Thianthrene 5-Oxide as a Probe of the Electrophilicity of Hemoprotein Oxidizing Species†

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ABSTRACT: Thianthrene 5-oxide (T-5-O), which is oxidized to the 5,10- and 5,5-dioxides, respectively, by electrophilic and nucleophilic agents, has been used to determine the electronic properties of hemoprotein oxidizing species. Cytochrome P450 oxidizes T-5-O to the 5,10- rather than the 5,5-dioxide but oxidizes the 5,5-dioxide rapidly and the 5,10-dioxide slowly to the 5,5,10-trioxide. Chloroperoxidase oxidizes T-5-O to the 5,10-dioxide but very poorly oxidizes it further to the 5,5,10-trioxide. It does, however, readily oxidize the 5,5-dioxide to the trioxide. The oxidizing species of cytochrome P450 and chloroperoxidase are thus comparably electrophilic, but the former is more powerful. T-5-O is not detectably oxidized by horseradish peroxidase/H_2O_2 but is oxidized exclusively to the 5,5-dioxide when the peroxide is replaced by dihydroxyfumaric acid (DHFA). The oxygen incorporated into the 5,5-dioxide in this reaction derives from molecular oxygen. This is consistent with the involvement of a DHFA-derived co-oxidizing species. Oxidation of T-5-O by human hemoglobin and H_2O_2 yields the 5,5- and 5,10-dioxides and the 5,5,10-trioxide. The oxygen in these products derives primarily (>80%) from H_2O_2. Hemoglobin and H_2O_2 thus form both a P450-like electrophilic oxidant (5,10-dioxide) and a peroxide-derived nucleophilic oxidant (5,5-dioxide). A large difference in the cis:trans ratios of the 5,10-dioxides produced from T-5-O by cytochrome P450 (1.3:1) and chloroperoxidase (2.5:1) vs hemoglobin (0.1:1) suggests that the hemoglobin active site severely constrains the geometry of the electrophilic oxidation. T-5-O is shown by these results to be a useful probe of the electronic properties of biological oxidants.

Peroxidation, peroxygenation, monooxygenation, and dioxygenation are among the diverse catalytic functions supported by the heme prosthetic group. All of these catalytic functions are thought to involve oxidation of the iron to a ferryl [Fe^{IV}=O] complex and the porphyrin or the protein to a radical species (Ortiz de Montellano, 1987). In cytochrome P450- and chloroperoxidase-catalyzed monooxygenations, the ferryl oxygen is transferred to the substrate (Ortiz de Montellano, 1986, 1987; Ortiz de Montellano et al., 1987; Kobayashi et al., 1986). The dominance of this ferryl oxygen transfer mechanism is due, in part, to the fact that the heme group in both proteins is coordinated to a cysteine thiolate ligand (Dawson & Sono, 1987). The ferryl oxygen, however, is not as readily transferred to substrates by hemoproteins with an imidazole rather than a thiolate heme ligand. Indeed, the ferryl oxygen appears not to be the immediate oxidant in many of the reactions catalyzed by these hemoproteins.

Horseradish peroxidase catalyzes the dihydroxyfumarate- and oxygen-dependent hydroxylation of aromatic compounds (Mason et al., 1957; Buhler & Mason, 1961; Klibanov et al., 1981). DHFA[1] cannot be replaced by H_2O_2, but there is some disagreement as to whether low concentrations of endogenously produced H_2O_2 facilitate catalysis (Halliwell & Ahluwalia, 1976; Halliwell, 1977; Dordick et al., 1986). The formation of H_2O_2 is consistent with the fact that the reaction is not tightly coupled, 12 mol of DHFA being consumed per mole of hydroxylated product. The oxygen atom incorporated into the hydroxylated aromatic product has been shown in one instance to derive from molecular oxygen (Mason, 1958). In contrast to hydroxylation by cytochrome P-450, the hydroxyl group is introduced without migration of the aromatic hydrogen to a vicinal position (the NIH shift) (Daly & Jerina, 1970). Product distributions, notably the formation of all three isomers of nitrophenol from nitrobenzene, are most consistent with a radical oxidizing species (Buhler & Mason, 1961). The finding that superoxide dismutase inhibits, and mannitol partially inhibits, the hydroxylation of phenols by horseradish peroxidase and DHFA has led to the proposal that the hydroxyl radical is the oxidizing species (Halliwell & Ahluwalia, 1976; Halliwell, 1977; Dordick et al., 1986). Accumulation of compound III, formed by reaction of the ferric hemoprotein with the superoxide radical anion, suggests that compound III may be an intermediate in the hydroxylation reaction (Halliwell, 1977; Dordick et al., 1986). Spectroscopic studies have provided evidence that DHFA is required for the formation of compound III and demonstrated that hydroxylation by compound III only occurs in the presence of DHFA. It has been argued from these results that the hydroxyl radical, formed as shown in Figure 1, is the actual oxidizing species, but its involvement is contradicted by a discrepancy between the concentrations of alcohol required to inhibit the DHFA-supported reactions and the concentrations required to inhibit the hydroxylation of *p*-nitrosodimethylaniline and other reactions mediated by radiolytically generated hydroxyl radicals (Bors et al., 1979). The ratio of hydroxylated products obtained from benzoic acid and nitrobenzene with radiolytically generated hydroxyl radicals also differs from that obtained with the DHFA/HRP system (Buhler & Mason, 1961).

Two or three mechanisms are required to explain the H_2O_2-dependent epoxidation of olefins by hemoglobin and myo-

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[1] Abbreviations: heme, iron protoporphyrin IX regardless of the oxidation and ligation state; T-5-O, thianthrene 5-oxide; T-5,5-dioxide, thianthrene 5,5-dioxide; *cis*-T-5,10-dioxide, *cis*-thianthrene 5,10-dioxide; *trans*-T-5,10-dioxide, *trans*-thianthrene 5,10-dioxide; T-5,5,10-trioxide, thianthrene 5,5,10-trioxide; DHFA, dihydroxyfumaric acid; HRP, horseradish peroxidase.

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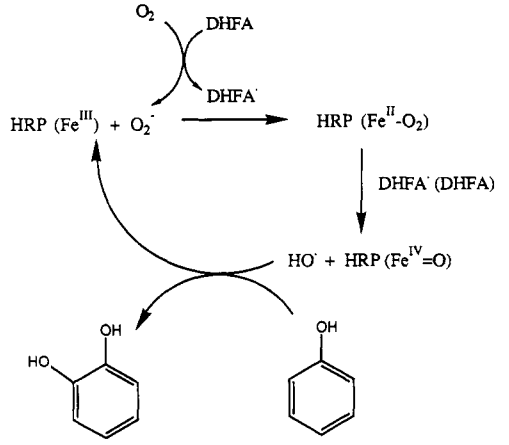


FIGURE 1: Catalytic cycle involving a hydroxyl radical proposed for oxidations supported by horseradish peroxidase and DHFA (Dordick et al., 1986).

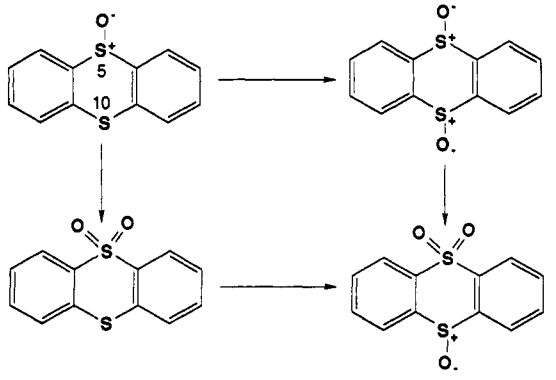


FIGURE 2: Oxidation of thianthrene 5-oxide (T-5-O) to the 5,5-dioxide, 5,10-dioxide, and 5,5,10-trioxide.

globin (Ortiz de Montellano & Catalano, 1985; Catalano & Ortiz de Montellano, 1987). One of these mechanisms is a cytochrome P450-like ferryl oxygen transfer to the olefin because the reaction proceeds with retention of the olefin stereochemistry and incorporation of an oxygen from the peroxide into the product. However, a fraction of the epoxidation of styrene and stilbene proceeds with loss of stereochemistry and incorporation of oxygen from O_2 rather than H_2O_2 (Ortiz de Montellano & Catalano, 1985; Catalano & Ortiz de Montellano, 1987). A co-oxidation mechanism in which the substrate is oxidized by the peroxy radical formed when oxygen adds to the protein radical was postulated to explain these findings. This rationalizes both the source of the epoxide oxygen and the loss of stereochemistry. Incorporation of oxygen from the medium into the tetrol produced by epoxidation of 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene by hemoglobin suggests the existence of a third mechanism, possibly one that involves oxidation of the polycyclic aromatic hydrocarbon to a radical cation (Catalano & Ortiz de Montellano, 1987).

We describe here a study of the electronic properties of the oxidizing species of cytochrome P450, chloroperoxidase/H_2O_2, HRP/DHFA, and hemoglobin/H_2O_2 using thianthrene 5-oxide (T-5-O). T-5-O, a probe which has been used to investigate the electronic character of *chemical* oxidants, is preferentially converted to the 5,10- and 5,5-dioxides, respectively, by electrophilic and nucleophilic oxidants (Figure 2) (Adam et al., 1984, 1987, 1991; Ballisteri et al., 1991). Electrophilic and nucleophilic oxidants also preferentially oxidize the 5,5- and 5,10-dioxides, respectively, to the 5,5,10-trioxide. The primary exception to this general trend is oxidation of T-5-O to the 5,5-dioxide by electrophilic radicals with a high ($E_{ox} > \sim 1.76$ V) oxidation potential. Formation of the nominally nucleophilic product by these electrophilic radicals involves electron transfer to give the T-5-O radical cation and the reduced oxidant that collapse, in a second step, to the observed product (Adam et al., 1991; Ballisteri et al., 1991). T-5-O is potentially very useful as a biological probe because the nucleophilic and electrophilic oxidation sites are part of a single, nearly symmetric molecule. This minimizes problems caused by differential substrate binding and rate-determining steps other than actual substrate oxidation. The results, some of which have been communicated in an abstract (Alvarez & Ortiz de Montellano, 1989), clarify the electronic nature of several hemoprotein oxidizing agents and demonstrate the utility of T-5-O as a biological probe.

EXPERIMENTAL PROCEDURES

Materials. Horseradish peroxidase (type VI), chloroperoxidase from *Caldariomyces fumago*, human hemoglobin (type IV), NADP, superoxide dismutase, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, 30% H_2O_2, and DHFA were purchased from Sigma (St. Louis, MO). $^{18}O_2$ (97.6 atom % ^{18}O) was purchased from MSD Isotopes. [^{18}O]H_2O_2 was prepared from $^{18}O_2$ according to the method of Sawaki and Foote (1979) or was purchased from ICON (Summit, NJ). Iodimetric titration established that the [^{18}O]H_2O_2 concentrations were 62 and 800 mM, respectively, for the synthetic and commercial samples (Pesez & Bartos, 1974). The ^{18}O content of the peroxide was determined by gas chromatography–mass spectrometry of the menadione epoxide generated by reaction of the peroxide with menadione under basic conditions (Ortiz de Montellano & Catalano, 1985). The ^{18}O contents were 52.8% and 97.9%, respectively, for the synthetic and commercial samples. T-5-O and T-5,5,10-trioxide were synthesized by oxidizing thianthrene (Aldrich Chemical Co.) in acetic acid with, respectively, nitric acid or chlorine (Gilman & Swayampati, 1955a). T-5,10-dioxide was obtained by stopping the oxidation of thianthrene with chlorine when the dioxide precipitated out (Gilman & Swayampati, 1955a). T-5,5-dioxide was prepared by reducing the 5,5,10-trioxide with zinc dust in acetic acid (Gilman & Swayampati, 1955b). The thianthrene oxides were shown to be pure by high-pressure liquid chromatography. The compounds had infrared, NMR, and mass spectra consistent with the assigned structures and melting points in good agreement with literature values: T-5-O, 142–143 °C [lit. 143–143.5 °C (Gilman & Swayampati, 1955a)]; T-5,5-dioxide, 164–165 °C [lit. 168–169 °C (Gilman & Swayampati, 1955b)]; *cis*-T-5,10-dioxide, 281–282 °C [lit. 284 °C (Hosoya, 1966)]; *trans*-T-5,10-dioxide, 250–251 °C [lit. 249 °C (Hosoya, 1966)]; and T-5,5,10-trioxide, 220–221.5 °C [lit. 221.5–222.5 °C (Gilman & Swayampati, 1955a)]. The absorption maxima for the compounds were as follows: T-5-O, λ_{max} = 201, 245 nm; T-5,5-dioxide, λ_{max} = 225, 263, 281, 311 nm; *cis*-T-5,10-dioxide, λ_{max} = 213 nm; *trans*-T-5,10-dioxide, λ_{max} = 213 nm; and T-5,5,10-trioxide, λ_{max} = 217, 243, 275 nm. 1-Aminobenzotriazole was synthesized by Dr. Ralph Stearns according to the procedure of Campbell and Rees (1969). All buffers were Chelex-treated to remove trace metal ions.

Absorption spectra were recorded on a Hewlett-Packard 8450A diode array spectrophotometer. NMR spectra were obtained on a Varian FT-80 instrument. High-pressure liquid chromatography was performed on a system consisting of two Beckman 110A pumps, an Altex gradient controller, and a Hewlett-Packard 1040A diode array detector interfaced with

a Hewlett-Packard workstation. Electron impact mass spectra were obtained on a Kratos MS-25 instrument at 70-eV ionizing current.

Product Analysis. The identity of the products was routinely confirmed by comparison of their retention times and absorption spectra with those of authentic samples. The mass spectra of the metabolites were also compared with those of the standards in early experiments. A standard curve was prepared using phenyl sulfone, which is well separated on HPLC from the thianthrene oxides, as an internal standard. Known amounts of the sulfone and of each of the thianthrene oxides were added to phosphate buffer, the mixture was extracted into methylene chloride, and the solvent was removed on a rotary evaporator. The residues were taken up in the mobile phase used for HPLC analysis (hexane/tetrahydrofuran/methanol 95:5:0.5). The HPLC analysis was carried out isocratically on a Partisil silica gel column at a solvent flow rate of 3 mL/min with the variable-wavelength detector set at 215 nm. The standard curves were based on five points each and had correlation coefficients greater than 0.970. The same workup protocol and HPLC system was used to analyze the products formed in each of the systems studied in this paper.

The *cis*- and *trans*-T-5,10-dioxide isomers are not resolved in the above HPLC system but are resolved by elution from the same column with 2.9% methanol in methylene chloride at a flow rate of 0.35 mL/min. This gives retention times of approximately 10, 12, 14, and 16 min for the 5,5-dioxide, 5,5,10-trioxide, 5-oxide, and *cis*-5,10-dioxide, respectively. The *trans*-5,10-dioxide has a much longer retention time in this system and is therefore eluted by increasing the flow rate to 2 mL/min after the cis isomer elutes from the column. The concentrations of the two isomers were calculated using the formula $(\text{cis/trans})_{\text{concentration}} = 0.2940 \times (\text{cis/trans})_{\text{area}}$ derived from a standard curve prepared with known amounts of the two isomers.

Microsomal Cytochrome P450 Incubations. Liver microsomes were prepared from Sprague-Dawley male rats pretreated with sodium phenobarbital (80 mg kg^{-1} day^{-1} for 4 days) as previously reported (Ortiz de Montellano et al., 1981). Incubations contained 1 μM microsomal cytochrome P450, 200 μM T-5-O, 1 mM NADPH, 3 mM glucose 6-phosphate, 2 mM $MgCl_2$, 14 units of glucose-6-phosphate dehydrogenase, 150 mM KCl, and 1.5 mM DETAPAC in 7 mL of Chelexed 100 mM phosphate buffer (pH 7.4). Incubations were initiated by adding the NADPH regenerating system and were carried out at 37 °C. Aliquots withdrawn at appropriate time periods were combined with 17.5 μL of 10 mM phenyl sulfone before the reaction was quenched by extraction into 7 mL of methylene chloride. The extracts were concentrated to dryness on a rotary evaporator, and the residue was taken up in the mobile phase used for HPLC analysis. Control incubations were carried out in the absence of the NADPH regenerating system. The T-5-O was replaced in some incubations by T-5,5-dioxide (50 μM) or either *cis*- (100 μM) or *trans*-T-5,10-dioxide (130 μM). In some experiments, the complete liver microsomal mixture except for T-5-O was incubated with two different concentrations (500 μM or 5 mM) of 1-aminobenzotriazole for 17 min before T-5-O was added, and the mixture was incubated for a further 10 min.

Chloroperoxidase Incubations. A solution of chloroperoxidase (6.66 μM), H_2O_2 (233 μM), and 50 μM T-5-O in 10 mL of 100 mM acetate buffer (pH 5.0) was incubated at 25 °C. Phenyl sulfone was added as an internal standard to aliquots withdrawn from the incubation at various time points before they were extracted with methylene chloride. The methylene chloride extracts were concentrated on a rotary evaporator and analyzed by high-pressure liquid chromatography. Analogous incubations were carried out with *trans*-T-5,10-dioxide (50 μM), *cis*-T-5,10-dioxide (50 μM), and T-5,5-dioxide (13.5 μM) as the substrate.

Horseradish Peroxidase/DHFA Incubations. Horseradish peroxidase (200 nM), 50 μM T-5-O, and 3 mg of DHFA in 20 mL of Chelex-treated 50 mM sodium acetate buffer (pH 5.0) were incubated at 25 °C with oxygen continuously bubbled through the incubation mixture. Additional DHFA (1.5 mg) was added in some experiments after 10 min. Aliquots (1 mL) removed at the desired time points were combined with 5 μL of a 10 mM phenyl sulfone solution and were then immediately extracted with methylene chloride. In large-scale incubations, 15 mg of DHFA was added initially and every hour thereafter. The extracts were taken to dryness on a rotary evaporator, and the product residues were analyzed by high-pressure liquid chromatography. In some incubations, the T-5-O was replaced by the 5,5-dioxide (50 μM), *cis*-5,10-dioxide (50 μM), or *trans*-5,10-dioxide (50 μM). Some incubations contained superoxide dismutase (2 mg, 7100 units). Methanol (49, 124, 247 mM) was added to a set of smaller (5 mL) incubations, and 1-mL aliquots were periodically removed and analyzed for metabolite formation. Control incubations were generally run in the absence of horseradish peroxidase.

In some experiments, compound III was preformed by aerobic incubation of horseradish peroxidase with DHFA. The reaction was then passed through a Sephadex G-25 column to remove excess DHFA and other small molecules. The compound III solution thus obtained was diluted with an equal amount of buffer and was incubated with and without 50 μM T-5-O.

Incubation of Horseradish Peroxidase/DHFA with T-5-O and $^{18}O_2$. Approximately 25 mL of a 1 μM solution of horseradish peroxidase in 50 mM sodium acetate buffer (pH 5.0) was placed in a three-neck round-bottom flask equipped with a pressure-equalizing funnel containing 45 mg of DHFA in 10 mL of the same buffer. An ampule containing 250 mL of $^{18}O_2$ and a vacuum manifold were attached to the other two necks. The system was repeatedly evacuated and purged with nitrogen and was finally left under a slightly negative pressure of nitrogen. The vigorously stirred mixture was then cooled in an ice bath, the oxygen ampule was opened, and one-third of the DHFA was added to initiate the reaction. The remaining DHFA was added in two further equal portions after the first and second hours of the incubation. The total incubation time was 3 h. The mixture was then extracted with methylene chloride, and the organic products were separated by high-pressure liquid chromatography. The T-5,5-dioxide product was collected and analyzed by mass spectrometry.

Hemoglobin Incubations. To a solution of human hemoglobin (40 μM) and T-5-O (50 μM) in 5 mL of 200 mM phosphate buffer (pH 7.4) was added enough H_2O_2 to bring its final concentration to 600 μM. The mixture was incubated at 25 °C. Aliquots were removed at various time points and were combined with 5 μL of 10 mM phenyl sulfone prior to extraction into methylene chloride. Identical control incubations were carried out in the absence of hemoglobin. The methylene chloride extracts were concentrated and analyzed by high-pressure liquid chromatography. Analogous experiments were also carried out in which the T-5-O was replaced

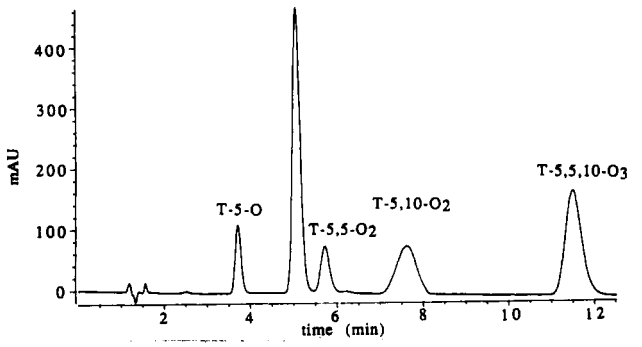


FIGURE 3: High-pressure liquid chromatogram of the possible reaction products formed in the catalytic oxidation of T-5-O. The incubation and chromatographic conditions are given under Experimental Procedures. The identity of each peak is indicated. The unlabeled peak is the internal standard phenyl sulfone.

by equal concentrations of *trans*- or *cis*-T-5,10-dioxide or T-5,5-dioxide. Superoxide dismutase (2 mg, 7200 units) was added to some incubations. To determine whether the oxygen comes from molecular oxygen, 80 mg of hemoglobin was added to 20 mL of Chelex-treated 200 mM phosphate buffer (pH 7.4) containing 50 μM T-5-O. After several evacuation/argon purge cycles, $^{18}O_2$ was admitted to the flask by breaking the gas ampule seal. The reaction was started by adding 150 μL of 0.1 M H_2O_2. After 20 min, the reaction was worked up and the products purified by HPLC as described above. The 5,5-dioxide, 5,10-dioxide, and 5,5,10-trioxide products were then analyzed by mass spectrometry. Analogous experiments with 40 mg of hemoglobin in a total of 10 mL of buffer containing 100 μM T-5-O were used to determine whether the oxygen came from H_2O_2. These reactions were initiated, without the argon purge step, by adding either 350 μL of 62 mM [^{18}O]H_2O_2 or 70 μL of 100 mM [^{18}O]H_2O_2.

RESULTS

Cytochrome P450. T-5-O is oxidized by liver microsomes from phenobarbital-pretreated rats in the presence of NADPH to T-5,10-dioxide and T-5,5,10-trioxide (Figure 3). The identities of these two products were established by cochromatography with authentic samples and by comparison of their distinctive absorption spectra with those of authentic materials. Product formation is decreased by >80% if the liver microsomes are preincubated for 17 min with 5 mM 1-aminobenzotriazole, a mechanism-based inactivator of cytochrome P450 (Ortiz de Montellano & Mathews, 1981) that does not inhibit the microsomal flavin monooxygenase (Cashman, 1987). Quantitative study of the time course of the oxidation shows that disappearance of T-5-C is paralleled by appearance of the 5,10-dioxide and a small amount of the 5,5,10-trioxide (Figure 4). No more than a trace of T-5,5-dioxide is observed at any point in these incubations. The initial rate of 5,10-dioxide formation is approximately 32 nmol (nmol of P450)$^{-1}$ min^{-1}. The T-5,10-dioxide that is obtained consists of a 57:43 cis:trans isomer mixture (Table I). Incubation of T-5,5-dioxide or either *cis*- or *trans*-5,10-dioxide with the liver microsomal system establishes that the three dioxides are converted to the trioxide at different but readily observable rates (Figure 5). Initial rates of approximately 8, 4, and 2 nmol (nmol of enzyme)$^{-1}$ min^{-1} were estimated for the 5,5-, *cis*-5,10-, and *trans*-5,10-dioxides, respectively. The 5,5-dioxide is thus oxidized more rapidly to the trioxide than either the *cis*- or *trans*-5,10-dioxide, and the *cis*-5,10-dioxide is oxidized more rapidly than the *trans*-5,10-dioxide.

Chloroperoxidase. Chloroperoxidase oxidizes T-5-O to the 5,10-dioxide in high yield without detectably forming the 5,5-dioxide or 5,5,10-trioxide (Figure 6). No oxidized products are obtained in control incubations without the enzyme. The 5,10-dioxide is formed in a time-dependent manner at 25 °C at a rate of approximately 1.5 nmol (nmol of enzyme)$^{-1}$ min^{-1}, a rate that corresponds well with that for disappearance of the 5-oxide (Figure 6). Analogous incubations with the more highly oxidized thianthrenes show that the 5,5-dioxide is readily converted by chloroperoxidase to the 5,5,10-trioxide (Figure 7). The initial rate of the reaction is approximately 0.06 nmol (nmol of enzyme)$^{-1}$ min^{-1}, but the concentration of the

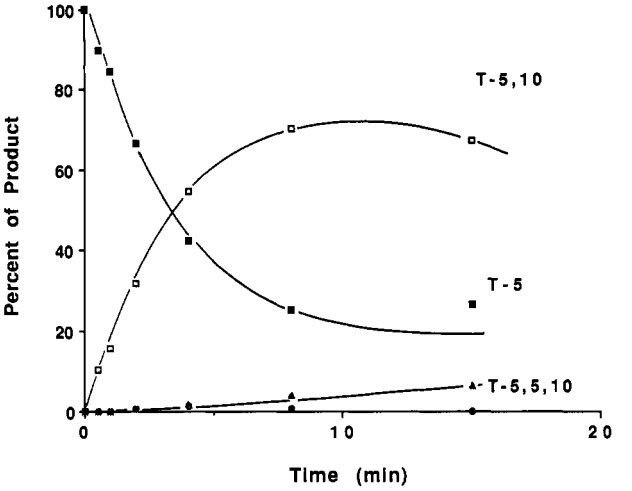


FIGURE 4: Time course for the microsomal cytochrome P450-catalyzed oxidation of T-5-O (■) to T-5,10-dioxide (□) and T-5,5,10-trioxide (▲). T-5,5-dioxide (●) is not detectably formed.

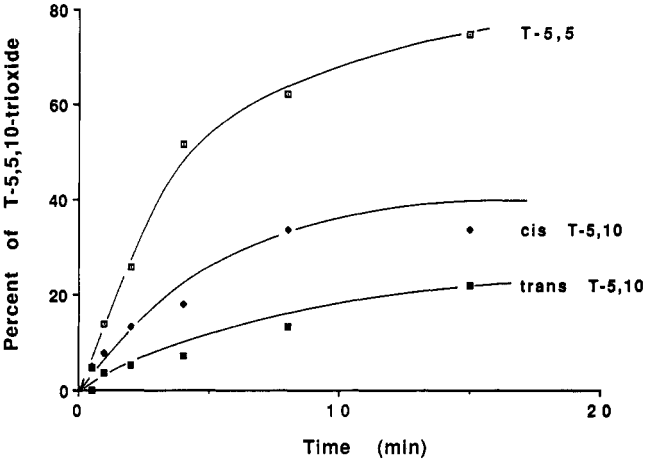
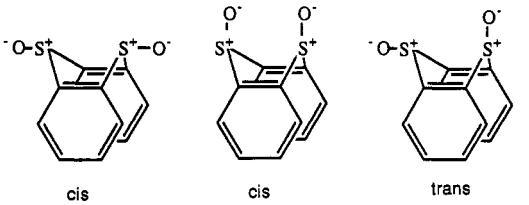


FIGURE 5: Time courses for the microsomal cytochrome P450-catalyzed oxidations of T-5,5-dioxide (□), *cis*-T-5,10-dioxide (♦), and *trans*-T-5,10-dioxide (■) to T-5,5,10-trioxide. The incubation details are given under Experimental Procedures.

Table I: Isomeric Composition of the T-5,10-dioxide Product[a]



enzyme system	% cis	% trans	cis/trans
cytochrome P450	57.1 ± 0.9	42.9 ± 0.9	1.3
chloroperoxidase	71.3 ± 1.3	28.7 ± 1.3	2.5
hemoglobin	9.3 ± 2.0	90.7 ± 2.0	0.1

[a] Values are given ± standard deviation.

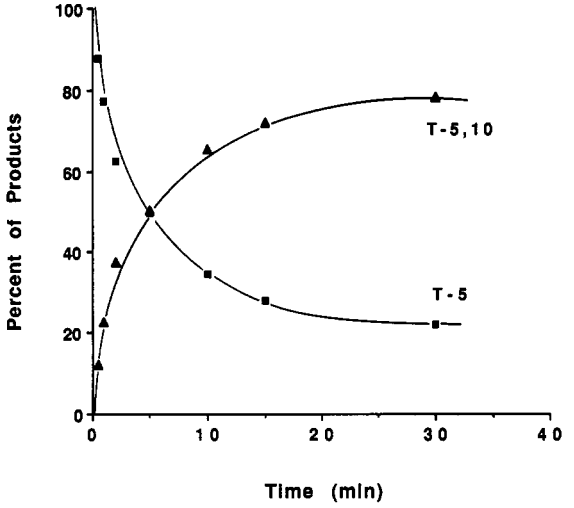


FIGURE 6: Time course for the chloroperoxidase-catalyzed formation of T-5,10-dioxide (▲) from T-5-O (■). The incubation details are given under Experimental Procedures.

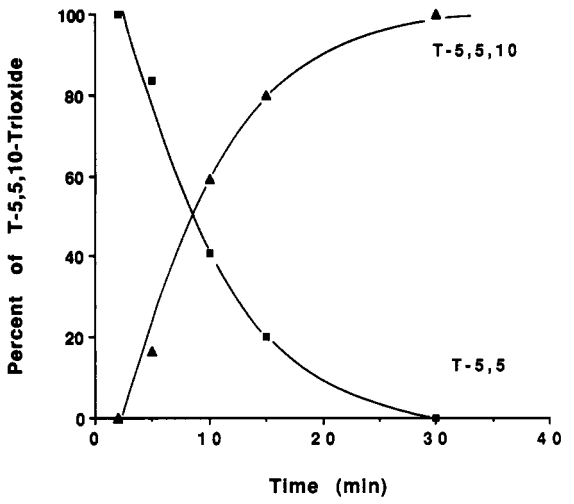


FIGURE 7: Time course for chloroperoxidase-catalyzed oxidation of T-5,5-dioxide (■) to T-5,5,10-trioxide (▲). The incubation details are given under Experimental Procedures.

substrate, which was only 13.5 μM due to solubility problems, may not have been sufficiently high to saturate the enzyme. The *cis*-5,10-dioxide is converted to the trioxide at a rate no greater than 0.03 nmol (nmol of enzyme)$^{-1}$ min^{-1}, whereas only traces of the 5,5,10-trioxide could be detected with the *trans*-5,10-dioxide.

Horseradish Peroxidase/Dihydroxyfumaric Acid. No reaction products were detected when horseradish peroxidase was incubated with T-5-O and H_2O_2. However, incubation of T-5-O with horseradish peroxidase and DHFA without added H_2O_2 produces T-5,5-dioxide (Figure 8). The 5,10-dioxide and 5,5,10-trioxide were not detectably formed in these incubations. T-5,5-dioxide formation tapers off after a few minutes. Product formation recommences, however, if fresh DHFA is added to the incubation mixture (Figure 8) and can be continued for hours if DHFA is added periodically. The reaction is thus limited by exhaustion of DHFA. The reaction consumes approximately 5–7 nmol of DHFA/mol of 5,5-dioxide formed. The initial rate of formation of T-5,5-dioxide can be estimated from the data to be approximately 5.7 nmol (nmol of enzyme)$^{-1}$ min^{-1}. Control incubations without horseradish peroxidase show that the 5,5-dioxide is formed but at a much lower rate due to a reaction supported solely by DHFA. Superoxide dismutase inhibits, but added H_2O_2

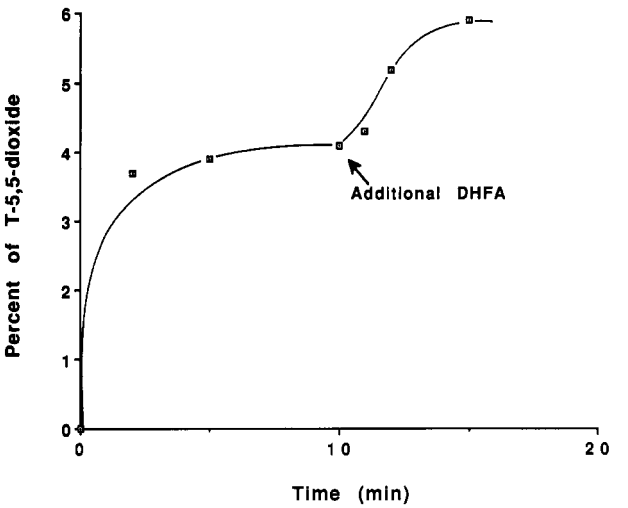


FIGURE 8: Time course of the formation of T-5,5-dioxide from T-5-O in incubations with horseradish peroxidase and DHFA. The arrow indicates the time point at which a supplement of DHFA was added to the incubation. The details of the incubation system are given under Experimental Procedures.

has little effect on, product formation (not shown). To determine the source of the oxygen atom incorporated into the product, a large-scale incubation of T-5-O, horseradish peroxidase, and DHFA was run under an atmosphere of $^{18}O_2$, and the T-5,5-dioxide produced was isolated and compared by mass spectrometry with material obtained under a normal $^{16}O_2$ atmosphere. Nearly complete shift of the T-5,5-dioxide molecular ion from m/z 248 to 250 in the incubation with labeled oxygen clearly establishes that the oxygen incorporated into the product is from molecular oxygen. Concentrations of methanol in the millimolar range inhibit the formation of T-5,5-dioxide by the HRP/DHFA system in a dose-dependent fashion but do not lead to the appearance of other identifiable T-5-O metabolites (not shown). The inhibition presumably involves transfer of a hydrogen from the carbon atom of methanol to the oxidizing species, but detailed studies of the inhibition mechanism have not been carried out.

No 5,5,10-trioxide was obtained in incubations of the 5,5-dioxide with horseradish peroxidase and DHFA. However, both isomers of the 5,10-dioxide were converted to the trioxide in this system. The time course for conversion of the *cis*-5,10-dioxide to the trioxide (Figure 9) shows that the reaction is also limited by exhaustion of DHFA because product formation recommences when DHFA is added after product formation levels off. The initial rate of product formation appears to be approximately 7.6 nmol (nmol of enzyme)$^{-1}$ min^{-1}. The *trans*-5,10-dioxide is also oxidized to the trioxide but slowly enough that it has not been possible to accurately estimate the rate.

Dordick et al. showed that compound III is stable and does not hydroxylate aromatic compounds unless DHFA is present (Dordick et al., 1986). To determine the reactivity of compound III itself with T-5-O, compound III was formed by incubating horseradish peroxidase with DHFA and passing the reaction mixture through Sephadex G-25 to remove excess DHFA and other small redox-active substances. The compound III thus obtained was reasonably stable, very little decay being observed after 6 min. This contrasts with a half-life of approximately 3 min for compound III in the presence of DHFA. Addition of T-5-O to compound III did not accelerate the rate of decay of the iron–oxygen complex and did not give rise to oxygenated products. The requirement for HRP and

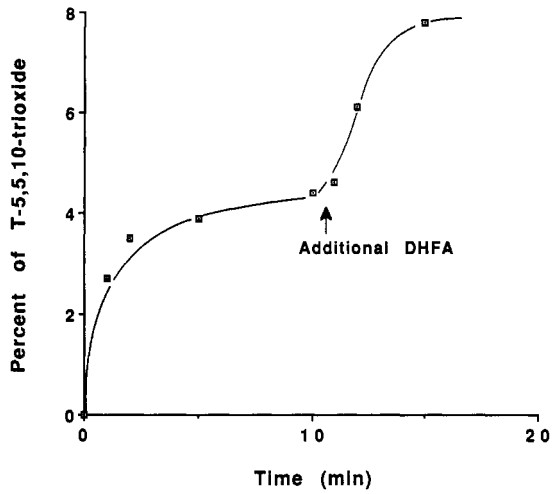


FIGURE 9: Time course of the formation of T-5,5,10-trioxide in incubations of *cis*-T-5,10-dioxide with horseradish peroxidase and DHFA. The arrow indicates the time point at which a supplement of DHFA was added to the incubation.

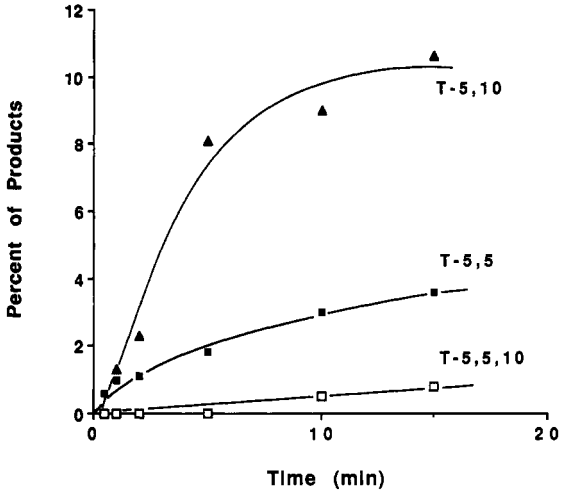


FIGURE 10: Time course of the oxidation of T-5-O by human hemoglobin and H_2O_2. The incubation details are given under Experimental Procedures.

DHFA for the oxidation of T-5-O thus cannot be met by compound III alone.

Hemoglobin. Oxidation of T-5-O by human hemoglobin and H_2O_2 results in time-dependent formation of T-5,5-dioxide, -5,10-dioxide, and 5,5,10-trioxide (Figure 10). The initial rate of T-5,5-dioxide formation is 50 nmol min^{-1} (mg of hemoglobin)$^{-1}$ and that for T-5,10-dioxide 160 nmol min^{-1} (mg of hemoglobin)$^{-1}$. The ratio of the 5,5- to 5,10-thianthrene products in the incubation is 1:5. Incubation of the 5,5-dioxide or the *cis*- or *trans*-5,10-dioxide with hemoglobin and H_2O_2 shows that all three dioxides are converted to the trioxide. The 5,5-dioxide and *cis*-5,10-dioxide are converted to the trioxide much more rapidly than the *trans*-5,10-dioxide (not shown). Control incubations demonstrate that both hemoglobin and H_2O_2 are required for these transformations to occur. Little difference is seen in the product distribution or yield when the incubations are run under hypoxic conditions, except perhaps for a slight decrease in the yield of the 5,5-dioxide, or in the presence of superoxide dismutase (not shown). This suggests that neither molecular oxygen nor the superoxide radical is involved in formation of any of the products. This is confirmed by mass spectrometric analysis of the products formed under an atmosphere of $^{18}O_2$. None of the products showed significant (>5% above natural abundance background) incorporation of labeled oxygen. In contrast, the oxygen incorporated into T-5,5-dioxide derives primarily or exclusively from the peroxide. Thus, the metabolite produced in an incubation with $[^{18}O]H_2O_2$ was 92% ^{18}O-labeled (after correction for the specific ^{18}O content of the peroxide). The somewhat lower value of 85% was found in a second experiment with a different sample of labeled peroxide. The ^{18}O contents of the 5,10-dioxide product were found to be 90% and 94% in two different experiments. It appears, on the basis of these experiments, that most, if not all, of the sulfoxide oxygen derives from H_2O_2.

T-5,10-dioxide Isomers. Cytochrome P450, chloroperoxidase, and hemoglobin yield very different proportions of the *cis*- and *trans*-T-5,10-dioxides (Table I). Chloroperoxidase produces more of the cis isomer, microsomal cytochrome approximately equal amounts of both isomers, and hemoglobin far more of the trans than the cis isomer. These differences in the stereochemistry of the T-5,10-dioxide product must be due to intrinsic differences in the stereospecificities of the oxidizing agents or steric constraints imposed on the reaction by the protein structure. Since the 5,10-dioxide is thought to be produced in all cases by reaction with electrophilic ferryl oxygen species, it is likely that the stereochemical differences reflect protein control of the reaction.

DISCUSSION

T-5-O is rapidly oxidized by microsomal cytochrome P450, as expected for an electrophilic oxidant, to the 5,10- but not the 5,5-dioxide (Figures 3 and 4). The synthetic 5,5-dioxide is also more rapidly oxidized to the 5,5,10-trioxide than either isomer of the 5,10-dioxide (Figure 5). The approximate relative rates of oxidation of the various thianthrene species are as follows: T-5-O, 35; 5,5-dioxide, 8; *cis*-5,10-dioxide, 4; and *trans*-5,10-dioxide, 2 nmol (nmol of enzyme)$^{-1}$ min^{-1}. The regiospecificity of the oxidation of T-5-O is thus consistent with earlier data showing that the V_{max} values for the cytochrome P450-catalyzed oxidation of four thioanisoles (ρ = –0.16 vs σ^+) (Watanabe et al., 1981) and four sulfoxides (ρ = –0.2 vs σ^+) (Watanabe et al., 1982) are linearly related to the oxidation potentials of the substrates and with the evidence implicating an electron-deficient ferryl oxygen in the catalytic action of cytochrome P450 (Ortiz de Montellano, 1986). The one-electron oxidation potential of T-5-O is 1.76 V (Adam et al., 1991; Evans & Blount, 1977).

Chloroperoxidase likewise oxidizes T-5-O to the isomeric 5,10-dioxides but not detectably to the 5,5-dioxide (Figure 6). This indicates that its oxidizing species is highly electrophilic. Chloroperoxidase oxidations have been postulated to involve P450-like ferryl oxygen transfer to substrates because the label from $[^{18}O]H_2O_2$ is incorporated into the sulfoxides formed from thioanisole and *p*-methylthioanisole (Kobayashi et al., 1986) and the epoxide produced from styrene (Ortiz de Montellano et al., 1987). The major difference between the chloroperoxidase and cytochrome P450 catalytic species appears to be that the latter is a more powerful oxidizing agent. Chloroperoxidase thus readily oxidizes the 5,5-dioxide to the trioxide (Figure 7) but, unlike cytochrome P450, very poorly oxidizes the 5,10-dioxide isomers. This may be due to differences in the reactivities of the two ferryl species caused by, for example, the proposed higher polarity of the chloroperoxidase active site (Ortiz de Montellano et al., 1987; Dawson & Sono, 1987). Alternatively, chloroperoxidase may be a weaker oxidant because it is sensitive to self-inactivation by H_2O_2 in the absence of a good substrate. Chloroperoxidase has been reported to be inactivated during the catalytic

turnover of substituted thioanisoles (Kobayashi et al., 1986) and may be inactivated more rapidly when T-5-O is replaced by the less readily oxidized 5,10-dioxides. The clear-cut preference for oxidation of the electron-rich sulfur in T-5-O conflicts with the reported absence of a relationship between the sulfoxidation rates and the electrochemical oxidation potentials in the chloroperoxidase-catalyzed oxidation of benzyl methyl sulfide ($E_{1/2}$ = 1.71 vs SCE), thioanisole ($E_{1/2}$ = 1.44), and thiobenzamide ($E_{1/2}$ = 1.30) (Doerge, 1986) or the sulfoxidation rates for anisole and its *p*-Me, *p*-MeO, and *p*-iPrO derivatives (Kobayashi et al., 1986). It agrees, however, with a later study of the oxidation of nine para-substituted thioanisoles which found a moderately good correlation (ρ = −1.40, correlation coefficient r = 0.824) between the log of the oxidation rate and the σ substituent constant (Kobayashi et al., 1987).

The hydroxyl radical has been proposed as the oxidizing species in the DHFA/HRP system, but the primary evidence for its involvement is the inherently ambiguous observation that the reaction is inhibited by agents that react with the hydroxyl radical. The fact that T-5-O is exclusively oxidized in this system to the 5,5-dioxide (Figure 8) and that the 5,10- but not the 5,5-dioxide is oxidized to the 5,5,10-trioxide (Figure 9) rules out a P450- or chloroperoxidase-like electrophilic ferryl oxygen species. The hydroxyl radical is relatively nonselective as an oxidant, but to the extent that its reactivity is governed by its electronic properties, it should behave as an electrophile rather than a nucleophile. In agreement with this analysis, the oxidation of T-5-O by the hydroxyl radical using the Fenton reaction and ^{60}Co radiolysis has been found to yield products other than the 5,5-dioxide (unpublished results). Inclusion of 50 mM acetate in the buffer, as in the DHFA/HRP reactions, suppresses substrate oxidation in the radiolysis experiments. It is therefore unlikely that the species that cleanly oxidized T-5-O to the 5,5-dioxide in the DHFA/HRP system is the free hydroxyl radical.

One alternative for the oxidizing species in the DHFA/HRP system is the iron–dioxygen [$Fe^{II}O$–O^-] complex expected from electron transfer to compound III. This would be consistent with the finding that compound III does not mediate aromatic ring hydroxylation unless excess DHFA is present (Dordick et al., 1986). This possibility is also supported by the recent finding that an iron–peroxide complex, albeit with non-heme iron, catalyzes the hydrolysis of peptide bonds (Rana & Meares, 1991). Another alternative is that the primary function of the peroxidase is to accelerate the oxidation of DHFA, using endogenously generated peroxide, to a radical that binds oxygen and co-oxidizes the substrates. The first steps of such a mechanism would be comparable to those proposed for the HRP-catalyzed oxidation of malonaldehyde (MacDonald & Dunford, 1989). The fact that the oxygen incorporated into the 5,5-dioxide derives quantitatively from molecular oxygen is consistent with both mechanisms, but the finding that DHFA is rapidly consumed in the reaction and that low yields of the same product are produced in control incubations with DHFA but no enzyme is more consistent with a DHFA-mediated co-oxidation mechanism (Figure 11).

The oxidation of T-5-O by human methemoglobin and H_2O_2 produces both the 5,5- and 5,10-dioxide metabolites (Figure 10). Of the enzyme systems examined, only hemoglobin/H_2O_2 oxidizes T-5-O with comparable efficiency to both the 5,5- and 5,10-dioxides. This indicates that the enzyme produces an electrophilic and either a nucleophilic or a high oxidation potential radical oxidant. Failure to incorporate an atom of oxygen from labeled O_2 into either product rules

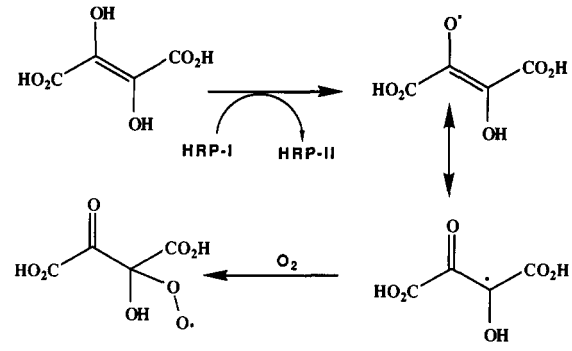


FIGURE 11: Formation of a hypothetical DHFA-peroxyl radical that co-oxidizes T-5-O in the HRP/DHFA system. The oxidizing species could be the peroxy radical or peroxy anion.

out formation of the 5,5-dioxide by the protein co-oxidation mechanism previously invoked to explain the methemoglobin-catalyzed incorporation of an atom of oxygen from molecular oxygen into the styrene oxide formed from styrene (Ortiz de Montellano & Catalano, 1985; Catalano & Ortiz de Montellano, 1987). The finding that the oxygen of the 5,10-dioxide derives primarily or exclusively from H_2O_2 is readily explained by the involvement of an electrophilic, P450-like, ferryl species. A ferryl species is known to be formed in the reaction of methemoglobin with H_2O_2, and evidence for its involvement in olefin oxidation has been obtained (Ortiz de Montellano & Catalano, 1985; Catalano & Ortiz de Montellano, 1987). The incorporation of oxygen from the peroxide but not molecular oxygen into the 5,5-dioxide product is more difficult to rationalize. The possibility that this oxygenation reaction is mediated by the superoxide radical anion produced by autoxidation of the hemoglobin ferrous dioxygen complex is not consistent with the observation that superoxide dismutase does not alter either the extent of product formation or the product distribution. The reaction could be mediated by the ferrous dioxygen complex itself but only after it is activated in some manner, possibly by transfer of an electron to produce the Fe^{II}–O–O^- species, because control experiments show that the ferrous dioxygen complex itself (i.e., oxyhemoglobin) is inert. It is of interest, in the present context, that chlorpromazine, a substrate that resembles T-5-O, binds to and perturbs the heme crevice of hemoglobin (Bhattacharyya et al., 1989, 1990), is oxidized by hemoglobin/H_2O_2, and binds covalently to the protein as a result of its catalytic turnover (Kelder et al., 1991; Magee & Marletta, 1989).

The T-5,10-dioxide cis:trans product ratio slightly favors the cis isomer and is similar for the cytochrome P450 and chloroperoxidase reactions but overwhelmingly favors the trans isomer in the hemoglobin reaction (Table I). This difference could reflect differences in the intrinsic properties of the oxidizing agent or differences in the constraints imposed on the reaction by the hemoprotein active sites. Derivation of the oxygen from the peroxide in the product generated by all three hemoproteins, presumably by oxygen transfer from the ferryl complex, provides no support for an essential difference in the oxidizing species. The differences in the isomer ratio are therefore more likely to be caused by differences in the active sites of the hemoproteins. Microsomal cytochrome P450 enzymes are suggested by their broad substrate specificities (Guengerich, 1987), propensity to oxidize alternative sites when a favored substrate site is deuterated (Foster, 1985), and structural similarities to cytochrome $P450_{cam}$, for which a crystal structure is available (Poulos et al., 1987), to enclose their substrates in large, malleable active sites within which there may be a substantial degree of rotational freedom. T-

5-O oxidation could therefore be relatively unconstrained in the cytochrome P450 active site. On the other hand, the crystal structures of sperm whale myoglobin (Takano, 1977a,b) and human hemoglobin (Fermi & Perutz, 1977) indicate that the heme is located near the surface in a small and congested crevice that is capped by a histidine residue. The crystal structures of a myoglobin phenyl–iron complex (Ringe et al., 1984), a complex of myoglobin with ethyl isocyanide (Johnson et al., 1989), and a complex of methemoglobin with imidazole (Bell et al., 1981) indicate, however, that the histidine is readily displaced, making the heme accessible to relatively large ligands. Nevertheless, the crystal structures suggest that displacement of the histidine by T-5-O will only allow a corner of the substrate to actually enter the heme crevice. This could severely constrain the oxidation geometry and lead to the observed bias for the trans isomer.

The oxidation of T-5-O, as shown here, is a useful tool for characterizing biological oxidizing species. It readily distinguishes the oxidations mediated by the electrophilic ferryl oxygen of cytochrome P450 and chloroperoxidase from the electrophilic oxidant of hemoglobin and the reactions mediated by nucleophilic oxidants. The latter oxidizing agents remain to be fully characterized, but the present results provide valuable insight into their electronic properties and eliminate a number of otherwise viable alternatives. T-5-O should prove similarly useful in the characterization of other heme and non-heme biological redox systems.

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