Mechanism of S-Oxidation Reactions Catalyzed by a Soybean Hydroperoxide-Dependent Oxygenase†

Elizabeth Blée*,‡ and Francis Schuber§

Laboratoire d'Enzymologie Moléculaire et Cellulaire and Laboratoire de Chimie Enzymatique (CNRS UA 1182), Université Louis Pasteur, Institut de Botanique, 28, rue Goethe, 67000 Strasbourg, France

Received November 29, 1988; Revised Manuscript Received March 3, 1989

ABSTRACT: Detergent-solubilized and partially purified soybean sulfoxidase catalyzes oxygen transfer from 13(*S*)-hydroperoxylinoleic acid to para-substituted thioanisoles. This enzyme has the spectral characteristics of a hemoprotein. A linear Hammett plot ($r^2 = 0.940$), with a slope of $\rho = -0.65$, was found between the log of maximal rates of sulfoxide formation and the substituent constants σ^+. The stereochemical course of the enzymatic oxygenation of methyl *p*-tolyl sulfide was determined, and the (*S*)-sulfoxide was produced with about 90 enantiomeric excess %. A mechanism, consistent with our results, is proposed for the sulfoxidase that presents catalytic properties of a peroxygenase; it involves (i) a heterolytic cleavage of the hydroperoxide O–O bond to yield a compound I type oxo heme that reacts with the substrate to form, in the rate-limiting step, a sulfenium radical cation intermediate and (ii) a rapid and stereoselective oxygen transfer leading to the sulfoxide. Our data do not rule out, however, the occurrence of a one-step mechanism in the oxygen transfer from compound I to the sulfide substrates.

We have recently reported the occurrence in soybean microsomes of a hydroperoxide-dependent oxidase catalyzing the sulfoxidation of methiocarb [4-(methylthio)-3,5-xylyl methylcarbamate], an aryl sulfide containing pesticide (Blée & Durst, 1986, 1987). Although we confirm here the hemoprotein character of this enzyme, its spectral properties and cofactor requirements (Blée & Durst, 1986) are quite distinct from those of cytochrome P-450 dependent mixed-function oxidases or FAD[1]-dependent monooxygenases (Takahashi et al., 1978; Oae et al., 1985) or the peroxidases (HRP, CPO, LPX), which are also known to oxidize sulfide xenobiotics (Kobayashi et al., 1986; Doerge, 1986). The S-oxidation of organic sulfides by hemoproteins is generally believed to involve high-energy-state intermediates referred to as compound I, i.e., $(Fe{=}O)^{3+}$, and compound II, i.e., $(Fe{=}O)^{2+}$, or similar species (Blake & Coon, 1980; Wagner et al., 1983). Essentially two modes of oxygen transfer from the intermediary complex, formed by the usual heterolytic cleavage (Ortiz de Montellano, 1987) of the hydroperoxide bond (eq 1), to the sulfide can be proposed: (i) a one-step oxygen transfer, which occurs via a two-electron oxidation mechanism (eq 2)

$$Fe^{3+} + ROOH \rightarrow (Fe{=}O)^{3+} + ROH \quad (1)$$

$$(Fe{=}O)^{3+} + S \rightarrow Fe^{3+} + SO \quad (2)$$

and (ii) a two-step oxygen transfer involving a radical cation intermediate (eq 3 and 4)

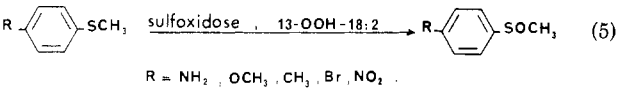
$$Fe^{3+} + ROOH \rightarrow (Fe{=}O)^{3+} + ROH \quad (1)$$

$$(Fe{=}O)^{3+} + S \rightarrow (Fe{=}O)^{2+} + S^{\bullet+} \quad (3)$$

$$(Fe{=}O)^{2+} + S^{\bullet+} \rightarrow Fe^{3+} + SO \quad (4)$$

In this latter reaction sequence, sulfides undergo oxygenation by way of a one-electron transfer mechanism. A two-step oxygen-transfer reaction mechanism was reported for NADPH-dependent cytochrome P-450 (Watanabe et al., 1980) and LPX (Doerge, 1986) catalyzed sulfoxidation reactions and was strongly suggested for HRP and CPO (Kobayashi et al., 1987). Unlike these enzymes, the prostaglandin synthase was speculated to oxidize sulindac sulfide and methyl phenyl sulfide via direct oxygen transfer from compound I as shown in eq 2 (Egan et al., 1981); however, the mechanism for that peroxidase is still not determined.

We have previously demonstrated with ^{18}O-labeling studies that the soybean sulfoxidase catalyzes an oxygen transfer from the fatty acid hydroperoxides to the sulfides (Blée & Durst, 1987). This observation raises important questions on the nature of the reaction of the hemoprotein with the hydroperoxide and whether compound I or II like species act as the ultimate oxygen donors for the sulfide substrates. The answers should provide a better understanding of the mode of action of this enzyme and allow its classification among the oxygenases. We have addressed this problem by the use of linear free energy relationships. This paper reports the substituent effect on rates of S-oxidation of para-substituted thioanisoles, catalyzed by a solubilized and partially purified soybean sulfoxidase (eq 5), and the determination of the stereochemical



(5)

course of the sulfoxidation reaction. The results obtained suggest a one-electron transfer mechanism for this S-oxygenation and underline the uniqueness of this enzyme.

Materials and Methods

Materials. Para-substituted thioanisoles were purchased from Aldrich (Strasbourg, France). Sulfoxides were prepared by oxidation of the parent sulfides with *m*-chloroperbenzoic acid, purified by chromatography on silica gel plates, and identified by mass spectrometry. (*R*)-(+)-Methyl *p*-tolyl sulfoxide was a gift from Drs. C. Mioskowski and P. Pflieger.

† This work was partially supported by a CNRS grant (Contrat Stimulation 1985-4585) to E.B.

* Address correspondence to this author at the Laboratoire d'Enzymologie Moléculaire et Cellulaire.

‡ Laboratoire d'Enzymologie Moléculaire et Cellulaire.

§ Laboratoire de Chimie Enzymatique.

[1] Abbreviations: HRP, horseradish peroxidase; CPO, chloroperoxidase; LPX, lactoperoxidase; FAD, flavin adenine dinucleotide; ee, enantiomeric excess.

0006-2960/89/0428-4962$01.50/0 © 1989 American Chemical Society

13(*S*)-Hydroperoxylinoleic acid was prepared from linoleic acid (Sigma) and purified on silica columns as described by Dix and Marnett (1985). Emulphogene BC-720 was from GAF (Louvres, France).

Enzyme Purification. Microsomes were prepared from soybean (*Glycine max*) seedlings as described previously (Blée & Durst, 1986). All subsequent steps were performed at 4 °C. The membrane fraction was carefully washed by successive resuspensions, followed by centrifugations at 100000*g* for 60 min, in 0.1 M sodium pyrophosphate and 0.01 M potassium phosphate buffers, pH 7.4. Washed microsomes (50 mg of protein), resuspended in 20 mL of a 10 mM sodium acetate buffer, pH 5.5, containing 20% glycerol (buffer A), were treated with emulphogene (final concentration 1% w/v) at 4 °C for 30 min. The mixture was then centrifuged at 100000*g* for 45 min. The supernatant was applied to a 2 × 12 cm column of CM-Sepharose CL-6B (Pharmacia) equilibrated with buffer A containing 1% emulphogene. After the column was washed with buffer B (buffer A containing 0.1% emulphogene) to eliminate the first protein peak, sulfoxidase activity was eluted with a linear NaCl gradient (0 → 0.3 M) in buffer B (2 × 30 mL). The flow rate was 0.5 mL/min and 3-mL fractions were collected. The fractions containing the peak of sulfoxidase activity were pooled (6 mL) and used as the source of enzyme. Protein concentrations were determined (Bradford, 1976) with bovine serum albumin as standard.

Enzyme Assay. Sulfoxidase activity was assayed by using [^{14}C]methiocarb as substrate, as described previously (Blée & Durst, 1986). This activity was also routinely measured (e.g., during the purification procedure) for its peroxygenase activity (Ishimaru & Yamazaki, 1975), with aniline as substrate. In this case, the formation of nitrosobenzene was followed spectrophotometrically at 310 nm (Blée & Durst, 1987). Peroxidase activity was assayed by monitoring the oxidation of guaiacol in the presence of H_2O_2 (Maehly & Chance, 1954). Lipoxygenase activity was measured by following the increase of absorbance at 234 nm, resulting in the transformation of linoleic acid into its corresponding hydroperoxide (Ben-Aziz et al., 1971).

Heme Determination. The presence of cytochrome P-450 and protoheme in the purified sulfoxidase fraction was determined spectrophotometrically (Shimadzu Model MPS-2000 spectrophotometer) according to the method of Omura and Sato (1964). The amount of heme bound to protein in the enzyme preparation was determined fluorometrically according to the method of Sassa and Kappas (1977), using hemin solutions as standards. Fluorometric spectra (560–700 nm) were recorded on a Perkin-Elmer MPF-66 fluorescence spectrophotometer (excitation 400 nm, excitation slit 5 nm, and emission slit 10 nm).

Kinetics. The initial rates of S-oxidation of different para-substituted thioanisoles were determined with the partially purified sulfoxidase. Enzyme (4–20 μg of protein), added to a 10 mM sodium acetate buffer, pH 5.5, containing 0.05% emulphogene and 0.25 mM hydroperoxylinoleic acid (total volume 0.5 mL), was preincubated for 30 s at 30 °C in a tightly capped tube under argon atmosphere. The reaction was initiated by addition of substrate (40–500 μM) and, after 2 min at the same temperature, stopped by freezing in liquid nitrogen. Controls were performed in parallel with boiled enzyme. 1-Naphthol (internal standard) was then added, followed by the extraction of the reaction mixture with 3 × 1 volume of ethyl acetate. After removal of the solvent under a stream of nitrogen, the products were redissolved in 0.1 mL of methanol. The reaction products were separated by HPLC on a 3.9 × 300 mm reverse-phase μBondapak C_{18} column (Waters Associates), operated at ambient temperature, under isocratic elution conditions using 70% methanol in water at a flow rate of 0.8 mL/min. The compounds were detected by their absorbance at 254 nm and identified by their retention times. For quantitative estimates, the peak areas were integrated and normalized by comparison with the internal standard using the molar extinction coefficient (in M^{-1} cm^{-1}), which were determined at 254 nm for authentic thioanisole sulfoxides standards and for 1-naphthol: *p*-OCH_3, 12 540; *p*-NH_2, 8570; *p*-Br, 3900; *p*-NO_2, 1777; *p*-CH_3, 1660; and 1-naphthol, 843. Under these experimental conditions sulfoxidation rates were found to be linear with time and proportional to enzyme concentration. Control experiments demonstrated that sulfoxides were the only products formed in the reaction. The reaction mixtures were first analyzed by TLC on silica plates (using chloroform/ethyl acetate, 1:2 v/v, as eluant) and the bands corresponding to sulfoxides and sulfones eluted and analyzed by HPLC; under these conditions no sulfones could be detected. Similarly, no S-dealkylation products could be found by HPLC analysis or Ellman's reagent (Riddles et al., 1978). The contribution to rates of nonenzymatic oxidation (control) was found to be lower than 10% of the enzymatic reaction and subtracted; the kinetic parameters were determined according to Wilkinson (1961). The results given are means of at least duplicate experiments that do not differ by more than 10%.

Stereochemical Studies. Methyl *p*-tolyl sulfide (3.5 mM) was added to a reaction mixture composed of 10 mM sodium acetate buffer, pH 5.5, 3 mM hydroperoxylinoleic acid, 0.1% emulphogene (final volume 1 mL), and partially purified sulfoxidase (400 μg of protein). The reaction was carried out at 25 °C, for 5 min, under argon. In controls, an identical incubation was performed without enzyme. The products were then extracted by 3 × 1 volume of ethyl acetate; after evaporation of the organic phase the sulfoxide was purified by thin-layer chromatography using chloroform/ethyl acetate (1:2 v/v) as solvent. The resolution of the methyl *p*-tolyl sulfoxide enantiomers was achieved by HPLC on a Pirkle column (Pirkle et al., 1981) containing (*R*)-(−)-*N*-(3,5-dinitrobenzoyl)-α-phenylglycine as the chiral stationary phase (type A-1, ionically linked; 5 μm, modified Spherisorb), obtained from Regis Chemical Co. The column (4.6 × 250 mm) was eluted isocratically with 2-propanol–hexane (5:95 v/v) at a flow rate of 2 mL/min. The compounds were detected by their absorbance at 254 nm, and their retention times, alone or coinjected, were compared with that of an authentic sample of (*R*)-(+)-methyl *p*-tolyl sulfoxide. Since some sulfoxides are known to racemize slowly with time (Pitchen et al., 1984), the ee percents were calculated from the peak areas obtained with freshly prepared samples. The enantiomeric composition of the enzyme-generated sulfoxide was highly reproducible (within 2.5%, $n = 4$).

Results and Discussion

Partial Purification and Characterization of the Sulfoxidase. In order to obtain unequivocal information on the mechanism of the sulfoxidase, we have solubilized and partially purified the enzyme from soybean microsomes. This was necessary since this plant material contains several enzymatic activities susceptible to catalyze cooxidations of organosulfur compounds in the presence of fatty acids or fatty acid hydroperoxides (unpublished results). The membrane preparations were carefully washed with a pyrophosphate buffer and a hypotonic medium; this preliminary step virtually eliminated soluble peroxidases and lipoxygenases. The sulfoxidase was

found to be extremely labile during the purification procedure, and the presence of glycerol (20% v/v) protected, to some extent, this activity. It should be noted that diethyl dithiocarbamate known to stabilize, e.g., prostaglandin H synthase (Lambeir et al., 1985), inhibits the sulfoxidase activity. Solubilization of the enzyme could be achieved in relatively good yield (40%) with emulphogene, a nonionic detergent (Helenius & Simons, 1975), according to conditions outlined previously (Blée & Durst, 1987). After an ion-exchange chromatography step on CM-Sepharose, a purification of about 20-fold was obtained. This sulfoxidase preparation, which was obtained from the washed microsomes with an average yield of 30%, was used for the present study (a complete purification scheme will be reported elsewhere). It was found devoid of membrane-bound lipoxygenase isoenzymes that are associated with the soybean microsomes (Blée, unpublished results).

The dithionite-reduced sulfoxidase fraction did not exhibit an absorbance at 450 nm in presence of CO, nor a peak at 420 nm, revealing the absence of active or denatured cytochrome P-450. In the presence of pyridine, however, this fraction showed a band at 556 nm characteristic of the pyridine–ferrohemoprotein complex of cytochrome *b* type protoheme (Lemberg & Barrett, 1973). The presence of heme was confirmed fluorometrically; i.e., the spectra obtained with the enzymatic preparation were typical of that of hemin (not shown); the amount estimated by this method (about 5 nmol of heme/mg of protein) was in excellent agreement with that calculated from the absorbance at 408 nm using $\epsilon = 115$ mM^{-1} cm^{-1} (Torii & Ogura, 1968). The fluorometric method also gave evidence that, during the purification procedure by ion-exchange chromatography, the activity of the sulfoxidase was correlated with the heme content of the fraction. Optical spectra of the native sulfoxidase indicated maximal absorption peaks at 630, 500, and 408 nm, which are α, β, and Soret bands typical of high-spin (out-of-plane) Fe(III) heme. Difference spectra between native and dithionite-reduced enzyme revealed peaks at 425 and 555 (broad) nm, whereas spectra with dithionite-reduced fractions gave, in the presence of CO, a band at 412 nm. Taken together, these spectral data are very similar to those of peroxygenase, a hemoprotein described by Ishimaru (1979) in pea seedlings.

Substituent Effects on Thioanisole Oxidation Rates. So far our study on the sulfoxidase was carried out with methiocarb, a sulfide function containing pesticide (Blée & Durst, 1986, 1987); in the present work, in order to probe the sensitivity of the sulfoxidation to charge development in the transition state, we have chosen para-substituted thioanisoles as substrates. The partially purified soybean sulfoxidase was found to efficiently catalyze a fatty acid hydroperoxide-dependent oxidation of these molecules into their corresponding sulfoxides [e.g., as determined from its V_{max}, vide infra, the oxidation rate of methyl *p*-tolyl sulfide is about 170 nmol min^{-1} (nmol of heme)$^{-1}$]. No further transformations into sulfones or S-dealkylation reactions could be detected. This enabled us to determine the effect of electron-donating or -withdrawing substituents on the sulfoxidation rates. For each substrate the values of the kinetic parameters K_M and V_{max} were determined (Table I). The maximal rates of oxidation of the para-substituted thioanisoles catalyzed by the sulfoxidase were found to increase with the electron-donating ability of the ring substituent. Both reaction mechanisms proposed for the sulfoxidation, i.e., eq 2 and eq 3 and 4 (see the introduction), predict such a correlation. Figure 1 shows a linear free energy plot of the values of V_{max} as a function of the electrophilic

Table I: Kinetic Constants for Oxidation of Para-Substituted Thioanisoles by Soybean Sulfoxidase

substituent	K_M (μM)	V_{max} [μmol min^{-1} (mg of protein)$^{-1}$]
$-NH_2$	570	5.03
$-OCH_3$	210	3.61
$-CH_3$	245	0.85
$-Br$	130	0.81
$-NO_2$	59	0.23

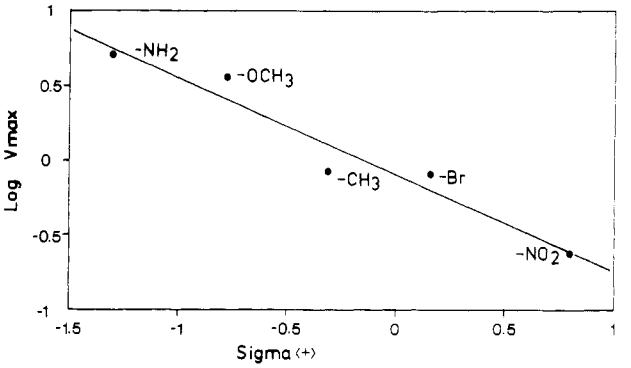


FIGURE 1: Linear free energy plot of V_{max} values for the formation of para-substituted thioanisole sulfoxides catalyzed by a solubilized and partially purified microsomal soybean sulfoxidase. Initial rates were determined in a 10 mM sodium phosphate buffer, pH 5.5 at 30 °C, in the presence of 40–500 μM substrate, 250 μM hydroperoxylinoleic acid, and 4–20 μg of enzyme preparation. Logarithms of maximal rates [in μmol min^{-1} (mg of protein)$^{-1}$], determined as described under Materials and Methods, are plotted against σ_p^+ constants (Williams, 1984).

substituent constants σ_p^+. A relatively good correlation was obtained ($r^2 = 0.940$), giving a slope value (ρ) of −0.65. Use of the Hammett parameter σ_p gave a somewhat poorer correlation with $r^2 = 0.869$ and $\rho = -0.91$. These data are consistent with a mechanism in which the substrate undergoes an electron abstraction to generate a transient sulfenium radical cation intermediate. The rates of reactions involving radicals are characterized by their correlation, in most cases, with σ^+ constants, and by the small absolute magnitudes of the ρ values (Howard & Ingold, 1963; Kennedy & Ingold, 1966; Mulder et al., 1988; Alder et al., 1971). The slope factor ρ is a measure of the extent of charge development in the transition state, and its sign, positive or negative, depends on whether the electron-abstracting species acts as an electrophile or a nucleophile. The negative value found here is consistent with an electron demand on the substrate in the transition state and an electrophilic reaction center forming the sulfenium radical cation, as would be expected for the reaction of $(Fe{=}O)^{3+}$ with the sulfide (eq 3). The good rate correlation observed with σ^+ indicates also that, at least for the substrates studied here, the rate-limiting step of the sulfoxidation reaction is the conversion of the sulfide into a radical cation intermediate. In contrast, the rates of the reaction proceeding via a concerted insertion of the oxygen by compound I (eq 2) into the sulfides would be expected to be better correlated with σ values. Our results on the oxygenation of sulfide compounds by the soybean sulfoxidase seem, therefore, less in favor of such a nucleophilic mechanism, which was, for example, found operative in sulfide oxidations by H_2O_2 (Modena & Mariola, 1957) or proposed for sulfoxidations catalyzed by dopamine β-hydroxylase (May et al., 1981), where much larger negative ρ values were observed (i.e., $\rho = -1.17$ and −3.6, respectively). Similar arguments in favor of the occurrence of sequential one-electron transfer processes were presented in sulfoxidations mediated by cytochrome P-450 (Watanabe et al., 1980, 1982),

by HRP (Kobayashi et al., 1987), or by lactoperoxidase (Doerge, 1986).

Although structure–reactivity correlations can provide good insights into the nature of the intermediates in chemical and in enzyme-catalyzed reactions (Douglas & Wilson, 1984; Kirsch, 1972), they represent only one parameter among others that could allow us to distinguish between the two mechanisms proposed for the sulfoxidase reaction. For example, one cannot exclude that the reaction occurs, depending on the lifetime of the intermediate (Jencks, 1980, 1985), via a borderline mechanism or that the mechanism is dependent on the nature of the ring substituents; in both cases the interpretation of the Hammett plots could be misleading. Moreover, in an exhaustive literature survey, Miller et al. (1986) have indicated the difficulty of finding reliable parameters (substituent effects, solvent effects, isotope effects, product analysis) for distinguishing, in the oxidation of sulfides into sulfoxides, mechanisms occurring via nucleophilic displacements or via single-electron transfer reactions. At the present stage of our investigation, we can only suggest that the oxidation catalyzed by the soybean sulfoxidase involves some single-electron transfer character (eq 3 and 4).

Stereochemical Course of the Sulfoxidation and Mechanistic Implications. The reaction given in eq 1 (introduction) implicates an initial heterolytic cleavage of the O–O bond in hydroperoxylinoleic acid. Such a process occurs readily, e.g., with the peroxidases, which possess in their active site a juxtaposition of catalytic groups (histidine and arginine residues) that greatly facilitate heterolysis by transferring the hydroperoxide proton to the leaving group (Poulos & Kraut, 1980). Thus the linoleic acid alcohol will be released and compound I formed. Oxygenation of sulfides by compound I or compound II can then be achieved by either of the subsequent pathways described earlier (eq 2 or eq 3 and 4) or, alternatively, follow the reaction scheme reported by Kobayashi et al. (1987) for peroxidases such as HRP and CLP. In this case compound II, obtained in reaction 3, can react with another sulfide molecule, forming a radical cation (eq 6), which after disproportionation (eq 7) yields a dication. This latter species finally reacts with water, giving the corresponding sulfoxide (eq 8).

$$(Fe{=}O)^{2+} + ArSCH_3 + 2H^+ \rightarrow Fe^{3+} + ArS^{\bullet+}CH_3 + H_2O \quad (6)$$

$$2(ArS^{\bullet+}CH_3) \rightarrow ArS^{2+}CH_3 + ArSCH_3 \quad (7)$$

$$ArS^{2+}CH_3 + H_2O \rightarrow ArSOCH_3 + 2H^+ \quad (8)$$

Fundamental differences exist between mechanisms 2–4 and 6–8. First, in the former reactions the oxygen atom of the sulfoxide group originates from the hydroperoxide, whereas in the latter it comes from water. Second, a racemic sulfoxide will be expected in eq 8, whereas, inversely, a chiral sulfoxide might be anticipated in eq 2 and 4. On the other hand, one cannot exclude the fact that the unknown active site of the soybean sulfoxidase, in contrast to the peroxidases, does not possess a structure that catalyzes a heterolytic cleavage of the O–O bond in the hydroperoxide (eq 1). In this case, homolytic scission occurs, resulting in the direct formation of compound II, which could then react with the sulfides according to eq 6–8.

It is of importance to know which mechanism describes best the soybean sulfoxidase activity. At this point we must mention that our previous data have demonstrated that the sulfoxide oxygen atom originates, in the enzyme-catalyzed reaction, from the hydroperoxide and is unexchangeable with that of water molecules (Blée & Durst, 1987). This would a priori exclude the mechanism represented by eq 6–8 and favor eq 2–4. In this latter event, chiral sulfoxides might be anticipated as products of the sulfoxidase-catalyzed reactions. In order to distinguish these different mechanistic possibilities, we have therefore investigated the chiral selectivity in the oxidation catalyzed by purified soybean sulfoxidase. Methyl *p*-tolyl sulfide was chosen as a model substrate, and the enantiomeric composition of the sulfoxide formed by incubation, under standard conditions, with the enzyme was determined by HPLC using a chiral stationary phase according to Pirkle et al. (1981). Under our experimental conditions the *R* and *S* enantiomers of methyl *p*-tolyl sulfoxide were separated with a net retention time ratio of $\alpha = 1.03$ (Figure 2). Comparison with an authentic sample of (*R*)-(+)-methyl *p*-tolyl sulfoxide indicated that the sulfoxide obtained enzymatically (Figure 2) had the *S* configuration with a 78 ± 2.5 ee % ($n = 4$). In contrast, the spontaneous oxidation observed with the control (without enzyme) led to a racemic sulfoxide. This lack of stereospecificity is expected from chemical sulfoxidations involving, e.g., hydroperoxides and trace metals. When taking into account the contribution of this nonenzymatic process, which under our experimental conditions represented about 10 ± 2% of the overall sulfoxide formation, we could estimate that the partially purified soybean sulfoxidase catalyzed the oxygenation of methyl *p*-tolyl sulfide with a very high stereoselectivity; i.e., the ee was at least 90% in favor of the *S* enantiomer. This finding can be compared to the enantioselectivity in the formation of sulfoxides catalyzed by other oxygenases. Cytochrome P-450 dependent monooxygenases are known to produce chiral sulfoxides; for example, a 58–68 ee % was observed in the formation of (*S*)-ethyl *p*-tolyl sulfoxide by different isoenzymes of purified rat liver NADPH-dependent cytochrome P-450 (Waxman et al., 1982). Dopamine β-hydroxylase, a nonhemic monoxygenase, oxidized with high stereoselectivity phenyl 2-aminoethyl sulfide into the (*S*)-sulfoxide (May & Phillips, 1980). An opposite chirality, i.e., *R* enantiomer, is observed in the formation of the alkyl aryl sulfoxides catalyzed by a FAD-dependent monooxygenase purified from hog liver microsomes (90 ee %; Light et al., 1982) and by CPO (12.7 ee %; Kobayashi et al., 1987). In contrast, HRP induced no chirality in the formation of methyl *p*-tolyl sulfoxide (Kobayashi et al., 1987). It is interesting to note that the sulfoxide enantiomer obtained with the soybean sulfoxidase is similar to the one formed by mammalian cytochrome P-450 dependent oxidases. The high stereoselectivity of the oxo transfer observed in the present work, in addition to the incorporation of oxygen from the hydroperoxide into the substrate (Blée & Durst, 1987), may implicate a direct access of the sulfide to the ferryl oxygen group, as has been suggested in the reactions catalyzed by chloroperoxidase and cytochrome P-450 dependent enzymes (Ortiz de Montellano, 1987). In contrast, the lack of stereospecificity in sulfoxidation catalyzed by HRP might be the result of a reaction occurring at the heme edge. Moreover, in the hypothesis of a one-electron transfer process in the sulfoxidase-catalyzed reaction, we must envisage a tight coupling of the oxygenation step (eq 4) with the electron abstraction (eq 3), i.e., trapping of the radical intermediate before its diffusion out of the active site. This would be in agreement with the lack of S-dealkylation observed in the sulfoxidation reaction, with the inability of the sulfoxidase to support a one-electron peroxidation of guaiacol (not shown), and with the observation that a compound such as cyclopropyl phenyl sulfide is an excellent substrate of the sulfoxidase and not a mechanism-based inhibitor (E. Blée, unpublished results). A similar strong tendency of the in-

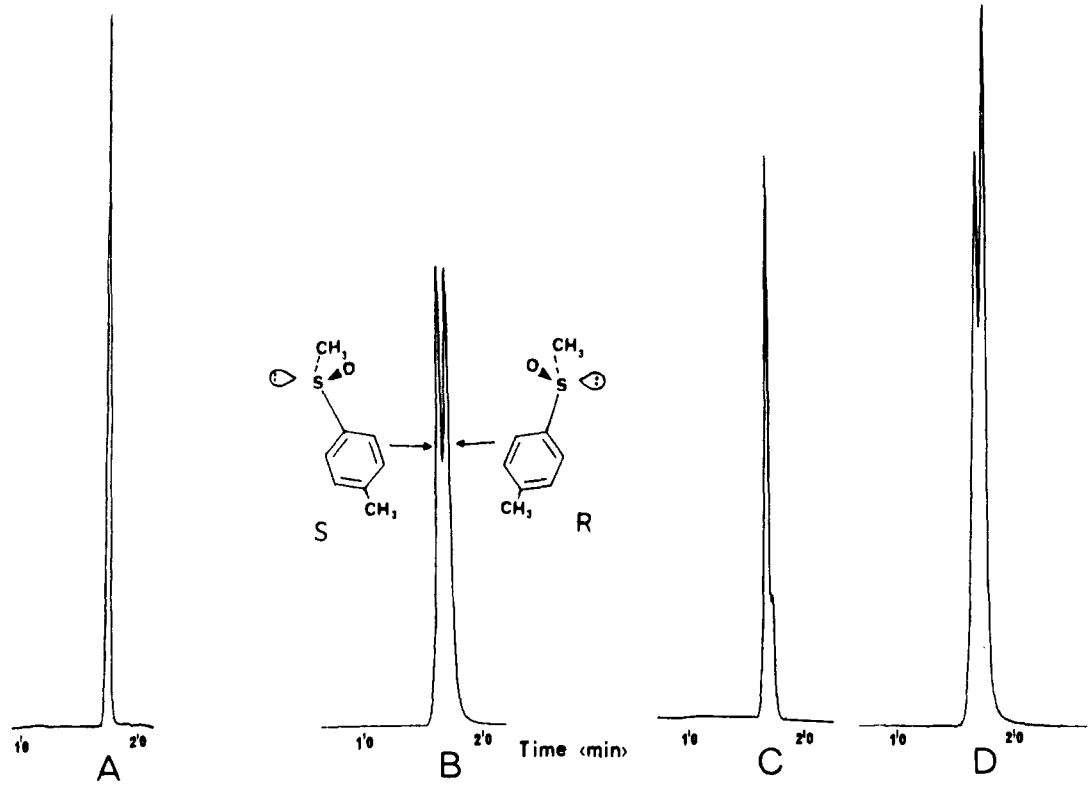


FIGURE 2: Enantioselective chromatography of methyl *p*-tolyl sulfoxide prepared with the soybean sulfoxidase. The isomers were separated by high-performance liquid chromatography using a chiral Pirkle type A-1 column with detection at 254 nm as described under Materials and Methods. (A) (*R*)-(+)-Methyl *p*-tolyl sulfoxide (standard); (B) racemic sulfoxide prepared chemically; (C) sulfoxide product obtained by incubation with partially purified sulfoxidase; (D) enzymatic sulfoxide coinjected with (*R*)-(+)-methyl *p*-tolyl sulfoxide.

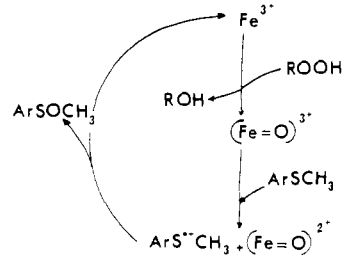


FIGURE 3: Hypothetical mechanism for S-oxygenation of sulfide substrates by membrane-bound soybean sulfoxidase.

termediary sulfur cation radical toward sulfoxidation was noted with cytochrome P-450 (Guengerich & MacDonald, 1984).

Our stereochemical results are, in addition, clearly in favor of the mechanisms represented by eq 2–4 and confirm that the hydroperoxide reacts with the hemoprotein according to a heterolytic pathway yielding first a compound I like species (eq 1). According to considerations developed by Lee and Bruice (1985), a homolytic scission of the O–O bond in hydroperoxylinoleic acid should be expected when reacting nonenzymatically with an iron(III) porphyrin [see also Labeque and Marnett (1988)]. Our data therefore suggest the occurrence of an additional catalytic contribution of the active site of the sulfoxidase to the cleavage reaction in analogy with the mechanism operating, e.g., for the peroxidases (Poulos & Kraut, 1980).

Conclusion

The present results on the mechanism of the 13(*S*)-hydroperoxylinoleic acid dependent oxidation of para-substituted thioanisoles by a partially purified soybean sulfoxidase are consistent with a heterolytic reaction of the hydroperoxide with the hemoprotein to yield a compound I type species. Hammett plots suggest that the oxidation of the substrates involves some single-electron transfer character with the possible formation of a sulfenium radical cation, this intermediate being ultimately transformed, according to a highly asymmetric process, into the corresponding sulfoxide. Such a hypothetical pathway, which is illustrated in Figure 3, has been adapted from that described for mammalian NADPH-dependent cytochrome P-450 enzymes (Watanabe et al., 1980). The important stereoselectivity observed in the oxygen-transfer step indicates that this putative intermediate must be very reactive. One cannot, therefore, exclude that we are dealing with a borderline reaction in which it becomes difficult to distinguish a two-step (via a sulfenium radical cation intermediate) from a one-step (direct insertion) oxygen-transfer mechanism. Sulfoxidase, like cytochrome P-450, transfers its ferryl oxygen to sulfides, acting in this way as a peroxygenase. The fact that the sulfoxidase, like chloroperoxidase and cytochrome P-450 (Ortiz de Montellano, 1987), might operate through unstabilized ferryl oxygen species in direct contact with the substrate can explain the fragility of this enzyme in the absence of acceptor substrates and during its catalytic cycle (Blée & Durst, 1987).

Acknowledgments

We thank Drs. C. Mioskowski and P. Pflieger for their generous gift of (*R*)-(+)-methyl *p*-tolyl sulfoxide, Dr. G. Duportail for his help in the fluorometric determination of hemin, and Dr. F. Durst for his constant interest in this work.

Registry No. *p*-Aminothioanisole, 104-96-1; *p*-methoxythioanisole, 1879-16-9; *p*-methylthioanisole, 623-13-2; *p*-bromothioanisole, 104-95-0; *p*-nitrothioanisole, 701-57-5; methyl *p*-tolyl sulfide, 623-13-2; sulfoxidase, 73699-14-6.

References

Alder, R. W., Baker, R., & Brown, J. M. (1971) in *Mechanism in Organic Chemistry*, pp 1–77, Wiley, London.

Ben-Aziz, A., Grossman, S., Ascarelli, I., & Budowski, P. (1971) *Phytochemistry 10*, 1445–1452.

Blake, R. C., & Coon, M. J. (1980) *J. Biol. Chem. 255*, 1520–1528.
Blée, E., & Durst, F. (1986) *Biochem. Biophys. Res. Commun. 135*, 922–927.
Blée, E., & Durst, F. (1987) *Arch. Biochem. Biophys. 254*, 43–52.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
Dix, T. A., & Marnett, L. J. (1985) *J. Biol. Chem. 260*, 5351–5357.
Doerge, D. (1986) *Arch. Biochem. Biophys. 244*, 678–685.
Douglas, K. T., & Wilson, M. T. (1984) The Chemistry of Enzyme Action, *New Compr. Biochem. 6*, 127–201.
Egan, R. W., Gale, P. H., Baptista, E. M., Kennicott, K. L., Vandenheuvel, W. J. A., Walker, R. W., Fagerness, P. E., & Kuehl, F. A. (1981) *J. Biol. Chem. 256*, 7352–7361.
Guengerich, F. P., & MacDonald, T. L. (1984) *Acc. Chem. Res. 17*, 9–16.
Helenius, A., & Simons, K. (1975) *Biochim. Biophys. Acta 415*, 29–79.
Howard, J. A., & Ingold, K. U. (1963) *Can. J. Chem. 41*, 1744–1751.
Ishimaru, A. (1979) *J. Biol. Chem. 254*, 8427–8433.
Ishimaru, A., & Yamazaki, I. (1977) *J. Biol. Chem. 252*, 6118–6124.
Jencks, W. P. (1980) *Acc. Chem. Res. 13*, 161–169.
Jencks, W. P. (1985) *Chem. Rev. 85*, 511–527.
Kennedy, B. R., & Ingold, K. U. (1966) *Can. J. Chem. 44*, 2381–2385.
Kirsch, J. F. (1972) in *Advances in Linear Free Energy Relationships* (Chapman, N. B., & Shorter, J., Eds.) pp 369–400, Plenum Press, London.
Kobayashi, S., Nakano, M., Goto, T., Kimura, T., & Schapp, A. P. (1986) *Biochem. Biophys. Res. Commun. 135*, 166–171.
Kobayashi, S., Nakano, M., Kimura, T., & Schapp, A. P. (1987) *Biochemistry 26*, 5019–5022.
Labeque, R., & Marnett, L. J. (1988) *Biochemistry 27*, 7060–7070.
Lambeir, A. M., Markey, C. M., Dunford, H. B., & Marnett, L. J. (1985) *J. Biol. Chem. 260*, 14894–14896.
Lee, W. A., & Bruice, T. C. (1985) *J. Am. Chem. Soc. 107*, 513–514.
Lemberg, R., & Barrett, J. (1973) in *Cytochromes*, pp 8–16, Academic Press, New York.
Light, D. R., Waxman, D. J., & Walsh, C. (1982) *Biochemistry 21*, 2490–2498.
Maehly, A. C., & Chance, B. (1954) in *Methods of Biological Analysis* (Glick, D., Ed.) Vol. 1, pp 357–408, Interscience, New York.
May, S. W., Phillips, R. S., Mueller, P. W., & Herman, H. H. (1981) *J. Biol. Chem. 256*, 8470–8475.
Miller, A., Bischoff, J., Bizub, C., Luminoso, P., & Smiley, S. (1986) *J. Am. Chem. Soc. 108*, 7773–7778.
Modena, G., & Mariola, L. (1957) *Gazz. Chim. Ital. 87*, 1306–1316.
Mulder, P., Saastad, O. W., & Griller, D. (1988) *J. Am. Chem. Soc. 110*, 4090–4092.
Oae, S., Mikami, A., Matsuura, T., Ogawa-Asada, K., Watanabe, Y., Fujimori, K., & Iyanagi, T. (1985) *Biochem. Biophys. Res. Commun. 131*, 567–573.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.
Ortiz de Montellano, P. R. (1987) *Acc. Chem. Res. 20*, 289–294.
Pirkle, W. H., Finn, J. M., Schreiner, J. L., & Hamper, B. C. (1981) *J. Am. Chem. Soc. 103*, 3964–3966.
Pitchen, P., Dunach, E., Deshmukh, M. N., & Kagan, H. B. (1984) *J. Am. Chem. Soc. 106*, 8188–8193.
Poulos, T. L., & Kraut, J. (1980) *J. Biol. Chem. 225*, 8199–8205.
Riddles, P. W., Blakeley, R. L., & Zener, B. (1978) *Anal. Biochem. 94*, 75–81.
Sassa, S., & Kappas, A. (1977) *J. Biol. Chem. 252*, 2428–2436.
Takahashi, T., Kim, Y., Fukushima, D., Fujimori, K., Oae, S., & Iyanagi, T. (1978) *Heterocycles 10*, 229–235.
Torii, K., & Ogura, Y. (1968) *J. Biochem. (Tokyo) 64*, 171–179.
Wagner, G. C., Palcic, M. M., & Dunford, H. B. (1983) *FEBS Lett. 156*, 244–248.
Watanabe, Y., Iyanagi, T., & Oae, S. (1980) *Tetrahedron Lett. 21*, 3685–3688.
Watanabe, Y., Oae, S., & Iyanagi, T. (1982) *Bull. Chem. Soc. Jpn. 55*, 188–195.
Waxman, D. J., Light, D. R., & Walsh, C. (1982) *Biochemistry 21*, 2499–2501.
Wilkinson, G. N. (1961) *Biochem. J. 80*, 324–332.
Williams, A. (1984) in *The Chemistry of Enzyme Action* (Page, M. I., Ed.) pp 127–203, Elsevier, Amsterdam.