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Stereoselectivity of Chloroperoxidase-Dependent Halogenation†

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ABSTRACT: The stereoselectivity of chloroperoxidase halogenation of four substrates has been examined. Chloroperoxidase catalyzes the bromination, but not chlorination, of racemic 2-*exo*-methylbicyclo[2.2.1]hept-5-ene-2-*endo*-carboxylic acid (to the δ-lactone) and racemic bicyclo-[3.2.0]hept-2-en-6-one (to the 2-*exo*-bromo-3-*endo*-hydroxybromohydrin). These products are obtained in near quantitative yield and are racemic. The circumstances of the bromination strongly suggest that halogenation does not occur at the active site but rather by chloroperoxidase-catalyzed formation of Br_2 and its release into solution. The inability of chloroperoxidase to halogenate these two alkenes at its active site most probably derives from a steric exclusion from the active site. The stereoselectivity of two additional substrates that undergo active site chlorination was determined. Methionine is quantitatively converted to a 50:50 ratio of the two methionine sulfoxide diastereomers. 2-Methyl-4-propylcyclopentane-1,3-dione is quantitatively chlorinated to 2-chloro-2-methyl-4-propylcyclopentane-1,3-dione. On the basis of optical rotation and proton nuclear magnetic resonance, this product is present as a 40:60 ratio of the racemic diastereomers. It is concluded that active site chlorination by chloroperoxidase proceeds without appreciable stereoselectivity.

Enzyme-catalyzed transfer of an electrophilic halogen to an acceptor molecule is a common biological occurrence. It is an event in the elaboration of secondary metabolites by marine plants; and in eukaryotes, it occurs during the synthesis of the thyroid hormones and during the oxidative destruction of microorganisms by the phagocytes. In these instances, the electrophilic halogen is obtained by a hydrogen peroxide dependent oxidation of the halide anion, catalyzed by a haloperoxidase enzyme. The best studied of these enzymes is the chloroperoxidase of the fungus *Caldariomyces fumago* (Hollenberg & Hager, 1978). Within this organism, chloroperoxidase catalyzes the sequential halogenation, at C-2, of 1,3-cyclopentanedione, on the pathway to the antibiotic caldariomycin (2,2-dichloro-*trans*-1,3-cyclopentanediol; Shaw & Hager, 1959; Beckwith et al., 1963). Hager and co-workers have provided a wealth of information on chloroperoxidase, including aspects of its protein chemistry (Morris & Hager, 1966a,b) coenzyme environment (Rutter & Hager, 1982), specificity (Thomas et al., 1970), kinetic mechanism (Libby et al., 1982), and chemical mechanism of halogen activation and transfer (Hollenberg et al., 1974; Araiso et al., 1981). Chloroperoxidase contains an active site ferriprotoporphyrin IX prosthetic group in an environment similar to that found in the cytochrome P-450 monooxygenases (Dawson et al.,

† From the Department of Chemistry, University of Minnesota, Minneapolis, Minnesota 55455. *Received January 1, 1983.* We thank the Petroleum Research Fund (administered by the American Chemical Society) for initial support of this research and the National Institute of General Medical Sciences for sustaining support. Grants from the National Science Foundation in support of the high-field nuclear magnetic resonance and amino acid analyzer facilities of the Department of Chemistry, University of Minnesota, are also acknowledged.

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1976; Hollenberg et al., 1980). Consequently, this enzyme remains of interest due to its unique ability to catalyze (dependent on reaction conditions) halogenation, classical peroxidase reactions (Thomas et al., 1970), and (apparent) oxygen activation and transfer (Corbett et al., 1978, 1980; Kedderis et al., 1980; Ashley & Griffin, 1981). Chloroperoxidase is an enzyme having both mechanistic versatility and an unusually rapid velocity for catalytic turnover (Kedderis et al., 1980; Libby et al., 1982).

Although a systematic evaluation of the substrate specificity of chloroperoxidase has never been done, there are many reports of its acceptance of substrate structure other than 1,3-cyclopentanedione. Thus, efficient halogenation has been observed for β-keto acids (Hager et al., 1966; Thomas et al., 1970), activated aromatic compounds (Brown & Hager, 1967; Corbett et al., 1980), thiols (Silverstein & Hager, 1974; Libby et al., 1982), and the analgesic antipyrine (Ashley & Griffin, 1981). Chloroperoxidase also catalyzes the peroxide-dependent N-demethylation of anilines (Kedderis et al., 1980) and antipyrine (Ashley & Griffin, 1981), N-hydroxylation of *p*-chloroaniline (Corbett et al., 1980), and C-2-oxidation of indole of oxindole (Corbett & Chipko, 1979). The mechanism of these latter reactions is not known but may involve chloroperoxidase-initiated free-radical reactions (Ashley & Griffin, 1981). Lastly, chloroperoxidase catalyses a peroxide-dependent halogenation of alkenes, to the halohydrins, although at much slower rates than any of the preceding reactions (Neidleman & Levine, 1968; Kollonitsch et al., 1970). Our interest in the substrate specificity of chloroperoxidase derived from this apparent mechanistic versatility and broad substrate specificity. Specifically, due to the rapid velocity of chloroperoxidase halogenation (1540 s^{-1} for chlorination of monochlorodimedone; Hollenberg & Hager, 1978), it seemed reasonable to anticipate that chloroperoxidase might be useful for the kinetic resolution of a racemic substrate, by preferential halogenation of one enantiomer. A second goal was the comparison of the mechanism for halogen and for oxygen transfer. Since the activated halide is electrophilic (Brown & Hager, 1967), its transfer to a substrate capable of carbocation-induced rearrangement might provide a rearranged product. If this substrate were also capable of accepting activated oxygen from the chloroperoxidase active site, then the presence or absence of the rearrangements would describe the transition state for oxygen transfer. In this paper, we describe experiments indicating that neither goal is attainable for this enzyme. Contrary to our expectations at the start of this problem, chloroperoxidase exerts selectivity with respect to substrate reaction at its active site. Further, it has been found that active site halogenation proceeds without appreciable discrimination between the stereoheterotopic faces of the reacting functional group.

Experimental Procedures

Chloroperoxidase was obtained as the partially purified ("crude") enzyme from Sigma. It had an absorbance ratio at 280 to 403 nm of 1.3, to be compared to a ratio of 0.72 for the homogeneous enzyme (Kedderis et al., 1980). This indicates a purity of approximately 0.5 that of homogeneous enzyme. Mechanistic studies have been done on chloroperoxidase of comparable purity, without difference in behavior from that of the homogeneous enzyme (Ashley & Griffin, 1981). The enzyme was stored at 4 °C in 2.7 M $(NH_4)_2SO_4$, as received. All reagents were used as received from commercial suppliers. The preparation of the enzyme substrates and the procedures for their conversion to products by chloroperoxidase are described:

2-exo-Methylbicyclo[2.2.1]hept-5-ene-2-endo-carboxylic acid (**1**) was obtained by the procedure of Berson & Ben-Efraim (1959). The acid was neutralized with KOH and used as the K^+ salt. Authentic bromo lactone (**2**) was obtained by the method of van Tamelen & Schamma (1954).

Bicyclo[3.2.0]hept-2-en-6-one (**3**) was obtained by the procedure of Grieco (1972). Aqueous solutions containing millimolar concentrations of this material are easily obtained, presumably due to the ease of ketone hydration.

2-exo-Bromo-3-endo-hydroxybicyclo[3.2.0]heptan-6-one (**4**) was prepared by the method of Grudzinski & Roberts (1975), mp 87.5–90 °C (lit. mp 87–89 °C).

2-Methyl-4-propyl-1,3-cyclopentanedione (**5**) was obtained by the procedure described by Mellor & Pattenden (1979). To 50 mL of dry (CaH_2), distilled tetrahydrofuran is added 1.0 g (8.9 mmol) of 2-methyl-1,3-cyclopentanedione dissolved in 7.5 mL of dry hexamethylphosphoramide. To this solution, cooled to −78 °C, is added 2.1 equiv of *n*-butyllithium in hexane solution over 3 min. After being stirred at this temperature for 20 min, 1.3 molar equiv of iodopropane is added over 5 min. The solution is stirred for 1 h at −78 °C and 30 min at −20 °C, quenched with 15 mmol of dilute aqueous HCl, and extracted with ether. The combined ether extracts are washed with Na_2CO_3, and the ether is discarded. The aqueous solution is acidified, extracted with ether, and then dried with $MgSO_4$. Evaporation provides 750 mg (55%) of the product, which may be recrystallized from ethyl acetate–hexane: mp 74–76 °C; ^{1}H NMR[1] ($CDCl_3$) δ 0.92 (t, 3 H), 1.23–1.50 (m, 4 H), 1.66 (s, 3 H), 1.75–1.90 (m, 1 H), 2.25 (d, 1 H), 2.71 (dd, 1 H), 10.6 (br s, 1 H); high-resolution EIMS (70 eV), *m/z* 154.1018 (calcd for $C_9H_{14}O_2$: 154.0994); low-resolution EIMS (70 eV), *m/z* (relative intensity) 154 (15.4), 125 (9.8), 112 (100), 111 (16.3), 94 (17.4), 83 (17.4), 56 (18.6), 55 (12.8), 43 (21.9), 41 (18.6), 39 (10.4); UV max (CH_3OH) 250 nm (ϵ 15 200 M^{-1} cm^{-1}). As expected, this compound has no optical rotation. It is stored at −20 °C in a desiccator.

4-(2-Ethoxyethyl)-2-methyl-1,3-cyclopentanedione, mp 74–76 °C, was from 2-bromoethyl ethyl ether addition to the dienolate; low-resolution EIMS (70 eV), *m/z* (relative intensity) 184 (M^+, 18.5), 154 (2.2), 138 (22.1), 127 (21.8), 112 (100), 94 (10.4), 83 (65.7), 59 (40.1), 43 (58.6).

Methionine, methionine sulfoxide, and methionine sulfone were purchased from Sigma. A diastereomeric composition of 50/50 for the commercial methionine sulfoxide was indicated by its molar rotation $[M]^{29}_D = +58.6°$ (0.05 M, 1.0 N HCl), lit. $[M]^{29}_D$ +58.8° (Lavine, 1947). Separation of the methionine sulfoxide diastereomers was accomplished on a 0.9 × 30 cm W2H cation resin column on a Beckman 118BL amino acid analyzer, using 0.2 M sodium citrate pH 3.0 buffer containing 2% benzyl alcohol, at a column temperature of 78 °C. Under these conditions, the methionine sulfoxide diastereomers had retention times of 86 and 91 min, and methionine sulfone a retention time of 96 min.

Chloroperoxidase-catalyzed halogenation was accomplished by either of two procedures, depending on whether a specific (halogenation at the active site) or nonspecific (halogenation in solution) substrate is used. Chlorination of 2-methyl-4-propyl-1,3-cyclopentanedione and methionine is specific, while bromination of 2-*exo*-methylbicyclo[2.2.1]hept-5-ene-2-*endo*-carboxylic acid and bicyclo[3.2.0]hept-2-en-6-one is nonspecific.

2-Chloro-2-methyl-4-propyl-1,3-cyclopentanedione (**6** *and* **7**). 2-Methyl-4-propyl-1,3-cyclopentanedione (39 mg, 0.25

[1] Abbreviations: NMR, nuclear magnetic resonance; EIMS, electron-impact mass spectroscopy.

mmol) is dissolved in 40 mL of 0.10 M potassium phosphate pH 2.75 buffer by gentle warming. After this cooled, 2.5 mL of 2.0 M KCl and 50 μL of 10 M H_2O_2 solutions are added, and the reaction is initiated by chloroperoxidase (200 μL, 120 μmol min^{-1} total activity). After 3 min, 10 mL of a saturated NaCl solution is added and the product extracted with a single 50-mL portion of dichloromethane. Drying ($MgSO_4$) and evaporation of the solvent provides 2-chloro-2-methyl-4-propyl-1,3-cyclopentanedione (30 mg, 61%) as an oil. The structural assignment was confirmed by the electron impact mass spectrum, which showed the appropriate ratio of $(M^+ + 2)/z$ to M^+/z for the monochlorinated product: low-resolution ratio of m/z 190/188 of 0.362; anticipated ratio for $C_9H_{13}O_2Cl$ is 0.334. Proton NMR indicates that this product is diastereomeric.

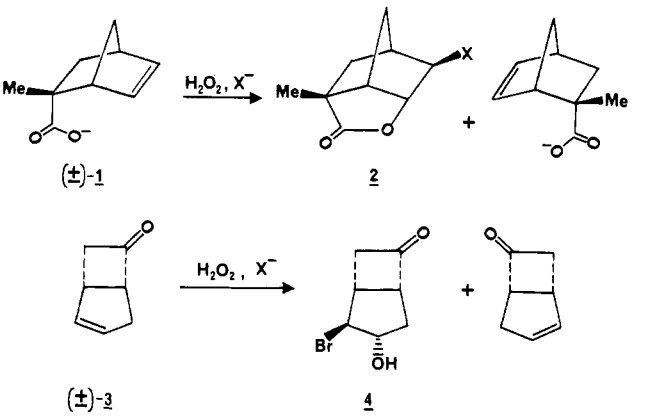
Methionine Sulfoxides. Methionine (4.0 mg, 25 μmol) was dissolved in 20 mL of 0.1 M potassium phosphate pH 2.75 buffer, and to this was added 0.25 mL of 2.0 M KCl and 5 μL of 10 M H_2O_2. The reaction was initiated by chloroperoxidase (12 μmol min^{-1} activity under standard assay conditions). After 10 min, the solution was adjusted to pH 7.0 with sodium hydroxide, and catalase was added to remove any remaining H_2O_2 and, thus, quench the reaction. The extent of the reaction was measured by liquid chromtographic separation of the methionine sulfoxides from methionine, using an Ultrasphere C_{18} reverse-phase column with 0.9 mL min^{-1} 0.1% H_3PO_4 in water as the mobile phase (Libby et al., 1982). Under these conditions, the methionine sulfoxides elute at 3.6 min, and methionine elutes at 10.2 min.

2-exo-Bromo-3-endo-hydroxybicyclo[3.2.0]heptan-6-one. After some experiment, it was found that an initial sequential addition of enzyme and hydrogen peroxide gave optimal yields. Bicyclo[3.2.0]hept-2-en-6-one (116 mg, 1.07 mmol) was dissolved in 20 mL of 0.25 M potassium phosphate pH 2.75 buffer, and to this was added 3 mL of a 2.0 M KBr solution. Chloroperoxidase (500 μL total, 300 μmol min^{-1} total activity) was added in five equal portions at 0, 1, 2, 3, and 4 min elapsed time. Thirty seconds after each enzyme addition, 100 μL of 10 M H_2O_2 was added, continuing at 1-min intervals until 10 portions (1.0 mL total) were added. After 30 min, the reaction was acidified to pH 1.0 and the product extracted in three 75-mL portions of dichloromethane. Drying and evaporation of this solvent gave 172 mg (82%) of the bromohydrin as an oil. Purification by silica gel chromatography (2/1 chloroform/ethyl acetate) gave 46 mg of crystalline bromohydrin, mp 85–87 °C, having chromatographic properties and spectral properties (mass spectrum and NMR) identical with the authentic sample. Bromination of 2-*exo*-methylbicyclo[2.2.1]-hept-5-ene-2-*endo*-carboxylic acid by chloroperoxidase was done in an identical fashion. The isolated product was compared to, and found identical with, an authentic sample of the bromo lactone.

Results

The first compound examined as a chloroperoxidase substrate was racemic 2-*exo*-methylbicyclo[2.2.1]hept-5-ene-2-*endo*-carboxylic acid (**1**). This substrate offered several advantages for the study of the enantioselectivity and mechanism of chloroperoxidase halogenation. First, the π-bond of bicyclo[2.2.1]heptenes has unusually high reactivity (Huisgen et al., 1980). Electrophilic halogenation of the *endo*-norbornene carboxylate (**1**) by chloroperoxidase would provide, by analogy to the chemical reaction, the halo δ-lactone, **2** (Scheme I). The mechanism of the chemical reaction has been thoroughly studied and proceeds without skeleton rearrangement (Moriarty et al., 1979). The reaction type, halolactonization, is

Scheme I: Anticipated Kinetic Resolutions by Chloroperoxidase Halogenation[a]



[a] The choice of the reactive enantiomer is arbitrary.

moreover a reaction of considerable scope and utility (Dowle & Davies, 1979) and has increasingly been employed in synthesis to provide stereochemical control during functionalization [for examples, see Corey et al. (1970), Barlett & Myerson (1978), and Grieco et al. (1979)]. Last, the norbornene carboxylate was structurally similar to the bicyclic alkenes that were to be used subsequently to determine whether enzymatic halogenation proceeded with carbocation rearrangement of the substrate. Accordingly, the substrate, **1**, was reacted with chloroperoxidase under conditions corresponding to optimal halogenation of specific substrates (pH 2.75 buffer, with H_2O_2 as oxidant and Cl^- as halide; Hollenberg & Hager, 1978). Under no circumstances was the chloro δ-lactone observed as product. Rather, the chloroperoxidase suffered rapid and irreversible loss in its catalytic activity. In contrast, with Br^- as halide, conditions were readily identified that resulted in the chloroperoxidase-catalyzed conversion of **1** to the bromo δ-lactone *in quantitative yield.* The bromo lactone obtained neither at early time points nor at late time points showed optical activity. Likewise, continuous monitoring of the optical rotation by flow of the reaction solution through the polarimeter cell gave no indication of enantioselectivity at any point during the conversion. Chloroperoxidase does not, therefore, kinetically resolve this substrate.

As a consequence of this somewhat surprising outcome, a second substrate was examined, bicyclo[3.2.0]hept-2-en-6-one (**3**). Electrophilic halogenation of this substrate would provide, by analogy to the chemical reaction (Grudzinski & Roberts, 1975), the halohydrin, **4** (Scheme I). The preparation of the optically active bromohydrin, **4** ($X = Br^{-1}$), via chloroperoxidase kinetic resolution of racemic **3** would be of considerable value, as the bromohydrin enantiomers are used as intermediates in the total synthesis of chiral prostaglandin intermediates (Newton & Roberts, 1980; Newton et al., 1981; Howard et al., 1981). The outcome with this substrate with chloroperoxidase was identical with that of the norbornene carboxylate. No product was formed with chloride, while with bromide the bromohydrin was obtained in quantitative yield (Figure 1). Regardless of the reaction extent, the isolated bromohydrin was racemic, $[\alpha]^{22}_D$ +0.2° (*c* 0.74, $CHCl_3$) [lit. $[\alpha]^{22}_D$ +63° (Newton & Roberts, 1980)]. Chloroperoxidase does not kinetically resolve this substrate.

The complete absence of stereoselectivity in these two conversions raised the question of the enzyme's role in the halogenation process. The critical observation is the inability of the enzyme to chlorinate these two substrates, as opposed to the relative ease of bromination. As interception of the

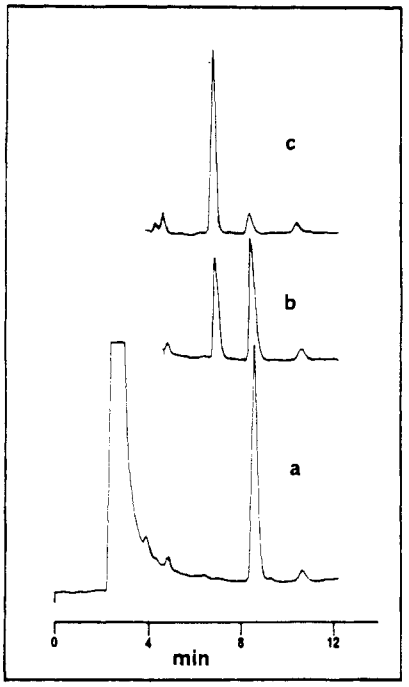


FIGURE 1: Time course of racemic bromohydrin formation from bicyclo[3.2.0]hept-2-en-6-one, catalyzed by chloroperoxidase in the presence of H_2O_2 and Br^{-1}. At various time points, 100-μL portions of the reaction solution are withdrawn and quenched with 100 μL of CH_3OH, and 50 μL of the quenched mixture is injected onto a 25 × 4.6 cm Ultrasphere C_{18} reverse-phase column. The mobile phase was 50/50 methanol/water, and the column effluent was monitored spectrophotometrically at 220 nm. Chromatogram a, obtained prior to H_2O_2 and enzyme addition, shows only bicyclo[3.2.0]hept-2-en-6-one at a retention time of 8.6 min. Chromatogram b at 20 min elapsed time shows an approximately 50% conversion, while chromatogram c at 40 min shows nearly complete conversion to the bromohydrin (retention time of 6.9 min).

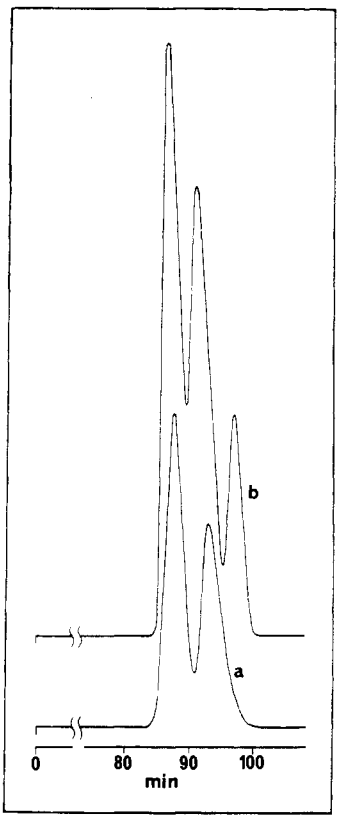
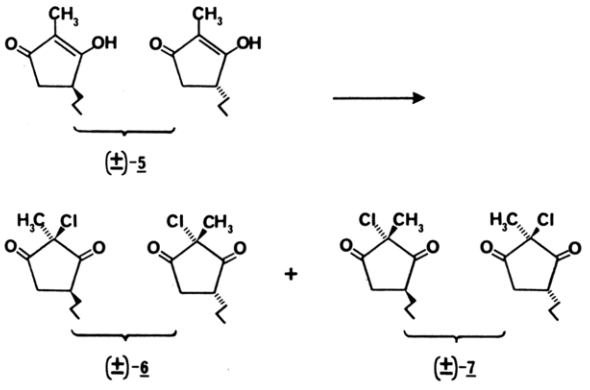


FIGURE 2: Determination of diastereomeric composition of methionine sulfoxide obtained from methionine chlorination by chloroperoxidase. Portions of the reaction mixture (1.0 mL) were injected directly onto the amino acid analyzer, and the effluent was monitored with ninhydrin. Chromatogram a, obtained before complete reaction had occurred, shows the presence of both methionine sulfoxide diastereomers (retention times of 86 and 91 min), at a combined yield corresponding to approximately 40% conversion of the methionine. The absolute configuration of the diastereomers has not been assigned. The diastereomers in chromatogram a are present in a 50:50 ratio; the difference in peak size is due to a difference in ninhydrin sensitivity. The chromatogram obtained at complete conversion (b) shows the same diastereomeric composition as well as indicating the presence of methione sulfone (retention time of 96 min). The methionine sulfone arises from nonenzymatic sulfoxide oxidation under the reaction conditions (Libby et al., 1982).

activated halogen is often the last, rate-limiting step in turnover (Libby et al., 1982), under the experimental conditions, there is no question that the activated chloro–heme complex is formed. Simply put, the enzyme must be unable to transfer the activated halide to either of the alkene substrates examined. Hager and co-workers have previously observed that treatment of chloroperoxidase with chloride and hydrogen peroxide, in the absence of substrate, results in the loss of enzyme catalytic activity (Shaw & Hager, 1961). This observation accounts for the loss in enzyme activity during our experiments with chloride, as neither alkene is a substrate. But how, then, are these substrates brominated? The answer is again found in the extensive studies by Hager's group. In contrast to chloride, bromide is capable of intercepting the activated bromo–heme complex to provide molecular bromine (Libby et al., 1982). Release of this bromine to solution would provide a halogenating reagent that would necessarily operate in an achiral fashion on the alkenes. The kinetic and chemical competence of bromine in these halogenations was established in a control experiment. Sequential addition of bromine, in concentrations equal to the hydrogen peroxide added during the enzymatic conversions, to an aqueous solution of the bicycloheptenone resulted in the appearance of the bromohydrin at a velocity and in yields nearly identical with those observed during the enzymatic reaction. The explanation for the formation of the racemic bromo compounds is enzyme-catalyzed production of Br_2 at the expense of H_2O_2 as an oxidant and its release into solution.

As neither alkene is a true chloroperoxidase substrate, an analysis of this enzyme's stereospecificity required a substrate that on the basis of kinetic evidence, would be certain to undergo *chlorination* at the active site (Libby et al., 1982). Of the known substrates for chloroperoxidase that fulfilled this criterion, only one offered the possibility of stereoisomer formation as a result of active site halogenation. This substrate is L-methionine. On the basis of catalytic turnover, L-methionine is one of the best substrates for chloroperoxidase, providing as the ultimate product methionine sulfoxide. The methionine sulfoxide most certainly derives from hydrolysis of a chlorosulfonium intermediate, obtained by chlorine atom transfer to sulfur at the active site (Silverstein & Hager, 1974; Libby et al., 1982). As nucleophilic displacement at the sulfur of sulfonium cations proceeds with inversion (Mislow et al., 1964; Johnson & McCombs, 1965), the chirality of the halosulonium cation intermediate should be transmitted to the stable sulfoxide. Thus if chloroperoxidase exhibits stereoselectivity for sulfur halogenation, a single diastereomer of L-methionine sulfoxide should be obtained; if stereoselectivity is not exhibited, both diastereomers should be obtained. Chloroperoxidase-catalyzed halogenation of methionine under standard conditions resulted in its quantitative conversion to methionine sulfoxide (Libby et al., 1982). Examination of this product on the amino acid analyzer indicated that both diastereomers were present, under conditions of either partial or

Scheme II: Stereochemical Outcome of 2-Methyl-4-propyl-1,3-cyclopentanedione Chlorination



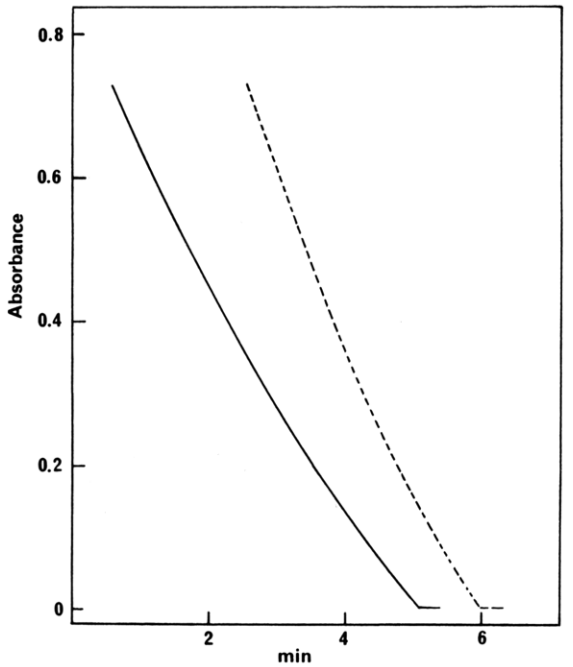


FIGURE 3: Progress curves for chloroperoxidase-catalyzed chlorination of monochlorodimedone [(—) monitored at 278 nm] and 2-methyl-4-propylcyclopentane-1,3-dione [(– –) monitored at 250 nm] under standard reaction conditions (20 mM KCl/0.2 mM H_2O_2 in 3.0 mL of 0.10 M pH 2.75 phosphate buffer). The initial substrate concentrations were approximately 0.1 mM, and identical quantities of enzyme were added to initiate each reaction. The velocity of both substrates (0.87 mmol min^{-1} mg^{-1}) and their K_ms (3 μM) are identical.

complete conversion (Figure 2). Using commercial methionine sulfoxide of known diastereomeric composition as a standard, the diastereomeric composition of the enzyme product was determined to be 50:50. Thus, the outcome of the halogenation of this specific substrate is uninfluenced by the C-2 chirality.

Since methionine sulfoxide was not the direct product of the enzymatic reaction, a second substrate was desired to confirm an absence of enzyme stereoselectivity. The compound used for this confirmation was 2-methyl-4-propyl-1,3-cyclopentanedione (**5**). This compound is very similar to the enzyme's natural substrate. Due to enolization, it has a single chiral center, but upon chlorination, a total of four stereoisomers may be obtained (Scheme II). The kinetics of chloroperoxidase chlorination were first examined. Under standard conditions, racemic **5** is rapidly and quantitatively consumed (Figure 3). Within experimental error, the velocity and K_m are identical with those for monochlorodimedone (2-chloro-5,5-dimethyl-1,3-cyclohexanedione). Chlorination of

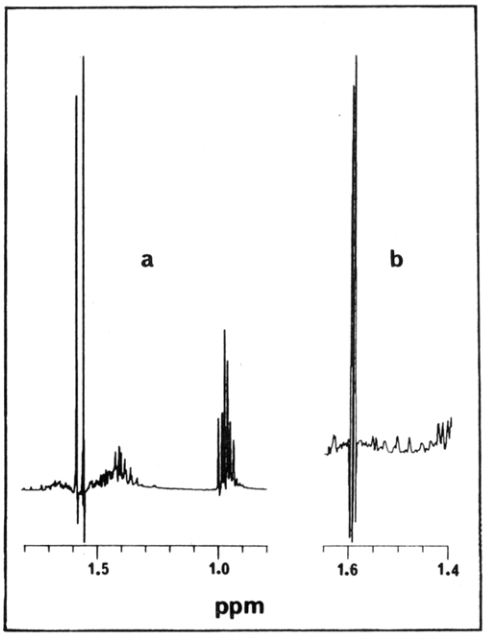


FIGURE 4: Lower energy resonances of ^1H NMR spectrum of 2-chloro-2-methyl-4-propylcyclopentane-1,3-dione obtained from chloroperoxidase-catalyzed chlorination (300 MHz, $CDCl_3$). Spectrum a is that of the product isolated from the enzymatic reaction. Evidence for diastereomers is found in the two pair of methyl singlets and methyl triplets. The diastereomeric composition is obtained from integration of the C-2 methyl singlets. An assignment of relative configurations has not been made. Spectrum b shows the C-2 methyl resonance of the faster eluting diastereomer in the presence of 0.25 equiv of the chiral shift reagent tris(di-*d*-campholylmethanato)europium. This reasonance is split into two singlets ($\Delta\Delta J$ = 1.71 Hz) of nearly equal intensity.

this substrate occurs within the active site (Libby et al., 1982); therefore, this must also be the case for 2-methyl-4-propyl-1,3-cyclopentanedione. Further, the progress curve shows no evidence of different kinetic constants for the two reacting enantiomers. Prompt extraction of a preparative-scale reaction provides the 2-chloro product in good yield. This product chromatographs as *two* close-moving spots (R_f 0.6, 4/1 $CHCl_3$/EtOAc on silica). The proton magnetic resonance spectrum of the isolated product provides evidence that the two spots correspond to diastereomers (Figure 4). Specifically, there appear two C-2 methyl singlets (δ 1.55 and 1.58) and two methyl triplets [from the propyl side chain, δ 0.95 (J = 7.3 Hz) and 0.97 (J = 7.3 Hz)]. Integration of the C-2 methyl singlets gives a diastereomeric composition of 40:60. The diastereomeric mixture was without optical activity, strongly suggesting that both diastereomers were racemic. This presumption was confirmed by separation of the two diastereomers (flash chromatography with 1/1 chloroform/hexanes on silica; Still et al., 1978). The proton NMR spectrum of the faster running diastereomer (no optical activity) showed one methyl triplet (δ 0.95) and one methyl singlet (δ 1.55). Upon addition of 0.25 equiv of the chiral shift reagent tris(di-*d*-campholylmethanato)europium, the C-2 methyl singlet split into two singlets of equal intensity (Figure 4). These are assigned to the two enantiomers. Last, chemical chlorination of 2-methyl-4-propyl-1,3-cyclopentanedione (Cl_2, 0.1 M HCl) gave a 55:45 ratio of the two diastereomers, different from the enzymatic reaction, further indicating that chlorination by chloroperoxidase had occurred at the active site. Thus, chloroperoxidase-catalyzed chlorination of 2-methyl-4-propyl-1,3-cyclopentanedione provides all four stereoisomers of 2-chloro-2-methyl-4-propyl-1,3-cyclopentanedione, in approximately equal yields of racemic diastereomers. Diaste-

reomers (40:60 ratio) are also obtained upon enzymatic chlorination of a 2-methylcyclopentanedione having a larger, more polar C-4 substituent (ethoxyethyl). We conclude that neither for methionine nor for cyclopentanediones, substrates that by kinetic evidence (Libby et al., 1982) undergo reaction at the active site, does chloroperoxidase exhibit appreciable stereoselectivity.

Discussion

In the one previous study of chloroperoxidase stereoselectivity, Kollonitsch et al. (1970) observed that chloroperoxidase-catalyzed chlorination of *cis*- and *trans*-propenylphosphonic acid gave the respective *threo*- and *erythro*-chlorohydrins as the racemates. This stereochemical outcome required that chlorine transfer had occurred with equal facility to the two faces of the double bond; and thus, chloroperoxidase was viewed as an unusual example of an enzyme that lacked biological stereoselectivity (Kollonitsch et al., 1970; Alworth, 1972). Although the results presented in this paper lead to the identical conclusion, at the start of these studies there was reason to believe that the propenylphosphonic acids were an equivocal measure of this enzyme's stereoselectivity. This conclusion derived from the reaction conditions reported by Kollonitsch et al. as necessary to produce the chlorohydrins. In particular, these suggest that specific, active site chlorination of these substrates did not occur. Both the reaction time (8 h) and molar ratio of substrate to enzyme (approximately 800) indicate that propenylphosphonic acid chlorination is slow. In contrast, the preparative-scale reactions reported by Hager (Hager et al., 1966; Brown & Hager, 1967) and in this paper correspond to much less demanding conditions (typical reaction time of less than 10 min and a molar ratio of substrate to enzyme of approximately 10^5). The more plausible explanation for the racemic chlorohydrins obtained from the propenylphosphonic acids is enzyme-catalyzed formation of Cl_2 and achiral chlorination outside of the enzyme. Chloroperoxidase is capable of Cl_2 formation but at velocities much less than Br_2 formation or specific chlorination (Libby et al., 1982). A rationalization for the exclusion of propenylphosphonic acid from reaction at the chloroperoxidase active site may be made on the basis of electrostatic grounds, steric hindrance, or an intrinsic unreactivity of the double bond. For this reason, the more detailed examination of chloroperoxidase reported in this paper was undertaken.

The stereochemical outcome of chloroperoxidase-catalyzed halogenation of four potential substrates has been determined. For the two bicyclic alkenes, the circumstances of their bromination strongly suggest that they are excluded from reaction at the active site. Rather, bromination is a consequence of enzyme-catalyzed formation and release of Br_2. What possible explanation exists for this exclusion? It is not likely electrostatics, as the bicyclo[3.2.0]heptenone is a neutral compound. Two possible explanations remain. Either their bicyclic topography provides a steric impedance to the approach of the π-bond to within bonding distance of the chlorine in the activated complex, or their unactivated alkene functional group is not susceptible to electrophilic attack by the activated chloro–heme complex. The possibility of limited steric access to the chloroperoxidase active site has been previously noted (Corbett & Chipko, 1979; Kedderis et al., 1980). In principle, the two explanations may be distinguished with a substrate having an unactivated alkene and a smaller steric requirement. We have examined cyclohex-2-enol as such a substrate and have found it neither a specific substrate (no product with Cl^- under preparative conditions) nor a competitive inhibitor (no alteration in the kinetics of monochlorodimedone chlorination at 10 mM cyclohex-2-enol). Possibly, potential substrates that possess out-of-plane substituents near the reacting functional group are unable to approach the activated chloro–heme complex. Nonetheless, the failure of chloroperoxidase to chlorinate cyclohex-2-enol under these circumstances does not decide with *absolute* certainty between the mechanistic possibilities of chlorination inside and outside of the active site. Since halogen transfer to the acceptor is rate determining for turnover, it is unreasonable to anticipate that the velocity for an unactivated alkene be equal to that of an activated alkene. It is probable that with longer reaction times, or greater quantities of enzyme, that some chlorinated product would be obtained. Yet, without stereochemistry to identify the product of active site chlorination and with the considerable experimental difficulties in measuring the rate of Cl_2 formation by chloroperoxidase (Libby et al., 1982), the portion of product derived from specific chlorination (rather than from HOCl) is undeterminable. It appears probable, however, that the major pathway for nonspecific chlorination is by way of enzyme-catalyzed formation of free HOCl (Geigert et al., 1983). For these reasons, mechanistic studies of chloroperoxidase that use a pseudosubstrate must be approached with considerable caution. In our opinion, the mechanistic uncertainty associated with unactivated alkene chlorination is sufficient to preclude further experimentation on one goal—whether halogenation (and perhaps oxygenation) of norbornenes proceeds with carbocation rearrangements.

A companion question is the stereoselectivity of the haloperoxidases as an enzyme class. This has not been addressed in this paper and will be a difficult problem. The remaining haloperoxidases are, generally, poorer activators of chloride and more prone to molecular halogen formation. Indeed, for some (such as myeloperoxidase) the synthesis of molecular halogen may be regarded as a natural catalytic function. For the moment, however, the possibility that the haloperoxidases may be generally lacking in stereoselectivity should be considered in interpreting experimental data. The isolation of product stereoisomers may not preclude the possibility of active site halogenation. Thus, the formation of diastereomeric leukotriene C-4 sulfoxides in the presence of myeloperoxidase catalyst (Lee et al., 1982) need not necessarily have occurred from free HOCl. A differentiation between the possibilities requires an independent determination of the myeloperoxidase kinetics and intrinsic stereoselectivity.

Hamilton (1976) expressed the anticipation that chloroperoxidase might offer to the organic chemist the advantage of a different selectivity than chemical halogenating reagents. It is now established that in terms of stereoselectivity, chloroperoxidase offers little over its chemical counterparts. It remains, however, an enzyme of undiminished intrigue.

Acknowledgments

We thank Professor George Barany for his advice in optimizing the methione sulfoxide diastereomer separation on the amino acid analyzer.

Registry No. **1**, 85719-73-9; **2**, 85761-21-3; **3**, 62182-73-4; **4**, 71963-06-9; **5**, 85719-74-0; **6**, 85719-75-1; **7**, 85719-77-3; 2-methyl-1,3-cyclopentanedione, 765-69-5; 4-(2-ethoxyethyl)-2-methyl-1,3-cyclopentanedione, 85719-76-2; methionine, 63-68-3; methionine sulfoxide, 454-41-1; chloroperoxidase, 9055-20-3; monochlorodimedone, 7298-89-7.

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