Mechanism of Inhibition of Horseradish Peroxidase by Cyclopropanone Hydrate*

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Cyclopropanone hydrate irreversibly inactivates horseradish peroxidase in a time-dependent manner in the presence of oxidizing agent, hydrogen peroxide. The inhibition reaction is a second order reaction of cyclopropanone hydrate with compound I, the 2-electron oxidized form of the peroxidase, and results in covalent modification of the heme cofactor. A new propionic acid side chain is substituted for one of the methane protons of the heme. A mechanism for inhibition is proposed to involve oxidative ring opening of cyclopropanone hydrate to give the primary free radical of propionic acid, which subsequently alkylates the heme. An isosorbyrin π cation intermediate is predicted by this mechanism, and this intermediate has been detected spectroscopically.

Cyclopropanone has been a useful moiety in the design of enzyme inhibitors. The keto form of cyclopropanone is highly electrophilic due to the ring strain in the unsaturated small ring (the chemistry of cyclopropanone has been reviewed by Wasserman et al., 1974). For example, only the hydrated form of cyclopropanone is observed in aqueous solution. Inhibition of aldehyde dehydrogenase by cyclopropanone hydrate has been examined in detail (Wiseman et al., 1980), and in this system, inhibition was found to be due to formation of a thiohemiketal between cyclopropanone and the prosthetic heme group of the enzyme. Suicide inactivation by precursors to cyclopropanone has been achieved for a number of enzymes (presumably) capable of oxidizing cyclopropyl amines or cyclopropyl alcohols to the corresponding imines or ketones. Inhibition of monoamine oxidase (Silverman and Hoffman, 1979), of an FAD-dependent alcohol oxidase (Mincey et al., 1980), and of a cytochrome P-450 N-demethylase (Hanzlik et al., 1979) has been reported. In addition, destruction of the heme of cytochrome P-450 enzymes by allenes has been demonstrated (Ortiz de Montellano and Kunze, 1980; Metcalf et al., 1981). Inhibition by the allenes was proposed to require oxidation to the cyclopropylimine is not possible (Silverman and Hoffman, 1981).

MATERIALS AND METHODS

Horseradish peroxidase, type X, a mixture of isozymes, was obtained from Sigma. Hydrogen peroxide, was from MCB Manufacturing Chemists, Inc. Cyclopropanone hydrate was prepared from the ethyl hemiketal (Wiseman and Abeles, 1979). The ethyl hemiketal was synthesized as described (Ruhlman, 1971; Solaini, 1976).

Hydrogen peroxide was standardized by titration with KMnO₄ (Bertn and Bergmeyer, 1974). Solutions of cyclopropanone hydrate were standardized by addition of an excess of NaOH, which results in rapid ring opening to give sodium propionate. The excess NaOH was then titrated with HCl. Horseradish peroxidase was assayed as described (Decker, 1977). The concentration of peroxidase solutions was determined using ε₉₀ = 96,000 M⁻¹ cm⁻¹ (Mauk and Girotti, 1974). All studies with peroxidase were performed in 0.1 M potassium phosphate buffer, pH 7.0, at 25°C.

Heme was prepared from inhibited peroxidase and characterized as follows. Enzyme, 75 μM in 6.5 ml, was incubated with 150 μM cyclopropanone hydrate and 300 μM H₂O₂. After 95% inhibition, the peroxidase solution was acidified to pH 6.5 and heme removed by extraction with 2-butane (Mauk and Girotti, 1974). Mass spectra were determined directly on the derivatized heme at the Middle Atlantic Mass Spectrometry Facility, Johns Hopkins University, by the fast atom bombardment technique using a Kratos MS-50 mass spectrometer. NMR spectra of the heme were obtained on a Varian FT-80A spectrometer in 25 μl of pyridine-d₅ containing an excess of SnCl₂.2H₂O (Caughey et al., 1975).

Reconstitution of apoperoxidase from the above extraction was as described (Mauk and Girotti, 1974). Heme isolated by the above method on a 4 times larger scale was derivatized in 1 ml of 3% sulfuryl acid in methanol at 0°C for 2 h. The esterified heme was extracted with methylene chloride after dilution of the methanol with water. The residue, after drying the methylene chloride over Na₂SO₄ and evaporation, was dissolved in 0.02 ml of Ar-saturated pyridine and diluted with 1 ml of Ar-saturated acetic acid. A saturated solution of FeCl₃ in concentrated HCl, 0.15 ml, was added. After 3 min at 50°C under Ar, 1 ml of chloroform was added and the result washed thoroughly with water. After drying with Na₂SO₄, the chloroform solution, which contained metal-free, esterified porphyrin, was chromatographed on 1 g of neutral alumina, Brockman activity III, with chloroform as eluent. This procedure is essentially as has been described (Smith and Fuhrhop, 1975). The NMR spectrum of this material was measured at 300 MHz with a Bruker WM 200 spectrometer in CDCl₃.

RESULTS

Kinetics and Stoichiometry of Inhibition—The kinetics of inhibition of horseradish peroxidase are presented in Fig. 1. The inhibition reaction has an absolute requirement for oxi-

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dizing agent, in this case hydrogen peroxide. Also inferred from Fig. 1 is the fact that the inhibition reaction is quite efficient, i.e. has a low partition ratio (Walsh, 1977). The results in Fig. 1 were obtained at only 1.0 molar equivalent of cyclopropanone hydrate and 1.7 molar equivalents of H₂O₂. A stoichiometry of 1.4 ± 0.3 mol of H₂O₂ and 2.0 ± 0.3 mol of cyclopropanone hydrate consumed/mol of peroxidase inactivated has been determined. The requirement for H₂O₂ implies that cyclopropanone hydrate is, in actuality, reacting with compound I, the 2-electron oxidized form of peroxidase. Indeed, when 1 eq of hydrogen peroxide was allowed first to react with peroxidase to form compound I, and cyclopropanone hydrate subsequently added, inhibition proceeded at the expected rate. Under pseudo-first order conditions, the rate of inhibition varied directly with cyclopropanone hydrate concentration up to 850 μm; a second order rate constant of 99 M⁻¹ s⁻¹ was calculated. Commensurate with the very fast formation of compound I (Dolman et al., 1975) relative to the rate of inhibition, the rate of inhibition was independent of the H₂O₂ concentration. When inhibited enzyme was isolated by chromatography over Sephadex G-25, no recovery of activity was observed after 48 h.

We conclude that inhibition is a simple second order, irreversible reaction between cyclopropanone hydrate and compound I of peroxidase.

Characterization of Inhibited Peroxidase—Conveniently, horse-radish peroxidase can be dissociated into heme plus apoenzyme under relatively mild conditions, pH 1.5 (Mauk and Girotti, 1974). When apoenzyme was prepared in this way from active peroxidase and peroxidase that had been inactivated with cyclopropanone hydrate, it was found that apoenzyme from both sources was active when reconstituted with heme from active peroxidase. This result indicated that cyclopropanone hydrate reacts with the heme and does not modify the protein of peroxidase. Covalent modification of the heme was confirmed by mass spectrometry. The mass spectrum was obtained without derivatization of the heme using the fast atom bombardment technique. A molecular ion of mass 688 was observed, which corresponds to loss of a hydrogen and addition of the elements of C₃H₅O₂.

The heme-inhibitor adduct was further characterized by NMR (Fig. 2A). The NMR spectrum of heme extracted under the same conditions from active peroxidase is provided for comparison (Fig. 2B). Assignment of peaks for the unmodified heme has been described (Caughey et al., 1975); unassigned peaks in the region 0.8 to 1.5 ppm are due to impurities. The spectrum of the modified heme shows a new multiplet at 5.4 ppm. Homonuclear decoupling of this multiplet indicated that it is coupled to a second multiplet at 1.8 ppm. These multiplets integrated for 2 protons each. Of the various 3-carbon moieties (see mass spectral results above) which might reasonably be derived from cyclopropanone hydrate, these two new peaks are most consistent with the presence of a new propionic acid side chain on the modified heme.

The second prominent feature of the spectrum of Fig. 2A is the presence of only three peaks instead of four in the region near 10 ppm. Integration confirmed that these three peaks arise from only 3 protons. The peaks in this region arise from the methine protons at the meso carbons, which implies that the heme is modified by covalent attachment of a propionic acid moiety at one of the meso carbons. We tentatively assign the specific structure presented in Fig. 2A to the modified heme. Modification at the particular meso position shown, the δ position, would be expected to cause significant and approximately equal chemical shifts of the 1- and 8- methyl substituents. This expectation would be consistent with the observed spectrum.

The NMR assignments for the cyclopropanone-derived substituent in Fig. 2A, were confirmed by esterification and removal of the iron from the modified heme. The porphyrin, after this derivatization, was purified by chromatography over alumina (see "Materials and Methods"). The NMR spectrum of the derivatized porphyrin is presented in Fig. 3. A peak at 5.5 ppm is again apparent. Upon homonuclear decoupling of this peak, the triplet at 3.0 ppm collapses to a singlet. Integration indicates that each of these two triplets accounts for 2 protons. The spectra in both Figs. 2A and 3 are therefore consistent with the presence of a new propionic acid side chain. Also consistent with Fig. 2A is the fact that only three signals for the methine protons are observed in Fig. 3 in the region near 10 ppm, which is again evidence for substitution at a methine position.

The peak assigned to the β-protons of the meso propionic acid substituent is unexpectedly far upfield at 1.8 ppm in Fig. 2A. This is in comparison to the chemical shift of 3.0 ppm observed in Fig. 3 for the same protons in the esterified, metal-
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An intermediate in the inhibition reaction. Pertinent spectra are presented in Fig. 4 for the Soret region near 400 nm and for the near IR region. Oxidation of cyclopropanone hydrate to compound I led to the loss of activity with a half-life of 1.5 min. This loss of activity was accompanied by a decrease in absorbance in the Soret region and the appearance of a new peak in the near IR region (spectrum at 8 min in Fig. 4). The spectrum observed for this (initial) product is characteristic of an isoporphyrin, a \( \pi \) cation. A similar spectrum has been reported for the model isoporphyrin (Structure 1) (Dolphin et al., 1970); this spectrum shows a relatively weak Soret band, \( \epsilon = 35,000 \text{ m}^{-1} \text{ cm}^{-1} \), and a strong band in the region 850 to 900 nm, \( \epsilon = 18,000 \text{ m}^{-1} \text{ cm}^{-1} \). In the case of the model compound, in which there is no proton at the meso carbon, the \( \pi \) cation is a stable, isolabel species. A structure for the proposed isoporphyrin product and a mechanism for its formation in the inhibition reaction are provided under “Discussion.”

The initial isoporphyrin was not stable. A second, and final product was formed from the isoporphyrin with a half-life of 45 min (spectrum at 250 min in Fig. 4). The absorption in the near-IR region has disappeared for this species and a strong Soret absorption is again apparent, indicating the presence of a fully conjugated porphyrin. This final product is the meso-substituted heme characterized above (structure in Fig. 2A).

**DISCUSSION**

A mechanism for reaction of horseradish peroxidase with cyclopropanone hydrate is presented in Fig. 5. Evidence for each step will be discussed in turn. The inhibition reaction is a simple second order reaction between compound I, the 2-electron oxidized form of peroxidase, and cyclopropanone hydrate. Compound I, species 2 in Fig. 5, is believed to be best represented as a \( \pi \) catiop radical with the second oxidizing equivalent being localized as Fe(IV) (Dolphin et al., 1971). An oxygen ligand to iron has been proposed (Hewson and Hager, 1979). Calculations predict that spin density in the \( \pi \) cation radical of peroxidase compound I is highest at the meso carbons and the nitrogen atoms (Fajer et al., 1970). As compound I is drawn in the scheme, the localization of spin density at the meso carbons is overemphasized. Compound I characteristically oxidizes substrates in 1-electron steps. The 1-electron oxidation of cyclopropanone hydrate is well known (Wasserman et al., 1974), is facile, and in all cases proceeds by hydrogen abstraction at oxygen and \( \text{C}_1-\text{C}_2 \) ring opening to yield a primary free radical intermediate. Opening of the ring is thought to be concerted with hydrogen abstraction (Gibson and DePuy, 1974). This known chemistry is the basis for the proposed transformation of Structure 2 to 3.

Collapse of the radical pair in Structure 3 would lead to covalent bond formation at the meso position. It is attractive to believe that the initial high radical density at the meso

Free porphyrin. In comparison to the shifts for the propionic acid substituents at \( \text{C}_8 \) and \( \text{C}_7 \); the shift of 3.0 ppm appears to be more normal. The peak for these protons is the only peak that shifts substantially under the conditions of Fig. 3 compared to Fig. 2A. Although we cannot offer a convincing explanation for this shift, the fact that iron is present in the case that the peak is shifted upfield argues that a specific interaction of the side chain with the metal or its ligands may be involved.

**Spectral Changes Accompanying Inhibition**—Reaction of cyclopropanone hydrate with compound I of peroxidase results in spectral changes which have allowed the detection of

![NMR spectrum in CDCl₃ at 200 MHz of heme extracted from inhibited peroxidase. The heme has been esterified with methanol and the iron has been removed (see “Materials and Methods”). Peak assignments are for peaks which are common to heme from inhibited and from active peroxidase. Peaks at 1.7 ppm with methanol and the iron has been removed (see “Materials and Methods”). Peak assignments are for peaks which are common to heme from inhibited and from active peroxidase. Peaks at 1.7 ppm due to water and at 1.3 and 0.9 ppm due to impurities have been omitted.](image1)

![Electronic absorption spectra of products of the inhibition reaction. Horseradish peroxidase, 0.021 mM, was reacted with 0.60 mM H₂O₂ to give compound I. Cyclopropanone hydrate was then added in a small volume to give a final concentration of 0.068 mM. Aliquots were removed and assayed for loss of activity, and spectra were scanned repeatedly to determine the time dependence of the spectral changes. Spectra shown are only those of stable or quasi-stable species (see text). Spectra in the Soret region were determined in a separate experiment from those in the near-IR region in order to allow sufficiently rapid scanning of the spectra. Note the difference in scales in the two regions. –––, peroxidase; - – – – , compound I; . . . . , spectrum 8 min after addition of cyclopropanone hydrate; ———, spectrum 250 min after addition of cyclopropanone hydrate.](image2)
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FIG. 5. Mechanism of inhibition of peroxidase by cyclopropanone hydrate. See “Discussion” for details.

positions controls the regiochemistry of covalent bond formation. If this is true, then collapse of the radical pair must occur faster than any possible reorganization of electron density in Structure 3 and may be concerted with ring opening.

Intermediate 4 is then the isoporphyrin $n$ cation which was detected as the initial quasi-stable product of the inhibition reaction. Observation of the electronic absorption spectrum of this intermediate is probably the single most important piece of evidence for the proposed mechanism. Loss of a proton from Structure 4 gives the final, stable modified heme (Structure 5). Loss of the proton is perhaps unexpectedly slow (half-life = 49 min), but in this case requires substantial movement of the new meso propionic acid side chain. This movement may be hindered by steric interactions with the enzyme. Additionally, coordination of iron by this new propionic acid substituent is possible in the isoporphyrin (Structure 4), as shown, but is not possible in the final product (Structure 5), due to the change in geometry at the meso carbon.

Meso substitution of heme has been observed in other systems. The degradation of heme to bile pigments involves hydroxylation at the meso position (O'hEocha, 1968), and the reaction of oxyhemoglobin and oxymyoglobin with arylhydrazines has recently been found to involve arylation at both nitrogen and the meso positions (Saito and Itano, 1981). Particularly relevant is the observation that the autoinactivation of horseradish peroxidase in the presence of excess oxidizing agent involves hydroxylation at the meso position; and a transient species with absorption at 940 nm, presumably an isoporphyrin intermediate, has been detected in this reaction (Bagger and Williams, 1971).

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