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S-Oxygenation of the thioether organophosphate insecticides phorate and disulfoton by human lung flavin-containing monooxygenase 2

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Abstract

Phorate and disulfoton are organophosphate insecticides containing three oxidizable sulfurs, including a thioether. Previous studies have shown that only the thioether is oxygenated by flavin-containing monooxygenase (FMO) and the sole product is the sulfoxide with no oxygenation to the sulfone. The major FMO in lung of most mammals, including non-human primates, is FMO2. The *FMO2*2* allele, found in all Caucasians and Asians genotyped to date, codes for a truncated, non-functional, protein (FMO2.2A). Twenty-six percent of individuals of African descent and 5% of Hispanics have the *FMO2*1* allele, coding for full-length, functional protein (FMO2.1). We have here demonstrated that the thioether-containing organophosphate insecticides, phorate and disulfoton, are substrates for expressed human FMO2.1 with K_m of 57 and 32 μM, respectively. LC/MS confirmed the addition of oxygen and formation of a single polar metabolite for each chemical. MS/MS analysis confirmed the metabolites to be the respective sulfoxides. Co-incubations with glutathione did not reduce yield, suggesting they are not highly electrophilic. As the sulfoxide of phorate is a markedly less effective acetylcholinesterase inhibitor than the cytochrome P450 metabolites (oxon, oxon sulfoxide or oxon sulfone), humans possessing the *FMO2*1* allele may be more resistant to organophosphate-mediated toxicity when pulmonary metabolism is an important route of exposure or disposition.

Keywords: FMO; Flavin-containing monooxygenase; Organophosphates; Insecticides; Lung; Toxicity

1. Introduction

Flavin-containing monooxygenase (FMO) is expressed as five gene families in mammals, each with a single member (FMOs 1–5). FMOs in different gene families exhibit 52–58% sequence identity and orthologs across species have 82–97% identity [1–3]. FMO2 is the major isoform found in lung of most mammals, including non-human primates [4–6]. This ''lung FMO'' was originally isolated and characterized from rabbit lung independently by Tynes et al. [7] and Williams et al. [8]. In the lung, FMO2 can account for 10% or more of the total microsomal protein [9]. One of the first observations concerning this lung-specific form of FMO was that it exhibited a substrate specificity demonstrably different from the major FMO in liver [7,8,10–12]. Subsequently, FMO2 has been characterized from mouse, rat, guinea pig, monkey and now human [4–6,13–17]. In humans the majority of individuals possess the *FMO2*2* allele, which codes for a truncated (471 amino acids) and non-functional protein (FMO2.2A). Twenty-six percent of individuals of African descent and 5% of Hispanics have at least one *FMO2*1* allele, coding for the full-length (535 amino acids), catalytically active protein (FMO2.1) [4,16–18]. It has yet to be determined if this genetic polymorphism in expression of FMO2 in human lung is important in the metabolism and toxicity of xenobiotics.

Phorate and disulfoton are thioether-containing organophosphate pesticides that have found wide use in agriculture, primarily on crops such as corn, potatoes, cotton and grains, including wheat. The EPA market estimates for 1999 usage include 2–3 million pounds of the active

Abbreviations: PKC, protein kinase C; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol

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ingredient of phorate in the US alone; the use of disulfoton is about half that of phorate [19]. Significant exposures to phorate and disulfoton can occur in both occupational settings and to the general public. Occupationally, the primary route of exposure is dermal and inhalation, whereas in the general population it is inhalation, diet and dermal [20,21]. As inhalation is an important route of exposure, a more thorough understanding of pulmonary pathways for bioactivation and/or detoxication in humans is important.

As with other organophosphates, cytochrome P450 (CYP)-dependent desulfuration to the oxon yields a toxic metabolite with much greater efficacy and potency in inhibition of acetylcholinesterase activity, the major target of organophosphate acute toxicity [22]. CYP can also catalyze formation of the sulfoxide and sulfone metabolites of the parent compound or the oxon [22]. Hodgson and colleagues have published extensive studies on the metabolism of phorate and other pesticides by CYP and FMO [23–32]. In the case of FMO, the sole metabolite is the sulfoxide which is not further oxygenated to the sulfone. FMO also demonstrates a stereoselectivity for formation of the (−) phorate sulfoxide, whereas CYP produces predominately the (+) phorate sulfoxide [27]. Phorate and phorate sulfoxide are both very weak inhibitors of acetylcholinesterase (IC_{50} of 3100 and 1500 μM, respectively), whereas phorate sulfone is 100 times more potent (40 μM). The oxons (formed only by CYP) are 1000-fold or more potent (IC_{50} of 3, 0.9 and 0.5 μM for phorate oxon, phorate oxon sulfoxide and phorate oxon sulfone, respectively) [27].

In the case of methiocarb, rat liver FMO (predominantly FMO1) exhibits enantioselectivity toward formation of the (A)-enantiomer which is a more potent acetylcholinesterase inhibitor than the parent compound and the (B)-enantiomer [33]. FMO does not catalyze desulfuration to the oxon. Once the sulfoxide is produced, in the presence of CYP, further metabolism produces the sulfone, the oxon sulfoxide and the oxon sulfone. A high rate of FMO-mediated thioether S-oxygenation, in individuals with the *FMO2*1* allele, relative to CYP mediated oxon formation in lung, should decrease toxicity for the majority of thioether-containing organophosphate insecticides.

As for CYP, it has been demonstrated that FMO substrate specificity differs between isoforms [8,10–12] and furthermore, that one cannot assume substrate specificity is consistent among orthologs [34]. For this reason, even though phorate and disulfoton have been shown to be substrates for FMOs, including human FMO1 and FMO3 [32] and FMO2 from mouse [13], it is important to characterize the activity of the human FMO2.1 toward these organophosphate insecticides and confirm the identity of the metabolite(s) produced. We confirm that, as with mouse FMO2, human FMO2.1 has high activity toward both of these organophosphates with K_m of 32 and 57 μM for disulfoton and phorate, respectively, and the sole metabolites are the S-oxides.

2. Materials and methods

2.1. Chemicals

Phorate and disulfoton were obtained from Chem Service Inc. NADPH, EDTA, potassium phosphate, FAD, phenylmethylsulfonyl fluoride (PMSF), glutathione (GSH) and trypan blue were from Sigma. Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. The Bac-to-Bac baculovirus expression system, all components of the BaculoDirect expression system, Sf9 insect cells and media were from Invitrogen.

2.2. Expression of FMO2 and preparation of insect cell microsomes

Human FMO2.1 was cloned in pFastBac1 and expressed in Sf9 insect cells with the Bac-to-Bac system, the complete details of which have been previously described [17]. In brief, the recombinant plasmid DNA was utilized in a transformation of DH10Bac competent cells. The recombinant bacmid was then used to produce primary baculovirus in Sf9 cells. The amplified tertiary or quaternary virus was the source of the protein used in this study. FAD is included in the media (10 μg/ml) during expression to enhance the yield of holoenzyme. At 96 h post-infection, the cells were harvested and microsomes prepared as described previously [17]. The microsomal protein content was determined by the Bradford method [35]. For FAD analysis, samples were prepared as described by Fader and Siegel [36] and quantified by the HPLC method of Klatt et al. [37] with the following modifications. Microsomal preparations were diluted 50× in buffer (0.1 M K_2HPO_4, 0.1 mM EDTA, pH 7.7), heated for 5 min at 95 °C, rapidly cooled on ice, and centrifuged at 10,000 × *g* for 30 min. The supernatants (20 μl) were injected on to a Shimadzu LC-10AD HPLC system equipped with a Waters C_{18} Novapak (3.9 mm × 150 mm) column eluted with 75% methanol and 25% 10 mM K_2HPO_4, pH 6.0 at 0.8 ml/min. Detection was by fluorescence (Shimadzu RF-10A) with excitation and emission wavelengths set at 450 and 520 nm, respectively. FAD standard curves were linear in the range of 25–1000 nM.

Rabbit FMO2 protein was produced for comparison with human protein using BaculoDirect expression. Rabbit FMO2 cDNA was amplified from a pFastBac1 derivative [5] via PCR with Pfu turbo and forward (5′-cacc**ATG**G-CAAAGAAGGTGGCAGT-3′) and reverse (5′-cggatcc**T-TA**GAACCATTGC-3′) rabbit FMO2-specific primers (start and stop codons are given in bold). Directional cloning into pENTR/SD/D-TOPO was mediated by topoisomerase, generating the pENTR·rF2 vector. After confirmation of the cDNA sequence, pENTR·rF2 DNA was recombined with BaculoDirect linear DNA to produce recombinant baculovirus DNA used to directly transfect

Sf9 cells and yield primary virus. Rabbit FMO2 protein was produced from tertiary or quaternary virus as already described for human FMO2.1.

2.3. Phorate and disulfoton as substrates of expressed human FMO2.1 and rabbit FMO2 as determined by substrate-dependent NADPH oxidation

Substrate-dependent NADPH oxidations were performed essentially as described previously [17]. Incubation mixtures (0.5 ml) containing 100 mM Tricine/0.1 mM EDTA (pH 9.5), 0.1 M NADPH, and 50 pmol/ml microsomal protein were preincubated in sample and reference cuvettes in a Cary 300 Bio UV–vis double beam spectrophotometer. Substrates, dissolved in ethanol, were added to the sample cuvette and the change in absorbance at 340 nm monitored for 4 min. Apparent K_m and k_{cat} were estimated from Lineweaver–Burk and Eadie–Hofstee plots of the data generated with at least four substrate concentrations (ranging from 5 to 200 μM) and two separate batches of each expressed protein.

2.4. HPLC, LC/MS and MS/MS of phorate and disulfoton metabolites

Standard microsomal incubations contained 100 mM Tricine, 1 mM EDTA (pH 9.5) and 50 μg total microsomal protein in a total volume of 100 μl. Substrates were added in ethanol (not exceeding 1 μl) at final concentrations of 200 μM. After a 3 min incubation at 37 °C, the reactions were initiated by the addition of 1 mM NADPH and the incubation continued for 15 min. Reactions were stopped by the addition of 100 μl methanol on ice, the mixtures transferred to Eppendorf tubes and centrifuged at 10,000 × *g* for 30 min at 4 °C. The supernatants were analyzed by HPLC and LC/MS/MS. Additional incubations were run with added GSH at concentrations ranging from 0.1 to 4 mM.

HPLC analysis was performed with a Waters 2690 pump equipped with a 996 diode array detector and a Waters Polarity C_{18} column (4 μm, 3.9 mm × 150 mm). Flow rates were 0.8 ml/min and column temperature was 35 °C. Samples were analyzed with an isocratic system of 60% CH_3CN:40% water for phorate and 70% CH_3CN:30% water for disulfoton. Detection was at 220 nm for phorate and 210 nm for disulfoton.

LC/MS and LC/MS/MS analysis used a Shimadzu HPLC with LC-10 pumps connected to a Perkin-Elmer Sciex API III mass spectrometer run in positive ion mode with an orifice voltage of +60 V, source temperature of 60 °C and scanning from *m/z* 150 to 400. Samples were introduced via the heated nebulizer interface set at 450 °C. Daughter-ion scanning in the MS/MS mode was used to obtain structural information with the collision energy set at 10 or 15 V for phorate and 12 V for disulfoton. LC conditions were the same as described above except temperature was ambient. Further confirmation of the chemical structure of phorate S-oxide was achieved with a Micromass Q-TOF mass spectrometer which allowed exact mass measurements for the metabolite fragment ions: *m/z* 199.0023 (calcd. for $C_5H_{12}O_2PS_2^+$: 199.0016), *m/z* 170.9710 (calcd. for $C_3H_8O_2PS_2^+$: 170.9703), *m/z* 153.0139 (calcd. for $C_4H_{10}O_2PS^+$: 153.0139), *m/z* 143.9382 (calcd. for $CH_4O_2PS_2^+$: 142.9390), *m/z* 125.9828 (calcd. for $C_2H_6O_2PS^+$: 124.9826), *m/z* 96.9504 (calcd. for $H_2O_2PS^+$: 96.9513). Except for the fragment ion with *m/z* 199, these ion peaks were also observed in the MS/MS spectrum of disulfoton S-oxide.

3. Results

Incubation of phorate or disulfoton (Fig. 1) with Sf9 insect cell microsomes expressing human FMO2.1 resulted in substrate-dependent NADPH oxidation. Kinetic analysis by double reciprocal plots (data not shown) demonstrated that disulfoton was a better substrate (K_m 32 μM, V_{max} 71 nmol/min/mg protein), compared to phorate (K_m 57 μM, V_{max} 63 nmol/min/mg). The metabolism of phorate and disulfoton by expressed rabbit FMO2 assayed under identical conditions yielded somewhat higher K_m values of 71 μM for both substrates and intermediate k_{cat} estimates of 39 and 44 min^{-1} μM^{-1} (Table 1). Phorate, but not disulfoton, is metabolized by expressed human FMO2.1 more efficiently than by rabbit FMO2 as indicated by the higher specificity constant achieved with human FMO2.1.

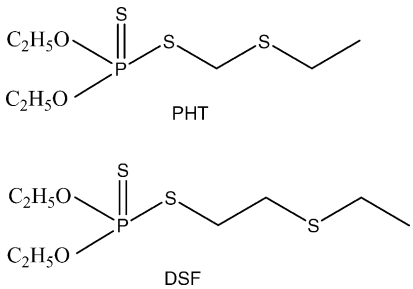

Fig. 1. Chemical structures of phorate (PHT) and disulfoton (DSF).

Table 1
Kinetic constants for FMO2-dependent S-oxygenation of phorate and disulfoton[a]

	Human FMO2.1			Rabbit FMO2		
	K_m[b]	k_{cat}[c]	S.C.[d]	K_m	k_{cat}	S.C.
Phorate	57	41	0.79	71	39	0.55
Disulfoton	32	20	0.61	71	44	0.62

[a] Values represent the mean of two different batches of expressed enzyme.
[b] K_m in μM.
[c] k_{cat} in min^{-1}.
[d] S.C. is the specificity constant expressed as k_{cat}/K_m.

For comparison, the K_m for phorate S-oxygenation in liver microsomes from mouse, pig and human was reported as 32.2, 12.3 and 53.1 μM, respectively, and the K_m for disulfoton was 3.4, 2.2 and 36.6 μM, respectively [25,32]. The relative role of FMO and CYP in S-oxide formation was estimated at 10 and 90%, respectively in human liver microsomes [32]. The relatively minor role for FMO in human liver microsomes appears to be due to the low expression of FMO1 in adult human liver and the relatively low activity of FMO3 toward phorate and disulfoton [32]. In liver microsomes from other mammals (in which FMO1 expression is much greater), the importance of FMO in S-oxide production is greater. This FMO contribution is expected to increase in extrahepatic tissues, such as lung, where the ratio of FMO/CYP is higher. Karoly and Rose report a K_m of 107 μM with phorate for expressed mouse FMO2 [13].

HPLC analysis of the metabolic profile of phorate and disulfoton following incubation with Sf9 cells expressing human FMO2.1 is depicted in Fig. 2. Consistent with

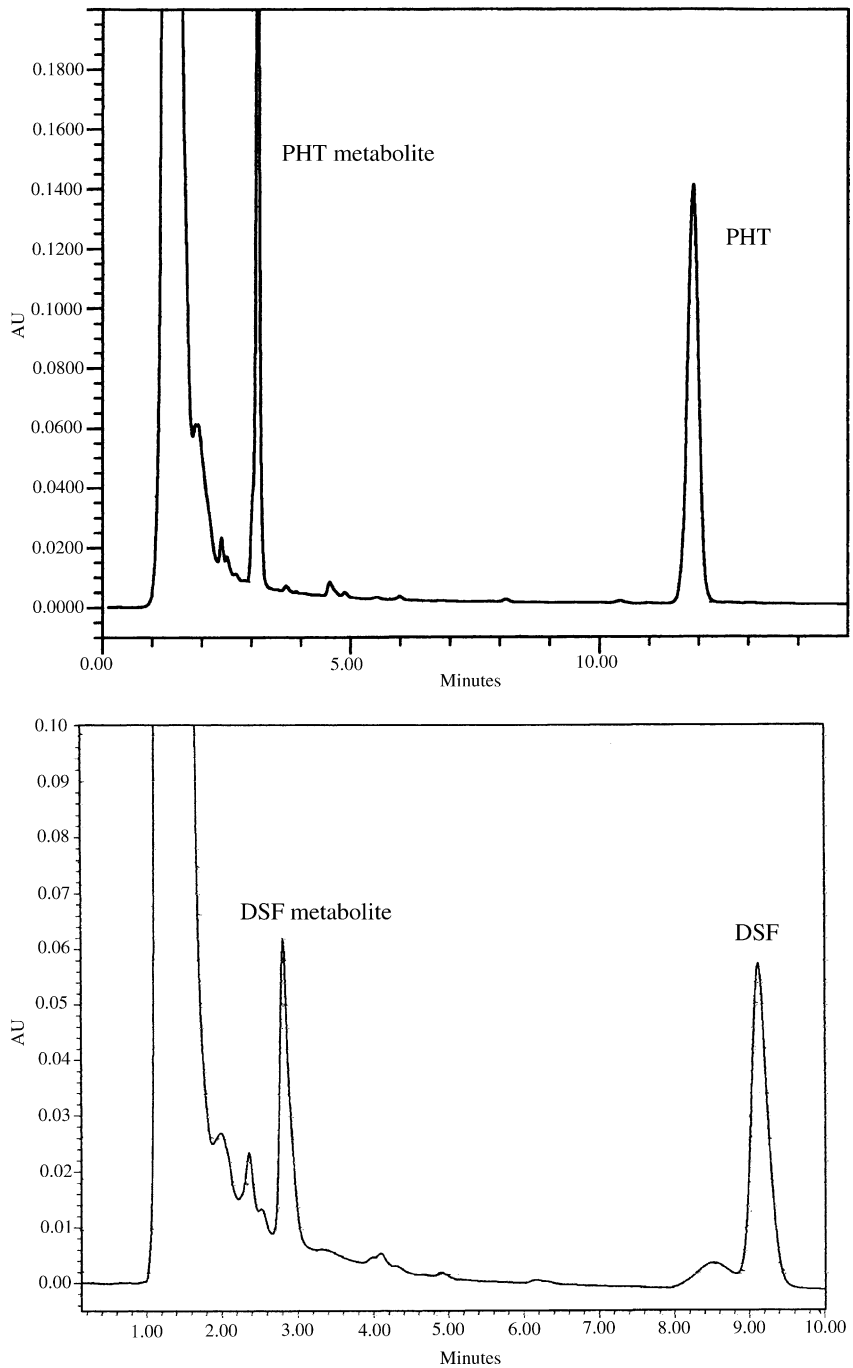

Fig. 2. HPLC chromatograms of phorate (PHT, upper panel) and disulfoton (DSF, lower panel) incubations with baculovirus-expressed FMO2.

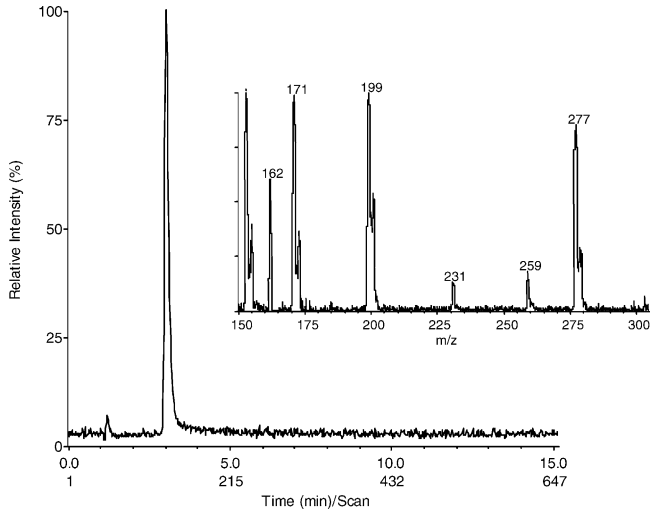

Fig. 3. LC/MS of phorate incubation showing extracted ion (mass 277) chromatogram and associated spectra of phorate sulfoxide.

previous reports [23–32], a single major polar FMO metabolite is evident. Hodgson and colleagues have shown that FMO specifically oxygenates the thioether of phorate and terminates at the sulfoxide. These studies had also shown that FMO exhibits stereoselectivity in producing predominantly the (−) sulfoxide of phorate, in contrast to CYP which is also capable of sulfoxidation of phorate but yields predominantly the (+) stereoisomer [27]. It is not unusual to observe stereoselectivity in CYP and FMO oxygenation of chiral sulfur-containing substrates [27,38–40]. In this study, we did not separate the (−) from the (+) sulfoxide metabolite. The sulfoxides of phorate and disulfoton appear to be fairly stable. Addition of glutathione to the incubation did not reduce the yield of the putative sulfoxide metabolite (data not shown).

LC/MS analysis confirmed the presence of a single polar metabolite following incubation of expressed human FMO2.1 with phorate (Fig. 3) or disulfoton (Fig. 4) and the MS analysis of the products (inset) confirmed addition of oxygen ($M + 1 + 16$). MS/MS analyses proved to be consistent with oxygenation at the thioether for both phorate (Fig. 5) and disulfoton (Fig. 6) which would agree with previous analysis of FMO-mediated phorate S-oxygenation in liver, lung and kidney microsomes [28]. The facile loss of a water molecule from the sulfoxide moiety in both pesticide metabolites is the reason that no stable fragment ions were produced in the MS/MS experiments that contained the oxygen atom introduced by the monooxygenase. In the case of phorate, the resulting positive charge on the *m/z* 259 ion can be distributed over both side-chain sulfur atoms due to tautomerism, hence there is no way of telling which of the two sulfur atoms was enzymatically oxygenated. On the other hand, the positive charge of the $[MH - H_2O]^+$ ion of disulfoton cannot be distributed over the two side-chain sulfur atoms in terms of resonance stabilization, and this difference may give a clue as to the position of the $>$S=O oxygen: the fragment with *m/z* 213 must contain the ethylene bridge linking the two side-chain sulfur atoms. Therefore, it seems plausible that a five-membered dithiaphospholanium ring is formed from disulfoton's $[MH - H_2O]^+$ ion as shown in Fig. 6. This fragmentation pathway supports oxygenation of the sulfur atom most distant from the phosphor atom because oxygenation of the proximal sulfur atom would not give rise to ions with an intact ethylene bridge.

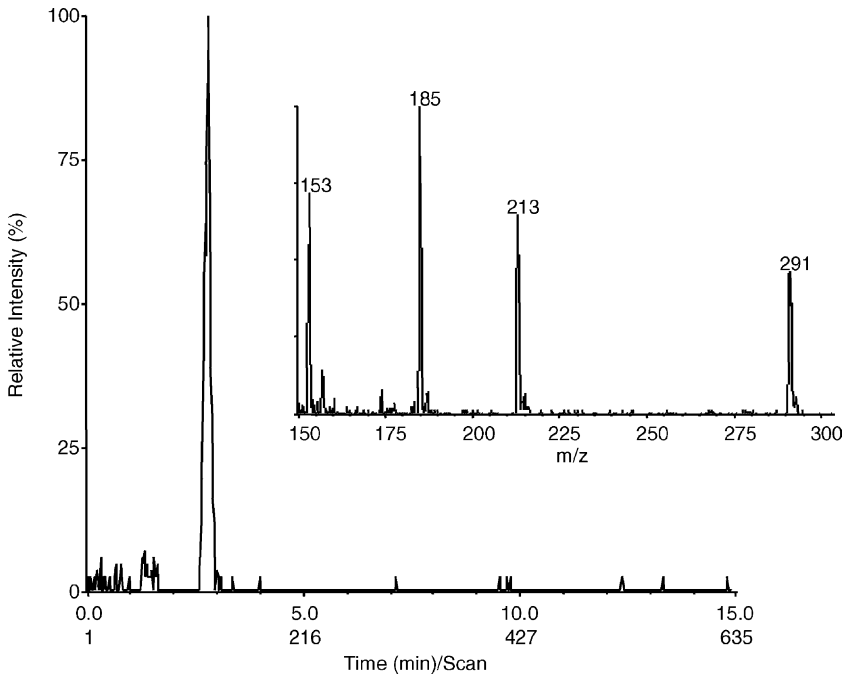

Fig. 4. LC/MS of disulfoton incubation showing extracted ion (mass 291) chromatogram and associated spectra of disulfoton sulfoxide.

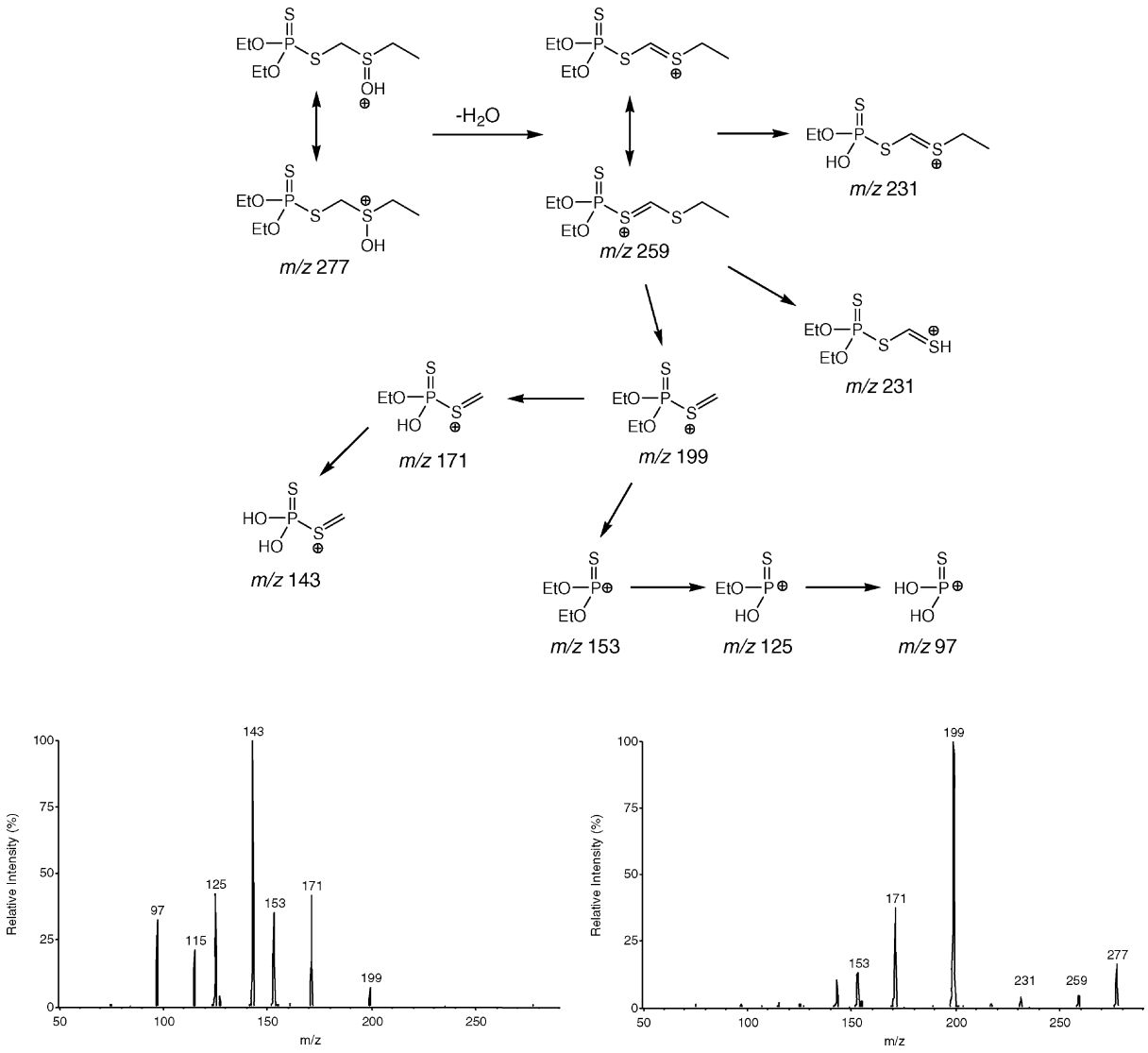

Fig. 5. MS/MS spectra of phorate sulfoxide (at 10 and 15 V) and derivation of fragments.

4. Discussion

FMOs have been shown to play a significant role in the oxidative metabolism of many pesticides. Among this class of chemicals, thioether-containing organophosphates such as phorate and disulfoton are among the best substrates [13,22–33,41–43]. Phorate and disulfoton are S-oxygenated by both CYP and FMO. Oxidative desulfuration to yield the oxon (a more toxic metabolite) is catalyzed by CYP and not FMO. Sulfoxidation of the thioether (usually markedly less toxic than the oxon) can be catalyzed by both FMO and CYP [22–33,41–43]. In extrahepatic tissues, such as kidney and lung where the ratio of FMO/CYP is higher, FMO appears to be the predominant enzyme in sulfoxide production [28]. In the case of the mouse, the sulfoxide represents 85% of the total phorate metabolites produced by liver microsomes, but 96% in the lung [28]. Sulfoxides can be further converted to sulfones and this second oxygenation can again be catalyzed by both monooxygenases, but sulfoxides are usually much poorer substrates for FMO and often only the first oxygenation of the thioether takes place. No sulfone metabolites are found following incubation of phorate with rat lung microsomes [28] or with the human FMO2.1 (present study). Sulfoxides and sulfones of phorate and disulfoton can also be converted to the respective oxons by CYP [28]. Bioactivation of organophosphates occur through a CYP-mediated oxidative desulfuration to the oxon which are very potent acetylcholinesterase inhibitors [27]. The sulfoxide is less toxic in vivo than would be predicted based on its inhibition of acetylcholinesterase in vitro (roughly equivalent to the parent compound) [44]. This, plus the lack of production of the oxons or sulfone metabolites (100–1000-fold more potent that the parent or the sulfoxide), suggests that a relatively high rate of FMO-dependent sulfoxide formation, relative to CYP-oxon production, in tissues such as lung or kidney could be protective.

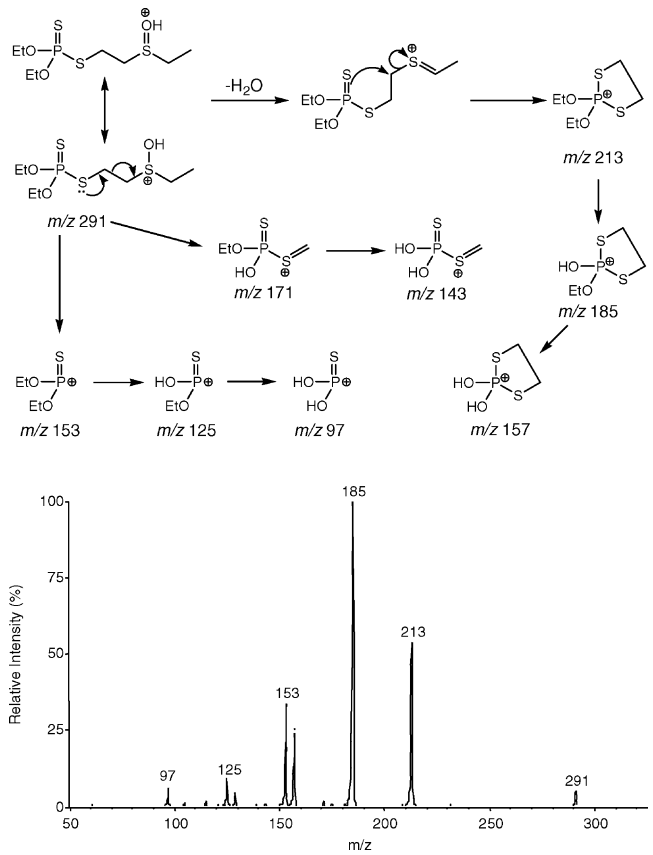

Fig. 6. MS/MS spectra of disulfoton sulfoxide and derivation of fragments.

In humans, the role of the lung in metabolism and toxicity of xenobiotics is still uncertain (reviewed in [45,46]). Compared to common laboratory animals, the expression of CYPs and FMO is low (1–5% that of a rat or rabbit) [46]. However, the lung receives 100% of the cardiac output and can function as a first pass metabolism organ for drugs administered by almost every route except orally. In addition, there are over 40 cell types in the lung and it may be that the level of expression in certain important cell types in the lung may be sufficient to significantly influence the pharmacological and/or toxicological outcome following exposure to certain drugs and xenobiotics. The expression of various CYPs in human lung has been documented by mRNA, protein and/or catalytic activity. The CYPs that appear most significant in human lung are 1A1 (smokers only), 1B1, 2B6, 2E1, 2J2, 2S1 and 3A5 [45,46], although in relatively low amounts. The total specific content of all CYPs combined in human lung is estimated at 2–10 pmol/microsomal protein [46]. In contrast to CYP, functional FMO2 does not appear to be expressed in most individuals. All Caucasians and Asians genotyped to date do not have the *FMO2*1* allele, but rather the *FMO2*2* allele which codes for a truncated and non-functional protein. However, 26% of African-Americans and 5% of Hispanics do possess at least one *FMO2*1* allele and express full-length, enzymatically active protein [4,16–18]. The initial estimate of the specific content of FMO2 in an African-American individual genotyped as *FMO2*1/FMO2*2* was 8.8 pmol/mg microsomal protein [17]. This finding, plus the fact that the CYPs most important in sulfoxidation of phorate and disulfoton in human liver (1A2, 3A4 and 2C9) are not expressed to a significant extent in lung [45,46], strengthen the hypothesis that FMO2 in individuals with the *FMO2*1* allele could be important in the metabolism of this class of insecticides in the lung and that FMO-mediated sulfoxidation should be protective.

In summary, we report here that human FMO2 (FMO2.1) is similar to FMO1 from mouse, pig and human and FMO2 from mouse in having high activity toward organophosphates such as phorate (but to a lesser degree with disulfoton). We confirm the production of a single major polar metabolite identified as the sulfoxide, consistent with previous results [23–33]. Our results suggest that the metabolism and toxicity of thioether-containing organophosphate insecticides in individuals expressing the *FMO2*1* allele may be strikingly different than in individuals not expressing an active FMO enzyme. In the case of organophosphate insecticides such as phorate and disulfoton, the *FMO2*1* allele may provide protection, whereas, in the case of exposure to environmental chemicals for which FMO activity results in a more toxic metabolite, such as S-oxygenation of thioureas to sulfenic acid metabolites [47], these individuals may have an enhanced risk for pulmonary toxicity.

Acknowledgments

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