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Cooxidation of Styrene by Horseradish Peroxidase and Glutathione

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SUMMARY

Styrene is oxidized to styrene oxide and benzaldehyde in the presence of glutathione, horseradish peroxidase, and hydrogen peroxide. Styrene oxide is not formed if any one of these reaction components is omitted or if oxygen is exluded from the reaction. The oxygen atom in the styrene oxide derives from molecular oxygen rather than from hydrogen peroxide. Oxidation of *trans*-[1-^{2}H]styrene yields the epoxide in which the deuterium stereochemistry is completely scrambled. The results indicate that the glutathione thiyl radical directly or indirectly activates molecular oxygen to a species that cooxidizes styrene. The one-electron oxidation of glutathione thus may promote the cooxidation of physiological substrates.

HRP, a prototype for the family of enzymes that includes cytochrome *c* peroxidase, myeloperoxidase, lactoperoxidase, and thyroid peroxidase, is oxidized by hydrogen peroxide to a ferryl [(Porphyrin$^{+\cdot}$)Fe(IV)=O] species known as compound I (1). Compound I is reduced stepwise to compound II [(porphyrin)Fe(IV)=O] and then to the ferric resting state [(porphyrin)Fe(III)] by sequential transfer of two electrons from oxidizable substrates. The catalytic cycle of HRP consequently generates two substrate-derived free radicals.

Oxidation of cysteine and glutathione by HRP yields the corresponding thiyl radicals (2, 3). These same thiyl radicals are produced by electron transfer from glutathione or cysteine to other free radicals (4–9) and by autooxidative, peroxidative, photochemical, and radiolytic processes (10–14). Glutathione radicals are primarily converted to the glutathione dimer (GSSG), although the sulfinic acid derivative [GS(O)OH] and other products have been detected (11, 14, 15). The formation of these metabolites is associated with the consumption of molecular oxygen (2–6). In the case of the glutathione dimer, collapse of the glutathione radical with a glutathione molecule is believed to yield a radical anion that transfers the extra electron to molecular oxygen. The sulfinic acid, in contrast, presumably derives from initial addition of the thiyl radical to a molecule of oxygen to give a thiyl peroxy radical (14, 16):

$$GS^{\cdot} + O_2 \rightarrow GSOO^{\cdot}$$

The biological formation of thiyl peroxy radicals has not been documented but is potentially of interest because thiyl peroxy radicals should be sufficiently reactive to oxidize lipids, proteins, and other physiological molecules. It is of interest, in this context, that glutathione and cysteine are reported to be mutagenic (17) and cytotoxic (10, 18, 19) under certain conditions. We demonstrate here that glutathione radicals support the oxygen-dependent cooxidation of styrene, a reaction that implicates a thiyl-activated peroxy radical as a reaction intermediate.

Experimental Procedures

Materials. HRP, glutathione, hydrogen peroxide, and mannitol were purchased from Sigma Chemical Co. (St. Louis, MO). Styrene, styrene oxide, benzaldehyde, and DMPO were obtained from Aldrich Chemical Co. (Milwaukee, WI). Chelex was obtained from Bio-Rad Laboratories (Richmond, CA). $^{18}O_2$ gas was obtained from MSD Isotopes (St. Louis, MO). The synthesis of *trans*-[1-^{2}H]styrene and $H_2{}^{18}O_2$ (42% ^{18}O) have been reported (20). Glass-distilled, deionized water was used for all of the incubations.

Incubations. Standard 10-ml mixtures containing reduced glutathione (10 μmol), H_2O_2 (11 μmol), styrene (0.4 mmol), and horseradish peroxidase (1 nmol) in 10 mM phosphate buffer (pH 7.4) were incubated for 15 min in a reciprocating water bath at 37°C. Incubations were initiated by adding the enzyme to the reaction mixture and were run in triplicate. 2-Undecanone (40 μg/incubation) was added as an internal standard at the end of the incubation period. The mixtures were extracted with ice-cold diethyl ether (twice in 5 ml) and the combined extracts, after washing with saturated NaCl solution and drying over anhydrous K_2CO_3, were analyzed by gas chromatography. The analyses were carried out on a 6-foot glass column packed with 3% OV-225 on 100/200 mesh Supelcoport on a Varian 2100 instrument fitted with flame ionization detectors. The oven temperature was programmed to rise from 80 to 160° at 10°/min. Standard curves were constructed by similarly analyzing extracts of incubations of styrene oxide with HRP

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ABBREVIATIONS: HRP, horseradish peroxidase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide.

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and H_2O_2. The results were essentially the same whether the buffers were passed through Chelex columns or not. Chelex-treated buffers were therefore employed only to confirm that removal of trace metals had little effect. Variations from the standard incubation conditions or procedures are specified in the text.

Product identification and $H_2{}^{18}O_2$ studies. The identities of the metabolites produced in the incubations were established by comparing their retention times and mass spectra with those of authentic samples. The labeled oxygen content of the styrene oxide obtained with labeled H_2O_2 (42% ^{18}O) (20) was determined on a Kratos MS-25 instrument by tandem gas chromatography-mass spectrometry. The incubations with labeled peroxide were the same as those with unlabeled peroxide except that the incubation volume was 20 ml.

Stereochemistry of styrene epoxidation. A solution of HRP (200 nM), H_2O_2 (3.0 mM), glutathione (2.0 mM), and *trans*-[1-^{2}H]styrene (40 mM) in 250 ml of 10 mM phosphate buffer (pH 7.4) that had been passed through a Chelex column was incubated at 37° for 60 min. The solution was then poured into 150 ml of ice-cold diethyl ether. The organic layer was recovered and the aqueous layer was extracted twice with 100 ml of ether. The combined extracts were washed with saturated sodium chloride solution, dried over potassium carbonate, and concentrated to a volume of approximately 1 ml. The concentrate was then chromatographed on a Lobar size B LiChroprep Si 60 (40–63 μm) silica gel column (E. Merck, Darmstadt) with 15% (v/v) diethyl ether:pentane as the eluting solvent. The column effluent was monitored with a Hitachi model 100-40 variable wavelength detector set at 265 nm. The peak that eluted with the retention volume of styrene oxide was collected and the presence of styrene oxide was confirmed by gas chromatographic analysis. The solvent was then carefully removed from the isolated sample and a solution of the residue in deuterated chloroform was analyzed by NMR spectroscopy on a General Electric GN 500-mHz instrument. NMR chemical shift values are reported in ppm relative to an internal tetramethylsilane standard.

Results

The incubation of styrene with glutathione, HRP, and H_2O_2 under aerobic conditions yields styrene oxide and benzaldehyde (Fig. 1). The structures of the two products are confirmed by the identity of their gas chromatographic retention times (Fig. 1) and mass spectra (not shown) with those of authentic standards. The formation of styrene oxide, the time course of which is given in Fig. 2, requires glutathione (Table 1, Fig. 3), HRP, and H_2O_2 (Table 1). The styrene oxide concentration increases nonlinearly for the first 30 min (Fig. 2), then ceases to increase even if further H_2O_2, glutathione, or styrene is added. Indeed, the concentration of styrene oxide eventually decreases due to side reactions with the protein. An incubation period of 15 min was therefore employed for the studies described in this paper. The requirement for glutathione was satisfied by concentra-

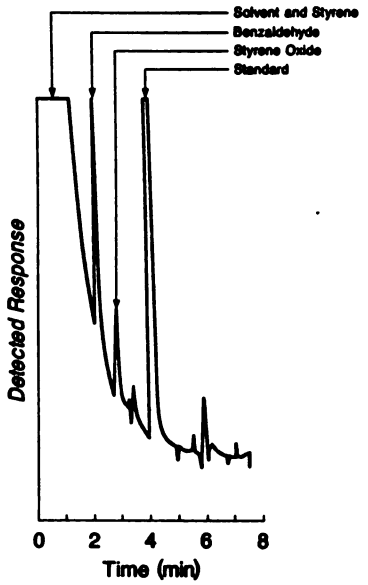

Fig. 1. Gas-liquid chromatographic analysis of the products from a standard incubation of styrene with glutathione, HRP, and hydrogen peroxide. The details of the standard incubation system are given in Experimental Procedures. Authentic samples of benzaldehyde and styrene oxide coelute, in that order, with the observed peaks.

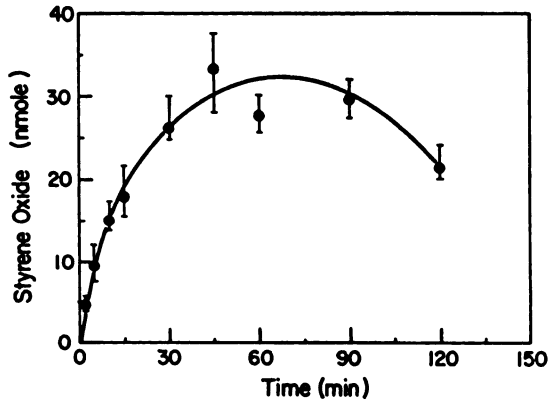

Fig. 2. Time course for the formation of styrene oxide. The incubations were carried out as described in Experimental Procedures. Standard deviations are given for the data points.

TABLE 1

Styrene oxide formation: Cofactor and inhibitor studies

Incubation system	Styrene oxide	Benzaldehyde
	% of control[a]	
Normal	100	100
−HRP	5	35
−Glutathione	ND[b]	25
$-H_2O_2$	2	25
$-O_2$ (argon)	ND	<10
+Mannitol (150 mM)	101	–[c]
+Superoxide dismutase	85[d]	–
+DMPO (100 mM)	50	–

[a] Percentage of control assumes 100% = 35 nmol of styrene oxide/nmol of enzyme/15 min.
[b] ND, not detectable.
[c] –, value not determined.
[d] The superoxide dismutase concentration was 240 units/ml; the H_2O_2 concentration in this incubation was held to 0.11 mM to minimize possible inactivation of the superoxide dismutase.

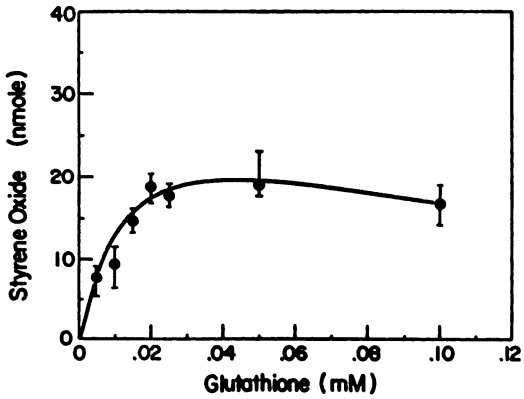

Fig. 3. Glutathione dependence of HRP-catalyzed styrene oxide formation. The incubations were carried out as described in Experimental Procedures. Standard deviations are given for the data points.

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tions as low as 5–10 μM and was saturated by concentrations of glutathione above 20 μM (Fig. 3). The formation of styrene oxide, which could not be readily measured at styrene concentrations below 5 mM, increased as the concentration of styrene was increased but leveled off due to saturation of the process at styrene concentrations above approximately 20 mM (Fig. 4).

The formation of styrene oxide requires molecular oxygen (Table 1) as well as HRP and glutathione. Styrene oxide is not formed if the incubations are carried out under an argon atmosphere. This result and the demonstration that the label from $H_2{}^{18}O_2$ is not incorporated into the styrene oxide (Table 2) indicate that molecular oxygen is the source of the oxygen in the metabolite. Neither mannitol, a hydroxyl radical scavenger, nor superoxide dismutase inhibits the production of styrene oxide, although DMPO, a radical spin trap, inhibits the reaction to some extent (Table 1). Benzaldehyde is also formed in a time-dependent manner (Fig. 5), but its formation is only

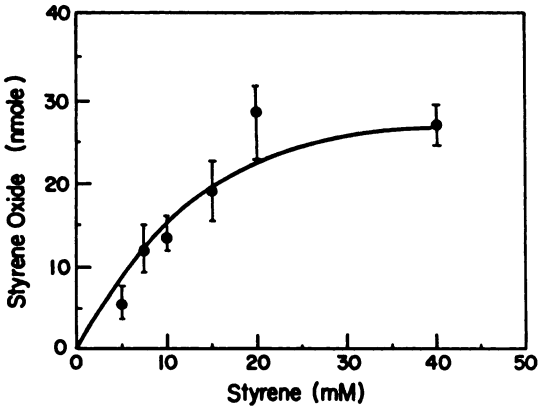

Fig. 4. Styrene dependence of HRP-catalyzed styrene oxide formation. The incubations were carried out as described in Experimental Procedures. Standard deviations are given for the data points.

TABLE 2
Source of the oxygen in styrene oxide

Precursor	Percentage of ^{18}O in precursor	Relative intensities of molecular ion peaks			
	m/z:	120	121	122	123
$H_2{}^{16}O_2$		100	18	1	0
$H_2{}^{18}O_2$	42	100	6	0	2

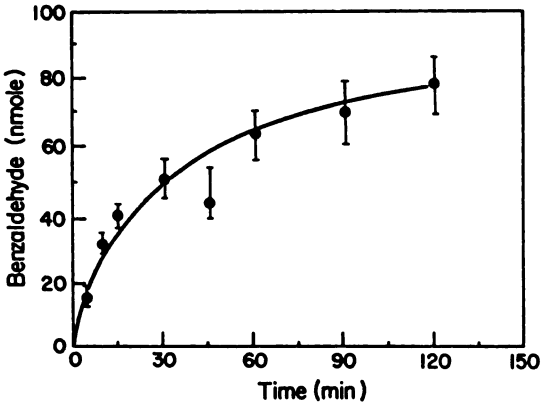

Fig. 5. Time course for the formation of benzaldehyde. The incubations were carried out as described in Experimental Procedures. Standard deviations are given for the data points.

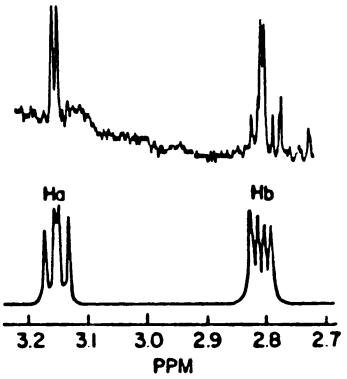

Fig. 6. Stereochemical outcome of the epoxidation reaction. The NMR signals of the two terminal epoxide protons of unlabeled styrene oxide are shown at the *bottom*. The *cis* and *trans* protons are labeled H_b and H_a, respectively. The signal at 3.15 is completely absent if the styrene oxide is prepared from the *trans*-deuterated olefin by oxidation with *m*-chloroperbenzoic acid (not shown). The protons of the deuterated epoxide appear in the NMR as doublets rather than doublets of doublets because deuterium substitution eliminates one of the scalar coupling interactions. The samples were obtained as described in Experimental Procedures.

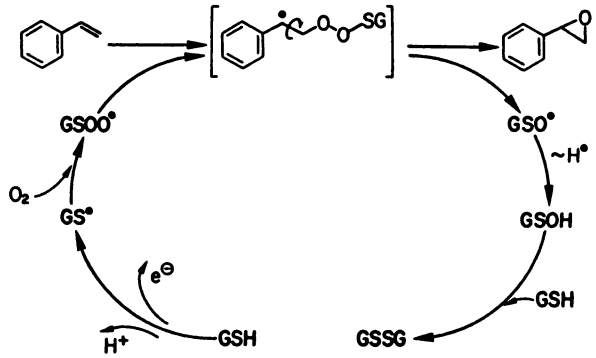

Fig. 7. Possible mechanism for the glutathione-dependent oxidation of styrene involving a thiyl peroxy radical as the oxidizing species. *GSH*, glutathione; GSSG, the glutathione dimer.

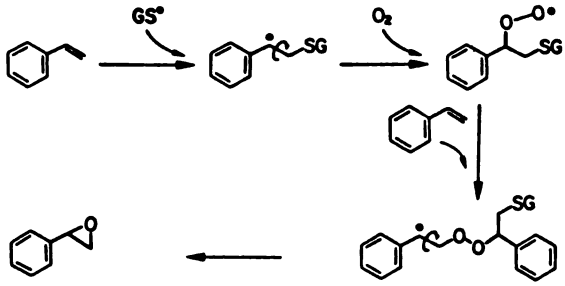

Fig. 8. Possible mechanism for the glutathione-dependent oxidation of styrene involving an alkylperoxy radical as the oxidizing species.

partially glutathione-, HRP-, and O_2-dependent (Table 1). The formation of benzaldehyde has not been investigated in detail.

Synthetic [1-^{2}H]styrene, shown by NMR and mass spectrometric analyses to be greater than 95% deuterium labeled in the *trans* position, is oxidized by the HRP-glutathione system to styrene oxide in which the stereochemistry of the deuterium is completely scrambled (Fig. 6). Integration of the signals in the spectrum due to the terminal protons of the *cis*- and *trans*-deuterated styrene oxide indicates that the isomers are present in essentially a 1:1 ratio.

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Discussion

The mechanism for the oxidation of styrene to styrene oxide must rationalize (a) the catalytic involvement of HRP; (b) the requirement for glutathione; (c) the activation of molecular oxygen to a species that reacts with styrene and transfers one of its oxygens to the π-bond; (d) the evidence that the oxidation is not directly mediated by hydrogen peroxide, superoxide, or the hydroxyl radical; and (e) the complete loss of stereochemistry that accompanies the epoxidation reaction. Two reasonable mechanisms can be envisioned that satisfy these constraints. In one of these mechanisms, the HRP-generated glutathione radical combines with oxygen to give a thiyl peroxy radical that reacts, in turn, with the styrene π-bond (Fig. 7). In the alternative mechanism, addition of a thiyl radical to the styrene double bond yields a carbon radical intermediate that is converted to an alkylperoxy radical by reaction with molecular oxygen. This alkylperoxy radical then cooxidizes a second molecule of styrene (Fig. 8). Both mechanisms are consistent with the evidence that HRP oxidizes cysteine and glutathione to the corresponding thiyl radicals (2, 3) and with the finding that the HRP-catalyzed oxidation of these residues is accompanied by the uptake of molecular oxygen (2–6). The first mechanism rationalizes the reported formation of sulfinic acid derivatives (11), whereas the second mechanism is consistent with the recent report that glutathione adds to the double bond of styrene (21) and with the fact that relatively high concentrations of styrene are required for epoxide formation. It is not possible at this time to clearly differentiate the two mechanisms or, indeed, to exclude the possibility that both are operative. Strong parallels exist between both of the proposed mechanisms and those that govern the cooxidation of arachidonic acid and xenobiotics catalyzed by prostaglandin synthase (22). The present results indicate that the glutathione radical is able, directly or indirectly, to engender a cooxidative species.

Acknowledgments

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