MOLECULAR PHARMACOLOGY, 26:348–352

Hydroperoxyflavin-Mediated Oxidations of Organosulfur Compounds

Model Studies for the Flavin Monooxygenase

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Received March 7, 1984; Accepted June 6, 1984

SUMMARY

Kinetic and product studies were carried out for the reaction of a synthetic hydroperoxyflavin with a series of organosulfur compounds as a model for the flavin-containing monooxygenase of mammalian liver (FMO). *S*-Oxidized products were identified, and the kinetics of the oxidation reactions were consistent with a mechanism involving attack of a sulfur nucleophile on the terminal oxygen atom of the hydroperoxyflavin. These results provide information about the substrate oxygenation step of FMO not available from steady-state enzyme kinetics.

INTRODUCTION

The exposure of mammals to the wide variety of organosulfur compounds used for agricultural, industrial, and medicinal purposes generally results in enzymatic *S*-oxidation of such xenobiotics (1). The major enzyme systems responsible for the oxidation of sulfur-containing compounds are Cyt P-450[3] monooxygenase and FMO. The relative participation of these monoxygenases in the biotransformation of organosulfur compounds is substrate-, species-, and organ-dependent (1, 2). In a recent study, the relative contribution of FMO and Cyt P-450 to the microsomal oxidation of thiobenzamide was thoroughly assessed in different organs from mouse and rat (2). It was concluded that both enzymes contribute significantly to the observed microsomal oxidation of thiobenzamide *in vitro*. These *S*-oxidation reactions generally impart increased water solubility to the xenobiotic, which leads to more efficient excretion, particularly if followed by a conjugation reaction (3). The enzymatic reactions can also produce oxidized intermediates that are more reactive than the parent compound. Reactions of these unstable intermediates with cellular macromolecules are thought to be the initial cause of the organ toxicity and genotoxicity observed *in vivo* (4).

In accord with the low substrate specificity of Cyt P-450, this family of enzymes has been shown to oxidize a broad spectrum of organosulfur compounds, including sulfides, sulfoxides, thioamides, thioureylenes, carbon disulfide, dithiocarbamates, and isothiocyanates (2, 5, 6). Cyt P-450-catalyzed oxidation of carbon disulfide and phosphorothioates has been reported to generate an electrophilic species with chemical reactivity characteristic of singlet atomic sulfur. This conclusion was reached following the observation that substrate-derived sulfur was incorporated into microsomal proteins in the form of a hydrodisulfide (4). On the other hand, Cyt P-450-catalyzed oxidation of thioacetamide produced a different electrophilic species, which caused the acetylation of lysine residues of microsomal proteins (4).

The microsomal FAD-containing monooxygenase is also known to oxidize a variety of sulfur-containing functional groups. Sulfides, disulfides, thioamides, thioureylenes, sulfoxides, sulfenes, and carbodithioic acids have been reported as substrates for pig liver FMO (6).

The catalytic mechanism for FMO was elucidated by spectral studies and was shown to involve the formation of 4*a*-hydroperoxy-FAD in an NADPH- and O_2-dependent reaction (7, 8). In subsequent studies, Ball and Bruice (9, 10) demonstrated the transfer of oxygen equivalents from a synthetic model compound (4*a*-hydroperoxy-3-methyllumiflavin) to secondary and tertiary amines and thioxane at rates ~10^4 greater than that by hydrogen peroxide. The products of the reactions were 4*a*-hydroxy-3-methyllumiflavin and secondary hydroxylamines, tertiary amine *N*-oxides or thioxane sulfoxide, respectively. These results and two studies of the oxidation of *p*-substituted thioanisole derivatives by synthetic hydro-

This research was supported by Grant CA 32385 awarded by the National Cancer Institute. This is contribution No. 5493 from the University of Florida Agricultural Experiment Station.

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[2] Recipient of Research Career Development Award ES 00120 from the National Institute of Environmental Health Sciences, Department of Health and Human Services.

[3] The abbreviations used are: Cyt P-450, Cytochrome P-450; FMO, flavoprotein monooxygenase; FlEtOOH, 4*a*-hydroperoxy-5-ethyl-3,8,10-trimethylisoalloxazine; FlEtOH, 4*a*-hydroxy-5-ethyl-3,8,10-trimethylisoalloxazine; HPLC, high-pressure liquid chromatography; TLC, thin-layer chromatography.

0026-895X/84/050348–05$02.00/0

peroxyflavins (11, 12) were consistent with a mechanism involving attack by sulfur or nitrogen nucleophiles on the terminal oxygen atom of the hydroperoxide.

Ziegler (1) and Poulsen (6) have compiled an extensive list of enzyme kinetic constants for organosulfur substrates of pig liver FMO, and these data have been useful for understanding sulfur functional group oxidations. These results were generated by steady-state enzyme kinetics and suffer from the limitation that the over-all rate-determining step appears to follow the substrate oxygenation step in the catalytic cycle (7, 8). This leads to an identical V_{max} for all substrates, albeit widely varying K_m values. The parameter k_{cat}/K_m has been described as the quantity which most accurately reflects the specificity of enzyme substrates, especially for comparative purposes (13). This parameter is analogous to a second-order rate constant when the substrate concentration is much less than K_m.

This study describes the kinetics and products of the nonenzymatic oxidation of a series of organosulfides (I–III), thiourea (IV), and thiobenzamide (V) by a model hydroperoxyflavin (cf. Fig. 1). These results permit the development of quantitative relationships between reaction rates, and provide information on the substrate oxygenation step of FMO-catalyzed oxidations.

METHODS

All solvents were dried and purified by redistillation according to standard methods (14). Anhydrous methanol was prepared by refluxing reagent-grade methanol over magnesium turnings with a few iodine crystals, followed by distillation. Organosulfur compounds were purchased from Aldrich Chemical Company (Milwaukee, Wis.) and purified by distillation or recrystallization. Phenylsulfoxide was synthesized by the method of Hojo and Masuda (15), thioanisole sulfoxide by the method of Johnson and Keiser (16), thiobenzamide *S*-oxide by the method of Hanzlik and Cashman (17), and formamidine sulfinic acid by the method of Havel and Kluttz (18). 3,8,10-Trimethylisoalloxazine was synthesized by the procedure of Yoneda *et al.* (19). The method of Ghisla *et al.* (20) was used to prepare 5-ethyl-3,8,10-trimethylisoalloxazinium perchlorate, which was allowed to react with hydrogen peroxide in a procedure similar to that of Kemal and Bruice (21) to form FlEtOOH. FlEtOOH (m.p. 118–119°) was characterized by its electronic spectrum, λ_{max}^{MeOH} =367 nm (ϵ = 8700 M^{-1} cm^{-1}), its chemical ionization mass spectrum [ions at *m/e* 319 (M+1) and 302 (M−16)], and elemental analysis performed by Galbraith Laboratories:

$C_{15}H_{18}N_4O_4$

Calculated: C 56.58, H 5.70, N 17.61
Found: C 56.47, H 5.81, N 17.63

FlEtOH was synthesized by the procedure of Ghisla *et al.* (20).

Kinetic measurements were conducted in anhydrous methanol at 30°. Solutions of FlEtOOH were prepared in anhydrous methanol, and the peroxidative equivalent was determined iodometrically (21). FlEtOOH was added to a methanolic solution of organosulfur compound to a final concentration varying from 0.05 to 0.2 mM. The kinetics of FlEtOOH disappearance were measured at 370 nm using a Beckman Model 35 recording spectrophotometer in stoppered cuvettes. The kinetics of the reactions between FlEtOOH and I-III were conducted under pseudo-first order conditions ([I–III] ≫ [FlEtOOH]), and the disappearance of FlEtOOH was monitored. It was determined that the reaction was first-order in both the sulfide and FlEtOOH concentrations. Pseudo-first order rate constants were computed using the Guggenheim method, which gave linear plots through three half-times of reaction (22). Pseudo-first order rate constants were linearly dependent on sulfide concentration (cf. Fig. 2), and bimolecular rate constants were calculated from the linear least-squares slope (cf. Table 1).

The kinetics of reaction between FlEtOOH and the thionosulfur compounds IV and V were measured under second-order conditions with the concentration of either IV or V greater than the concentration of FlEtOOH. The reaction showed first-order dependence on the concentration of FlEtOOH and second-order kinetics over-all to at least 90% completion of reaction. The time lag method of Sturdevant (23) was employed to calculate second-order rate constants of IV and V because of the rapid rates of reaction. The reaction of IV and FlEtOOH was measured spectrophotometrically by following the disappearance

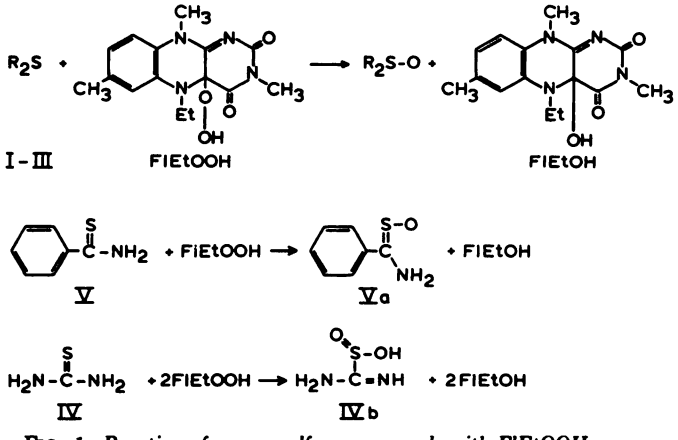

FIG. 1. *Reaction of organosulfur compounds with FlEtOOH*

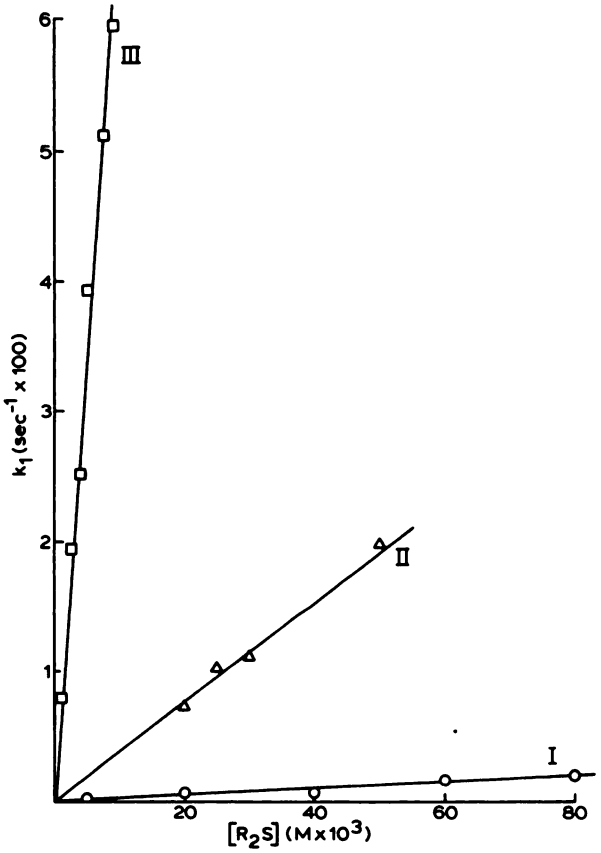

FIG. 2. *Plot of pseudo-first order rate constants (k_1) versus the concentration of sulfide substrates*

of FlEtOOH. The reaction of V and FlEtOOH was measured spectrophotometrically by following the formation of Va at 349 nm, which is an isosbestic point in the conversion of FlEtOOH to FlEtOH.

The products of the reaction between FlEtOOH and I–III were FlEtOH and the corresponding sulfoxide. These products from I and II were analyzed by HPLC on a μBondapak C-18 column (Waters Associates) using the solvent systems listed in Table 1. The solvent flow rate was 1.5 ml min^{-1} and component detections were made by UV absorbance at 254 nm. Quantitative calculations were made on the basis of component peak heights as compared with peak heights generated by known amounts of authentic standards. FlEtOH production following completion of the reaction was determined by HPLC on μBondapak C-18 with 50% acetonitrile/water with detection at 340 nm. FlEtOH was produced in quantitative yield from the reactions of I–V with FlEtOH. Dimethyl sulfoxide production was determined by fused silica capillary column GC/MS on Durabond-5 (J & W Scientific, Inc.) employing a Finnigan 4021 GC/MS/DS in the negative chemical ionization mode with methane as the ionizing gas. Thiobenzamide *S*-oxide was identified from reaction mixtures by TLC comparison with authentic material, and was quantitated spectrophotometrically (ϵ_{349}^{MeOH} = 6500 M^{-1} cm^{-1}). The thiourea oxidation product was identified after treatment of IV with more than two equivalents of FlEtOOH. The product, formamidine sulfinic acid, was identified by TLC using an iodine solution in carbon tetrachloride as a visualizing reagent (24).

RESULTS AND DISCUSSION

Table 1 lists the second-order rate constants (k_2) for the oxidation of organosulfur compounds by FlEtOOH. For the series of sulfides, k_2 decreases with aromatic substitution in the order I < II < III. This order is consistent with the nucleophilic mechanism previously demonstrated for FlEtOOH-mediated oxidations. Ball and Bruice (9, 10) determined bimolecular rate constants for the nonenzymatic oxidation of secondary aliphatic and secondary aromatic amines by a closely related hydroperoxyflavin in *t*-butanol. For comparison with these results on sulfur oxidations, it is assumed that the minor differences in flavin structures and alcoholic solvent have negligible effects on relative rates. The rate constants reported for di-*n*-butylamine and *N*-methylaniline were 8×10^{-2} and 9×10^{-4} M^{-1} sec^{-1}, respectively. This corresponds to a 90-fold decrease in reaction rate due to replacement of an alkyl group by a phenyl group. The analogous change in rate constants for the sulfides III and II is only a 14-fold decrease. Amine nucleophilicity is decreased to a greater extent than sulfide nucleophilicity by the adjacent aromatic system. Aryl substitution has a smaller effect on sulfide nucleophilicity because of the poorer $3p\pi$-$2p\pi$ orbital overlap in arylsulfides relative to the $2p\pi$-$2p\pi$ overlap in arylamines (25).

Reported values of k_{cat}/K_m for FMO-catalyzed oxidations of I–III (cf. Table 2) decrease in the same manner, although not as dramatically as the rate constants (k_2) for the reactions of I–III with FlEtOOH. The k_{cat}/K_m data for FMO presented in Table 2 provide a rough measure of the over-all interaction of substrate and enzyme, and allow comparisons to be made between substrates (13). The electronic effects of aromatic conjugation may be moderated by hydrophobic binding forces, since aromatic substitution generally leads to increased FMO substrate specificity (cf. Table 2, trimethylamine versus *N,N*-dimethylaniline). Thus, for the FMO-catalyzed oxidation of the sulfides (I–III), increasing substitution with aryl groups does not decrease FMO specificity as much as might be predicted from our model studies with FlEtOOH.

The value of k_2 for the dialkylsulfide III is 80-fold greater than the k_2 determined by Ball and Bruice (10) for the dialkylamine, di-*n*-butylamine. This reflects the difference in hard-soft acid-base chemistry between sulfur and nitrogen nucleophiles (26). Hybridized nonbonding pairs involving sulfur 3p orbitals extend farther and are more polarizable than are the corresponding 2p nonbonding orbital of nitrogen (25). The soft sulfur base reacts more readily with the soft acid (FlEtOOH) than does the hard nitrogen base.

These hard-soft acid-base considerations correlate with the reports that pig liver FMO substrates, which contain both nitrogen and sulfur, are often preferentially oxidized on sulfur (7). On the basis of this selectivity, Poulsen and Ziegler (7) have proposed that FMO is best described as an *S*-oxidase. Our results support this proposal, and it was found that oxidation of phenothiazine by FlEtOOH (data not shown) gave quantitative production of phenothiazine sulfoxide and FlEtOH but no detectable products of *N*-oxidation.

The rapid reaction between FlEtOOH and the thionosulfur compounds IV and V resulted in a significant amount (~10%) of reaction during the mixing process. This gave rise to large uncertainties in the product concentrations at early times; therefore, a time lag

TABLE 1

Kinetic constants and products for the reaction of organosulfur compounds with FlEtOOH

The rate constants and products were determined as described under Methods.

Compound	k_2	Product (yield)	Analytical method
	M^{-1} sec^{-1}		
Phenylsulfide (I)	6.2×10^{-2}	Phenylsulfoxide (77%)	HPLC 50% Methanol/water
Thioanisole (II)	4.4×10^{-1}	Thioanisole sulfoxide (100%)	HPLC 35% Methanol/water
Methylsulfide (III)	6.2	Dimethyl sulfoxide (−)[a]	GC/MS[b]
Thiourea (IV)	5.1×10^{1}	Formamidine sulfinic acid (−)[a]	TLC
Thiobenzamide (V)	1.2×10^{2}	Thiobenzamide *S*-oxide (98%)	TLC

[a] Percentage yield was not determined quantitatively.

[b] Gas chromatography/mass spectroscopy.

TABLE 2

Specificity constants for FMO

Substrate	k_{cat}/K_m[a]
	$M^{-1}\ min^{-1}$
Phenylsulfide (I)	0.88
Thioanisole (II)	5.00
Methylsulfide (III)	8.10
Thiourea (IV)	2.80
Trimethylamine	0.02
N,N-Dimethylaniline	20.90

[a] Data taken from ref 1.

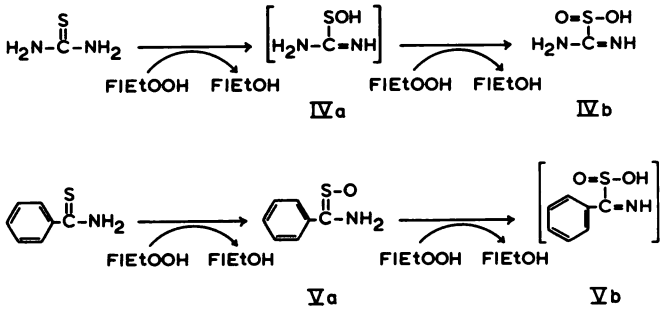

FIG. 3. *Proposed mechanism for the consecutive oxidations of thionosulfur compounds IV and V by FlEtOOH*

method was used to estimate the second-order rate constants. The method of Sturdevant (23) allows for the calculation of second-order rate constants when reactant concentrations are unequal and when initial and/or final properties of the solution are not known with precision. This procedure allowed calculation of average rate constants (Table 1) with 10–15% standard error (six replicates).

The production of Va was determined spectrophotometrically, and Va was formed in equimolar amounts with FlEtOH. The reactivity of IVa prevented its identification (27), but reaction of IV with excess FlEtOOH resulted in the formation of IVb (cf. Fig. 3). Rate data could not be determined for the second oxidation reactions of IV and V with FlEtOOH. The high absorbance of both Va and FlEtOOH in the 350–370 nm region precluded spectrophotometric measurements. The kinetics of the consecutive oxidation reactions between IV, IVa, and FlEtOOH were too complex for accurate determination. In order to compare parent compound reactivity, kinetics were performed with FlEtOOH as the limiting reagent. Quantitative determination of IVb was not possible under these conditions, but 2.0 mol of FlEtOH were produced for each mol of IV present in reaction mixtures. These results are consistent with consecutive oxidations of IV and V and are in accord with previous reports, which concluded that consecutive oxidations of thiono-sulfur compounds are necessary for expression of the hepatotoxic and genotoxic responses observed *in vivo* (4, 5, 27, 28). However, since the reactivity of oxygenated thionosulfur metabolites with cellular macromolecules has not been established, alternative pathways for the expression of organotoxicity and genotoxicity must not be excluded.

The high reactivity of the thiono-sulfur compounds (IV and V) with FlEtOOH relative to the sulfide-sulfur

FIG. 4. *Enhancement of sulfur nucleophilicity by resonance*

compounds (I–III) is undoubtedly due to through-resonance π-electron donation from the nitrogen atom in IV and V. The zwitterionic resonance form of Fig. 4 is shown to reflect the increased electron density on sulfur. In addition, the nonbonded electrons of thiono-sulfur probably have greater "p" character than sulfide-sulfur. These properties of thiones are consistent with increased nucleophilicity of IV and V with respect to I–III. The reason for the 2.4-fold greater oxidation rate of V relative to IV is unclear, although the presence of the second nitrogen atom in IV may decrease electron density on sulfur by an inductive effect.

The reaction of V with FlEtOOH was ~10^3 times faster than the reaction of V with hydrogen peroxide (28). This increased reactivity correlates with the greater leaving group potential of the 4*a*-oxyflavin relative to hydroxide ion in the nucleophilic displacement reaction (29).

The thionosulfur compounds IV and V are high specificity substrates for FMO, but their chemical reactivities with FlEtOOH do not correlate directly with literature values of steady state-derived substrate specificities (6). The high reactivity of IV with FlEtOOH, but not with FMO (relative to I–III), suggests that hydrophobic interactions at the enzyme-substrate binding site in some manner modulate the intrinsic substrate reactivity with active site-bound hydroperoxy-FAD.

The metabolic conversion of thiono-sulfur compounds to oxygenated derivatives by FMO is of potential significance in the production of proximate hepatotoxic and genotoxic metabolites *in vivo* (1, 4). The contribution of FMO to *S*-oxidation reactions could be particularly important in humans in view of the report that FMO activity is higher in human liver microsomes than it is in most species of laboratory animals (1).

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