METABOLISM OF DIALLYL DISULFIDE BY HUMAN LIVER MICROSOMAL CYTOCHROMES P-450 AND FLAVIN-CONTAINING MONOOXYGENASES

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ABSTRACT:

The metabolism of diallyl disulfide (DADS), a garlic sulfur compound, was investigated in human liver microsomes. Diallyl thiiosulfinate (allicin) was the only metabolite observed and its formation followed Michaelis-Menten kinetics with a \( K_m = 0.61 \pm 0.2 \text{ mM} \) and a \( V_{max} = 18.5 \pm 4.2 \text{ nmol/min/mg protein, respectively (mean \pm S.E.M., n = 4). Both flavin-containing monoxygenases and the cytochrome P-450 monoxygenases (CYP) were involved in DADS oxidation, but the contribution of CYP was predominant. The cytochrome P-450 isoforms involved in this metabolism were investigated using selective chemical inhibitors, microsomes from cells expressing recombinant CYP isoenzymes, and studying the correlation of the rate of DADS oxidation with specific monoxygenase activities of human liver microsomes. The chemistry of the sulfur-containing compounds of garlic (Allium sativum) is complex. Fresh garlic contains allicin (S-allylcysteine sulfoxide) from which many molecules are formed when a garlic clove is crushed. Among the transformation products, ajoene, sulfides, and allicin (diallyl thiiosulfinate or DADSO; Fig. 1) were noticed for their medicinal properties such as antimicrobial, antifungal, antitumor, antithrombotic, hypotensive, hypoglycemic, and hypolipemic properties (see Lin, 1989, for review).

Diallyl disulfide (DADS) is one of the major volatile degradative compounds of garlic formed from DADSO (Block, 1992; Augusti, 1996). It was found in the breath of human subjects who ate dry or fresh garlic (Cai et al., 1995). Many studies on animals showed its protective effects against chemically induced toxicity and against carcinogenesis (Ip et al., 1992; Reddy et al., 1993). The modulation of the metabolism of carcinogens by DADS was considered one of the possible mechanisms of its protective effect against the occurrence of cancer (Reddy et al., 1993). Previous studies in our laboratory demonstrated that the administration of DADS to rats modified the quantity and the activity of several hepatic drug metabolism enzymes (Haber et al., 1994; 1995). DADS produced an enhancement of the microsomal level of cytochrome P-450 monoxygenase (CYP2B1/2) and of the activities of UDP glucuronyltransferases and of glutathione S-transferases. It decreased the nitrosodimethylamine demethylase activity and the level of CYP2E1. In addition, there are many reports about the chemical analysis and biological activities of sulfur-containing compounds of garlic. However, few studies have dealt with the metabolism and pharmacokinetics of garlic constituents. One study performed in mice with radioactive DADS indicated that the uptake of radioactivity was highest in the liver at 90 min after i.p. injection (Pushpendran et al., 1980). In metabolic studies using perfused rat liver, DADS appeared to be converted to allyl mercaptan (Egen and Schwind et al., 1992). This information and previous results concerning the ability of DADS to modulate drug-metabolizing enzymes led us to hypothesize that DADS could be metabolized by cytochrome P-450 enzymes in the liver.

The flavin-containing monoxygenases (FMOs) play a role in xenobiotic and drug metabolism. These enzymes have been characterized in several mammalian species, including human. Among the identified isoenzymes, FMO3 is the predominant form in adult human liver. The FMOs are able to metabolize a wide variety of xenobiotics including thios, sulfides, and disulfides but the involvement of such enzymes in the metabolism of garlic compounds has not been reported.

In this study we investigated the in vitro metabolism of DADS in presence of human liver microsomes. We identified one metabolite formed and determined the kinetic parameters of the reaction. The contribution of both CYP and FMO were shown. Then we identified...
the CYP isoenzymes mainly involved in the reaction using several approaches: 1) effect of selective chemical inhibitors on DADS oxidation, 2) determination of DADS oxidation by microsomes from cells expressing recombinant CYP isoenzymes, and 3) study of the correlation of DADS oxidation with marker activities of CYP isoenzymes in different human liver microsomes.

**Experimental Procedures**

**Materials.** DADS (purity of 80%) was obtained from Aldrich Chemical Co. (Strasbourg, France). The other 20% were identified by HPLC as diallyl sulfide and diallyl trisulfide. DADS was used without purification for the determination of kinetic parameters and the study of the involvement of FMOs. DADSO was donated by Professor Auger, Institut de Chimie (Saint Quentin Fallavier, France). It was synthesized from the corresponding disulfide according to the method of Ferary (1996). 5,5-Dithiobis(2-nitrobenzoic acid), α-naphthoflavone, chloroxazone, coumarin, dextromethorphan, diethylthiocarbamate, NADPH, nifedipine, orphenadrine, quinidine, sulfaphenazole, tolbutamide, tranylcypromine, and tolbutamide were purchased from Sigma-Aldrich Chimie (Saint Quentin Fallavier, France). Dextrophan and 5-(+)-mephenytoin were purchased from Ultrase Chemicals (Salford, England). 1-Aminobenzotriazole was donated by Dr. Cabanne, Laboratoire de Phytopharmacologie, Institut National de la Recherche Agronomique (Dijon, France). [1-14C]Lauric acid and [14C] 5-(+)-mephenytoin were purchased from Amersham Pharmacia Biotech (Les Ulis, France). Microsomes from B-lymphoblastoid cell lines expressing individual human CYPs or baculovirus-infected insect cells expressing individual human FMOs were purchased from Gentest (Woburn, MA). All other chemicals and reagents used were of the highest commercial quality available.

**Human Liver Samples.** Human liver samples were provided by Professor Favre, Département de Chirurgie Digestive Thoracique et Cancérologique of the General Hospital of Dijon, France. The protocol was approved by a local ethics committee. The used tissues came from patients undergoing liver resection for various clinical reasons. The samples were frozen in liquid nitrogen and stored at −80°C until use for microsome preparation as described below. Biochemical characteristics of the microsomes are mentioned in Table 1.

**Preparation of Microsomal Fractions and Determination of Protein Content.** The microsomes were prepared as described previously (Haber et al., 1994). Microsomal proteins were quantified by the method of Bradford (1976) using bovine serum albumin fraction V as standard. Cytochrome P-450 was assayed according to Omura and Sato (1964).

**Microsomal Metabolism of DADS.** The reaction medium described below was the same for each experiment. This reaction medium consisted of human liver microsomes corresponding to 300 pmol of CYP, 1 nM DADS, 1 mM

### TABLE 1

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<sup>a</sup> pmol/min/nmol CYP
<sup>b</sup> nmol/min/nmol CYP

nd, not determined.
umes of ethanol were added to the incubation medium (total volume, 500 µl). After 30 min at 37°C, the reaction was stopped by adding 320 µl of acetonitrile. After 15 min of protein precipitation, the mixture was centrifuged at 10,500g for 10 min and 40 µl of the supernatant was analyzed by HPLC. The same protocol was applied with 25 pmol for each cdNA-expressed human CYP microsome. In case of cdNA-expressed human FMO3 microsomes, 100 µg of protein was added in a total volume of 125 µl. Many DADS concentrations were used to determine the kinetic parameters. Thermal inactivation of microsomes was performed by a preincubation of microsomes in Tris-HCl buffer for 10 min at 37°C in the absence of NADPH, or in the presence of NADPH for control microsomes. Then DADS and NAPD were added subsequently to start the incubation for 30 min.

Inhibition of DADS Metabolism. Inhibitors were added to be definitely included in the incubation mixtures before initiation of the reaction. Only with the mechanism-based inhibitors such as diethyldithiocarbamate or aminobenzotriazole, microsomes were preincubated for 10 min at 37°C before the addition of DADS. Non-hydrolysable inhibitors were dissolved in 100% ethanol and the following volumes of ethanol were added to be included in the incubation medium (total volume, 500 µl): 0.2 µl for sulfaphenazole and orphenadrine, 0.3 µl for coumarin, 0.4 µl for diethyldithiocarbamate, 0.5 µl for aminobenzotriazole and quinidine, 0.8 µl for α-naphthoflavone, and 2.5 µl for nifedipine. The human samples K51, K12, K25, KS28, K33, K40, KS41, and KS63 were used for the inhibitory experiments.

HPLC Analysis. HPLC analysis was carried out using a Waters (Saint Quentin-en-Yvelines, France) system equipped with a model 600 pump, a model 717 auto sampler, a model 996 photodiode array UV detector, and a GL Sciences Inc. (Tokyo, Japan) Interstil ODS-3 column (4.6 × 150 mm). The flow rate was 0.6 ml/min and the solvent-isocratic program was 30:70 (v/v, acetonitrile/water) for 20 min. The spectrum from 190 to 300 nm was used to detect DADS and its metabolites. The quantification was made at 254 nm. Data were processed by Waters Millennium software.

Determination of Kinetic Constants. K_m and V_max were determined with a range of substrate concentrations of 0 to 7.5 mM with human liver microsomes and FMO cDNA-expressed microsomes, and 0 to 5 mM with CYP cDNA-expressed microsomes. The values were estimated by fitting the Michaelis-Menten equation using a nonlinear regression program of SAS software (Cary, NC). The human samples K51, KS30, K33, and K40 were used in this experiment. The apparent V_max of FMO3 cDNA-expressed microsomes was determined considering that 100 µg of protein corresponded to approximately 100 pmol of enzyme. This consideration was based on the specific activity of recent lots of FMO3 from Gentest.

Enzyme Assays. Detailed information for these assays are given Table 2.

### Results

Identification of Metabolite. When DADS was incubated with human liver microsomes and NADPH, only one peak was detected by HPLC (Fig. 2). This peak was identified as DADSO by comparing its retention time in different HPLC gradients and its absorption spectrum with that of synthesized DADSO. No other metabolite was detected for various incubation times of DADS. When either NADPH or microsomes were omitted, no DADSO was detected.

Kinetics of Reaction. The formation of DADSO was linear over a period of 45 min. An incubation time of 30 min was therefore routinely used. A concentration of 1 mM NADPH was optimal for the reaction. The kinetic of formation of DADSO by liver microsomes was consistent with the Michaelis-Menten equation. The apparent K_m was 0.61 ± 0.2 mM and the apparent V_max was 18.5 ± 4.2 nmol/min/mg protein. Values are means ± S.E.M. for four samples.

Contribution of FMOs to DADS Oxidation. To evaluate the respective roles of FMOs and CYP in the oxidation of DADS, we initiated incubations of DADS in the presence of : 1) 1-amino-benzotriazole, a suicide inhibitor of CYPs (De Mentellano and Mathews, 1981; Fig. 3) shows the inhibition of DADS oxidation by this inhibi-
Effect of Human CYP Inhibitors on DADS Oxidation. To further assess which CYP isoenzymes are involved in DADS oxidation, the effects of the following chemical inhibitors were determined: α-naphthoflavone (specific of CYP1A), chlorzoxazone (CYP2E1; Amet et al., 1995), coumarin (CYP2A6; Harris et al., 1994), diethyl-dithiocarbamate (CYP2E1; Guengerich et al., 1991), mephenytoin (CYP2C19; Harris et al., 1994), nifedipine (CYP3A4; Harris et al., 1994), orphenadrine (CYP2B6; Chang et al., 1993), quinidine (CYP2D6; Harris et al., 1994), sulfaphenazole (CYP2C9; Baldwin et al., 1995), tolbutamide (CYP2C9; Harris et al., 1994), tranylcypromine (CYP2C19; Postlind et al., 1998), and troleandomycin (CYP3A4; Rodrigues, 1994).

The results are presented in Fig. 4. The strongest inhibitions were observed in the presence of diethyl-dithiocarbamate, followed by tranylcypromine. A slighter inhibition rate appeared with coumarin, chlorzoxazone, mephenytoin, nifedipine, and sulfaphenazole. Orphenadrine, tolbutamide, and troleandomycin had almost no effect on the DADS formation, and α-naphthoflavone and quinidine had no effect at all. To verify the selectivity of some of the inhibitors used, we measured PNPH activity, a marker of CYP2E1, in the presence of 30 μM tranylcypromine, or of 22 or 200 μM coumarin. The result is shown in Table 4. Tranylcypromine and coumarin, described as selective inhibitors of CYP2C19 and CYP2A6 respectively, were also inhibitors of PNPH activity. These results suggest that tranylcypromine and coumarin can inhibit other CYPs, at least CYP2E1.

Metabolism by Microsomes Containing cDNA-Expressed CYPs. The abilities of different human CYP enzymes to catalyze the S-oxidation of DADS to DADSO were investigated in many microsomes derived from cells expressing CYP DNA. CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 catalyzed the S-oxidation of DADS. CYP2D6, CYP2C19, and CYP2E1 were the most active enzymes. We can observe in Fig. 5, that every CYP isofrom followed a Michaelis-Menten kinetic except CYP2E1. For this latter enzyme, the DADS oxidase activity decreased quickly as soon as a certain amount of DADSO was produced. This could be explained by the CYP2E1 mechanism-based inhibitor property of DADSO. No detectable amounts of DADSO were found when using microsomes expressing CYP1A2, CYP3A4, or CYP4A11, or when using microsomes from B-lymphoblastoid cells transfected with an empty vector. The kinetic parameters were determined for CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (Table 5). CYP2E1 exhibited the smallest $K_m$ whereas CYP2D6, CYP2C19, and CYP2E1 had close $V_{max}$ values. The intrinsic clearance ($V_{max}/K_m$ ratio) was 2010 for CYP2E1 whereas it reached the maximum value of 108 for the other CYPs (Table 5).

Correlation Study between DADS Oxidation and Specific CYP Activities. The correlation between DADS oxidation and CYP marker activities was analyzed with a panel of at least 24 individual samples of human liver microsomes. The characteristic transformations by individual CYPs were EROD for CYP1A2, COH for CYP2A6, TDH for CYP2C9, MpH for CYP2C19, DOD for CYP2D6, (α-1)-LAH and PNPH for CYP2E1, NfO for CYP3A4, and (α)-LAH for CYP4A. These results are shown in Table 6. The best correlations (r) were found with PNPH, TDH, and (α-1)-LAH activities, whereas the correlations with EROD, COH, DOD, NfO, MpH, and (α)-LAH were very low.
Discussion

We have studied the metabolism of DADS in the presence of human liver microsomes and we have observed the appearance of DADSO formed by the oxidation of one sulfur atom. Both DADS and DADSO are natural compounds found in crushed garlic but the latter has been considered as the most important biologically active compound of garlic (Reuter, 1995; Augusti, 1996). Among several sulfides from *Allium* studied, DADS exhibited in vivo the strongest anticarcinogenic properties (Ip et al., 1992; Haber-Mignard et al., 1996) and protected against carcinogen-induced DNA strand breaks (Le Bon et al., 1997). These effects were attributed to in vivo modulation of hepatic drug-metabolizing enzymes by DADS (Haber et al., 1994). Knowing the in vitro DADS metabolism, we suppose that the in vivo effects of DADS are in fact due to DADSO.

In this study, we have observed the oxidation of DADSO. The in vivo metabolisms of diallyl sulfide (DAS; Brady et al., 1991, Chen et al., 1994) and of dipropyl sulfide (Nickson and Mitchell, 1994), two sulfur compounds from *Allium*, implicate a first step of oxidation to form a sulfoxide and a second one to form a sulfone. With DADS two steps of oxidation were not excluded, even if formation of diallyl thiosulfonate (DADSO2) was not observed in the incubation medium. In fact we have characterized DADSO as a mechanism-based inactivator of CYP2E1 (results not published). This should mean that DADSO could be oxidized to a reactive species (possibly...
DADSO₂ that should interact with the enzyme and should not be released from the active site of the enzyme.

Egen-Schwind et al. (1992) have studied the metabolism of DADSO in a perfused rat liver. They observed that while passing through the liver, DADSO was metabolized to DADS. The discrepancy could be explained by the models that were used in both studies. They did not add NADPH during the liver perfusion. Due to the sensitivity of DADSO to temperature, a dismutation of DADSO in DADS could be possible. We observed the formation of DADS when DADSO was incubated with microsomes at 37°C without NADPH. In addition, Jin and Baille (1997) studied the metabolism of DAS in rat. They proposed that the reduction of allyl sulfoxide to DAS was impossible with respect to the glutathione conjugates observed with rat fed with DAS or allyl sulfoxide.

Flavin or CYPs are the only enzymes present in microsomes that can catalyze NADPH- and oxygen-dependent oxidation of xenobiotics. The inhibition of CYP by 1-aminobenzotriazole, a suicide inhibitor, as well as the irreversible inactivation of FMO by heating induced a decrease of the rate of DADS oxidation. Moreover, the DADS oxidation was observed with microsomes prepared from cells expressing human FMO3. These results suggest a contribution of both CYP and FMO-containing monoxygenases. Some results allow the evaluation of the FMO implication in this oxidation: its $K_m$ is much higher than the one obtained for FMOs associated with CYP in human microsomes (10.15 versus 0.61 mM). The similar comparison made for the CYP 2C19 showed that in a competitive context like in human microsomes, only CYP2E1 would be involved. The apparent velocity of CYP2E1 was the highest one. We demonstrated that DADSO was a mechanism-based inhibitor of CYP2E1 (Martin, Teysseier and Siess, publication in preparation). This means that the more DADSO is produced, more CYP2E1 is inhibited. This observation may serve as an explanation for the low $V_{max}$ of CYP2E1.

In the inhibitory experiments, tranylcypromine, an inhibitor described as being specific of CYP2C19 (Postlind et al., 1998), strongly inhibited DADSO oxidase activity, suggesting the participation of this isoenzyme. However, the specificity of this inhibitor should be reconsidered because Draper et al. (1997) recently demonstrated that this molecule is among the strongest inhibitors of the coumarin hydroxylase activity (CYP2A6 activity marker), and we showed the PNPH activity inhibition by tranylcypromine. Therefore, CYP2C19 does not seem to be involved in DADSO oxidation.

DADS has a chemical structure similar to DAS; they differ only by one sulfur atom. These two molecules are metabolized by CYP2E1 and produce a mechanism-based inhibitor of CYP2E1. The metabolism of DADS and its pathway is one more similarity between these two molecules issued of garlic.

In conclusion, the oxidation of DADSO to DADS in human liver microsomes is mainly mediated by CYPs but also by FMOs. Among the CYP isoenzymes, CYP2E1 seems to be the most involved isoenzyme even if a few other isoenzymes are also able to catalyze this reaction.

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References


Dixit A and Roche TE (1984) Spectrophotometric assay of the flavin-containing monoxygenase calculated with cDNA-expressed CYP2E1, this isoenzyme exhibited the highest affinity and the highest intrinsic clearance among the isozymes tested. Even if the quantity of CYP2E1 in human microsomes was evaluated to 7% of whole CYP versus 18% for CYP2C and 29% for CYP3A (Shimada et al., 1994), the relative involvement of CYP2E1 is predominant.

Nevertheless, our results suggest the involvement of other CYPs. Many isoenzymes, such as CYP2A6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 were able to oxidize DADSO to DADS. Their $K_m$ values showed that in a competitive context like in human microsomes, only CYP2E1 would be involved. The apparent velocity of CYP2E1 was not the highest one. We demonstrated that DADSO was a mechanism-based inhibitor of CYP2E1 (Martin, Teysseier and Siess, publication in preparation). This means that the more DADSO is produced, more CYP2E1 is inhibited. This observation may serve as an explanation for the low $V_{max}$ of CYP2E1.


