. Two mechanisms are suggested for this MP8-dependent alkoxylating dehalogenation reaction. In one of these mechanisms the oxene resonant form of compound I of MP8 is suggested to react with methanol forming a cofactor-peroxide-alkyl intermediate. This intermediate reacts with the reactive π-electrons of the substrate, leading to the formation of the alkoxyphenols and the release of the fluorine substituent as fluoride anion.**

Microperoxidase-8 (MP8) is a fragment obtained from the enzymatic digestion of horse-heart cytochrome *c*. It consists of a heme attached to eight amino acids through thioether bonds. It is known that MP8 reacts with hydrogen peroxide to form intermediate compounds that could be analogous to compounds I and II of horseradish peroxidase (1, 2), although their exact nature remains to be unequivocally proved.

It has been shown that MP8 is able to convert a wide variety of organic compounds at the expense of hydrogen peroxide in a peroxidase type of reaction chemistry (3–5). Furthermore, recent work has demonstrated that MP8, especially in the presence of ascorbic acid, could, like cytochromes P450, mediate oxygen transfer reactions from hydrogen peroxide to substrates (6, 7). More recently, we showed that the MP8/H_2O_2 system catalyzes the dehalogenation of para-halogenated phenols, resulting in the formation of *p*-benzoquinones as the primary and only dehalogenated products (19). Experiments with ^{18}O-labeled $H_2{}^{18}O_2$ and $H_2{}^{18}O$ suggested the involvement of the solvent (water) in the mechanism of the H_2O_2-driven MP8-catalyzed dehalogenation.

In the present communication, the MP8/H_2O_2 catalyzed dehalogenation of 4-fluorophenol, 4-chlorophenol, 4-bromophenol, and 2-fluorophenol was investigated in nonaqueous medium, that is, solvents such as methanol and ethanol. The results obtained indicate that the MP8/H_2O_2 system catalyzes the dehalogenation of 4-fluorophenol, 4-chlorophenol, 4-bromophenol, and 2-fluorophenol in methanol and ethanol by a mechanism different from that in water, resulting in the formation of alkoxyphenols as the major dehalogenated products instead of benzoquinones. Two mechanisms that account for the formation of these products are proposed.

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0027-8424/97/944295-5$2.00/0
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MATERIALS AND METHODS

Chemicals. MP8 was prepared by the enzymatic digestion of horse-heart cytochrome *c* according to the procedure reported in the literature (8), and its concentration was determined by the chromogen method (8). 4-Fluorophenol, 4-chlorophenol, 4-bromophenol, 4-methoxyphenol, and sodium methoxide were purchased from Janssen Chimica (Beerse, Belgium). 2-Methoxyphenol, 3-methoxyphenol, 2-ethoxyphenol, 4-ethoxyphenol, hydroquinone, and *p*-benzoquinone were obtained from Aldrich. Hydrogen peroxide (vol/vol, 30%) was from Merck and was diluted before use either in methanol or in ethanol to obtain a 50 mM stock solution.

Incubation Conditions. In 1.8 ml of methanol (or ethanol), freeze-dried MP8 (136 μM final concentration), and 0.1 M (final concentration) of the halophenol substrate were dissolved. This reaction mixture was preincubated at 37°C for 2 min. The reaction was started by the addition of hydrogen peroxide stock solution diluted in the corresponding alcohol (2.5 mM final concentration). After 1-min incubation, the reaction was terminated by cooling the samples on ice. The incubation time was taken as 1 min because of the catalytic instability of MP8 (6, 9). This reaction mixture was then concentrated by evaporation to about 50 μl (methanol incubations) or about 20 μl (ethanol incubations).

In addition, the following control experiments were made. (*i*) MP8 was incubated with 4-fluorophenol using the same conditions as described above, but omitting hydrogen peroxide or MP8. (*ii*) MP8 (136 μM) was incubated with equimolar concentrations of hydroquinone and *p*-benzoquinone (10 mM final concentration) both in the absence and the presence of hydrogen peroxide (2.5 mM final concentration). Besides, these experiments with hydroquinone and *p*-benzoquinone were performed both under aerobic as well as anaerobic conditions. (*iii*) To investigate whether the alkoxylating dehalogenation might proceed through intermediate formation of methoxide as the reactive species, 0.1 M of 4-fluorophenol (final concentration) was incubated with 0.1 M (final concentration) of sodium methoxide in methanol. (*iv*) A control experiment to check whether there is an exchange of *p*-benzoquinone oxygen atoms with methanol was carried out by incubating 1.1 mM of *p*-benzoquinone in methanol. All these control reaction mixtures were incubated at 37°C for 1 min. Each of these reaction mixtures was concentrated by evaporation to about 50 μl and analyzed by gas chromatography-mass spectrometry (GC-MS) and in some cases by HPLC (as indicated).

Formaldehyde Determination. The concentration of the formaldehyde formed in the MP8-catalyzed alkoxylating dehalogenation of 4-fluorophenol was performed as described by Nash (10).

HPLC. A volume of 5 μl of each of the concentrated samples obtained from the reaction mixtures of either the para-

Abbreviations: MP8, microperoxidase-8; GC-MS, gas chromatography-mass spectrometry.

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halogenated phenol (4-fluorophenol, 4-chlorophenol, 4-bromophenol) or of 2-fluorophenol with MP8/H_2O_2 incubated in methanol or ethanol was diluted with 25 μl 6 M HCl acid aqueous solution containing ascorbic acid (4 mM final concentration). Ascorbic acid was added to inhibit the MP8-mediated dehalogenation of para-halogenated phenols known to occur in aqueous solution, but fully inhibited by ascorbic acid (19). Acidification improves the resolution of the HPLC peaks of the products from those of the substrates. From each of these diluted samples 10 μl was loaded onto a Lichrosorb RP8 column (100 $\times$ 3 mm). Elution was carried out at 1 ml/min starting with 100% water, maintaining 100% water for 1 min, followed by a linear gradient to give 27.7% of methanol in water in 10 min. UV detection was performed with a Waters 996 diode array detector.

Products were identified by comparing retention time and UV spectrum of product peaks to those of reference compounds (e.g., 2-methoxyphenol, 4-methoxyphenol, and 4-ethoxyphenol).

Analysis by GC-MS. Of the reference compounds dissolved in methanol or ethanol and of each of the concentrated samples obtained from the incubations in methanol and in ethanol 5 μl were injected into the GC-MS system.

The gas chromatograph (Hewlett–Packard model 5890) was equipped with a 30 m $\times$ 250 mm capillary DB 17 column. The carrier gas was helium at a flow rate of 20 ml/min. A temperature gradient from 70 to 200°C in 13 min was applied. The column was connected to a Hewlett–Packard model 59870 mass spectrometer.

RESULTS

Recent investigations on the MP8/H_2O_2-dependent dehalogenation of para-halogenated phenols, carried out in aqueous solutions, showed *p*-benzoquinone as the only dehalogenated product formed (19). Because the solvent (water) was shown to be involved in the reaction mechanism, we further extended the studies on MP8-dependent dehalogenation of halophenols to other media, namely, alcoholic solvents. Interestingly, in methanol and ethanol formation of *p*-benzoquinone was no longer observed.

When the MP8/H_2O_2 system was incubated with either of the para-halogenated phenols (4-fluorophenol, 4-chlorophenol, 4-bromophenol) or 2-fluorophenol in methanol, the main products formed were identified, respectively, as 4-methoxyphenol and 2-methoxyphenol. The formation of these products was identified by GC-MS and HPLC analysis, using authentic standards. A typical gas chromatogram of the reaction mixture obtained when 4-fluorophenol is incubated with MP8/H_2O_2 in methanol is presented in Fig. 1*A*. The peak with the retention time of 11.5 min can be identified as 4-methoxyphenol. The mass spectra of this product is shown in Fig. 1*B*. The results obtained from the HPLC analysis of the same reaction mixture confirms the formation of 4-methoxyphenol. Similarly, formation of 4-methoxyphenol from 4-chlorophenol and 4-bromophenol and formation of 2-methoxyphenol from 2-fluorophenol were obtained (data not shown). The rate of conversion of 4-fluorophenol was found to be about 170- and 1,000-fold more than the rates of conversion of 4-chlorophenol and 4-bromophenol, respectively. This result indicates that the ease of halogen elimination decreases in the order F > Cl > Br. This trend of halogen elimination is also observed for the MP8-dependent dehalogenation reaction of the same substrates in aqueous solutions (19); however, the differences observed for the ease of halogen substituent elimination are more pronounced in the alcoholic solvents. No product formation was observed when control experiments were performed in methanol, omitting either MP8 or H_2O_2 (data not shown). Similarly, no reaction of *p*-benzoquinone with methanol was observed when a control experiment was performed by incubating

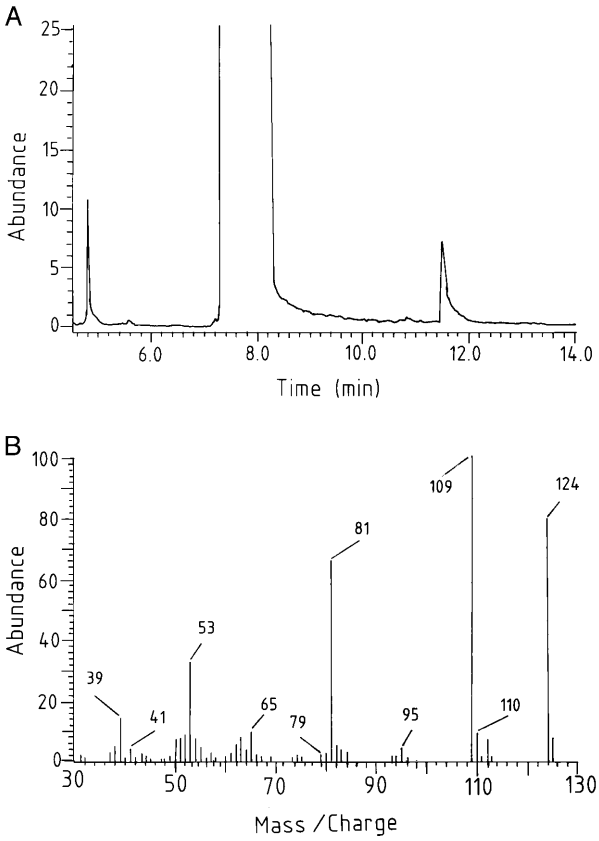

FIG. 1. (*A*) Gas chromatogram of the concentrated sample obtained from the incubation of MP8/H_2O_2 with 4-fluorophenol in methanol. The peak with the retention time of 4.8 min is an impurity present in the substrate preparation and was identified as 2-fluorophenol. (*B*) Mass spectrum of peak with retention time of 11.5 min identified as 4-methoxyphenol (*A*) and obtained when the MP8-dependent dehalogenation of 4-fluorophenol was performed in methanol. The yield of this product was 0.5 mM (0.5%).

p-benzoquinone in methanol for a time period equal to that used for the MP8-catalyzed reaction. This latter experiment was essential to investigate whether the formation of the alkoxyphenol might result from a reaction of *p*-benzoquinone with methanol, since this would imply the formation of the same initial product (i.e., *p*-benzoquinone) as in water. However, no reaction was observed.

Furthermore, the lack of formation of the alkoxyphenol products when MP8 is incubated with a mixture of equimolar concentrations of hydroquinone and *p*-benzoquinone both in the absence and the presence of hydrogen peroxide argues against a mechanism including the semiquinone as an intermediate in the reaction mechanism. Likewise, the incubation of the substrate, 4-fluorophenol in methanol in the presence of 0.1 M of sodium methoxide, did not give rise to the formation of 4-methoxyphenol. This indicates that the methoxide ion as such cannot displace the fluorine substituent by nucleophilic attack under our experimental conditions.

The principal product formed when 4-fluorophenol was incubated with MP8/H_2O_2 in ethanol was identified as 4-ethoxyphenol by GC-MS, using an authentic standard. Fig. 2*A* presents the gas chromatogram of this reaction mixture. The peak with the retention time of 12.4 min can be ascribed to 4-ethoxyphenol, whose mass spectrum is illustrated in Fig. 2*B*. Besides the peak with the retention of 4.8 min which, as already mentioned in Fig. 1*A*, is an impurity present in the substrate preparation and was identified as 2-fluorophenol, there is another peak with retention time of 8.9 min as well as other minor peaks. The identification of these products was not

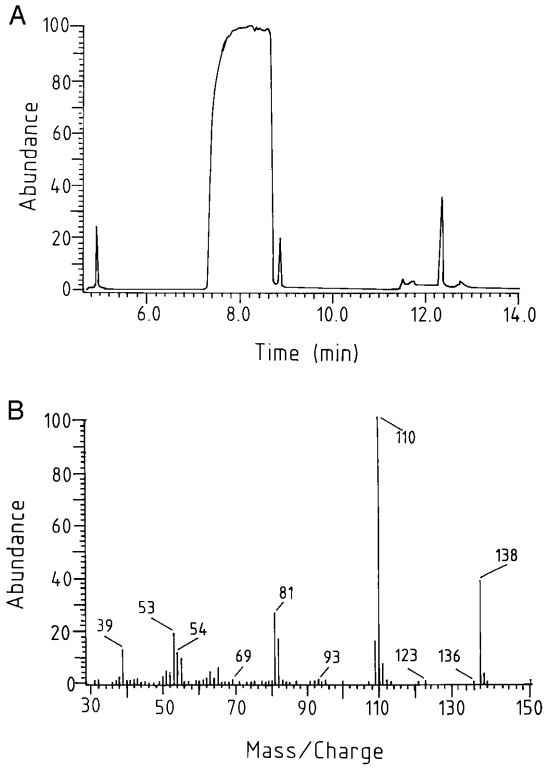

FIG. 2. (*A*) Gas chromatogram of the concentrated sample obtained from the incubation of 4-fluorophenol with MP8/H_2O_2 in ethanol. The peak with the retention time of 4.8 min is 2-fluorophenol present as an impurity in the substrate preparation. The peak with the retention time of 8.9 min as well as other minor peaks, observed in the chromatogram, are due to impurities present in the solvent. (*B*) Mass spectrum of peak with retention time of 12.4 min identified as 4-ethoxyphenol (*A*) and obtained when MP8-mediated dehalogenation of 4-fluorophenol was carried out in ethanol.

undertaken because they were shown to be impurities present in the solvent. Furthermore, in contrast to the formation of 2-methoxyphenol in methanol when 2-fluorophenol was used as a substrate, the corresponding product, 2-ethoxyphenol, was not detected in ethanol. This lack of 2-ethoxyphenol formation was not a surprise. First, the alkoxylating reaction catalyzed by MP8 takes place more rapidly in methanol than in ethanol. In fact, the rate of formation of 4-methoxyphenol compared with that of 4-ethoxyphenol is about 4-fold higher under the same experimental conditions. Second, the rate of 4-methoxyphenol formation compared with that of 2-methoxyphenol formation is 3-fold higher. Thus, formation of 2-ethoxyphenol can be expected to occur at a rate of about 12-fold lower than that of 4-methoxyphenol. This implies that 2-ethoxyphenol formation is possibly below the detection limit in the present experiments. It is also interesting to note that no product formation was detected when 3-fluorophenol was incubated with MP8/H_2O_2 in methanol or ethanol as the incubation medium.

Although the formation of other products was not found, it was essential to investigate a role of methanol as reductant in the catalytic process. Thus formaldehyde determinations were made in 1-min incubations of MP8 and hydrogen peroxide in methanol, in the presence and absence of 4-fluorophenol. Formation of formaldehyde was observed in three experiments. A slight excess of formaldehyde was observed with respect to the amount of the 4-methoxyphenol formed.

Together, the results of the present study indicate the occurrence of a reaction that can best be described as an alkoxylating dehalogenation, observed when a MP8/H_2O_2 system converts 2- or 4-halophenols in alcoholic media such as methanol and ethanol.

DISCUSSION

It is important to note that the results of the present study rule out several possible mechanisms for the H_2O_2-driven MP8-catalyzed conversion of 2- or 4-halophenols in alcoholic solvents.

First, it should be emphasized that the type of reaction products formed in addition to the halogen anion differs from the primary benzoquinone product formed when MP8/H_2O_2 conversion of the halophenols is performed in water. The dehalogenation performed in alcoholic solvents results in the formation of alkoxyphenols as the primary products. The observation that incubation of *p*-benzoquinone in methanol does not result in alkoxyphenol formation excludes the possibility that the benzoquinone is still the primary product but is converted in a subsequent reaction to give methoxyphenol. The formation of an alkoxyphenol implies that the reaction product formed in alcohols is formally two reducing-equivalents above the quinone-type products formed in water. This is especially important considering the overall electron balance of the reaction and taking into account the elimination of the halo substituent as a halogen anion. The possibility that in the alcoholic media the halogen is eliminated as a cation, leaving the two electron equivalents required for alkoxyphenol instead of benzoquinone formation, is contradicted by another observation of the present study. It was demonstrated that 4-fluorophenol is converted at a higher rate than 4-chlorophenol and 4-bromophenol. This argues against a mechanism involving the elimination of the halogen as a cation, since this would result in more difficult defluorination than dechlorination and debromination, which is not what is observed. Furthermore, a mechanism involving semiquinone intermediates could be eliminated based on the observation that upon addition of MP8/H_2O_2 to a methanol solution containing a mixture of benzoquinone and hydroquinone, in which a certain amount of semiquinone will be formed, no alkoxyphenol formation was observed. Likewise, the possible involvement of methoxide anions could be eliminated on the basis of a control experiment in which incubation of 4-fluorophenol with sodium methoxide did not result in 4-methoxyphenol formation.

Any working hypothesis suggested for this alkoxylating dehalogenation mediated by MP8 should explain (*i*) the involvement of high-valent iron–oxo intermediates formed from MP8 and H_2O_2; (*ii*) elimination of the halogen as an anion, the regioselectivity as well as the rates of substitution that follow the order F > Cl > Br; (*iii*) formation of formaldehyde from the MP8-dependent dehalogenation; and (*iv*) formation of the alkoxyphenol and not of the benzoquinone normally formed in an oxidative dehalogenation by a high-valent iron–oxo species (11, 12, 19). The two reaction mechanisms presented in Fig. 3 could account for these observations. Fig. 3*A* presents the first possible mechanism for the alkoxylating dehalogenation. In this mechanism the reactive heme-based species involved is suggested not to be the electrophilic compound I type iron–oxo species, but, instead, a methyl-peroxide intermediate. This intermediate could be formed when a molecule of the alcoholic solvent interacts with compound I. This reaction of compound I with the alcohol (Fig. 3*A*, step 1) implies an interaction of the oxygen of compound I bearing considerable negative charge (13) with the electron-rich alcoholic solvent, which might be unfavorable. However, one might argue that for this interaction a resonant form of compound I becomes of importance in which the iron is trivalent and the oxygen bears less negative charge. This trivalent-iron resonant form has been proposed as an alternative to the tetravalent iron π-cation radical form (13). This trivalent-iron resonant form of compound I has been suggested to account for chloroperoxidase-catalyzed N-oxidation of 4-chloroaniline and has been employed as the halogenating form of chloride peroxidase in which the electron-rich hypohalite ligand is coordinated to the

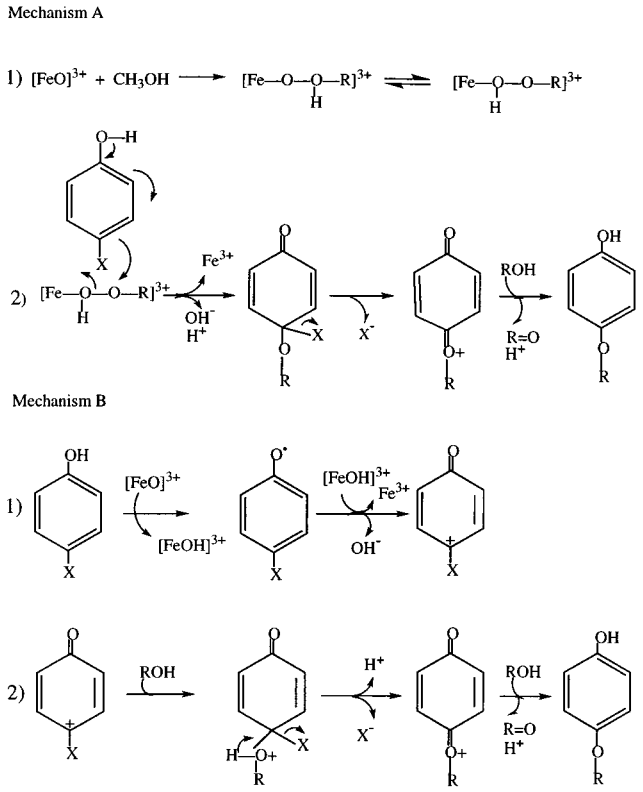

FIG. 3. Proposed mechanisms for the formation of the alkoxyphenols from the MP8-mediated dehalogenation of halophenols in alcoholic solvents. (*A*) Step 1: The formation of a MP8-alkylperoxide intermediate resulting from the reaction of compound I type with the alcohol. Step 2: The reaction of this alkylperoxide intermediate with the electron-rich centers of a halophenol which upon electronic rearrangement leads to the elimination of the halogen as an anion and the formation of an intermediate. This intermediate is further reduced by a solvent molecule resulting in the formation of the alkoxy product and an aldehyde. (*B*) An alternative mechanism for the product formation. Step 1: The formation of carbocation intermediates through the two consecutive one-electron oxidations of a halophenol by compounds I and II of the heme catalyst. Step 2: The nucleophilic attack of solvent molecules on these carbocation derivatives, followed by the release of the halogen as an anion and the formation of the same intermediate as in *A*, step 2, whose subsequent reduction by a solvent molecule leads to the final product formation.

trivalent iron–oxo complex of chloroperoxidase (14). Since it was also shown (15) that the approach of an aromatic substrate along the reaction pathway can cause considerable changes in spin density distribution of a high-valent iron–oxo species, one could even foresee that a change of medium may influence the distribution of the different resonant forms (16), making it possible to increase the contribution of the Fe^{3+} resonant form especially in less polar alcoholic solvents. Thus, the interaction of the alcohol with the high-valent iron–oxo or its trivalent–iron–oxo resonant form can give rise to formation of a methyl-peroxide intermediate as depicted in Fig. 3*A*, step 1. A nucleophilic attack of the relatively electron-rich sites of the substrate, the halophenol, on this methyl-peroxide intermediate, followed by heterolytic O—O bond cleavage (Fig. 3*A*, step 2) could lead to the formation of the intermediate shown in this pathway and the elimination of the halogen as an anion. Because two more electrons are required to account for the formation of the final product, the alkoxyphenol, it is proposed that these two equivalents are provided by the solvent molecules. In fact, the conversion of 4-fluorophenol in methanol by MP8/H_2O_2 is accompanied by the formation of formaldehyde. This detection of formaldehyde provides strong evidence for the view that the source of the two electrons required for the formation of the product, alkoxyphenol, are actually derived from the alcohol molecules.

Fig. 3*B* presents an alternative mechanism for the alkoxylating dehalogenation. In this case it is suggested that an electrophilic compound I type high-valent iron–oxo intermediate might be the reactive species catalyzing the alkoxylating dehalogenation. In this mechanism the electrophilic compound I type heme-based iron–oxo species here designated as $(FeO)^{3+}$ (Fig. 3*B*, step 1) could catalyze two consecutive one-electron oxidations of the halophenols, resulting in the formation of intermediate carbocation derivatives. Of course a compound II type $(FeOH)^{3+}$ intermediate can also be involved in these one-electron oxidations. The substrate oxidation is followed by nucleophilic attack of the solvent molecules on these intermediate derivatives and the elimination of the halogen as an anion. As a result of this reaction the same intermediate would be formed as in Fig. 3*A*, which is reduced by a solvent molecule resulting in the formation of the final product (Fig. 3*B*, step 2). Both reaction sequences outlined in Fig. 3 could account for the observations presented in this study and are in accordance with the presently accepted views on the reactivity of compound I and peroxide-heme intermediates.

Our proposal of the occurrence of a Fe^{3+}-peroxide-alkyl intermediate is to some extent supported by the recent observation that in mutant horseradish peroxidase an intermediate prior to the formation of compound I could be detected (17). It is noteworthy that a formation of alkylperoxo intermediates has been detected for simple iron porphyrins (18), which further supports this proposal. Further, both mechanisms account for the observed order of the halogen elimination as well as for the lack of reactivity of the 3-halo phenols which are in accord with an electrophilic attack.

Finally, it is clear that MP8 is an attractive biocatalyst that, as shown in this study, can be used in nonconventional media to convert substrates, which are poorly soluble in water, into products that may not be necessarily identical to those obtained in aqueous solutions.

In conclusion, the results of this work show that a MP8/H_2O_2 system mediates the dehalogenation of halophenols in alcoholic solvents, giving rise to the formation of alkoxyphenols as the major dehalogenated products. Two mechanisms are proposed for the formation of these products, one of which includes the possible existence of an alkoxylating peroxide-heme intermediate.

We thank Prof. Dr. P. J. Van Bladeren for his support. We gratefully acknowledge Dr. Alfin Vaz (University of Michigan) who pointed out the reducing role of the alcohol. This study was financially supported by European Union Biotech Grant B102-CT942052, by Training and Mobility for Research large scale NMR facility Grant ERB-CHGE-CT 94-0061, and by a grant from the Research School Environmental Chemistry and Toxicology (M&T) and Netherlands Central Organization for Applied Scientific Research (TNO) Nutrition and Food Research Institution (Zeist, The Netherlands).

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