KOR. J. ENVIRON. TOXICOL.
Vol. 15, No. 4, 139~145 (2000)

Substrate Specificity of the Human Flavin-containing Monooxygenase for Organic Selenium Compounds

Young Mi Kim

Department of Pharmacy, Duksung Women's University, Seoul, 132-714, Korea

사람 Flavin-containing monooxygenase의 셀레니움화합물에 대한 기질 특이성에 관한 연구

김 영 미

덕성여자대학교 약학대학 약학과

요 약

FMO (Flavin-containing Monooxygenase, EC1.14.13.8)는 다양한 종류의 식품, 약물이나 기타 외래 유래물질 (xenobiotics)을 산화시키는 NADPH와 O_2 의존성 약물대사 효소이다. 현재까지 5종의 subfamility가 존재하는 것으로 보고되어지고 있으며 그 중 가장 잘 알려진 FMO3는 대표적인 subfamiliy로서 주로 간에 존재한다. 사람 FMO에 관한 연구는 최근들어 활성화되기 시작했으며 질소, 황이나 인 등을 포함하는 친핵성 (*nucleophilic*) 화합물이 대표적인 기질로 보고되어 있다. 본 연구에서는 항 산화작용이 있는 것으로 알려진 selenium을 포함하고 있는 화합물에 대한 사람의 FMO3의 기질특이성을 알아보고자 하였다. 사람 FMO3를 baculovirus system을 이용하여 발현시킨 후 그 microsomal FMO3을 이용하여 thiocholine assay를 시행하였다. 그 결과 기질의 크기에 따라 활성의 차이가 있었으며 크기가 작은 selenium 화합물은 기존의 질소나 인 등을 포함하는 기질보다 더 낮은 K_m값을 보였다.

Key words : flavin containing monooxygenase, selenium compounds, baculovirus

INTRODUCTION

The microsomal flavin-containing monooxygenase (FMOs; EC 1.14.13.8) catalyze the flavin adenine dinucleotide (FAD)-, NADPH-, and O_2-dependent oxidation of numerous xenobiotics containing nitrogen, sulfur, phosphorous, or selenium heteroatoms (Scheme 1). Although primarily involved in the detoxication process, activation of compounds to a more reactive chemical species may occur and ultimately elicit a toxic response. In contrast with the numerous exogenous compouds identified as substrates for the FMOs, relatively few endogenous substrates are known; examples of these include cyteamine and the cysteine S-conjugates. The dietary compound trimethylamine is converted to its corresponding N-oxide by FMO; however, individuals deficient in FMO (presumably FMO3) may develop trimethylaminuria, a genetic disorder resulting in the excretion of the malodorous free amine (Poulsen LL *et al.*, 1995; Lang DH *et al.*, 1998).

To date, five FMO isoforms (designed FMO1-5) have been identified by amino acid or cDNA sequencing, each represented by a single gene. Orthologous

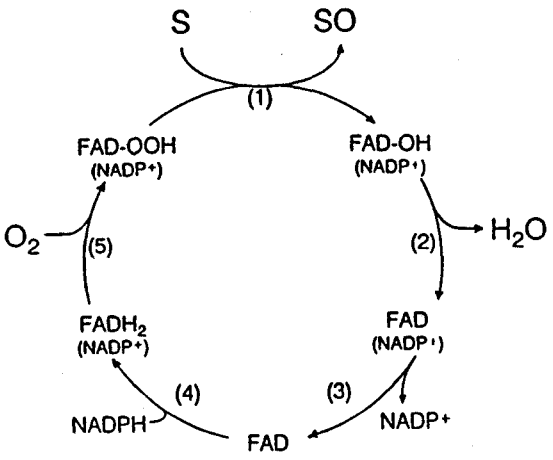
genes share at least 80% amino acid identity, whereas homologous FMOs are 52~57% identical. Highly related forms (greater than 98% identity) within a singly species are the result of allelic variation (Dolphin CT *et al.*, 1996).

In addition to the well-documented species and tissue-dependent expression of FMOs, endogenous factors such as developmental status or gender affect expression. Based on mRNA expression, the primary isoform expressed in adult human liver appears to be FMO3; however, FMO1 appears to be the predominant form expressed in human fetal liver. These FMOs are mostly found major organs of entry, such as liver and kidney, and have been purified from pigs, rats, mice, rabbits, and guinea pigs (Lemoine A *et al.*, 1990).

The quantitative description of structural elements controlling substrate specificities of enzymes catalyzing metabolism of xenobiotics is a major objective of predictive toxicology and drug metabolism. Although the metabolism of virtually all xenobiotics is catalyzed by more than one enzyme or at least by multiple isoforms whose expression can vary with species, tissue, and development, such a comprehensive analysis is extremely difficult.

The studies of John *et al.* demonstrated that rat liver microsomes catalyzed the oxidation of the methylselenide metabolite of the antioxidant. The reaction, which required NADPH and oxygen, was apparently catalyzed by a monooxygenase but the nature of the microsomal catalyst (s) was not explored in detal. A subsequent report (Chen GP. *et al.*, 1994) described the enzymic oxidation of this selenide to the selenoxide by purified pig liver microsome FMO.

In the present study, the baculovirus expression vector system was used to overexpress human FMO3 in insect cells for catalytic studies. The studies described in this paper were undertaken to define more precisely the contribution of human microsomal monooxygenases to oxidation of structurally different selenides.



Scheme 1. Major steps in the catalytic cycle of the porcine liver flavin-containing monooxygenase. In step (1), any soft nucleophile S that can contact the enzyme-bound 4a-hydroperoxyflavin is oxygenated to SO which is released immediately. After release of H_2O and $NADP^+$ in steps (2) and (3), the oxygenating form of the enzyme is regenerated by steps (4) and (5). FAD, flavin adenine dinucleotide.

MATERIALS AND METHODS

Chemicals

N-Benzylimidazole, acetylcholine chloride, and all the selenides (Scheme 2) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Methimazole, NADP+, glucose-6-phosphate, L-mesenteroides glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Co. (St. Louis, MO). All other reagents were of the highest grades available commercially.

Synthesis

Thiocholine chloride was prepared by the methanolysis of acetylthiocholine chloride as follows. 200 mg of acetylcholine dissolved in 2 ml of dry, oxygen -free methanol saturated with HCl was incubated at room temperature overnight. The reaction was usually complete after only 5 h but prolonged incubation had no detectable effect on yield of thiocholine.

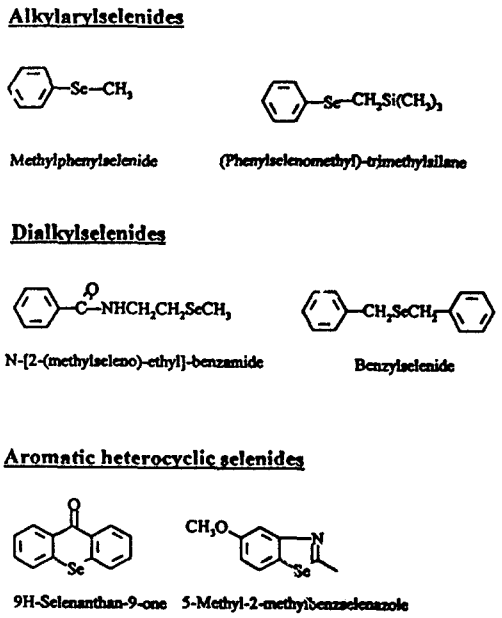
After incubation, methanol, methylacetate, and excess HCl were removed under vacuum or by purging with a stream of dry argon. Aqueous 10 mM solutions of thiocholine hydrochloride are stable for several months at 0~4°C.

Construction of expression and recombinant of Baculovirus-mediated FMO3

FMO3 recombinant baculovirus was constructed by minor modifications of the procedure described by Dolphin *et al.* (1998). The insert was excised by incubation with XbaI and HindIII, then gel-purified and ligated into pFastBac1 (GibcoBRL, Rockville MD) to give FMO3/pFastBac1. FMO3 recombinant baculovirus was generated by transfection of *Spodoptera frugiperda* (Sf)9 cells with the corresponding recombinant bacmid DNA, obtained via site-specific transposition using the Bac-to-Bac Baculovirus Expression System (GibcoBRL, Rockville, MD). For expression, 500 ml of Sf9 cells, grown to a density of 1×10^6 cells/ml in a 2 liter spinner flask, were infected with virus at a multiplicity of infection of 7 and incubated by stirring at 100 rpm in a stirring platform at 27°C for 72 hours. Cells were pelleted, resuspended in 50 ml of lysis buffer, consisting of 50 mM Tris-HCl (pH 7.4), 0.154 M KCl, 0.2 mM phenylmethylsulfonyl fluoride. The cells were broken by two passes through a pre-cooled French press cell disrupter at 800 psi. The lysed cells were centrifuged at 3,000 g for 15 min at 4°C. The supernatant was saved and microsomal fraction was obtained by centrifugation of the resulting supernatant at 100,000 g for 1 hour at 4°C. Microsomal pellets were resuspended in 10 ml of 10 mM HEPES (pH 7.5) buffer containing 0.154 M KCl, 1 mM EDTA, and 20% (v/v) glycerol. The preparation was stored in aliquots at −80°C until use. Protein concentration was determined by the method of Bradford with Bio-Rad (Hercules, CA) reagents using bovine serum albumin as a standard.

Western blot analysis

Proteins were separated by SDS-PAGE on 12.5% gels and transferred eletrophoretically to Immobilon -P membrane (Millipore). Blots were probed with a 1 : 4,000 dilution of rabbit anti FMO IgG followed by detection with a 1 : 4,000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase as described by the supplier (Bio-Rad).



Scheme 2. Structures of selenides used in this work.

Activity measurements

The oxidation of selenides catalyzed by microsomes was determined by following substrate-dependent thiocholine oxidation as described (Guo *et al.*, 1992). The reaction were carried out in 10 ml Erlenmeyer flasks in a metabolic shaker at 37°C in 0.1 M phosphate (pH 7.4), 0.25 mM $NADP^+$, 2.5 mM glucose-6-phosphate, 1.5 U glucose-6-phosphate deghydrogenase, 2 mM N-benzylimidazole and 150 μM thiocholine. After 3~4 min temperature equilibration, the microsomes were added and 1min later the reaction was initiated by adding the organoselemium compound. Aliquots (0.4 ml), routinely withdrawn at 0, 3, 6, and 9 min were transferred to tubes containing 0.04 ml 3.0 M trichloroacetic acid. The concentration of thiocholine in the protein free

supernatant fractions was determined as described earlier (Guo *et al.*, 1992).

RESULTS AND DISCUSSION

Since 1983, when baculovirus expression vector system technology was introduced, the baculovirus system has become one of the most versatile and powerful eukaryotic vector systems for recombinant protein expression. Baculovirus offer the following advantages over other expression vector systems. Safety, Ease of scale up, high levels of recombinant gene expression, accuracy and use of cell lines ideal for suspension culture (Haninig *et al.*, 1997).

To determine if the human FMO3 being expressed in the baculovirus vector system were full length, extracts were prepared from the insect cells carrying FMO3/Bacmid. This extract were analyzed by SDS-PAGE and western blotting with antisera prepared against FMO. The FMO3 could be seen on the stained gel (Fig. 1A), and the western blot showed band (~60 kDa) indicating that the human FMO3 was being expressed in the baculovirus system (Fig. 1B).

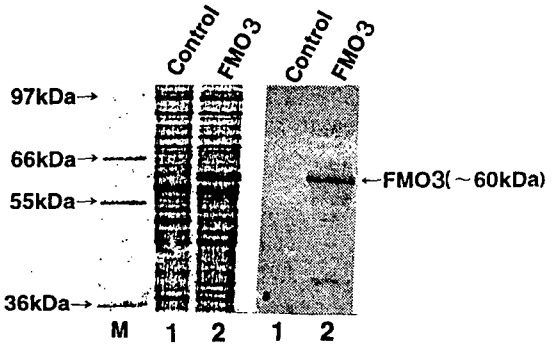


Fig. 1. Expression of Human FMO3 in the Baculovirus system.
A, samples (20 μg) of the microsomes from culture of Sf9 cells were electrophoresed on a polyacrylamide gel in the presence of SDS and stained with Coomassie Blue. B, samples (20 μg) of the microsomes from culture of Sf9 cells were electrophoresed on a polyacrylamide gel in the presence of SDS, transferred to Immobilon-P membrane, and visualized by immunostaining, as described under "Materials and Methods." The samples shown are Sf9 cells alone (*lane1*), FMO3 (*lane2*).

Initial studies carried out with microsomes isolated from Sf 9 cells infected FMO3 baculovirus indicated that this preparation catalyzed substrate-dependent oxidation of thiocholine. In the absence of substrate there was no detectable loss of thiocholine for up to 12 minutes incubation with the complete reaction medium. Microsomes isolated from control Sf 9 cells were also completely devoid of selenides oxidase activity.

All of the compounds in Scheme 1. are structures of selenides used in this work. While the K_m for methylphenylselenide, benzylselenide, and N-[2-(methylseleno)ethyl]benzamide are virtually the same (0.2~2 μM), the larger 9H-Selenanthan-9-one and 5-Methyl-2-methylbenzselenazole are virtually excluded from the enzyme-bound 4α-hydoperoxyflavin in human FMO3. Kinetic constants, calculate from rate of substrate dependent thiocholine oxidation catalyzed by microsomes, indicate that the dialkyl-, alkylaryl-selenides were oxidized at the almost same velocities (Table 1, Figs. 2-5). Aromatic heterocyclic selenides are apparently excluded

Table 1. K_m and V_{max} values for the oxidation of selenium compounds catalyzed by human FMO3 expressed in insect cells

Substrate	K_m	V_{max}
	μM	nmol substrate oxidized/min/mg
Methylphenylselenide	0.2	10.84±0.88
Benzylselenide	1.79	9.67±0.50
N-[2-(methylseleno)ethyl] benzamide	0.61	5.27±0.27
(Phenylselenomethyl) trimethylsilane	8.34	8.56±0.59
9H-Selenanthan-9-one		ND
5-Methyl-2-methylbenzselenazole		ND

Activities were measured by following substrate-dependent thiocholine oxidation as described in the text. Also see Guo *et al.* (1992). Values are means±S.E. of triplicate measurements for each preparation. Kinetic constants were obtained from reciprocal plots of velocity of substrate-dependent thiocholine oxidation versus substrate concentration above and below the concentration required to give half maximal velocity.
ND, Activity not detected at the highest concentrations soluble in the reaction medium.

from the catalytic site by bulky groups adjacent to the selenium atom. On the other hand, the side chain dialkyl-, alkylaryl- group of selenides are accepted by human FMO3 as are those of the structurally similar phenylthiourea or 1,3-diphenylthiourea. Theses data that pig FMO3 would be a good model for human FMO3. The size limits for substrates accepted by human FMO3 appear more similar to those observed for pig FMO3 (Chen *et al.* 1994).

In addition, thiocarbamides were examined for substrate activity with FMO3 (data not shown). Although, the K_m values for methimazole, thiourea, and phenylthioureaare virtually same (2~5 μM), the larger 1, 3 diphenylthiourea was not detected in human FMO3. Nucleophiles not much bigger than methimazole are readily accepted by FMO3. This leaves unanswered the question whether this form, like pig FMO3, can catalyze the oxidation of nucleophiles bearing even more bulky substituents. These data suggest that human FMO3, which accepts selenides much larger than methimazole.

The observation that the K_m and V_{max} for the selenides that can access the enzyme-bound oxidant of human FMO3 are essential the same suggest that tight binding enzyme-substrate complexes are not required for catalysis, and size restrictions measured

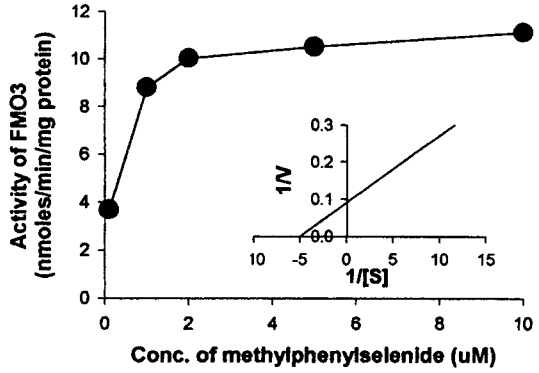


Fig. 2. The oxidation activity of methylphenylselenide catalyzed by human FMO3 expressed in the baculovirus system.

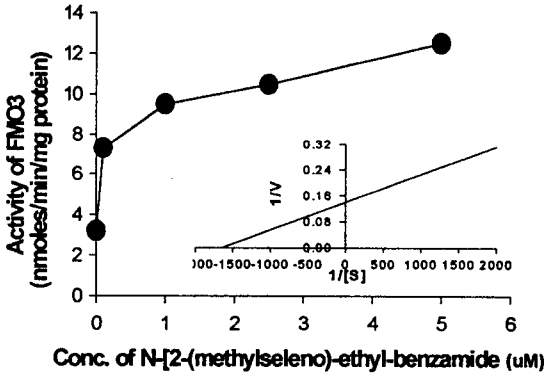


Fig. 4. The oxidation activity of N-[2-(methylseleno) ethyl]benzamide catalyzed by human FMO3 expressed in the baculovirus system.

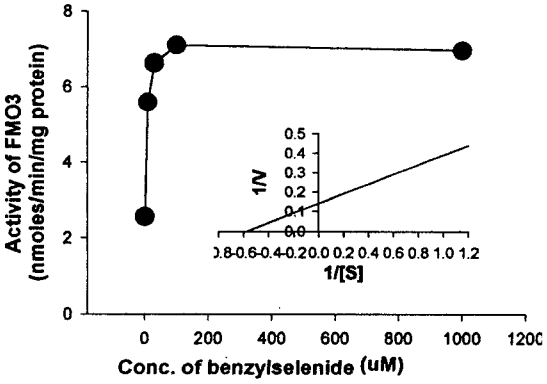


Fig. 3. The oxidation activity of benzylselenide catalyzed by human FMO3 expressed in the baculovirus system.

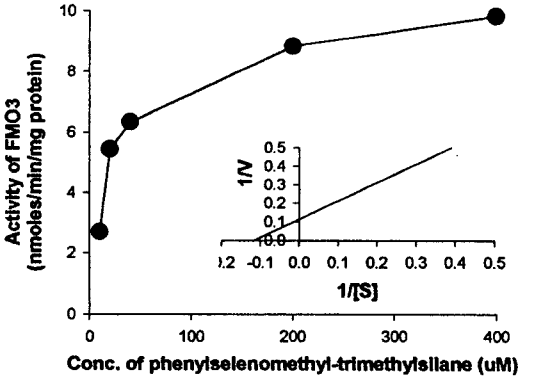


Fig. 5. The oxidation activity of (phenylselenomethyl) trimethylsilane catalyzed by human FMO3 expressed in the baculovirus system.

with selenides can be used to predict activity of other xenobiotic nucleophiles.

Despite these obvious limitations the studies reported in this paper indicate that size limitations may serve as an initial guide for predicting substrate activity in humans of a specific xenobiotic nucleophile bearing bulky substituents. The data also show that human FMO3 catalyzes the oxidation of xenobiotics considerably similar to those accepted by pig FMO3.

CONCLUSION

Six commercially available organic selenium compounds were examined for substrate activity with microsomes isolated from (Sf)9 cells infected with human FMO3 recombinant baculovirus. While none of the aromatic heterocyclic selenides tested showed detectable activity, all dialkyl- and alkylaryl-selenides free from ionic groups catalyzed the NADPH- and O_2-dependent oxidation. Kinetic constants demonstrate that (based on K_m) dialkyl-and alkylaryl-selenides are better substrates for human FMO3 than analogous nitrogen or sulfur compounds.

ABSTRACT

The flavin-containing monooxygenases (FMOs) (EC1.14.13.8) are NADPH-dependent flavoenzymes that catalyze oxidation of soft nucleophilic heteroatom centers in a range of structurally diverse compounds, including foods, drugs, pesticides, and other xenobiotics. In humans, FMO3 is quantitatively a major human liver monooxygenase. In the present study, the baculovirus expression vector system was used to overexpress human FMO3 in insect cells for catalytic studies. Six commercially available organic selenium compounds were examined for substrate activity with microsomes isolated from *Spodoptera frugiperda* (Sf)9 cells infected with human FMO3 recombinant baculovirus. While none of the aromatic heterocyclic selenides tested showed detectable activity, all dialkyl- and alkylaryl-selenides free from ionic groups catalyzed the NADPH- and O_2-dependent oxidation. Kinetic constants demonstrate that (based on K_m) dialkyl-and alkylaryl-selenides are better substrates for human FMO3 than analogous nitrogen or sulfur compounds.

ACKNOWLEDGEMENTS

This work was supported by a grant from Duksung Women's University in 1999.

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