Cooxidation of Styrene by Horseradish Peroxidase and Phenols: A Biochemical Model for Protein-Mediated Cooxidation†

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ABSTRACT: Styrene is oxidized to styrene oxide and benzaldehyde when incubated with horseradish peroxidase, H_2O_2, and 4-methylphenol. Styrene oxide is not formed in the absence of any of these reaction components or of molecular oxygen. The coupling products 2-(4-methylphenoxy)-1-phenylethane, 2-(4-methylphenoxy)-1-phenylethan-1-ol, and 2-(4-methylphenoxy)-2-phenylethan-1-ol are not formed, but the ortho-linked dimer of 4-methylphenol is a major product. The epoxide oxygen is labeled in the presence of $^{18}O_2$ but not $H_2{}^{18}O_2$. Styrene oxide formation is not inhibited by mannitol or superoxide dismutase. The stereochemistry of *trans*-[1-^{2}H]styrene is partially scrambled in the epoxide product. EPR signals attributable to the 2,4-dihydroxy-5-methylphenoxy radical, a product of the oxidation of 4-methylcatechol, are observed if Zn^{2+} is added to stabilize the radical. This radical is only detected in the presence of styrene. The results imply that styrene is epoxidized by the hydroperoxy radical generated by addition of molecular oxygen to the 4-methylphenoxy radical. The epoxidation mimics the chemistry proposed to occur in the protein-mediated cooxidation of styrene by hemoglobin and myoglobin.

The oxidation of arachidonic acid by prostaglandin synthase results in the cooxidation of a variety of organic substrates (Marnett, 1984; Marnett & Eling, 1983). Cooxidative processes are not restricted to the oxidation of unsaturated fatty acids by prostaglandin synthase, however, because arachidonic acid can be replaced by phenylbutazone (Reed et al., 1984) and prostaglandin synthase by hemin or other chemical or enzymatic systems (Dix et al., 1985; Yamamoto et al., 1986). Horseradish peroxidase, a case in point, supports the cooxidation of arachidonic acid by a variety of agents (Baumann et al., 1986) and of styrene by glutathione (Ortiz de Montellano & Grab, 1986). The N-dealkylation of tertiary aromatic amines by horseradish peroxidase also appears to proceed, in part, by a cooxidative process in which the carbon radical from one molecule of the alkylamine cooxidizes a second (Galliani et al., 1978; Kedderis et al., 1986). The key features of cooxidative processes, using styrene as the substrate, are summarized by the following set of reactions:

$$RH \rightarrow R^{\bullet}$$

$$R^{\bullet} + O_2 \rightarrow ROO^{\bullet}$$

$$ROO^{\bullet} + PhCH{=}CH_2 \rightarrow PhCHCH_2OOR$$

$$PhCHCH_2OOR \rightarrow PhCH\!\!<\!\!\begin{matrix}O\\ \\ \end{matrix}\!\!>\!\!CH_2 + RO^{\bullet}$$

The cosubstrate (RH) is oxidized to a radical (R•) that reacts with molecular oxygen. The resulting peroxy radical (ROO•) then adds to the double bond of styrene to give an organoperoxy carbon radical (PhĊHCH$_2$OOR) that decomposes to styrene oxide and the alkoxy radical (RO•).

Phenols, including 4-methylphenol, are oxidized by horseradish peroxidase to EPR-detectable[1] phenoxy radicals (Shiga & Imaizumi, 1975; Hewson & Dunford, 1976a). These radicals dimerize, polymerize, and undergo other typical radical reactions. The oxidation of 4-methylphenol by horseradish peroxidase and H_2O_2 (Hewson & Dunford, 1976a,b; Westerfield & Lowe, 1942) thus yields 2,2′-dihydroxy-5,5′-dimethylbiphenyl, the corresponding trimer of ortho-linked 4-methylphenol units, and a furan that stems from ortho–para rather than ortho–ortho dimerization of 4-methylphenoxy radicals (Hewson & Dunford, 1976a; Westerfield & Lowe, 1942; Booth & Saunders, 1956). Tyrosine is similarly oxidized to an ortho-linked dimer by horseradish peroxidase (Gross & Sizer, 1959). Products other than those that result from radical oligomerization are obtained if the ortho and/or para positions of the phenoxy radical are blocked. 2,3,5,6-Tetramethylphenol, a particularly relevant example, is oxidized not only to the para-linked dimer but also to 2,3,5,6-tetramethylquinone (Booth & Saunders, 1956). The quinone presumably derives from the hydroperoxide obtained by para addition of molecular oxygen to the phenoxy radical.

We have recently proposed that the H_2O_2-dependent oxidation of styrene by hemoglobin and myoglobin proceeds, in part, by a novel mechanism in which the olefin is cooxidized by a tyrosine vicinal to the prosthetic heme group (Ortiz de Montellano & Catalano, 1985). According to this mechanism, reaction of the hemoprotein with H_2O_2 results in oxidation of the iron to the tetravalent ($Fe^{IV}{=}O$) state and of the tyrosine to the corresponding phenoxy radical. Reversible binding of molecular oxygen to the phenoxy radical then yields a peroxy radical that cooxidizes the olefin. The investigation reported here of the cooxidation of styrene by horseradish peroxidase and 4-methylphenol demonstrates the chemical feasibility of this mechanism and clarifies some of its properties.

Experimental Procedures

Materials. Horseradish peroxidase and hydrogen peroxide were purchased from Sigma Chemical Co. (St. Louis, MO); styrene, styrene oxide, benzaldehyde, 2-undecanone, phenol, cresol, tyrosine, 4-methylcatechol, and mannitol were from Aldrich Chemical Co. (Milwaukee, WI); Chelex resin was from Bio-Rad (Richmond, CA); and $^{18}O_2$ was from MSD

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[1] Abbreviations: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide.

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Isotopes (St. Louis, MO). *trans*-[1-^{2}H]Styrene and $H_2{}^{18}O_2$ were synthesized and their isotopic contents determined as previously reported (Ortiz de Montellano & Catalano, 1985). Glass-distilled, deionized water passed through a Chelex column was used for all the biological work.

Synthesis of 2-(4-Methylphenoxy)-1-phenylethane. A mixture of 4-methylphenol (0.108 g, 1.0 mmol), 2-phenylethyl bromide (0.185 g, 1.0 mmol), tetrabutylammonium bromide (0.161 g, 0.5 mmol), and NaOH (0.5 mL of 2.0 N solution, 1.0 mmol) in 10 mL of a 1:1 mixture of water and CH_2Cl_2 was stirred at room temperature for 12 h. The mixture was then diluted with 10 mL of ether, and the organic layers were separated, washed with saturated NaCl solution, dried over $MgSO_4$, and concentrated on a rotary evaporator: EIMS, *m/e* 212 (M^+); ^{1}H NMR ($CDCl_3$) 7.28 (m, 5 H, Ph), 7.15 and 6.85 (d, J = 8.5 Hz, 4 H, C_6H_4), 4.13 (t, J = 7.5 Hz, 2 H, OCH_2), 3.07 (t, J = 7.5 Hz, 2 H, CH_2), 2.31 ppm (s, 3 H, Me).

Synthesis of 2-(4-Methylphenoxy)-1-phenylethan-1-ol and 2-(4-Methylphenoxy)-2-phenylethan-1-ol. Sodium (0.58 g, 0.025 mol) was added to a solution of 4-methylphenol (8.1 g, 0.075 mol) in 15 mL of dry tetrahydrofuran, and the mixture was refluxed under a nitrogen atmosphere. When the sodium had completely dissolved, styrene oxide (3.00 g, 0.025 mol) was added over a 3-min period and the mixture refluxed an additional 3 h. The mixture was then cooled and poured into an ice–water mixture containing 0.05 mol of NaOH. The resulting mixture was extracted with ether (2 × 25 mL), and the organic layer was washed with H_2O and saturated NaCl solution, dried over anhydrous $MgSO_4$, and concentrated on a rotary evaporator. The residue was purified by chromatography on 2000-μm silica gel GF plates with 20% ethyl acetate in hexane as the solvent. Two bands (R_f 0.75 and 0.50) were isolated. The top band is 2-(4-methylphenoxy)-1-phenylethan-1-ol: EIMS, *m/e* 228 (M^+); ^{1}H NMR ($CDCl_3$) 7.39 (s, 5 H), 6.94 (dd, J = 21.9, 8.5 Hz, 4 H), 5.07 (m, 1 H), 4.02 (m, 2 H), 2.80 (s, 1 H), 2.28 ppm (s, 3 H). Oxidation of this compound with pyridinium chlorochromate (Corey & Suggs, 1975) gave a ketone: ^{1}H NMR ($CDCl_3$) 7.7 (m, 5 H, Ph), 6.96 (dd, J = 19.6, 8 Hz, 4 H, C_6H_4), 5.22 (s, 2 H, CH_2), 2.27 ppm (s, 3 H, Me). The lower band is 2-(4-methylphenoxy)-2-phenylethan-1-ol: EIMS, *m/e* 228 (M^+); ^{1}H NMR ($CDCl_3$) 7.32 (s, 5 H, Ph), 6.86 (dd, J = 8.5, 18.9 Hz, 4 H, C_6H_4), 5.20 (m, 1 H, CH), 3.85 (m, 2 H, CH_2O), 2.21 ppm (s, 3 H, Me). Oxidation of this compound with pyridinium dichromate (Corey & Schmidt, 1979) gave an aldehyde: ^{1}H NMR ($CDCl_3$) 8.50 (s, 1 H, CHO), 7.25 (s, 5 H, Ph), 6.80 (dd, J = 19, 8.5 Hz, 4 H, C_6H_4), 5.21 (m, 1 H, CHCO), 2.14 ppm (s, 3 H, Me).

Analytical Procedures. Gas chromatography was performed on a Varian 2100 flame ionization instrument equipped with a 6-ft glass column packed with 3% OV-225 on 100/200-mesh Supelcoport. The injector and detector were held at 250 °C while the oven was programmed to rise linearly from 80 to 160 °C at 10 °C/min. Standard curves were obtained for quantitative product analyses by carrying a range of concentrations of authentic styrene oxide and benzaldehyde through the standard incubation procedure. Gas chromatography–mass spectrometry was performed on a Kratos AEI MS-25 instrument coupled to a Varian 3700 gas chromatograph equipped with a 30-m DB-1 column programmed to rise from 50 to 150 °C at 5 °C/min. High-field NMR spectra were obtained on a General Electric GN 500-MHz instrument in deuteriochloroform. NMR chemical shifts are reported in parts per million relative to internal tetramethylsilane. EPR spectra were recorded on a Varian E-104 instrument custom interfaced with an IBM personal computer.

Incubations. Standard incubation mixtures contained horseradish peroxidase (100 nM), H_2O_2 (1.1 mM), styrene (40 mM), and 4-methylphenol (1 mM) in 10 mL of Chelex-treated potassium phosphate buffer (pH 7.4). The incubations, started by adding the enzyme to the other components, were run in triplicate for 15 min in an agitating water bath held at 37 °C. The incubation time for the inhibitor and EPR studies was 60 min. The reactions were stopped by adding diethyl ether at −78 °C to the incubation mixtures. The internal standard 2-undecanone was then added, and the mixtures were extracted with ice-cold diethyl ether. The combined extracts were washed with brine, dried over anhydrous potassium carbonate, and analyzed by gas–liquid chromatography. Deviations from these standard conditions are indicated in the text. The identities of the metabolites produced in the 4-methylphenol–horseradish peroxidase system were established by comparing their gas chromatographic retention times and mass spectra with those of authentic samples.

Origin of the Oxygen Atom in Styrene Oxide. In experiments with labeled oxygen, large (20-mL) incubation mixtures containing styrene (50 mM), 4-methylphenol (2.0 mM), and horseradish peroxidase (200 nM) in Chelex-treated potassium phosphate buffer (10 mM, pH 7.4) were cooled to 0 °C and were alternatively purged with nitrogen and evacuated to 5 × 10^{-2} Torr five times. A 3% H_2O_2 solution was similarly purged. The incubation mixtures after the final evacuation were warmed to 37 °C, $^{18}O_2$ was introduced, and H_2O_2 was added by air-tight syringe to initiate the reaction. Experiments with labeled peroxide were similar in size but were not carried through the purging cycles. The incubations were worked up as usual and the extracts containing the styrene oxide analyzed by coupled gas chromatography–mass spectrometry.

Stereochemistry of the Epoxidation of trans-[1-^{2}H]Styrene. A solution of horseradish peroxidase (250 nM), H_2O_2 (5.0 mM), 4-methylphenol (2.5 mM), and *trans*-[1-^{2}H]styrene (50 mM) in 200 mL of Chelex-treated 10 mM phosphate buffer (pH 7.4) was incubated at 37 °C for 60 min. The incubation mixture was then poured into 100 mL of ice-cold diethyl ether, and the organic layer was separated and combined with two 100-mL diethyl ether washes of the aqueous layer. The combined ether layers were washed with saturated NaCl solution, dried over K_2CO_3, and concentrated to a volume of approximately 1 mL. The concentrate was chromatographed on a Lobar size B LiChroprep Si 60 (40–63 μm) silica gel column (E. Merck, Darmstadt) with 15% (v/v) ether/pentane. The column effluent was monitored with a Hitachi Model 100-40 variable wavelength detector set at 265 nm. The styrene oxide fraction was checked by gas-liquid chromatography before the solvent was carefully removed and the residue taken up in deuteriochloroform and examined by NMR spectroscopy.

EPR Studies. Incubations for EPR studies contained, in a total of 10 mL, horseradish peroxidase (200 nM), H_2O_2 (4.0 mM), styrene (40 mM), 4-methylphenol (4.0 mM), and $Zn(OAc)_2$ (350 mM) in 0.2 M acetate buffer (pH 5.5). Reaction mixtures were incubated at 25 °C for 60 min. Control incubations were carried out in the absence of styrene. 4-Methylphenol and styrene were not present in incubations that contained 4-methylcatechol (1.0 mM). The concentrations of horseradish peroxidase (100 nM) and H_2O_2 (2.0 mM) were also lower and the incubation period was shorter (30 min) in incubations with 4-methylcatechol. Deviations from these

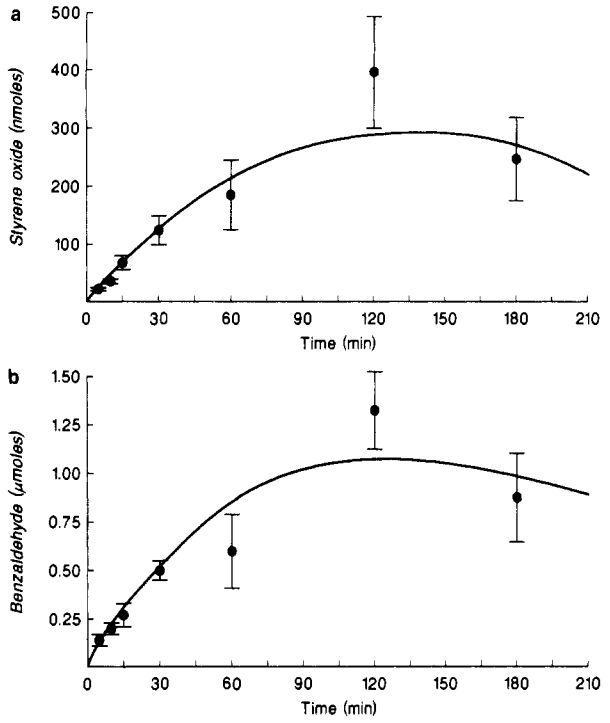


FIGURE 1: Time course for the formation of (a) styrene oxide and (b) benzaldehyde in incubations of styrene (40 mM) with horseradish peroxidase (100 nM), H_2O_2 (1.5 mM), and 4-methylphenol (1 mM). Standard deviations are indicated by the bars.

standard incubation protocols are noted in the text. Aliquots removed periodically from the incubation mixtures were subjected to EPR analysis. EPR parameters are given in the text.

RESULTS

Formation of Styrene Oxide and Benzaldehyde. Preliminary experiments established that styrene is oxidized to styrene oxide and benzaldehyde when incubated with horseradish peroxidase, H_2O_2, and either phenol, 4-methylphenol, tryptophan, or tyrosine. The structures of the two metabolites were confirmed by the identity of their chromatographic properties and mass spectra with those of authentic standards (not shown). 4-Methylphenol was selected rather than tyrosine for detailed investigation because it is not subject to the alternative reactions that are possible with the amine and carboxyl groups of tyrosine.

Styrene oxide (Figure 1a), benzaldehyde (Figure 1b), and the ortho-linked dimer of 4-methylphenol are produced in a time-dependent manner when styrene is incubated with 4-methylphenol, horseradish peroxidase, and H_2O_2. The 4-methylphenol dimer, which reflects dimerization of the 4-methylphenoxy radical, is a known product of the oxidation of 4-methylphenol by horseradish peroxidase (Hewson & Dunford, 1976a,b; Westerfield & Lowe, 1942). The dimerization occurs whether styrene is present or not and was therefore not investigated. The products expected (Scheme I) if the phenoxy radical adds to the styrene double bond, 2-(4-methylphenoxy)-1-phenylethane, 2-(4-methylphenoxy)-2-phenylethan-1-ol, and 2-(4-methylphenoxy)-1-phenylethan-1-ol were synthesized and specifically shown not to be detectably formed. The structures of these hypothetical adducts are shown in Scheme I. Styrene oxide and benzaldehyde are formed in an essentially linear manner for 30 min, after which time their formation begins to level off and eventually ceases (Figure 1). Metabolite formation is not resumed if more cresol or H_2O_2 is added to the mixture when metabolite production ceases (not shown), presumably because the enzyme is no longer active. Benzaldehyde and styrene oxide are produced in approximately a 4:1 ratio. Styrene oxide formation is strictly dependent on the presence of horseradish peroxidase, H_2O_2, *and* 4-methylphenol, but the yield of benzaldehyde is only decreased by about 35% if either horseradish peroxidase or H_2O_2 is omitted from the incubation. The formation of the two metabolites is differentiated most definitively, however, by the fact that benzaldehyde is formed equally well if the reaction is run under argon whereas the formation of styrene oxide is completely suppressed. The present studies focus on the formation of styrene oxide rather than benzaldehyde because cooxidative processes have an absolute requirement for molecular oxygen.

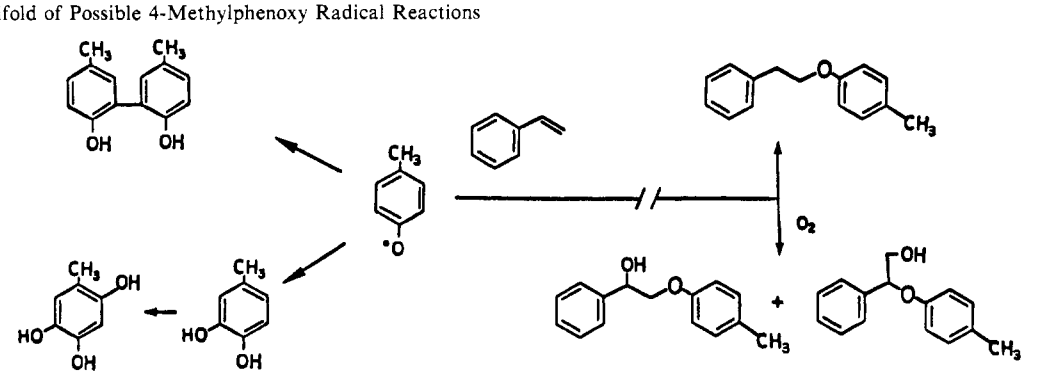
Table I: Incorporation of Labeled Oxygen into Styrene Oxide

	rel peak height in MS at m/z		
oxygen source	120	121	122
$H_2{}^{16}O_2/{}^{16}O_2$	100	12	
$H_2{}^{18}O_2$	100	8	
$^{18}O_2$	10		100

The formation of styrene oxide is completely blocked by the presence of 120 units/mL catalase, as expected from the requirement for H_2O_2, or by the inclusion of 100 mM ascorbic acid. Formation of the epoxide is slightly inhibited (11% decrease) in the presence of a 100 mM concentration of the spin trap DMPO. Epoxide formation, however, is slightly stimulated by a 250 mM concentration of the hydroxyl radical scavenger mannitol (21% increase) or by inclusion of 120 units/mL of superoxide dismutase (76% increase).

Source of the Oxygen in Styrene Oxide. The styrene oxide isolated from large-scale incubations of styrene with horseradish peroxidase and $H_2{}^{18}O_2$ (approximately 42 atom %) was isolated and analyzed by gas chromatography–mass spectrometry. The molecular ion region in its mass spectrum was essentially identical whether it was obtained from incubations with labeled or unlabeled peroxide (Table I). In contrast,

Scheme I: Manifold of Possible 4-Methylphenoxy Radical Reactions



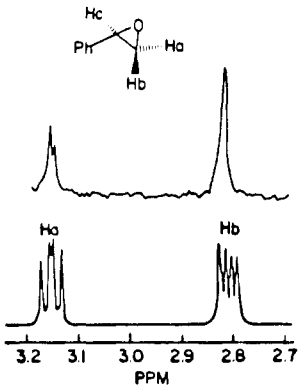


FIGURE 2: 500-MHz NMR signals due to the cis and trans epoxide protons of unlabeled styrene oxide (bottom) and styrene oxide isolated from incubations of *trans*-[1-^{2}H]styrene with horseradish peroxidase/H_2O_2/4-methylphenol (top). The signal at 3.15 ppm is that of the trans proton (cis-deuteriated isomer) and that at 2.8 ppm that of the cis proton (trans-deuteriated isomer) (see inset structure).

the styrene oxide isolated from an incubation carried out under $^{18}O_2$ (99 atom %) was 92% ^{18}O-labeled (Table I). The epoxide oxygen thus clearly derives from molecular oxygen rather than hydrogen peroxide.

Stereochemistry of Styrene Epoxidation. The epoxide produced from [1-^{2}H]styrene by the horseradish peroxidase/H_2O_2/4-methylphenol system was isolated and its stereochemistry determined by 500-MHz NMR spectroscopy. The starting deuterium-labeled styrene, as shown by mass spectrometric and NMR analyses, was greater than 95% monodeuteriated and bore the deuterium exclusively at the trans 1-position. In agreement with this, the NMR spectrum of styrene oxide generated chemically from the labeled styrene with *m*-chloroperbenzoic acid did not exhibit a trans proton signal at 3.15 ppm. However, the NMR spectrum of the epoxide obtained enzymatically from the same deuteriated styrene *does* exhibit a signal at this position (Figure 2). A doublet is observed rather than the AB quartet characteristic of unlabeled styrene oxide (Figure 2), as expected, because one of the scalar coupling constants is lost in the deuteriated compound. Complete retention of the deuterium label was confirmed by mass spectrometry. The ratio of the trans proton signal at 3.15 ppm (due to the cis-deuteriated isomer) to that for the cis proton at 2.81 ppm (due to the trans-deuteriated isomer) indicates that the styrene oxide consists of a 2:1 trans/cis isomer mixture (Figure 2).

EPR Detection of Oxidized 4-Methylphenol Products. Recent studies have shown that catechol radicals can be stabilized for EPR studies by complexation with Zn^{2+} or other divalent ions (Stegmann et al., 1981; Creber et al., 1982; Kalyanaraman & Sealy, 1982; Kalyanaraman et al., 1984). A strong EPR spectrum centered at g 2.0013 is thus obtained when authentic 4-methylcatechol is allowed to autoxidize in the absence of horseradish peroxidase or H_2O_2 (Figure 3). This spectrum is that of the Zn^{2+}-complexed 4-methylcatechol radical. The hyperfine coupling constants determined from the spectrum are as follows: 4-methyl, 5.4 G; H_3, 0.7 G; H_6, <0.5 G; H_5, 4.1 G. If 4-methylcatechol is incubated with horseradish peroxidase and H_2O_2, however, a second spectrum centered at g 2.0022 is superimposed on that for the 4-methylcatechol radical (Figure 3). The hyperfine coupling constants calculated from the second spectrum are as follows: methyl, 5.0 G; ring protons, <0.5 G. The species responsible for the second set of EPR signals is the Zn^{2+}-complexed 5-methyl-2,4-dihydroxyphenoxy radical. This latter species increases in importance as the initial concentration of 4-

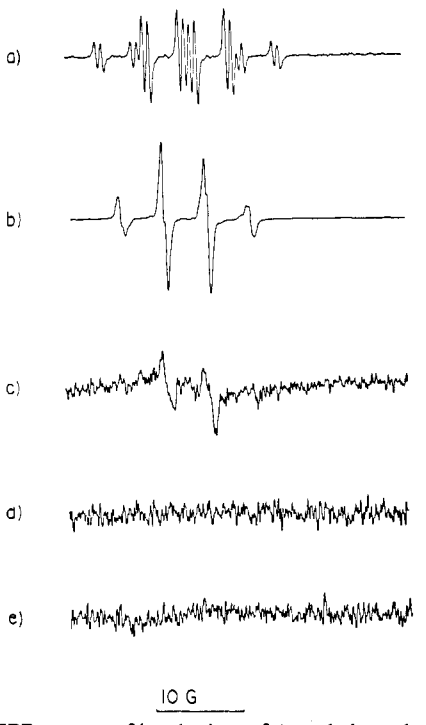


FIGURE 3: EPR spectra of incubations of 4-methylcatechol or styrene plus 4-methylphenol with horseradish peroxidase and H_2O_2 in the presence of $Zn(OAc)_2$: (a) 4-methylcatechol incubated aerobically without horseradish peroxidase or H_2O_2, g = 2.0013; (b) 4-methylcatechol incubated 60 min with the complete incubation system, g = 2.0022; (c) 4-methylphenol incubated 60 min with the complete incubation system, g = 2.0018; (d) 4-methylphenol incubated in the normal system but without horseradish peroxidase; (e) 4-methylphenol incubated in the normal system but without H_2O_2. EPR parameters: microwave power, 2 mW; modulation amplitude, 0.5 G; time constant, 0.128 s; scan time, 2 min; number of scans averaged, 10; scan range, 40 G; gain, (a) 1.25×10^4; (b) 4.0×10^3; (c) 1.0×10^5; (d) 1.0×10^5; (e) 1.0×10^5.

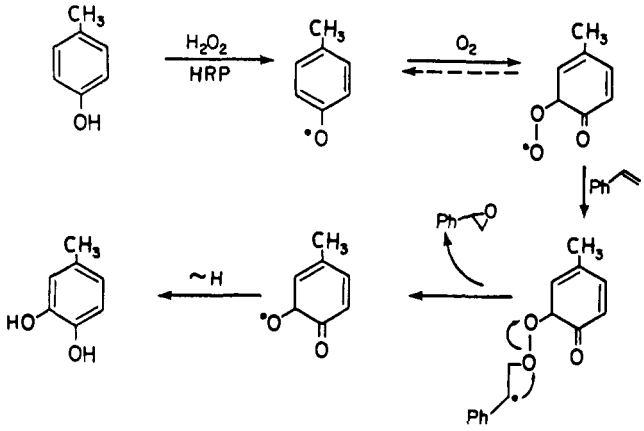
methylcatechol is decreased, to the point that it is the only detectable radical when the starting concentration of 4-methylcatechol is decreased to 0.2 mM.

Incubation of 4-methylphenol with horseradish peroxidase, H_2O_2, and $Zn(OAc)_2$ gives rise to a weak spectrum similar to that obtained with low concentrations of 4-methylcatechol (Figure 3). This spectrum is not observed in the absence of H_2O_2, horseradish peroxidase, or styrene. A correlation of the peak heights for incubations carried out with decreasing amounts of 4-methylcatechol with the peak heights obtained in standard incubations with 4-methylphenol plus styrene provides a rough estimate of about 1000 nmol of trihydroxy product formed from 4-methylphenol in 90 min. In the same period, approximately 450 nmol of styrene oxide is produced. Formation of the trihydroxy radical, which implicates 4-methylcatechol as a metabolite, in a quantity comparable to that of the styrene oxide that is formed provides strong support for a cooxidation mechanism.

Discussion

A number of independent criteria argue that the epoxidation of styrene reported here is the result of a cooxidative process. The requirement for 4-methylphenol, catalytically active horseradish peroxidase, and H_2O_2 rules our direct epoxidation of styrene by H_2O_2 or horseradish peroxidase plus H_2O_2. The absolute requirement for molecular oxygen and the finding that the epoxide oxygen derives from O_2 rather than H_2O_2 limit the possible mechanisms to those in which molecular oxygen is activated by the peroxidase/4-methylphenol system. The insensitivity of styrene oxide formation to high concentrations

Scheme II: Mechanism Proposed for the 4-Methylphenol-Mediated Cooxidation of Styrene



of mannitol or superoxide dismutase indicates that the catalytically activated oxygen species is not the superoxide or hydroxyl radical. The requirement for H_2O_2, inhibition by catalase, and lack of inhibition by superoxide dismutase also clearly differentiate the present reaction from the hydroxylations catalyzed by horseradish peroxidase in the presence of dihydroxyfumaric acid (Dordick et al., 1986). Inhibition of the reaction by DMPO and ascorbate is consistent with a radical process. The absence of products from addition of the 4-methylphenoxy radical to styrene rules out relay cooxidation mechanisms in which oxygen is activated by the carbon radical that results from addition of the phenoxy radical to the olefin. This contrasts with the cooxidation of styrene by horseradish peroxidase and glutathione which may, in part, involve such a relay mechanism (Ortiz de Montellano & Grab, 1986). Finally, detection of the radical signal expected from oxidation of a low concentration of 4-methylcatechol by the peroxidase confirms that oxygen is actually inserted into the 4-methylphenol structure during the catalytic sequence. The EPR signal is not that of the 4-methylcatechol radical itself but rather that of the triol obtained by addition of water to 4-methyl-*o*-quinone. This reaction, as shown by incubations with authentic 4-methylcatechol, occurs efficiently enough in situ even with a 0.2 mM concentration of the catechol to make the trihydroxy radical the only EPR-detectable species. The fact that the radical is not detected under comparable conditions in the absence of styrene reinforces the connection between radical formation and epoxidation. These collective results provide strong support for the cooxidation mechanism in Scheme II.

The evidence for a radical mechanism provided by product, cofactor, and inhibitor studies is strenghthened by the finding that the stereochemistry of trans-deuteriated styrene is partially scrambled in the styrene oxide. The epoxidation of olefins by cytochrome P-450, which involves transfer of the enzymatically activated ferryl oxygen to the olefin, proceeds with complete retention of the olefin stereochemistry (Ortiz de Montellano et al., 1983). In contrast, the epoxidation of styrene by the myoglobin–H_2O_2 system proceeds, as here, with partial loss of the olefin stereochemistry (Ortiz de Montellano & Catalano, 1985). The loss of stereochemistry indicates that free rotation about the carbon–carbon bond of styrene becomes possible at some stage in the epoxidation reaction. This is consistent with the existence of a radical intermediate (Scheme II). The fact that partial rather than complete loss of stereochemistry is observed suggests that closure of the peroxy radical intermediate to give the epoxide occurs on the same time scale as rotation about the carbon–carbon bond.

Cooxidation of styrene by the 4-methylphenol–horseradish peroxidase system demonstrates that phenoxy radicals, as postulated in earlier work to explain the role of molecular oxygen in the epoxidation of styrene by myoglobin, are capable of activating oxygen to an appropriate oxidative species. This oxidative species is shown by the data to be the peroxy radical obtained by addition of molecular oxygen to the ortho position of the phenoxy radical. Further work will be required to assess the biological and toxicological importance of cooxidation reactions mediated by tyrosine-centered protein radicals.

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