Journal of Biochemistry and Molecular Biology, Vol. 40, No. 6, November 2007, pp. 1021-1027

JOURNAL OF Biochemistry and Molecular Biology

Construction and Expression of Mutant cDNAs Responsible for Genetic Polymorphism in Aldehyde Oxidase in Donryu Strain Rats

Mayuko Adachi[1], Kunio Itoh[1], Akiko Masubuchi[1], Nobuaki Watanabe[2] and Yorihisa Tanaka[1,*]

[1]Department of Drug Metabolism and Pharmacokinetics, Tohoku Pharmaceutical University, Sendai, Japan

[2]Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi-Sankyo Co. Ltd., Tokyo, Japan

Received 18 May 2007, Accepted 22 August 2007

We demonstrated the genetic polymorphism of aldehyde oxidase (AO) in Donryu strain rats: the ultrarapid metabolizer (UM) with nucleotide mutation of (377G, 2604C) coding for amino acid substitution of (110Gly, 852Val), extensive metabolizer (EM) with (377G/A, 2604C/T) coding for (110Gly/Ser, 852Val/Ala), and poor metabolizer (PM) with (377A, 2604T) coding for (110Ser, 852Ala), respectively. The results suggested that 377G > A and/or 2604C > T should be responsible for the genetic polymorphism. In this study, we constructed an *E. coli* expression system of four types of AO cDNA including Mut-1 with (377G, 2604T) and Mut-2 with (377A, 2604C) as well as naturally existing nucleotide sequences of UM and PM in order to clarify which one is responsible for the polymorphism. Mut-1 and Mut-2 showed almost the same high and low activity as that of the UM and PM groups, respectively. Thus, the expression study of mutant AO cDNA directly revealed that the nucleotide substitution of 377G > A, but not that of 2604C > T, will play a critical role in the genetic polymorphism of AO in Donryu strain rats. The reason amino acid substitution will cause genetic polymorphism in AO activity was discussed.

Keywords: Aldehyde oxidase, Donryu rat, Expression, Mutant, Single nucleotide polymorphism (SNP)

Introduction

Aldehyde oxidase (AO, EC 1.2.3.1) is a major member of the molybdenum hydroxylase family along with xanthine oxidase. Both enzymes consist of a homodimer with a subunit molecular mass of about 150 kDa. Each subunit contains a molybdopterin cofactor, a FAD, and two different 2Fe-2S redox centers that are essential for catalytic activity. AO catalyzes the oxidation of a wide range of endogenous and exogenous aldehydes and *N*-heterocyclic aromatic compounds. The representative *N*-heterocyclic drugs that serve as substrates for AO are methotrexate, 6-mercaptopurine, cinchona alkaloids, and famciclovir (Beedham, 1985, 1987, 1997, 2002; Kitamura *et al*., 2006). In addition, AO can catalyze *in vitro* reduction of a variety of functional groups including sulfoxides, *N*-oxides, azo dyes, and *N*-hydroxycarbamoyl substituents in the presence of an appropriate electron donor (Kitamura *et al*., 2006). In fact, the atypical antipsychotic drug, ziprasidone, is mostly metabolized to its reductive ring-cleaved *S*-methyldihydroziprasidone by AO in human (Prakash *et al*., 1997; Beedham *et al*., 2003). Marked species differences have been well documented for the AO-catalyzed metabolism of drugs including methotrexate (Kitamura *et al*., 1999a; Jordan *et al*., 1999) and famciclovir (Rashidi *et al*., 1997). A large variation in rat strains has also been demonstrated in the oxidation activity of benzaldehyde (Sugihara *et al*., 1995) and methotrexate (Kitamura *et al*., 1999b). In addition, an interesting individual difference has been reported in Wistar rats (Gluekson-Waelsch *et al*., 1967) and in SD rats (Beedham, 1998).

RS-8359, (±)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7*H*-cyclopenta[*d*]-pyrimidine], is a reversible and selective MAO-A inhibitor (Yokoyama *et al*., 1989; Kumagae *et al*., 1991; Miura *et al*., 1993; Iwata *et al*., 1996), and has been developed as an antidepressant (Puchler *et al*., 1997; Plenker *et al*., 1997). One of the major metabolic pathways of RS-8359 is 2-oxidation on the pyrimidine ring to give the 2-keto metabolite. Cytosolic AO was identified as a responsible enzyme for the stereospecific formation of 2-keto metabolite from the (*S*)-enantiomer. Similar to the already reported findings regarding AO, we observed remarkable species differences, strain differences in rats, and individual differences in Donryu strain rats in the AO-catalyzed metabolism of RS-8359 (Takasaki *et al*., 2005; Itoh *et al*., 2005, 2006, 2007). Monkeys and humans have an extremely high AO activity that results in a substantially

*To whom correspondence should be addressed.
Tel: 81-22-727-0108; Fax: 81-22-275-2013
E-mail: ytanaka@tohoku-pharm.ac.jp

non-detectable plasma concentration of the (*S*)-enantiomer accompanied by a high rate of excretion of the 2-keto metabolite in urine. There was a 230-fold variation of V_{max} values between the highest activity in the Wistar-Imamichi strain rat and the lowest activity in the Slc-Wistar strain rat (Sasaki *et al.*, 2006). The AO-catalyzed 2-oxidation activity of (*S*)-RS-8359 in Donryu strain rats was divided into three groups: ultrarapid metabolizer (UM), extensive metabolizer (EM), and poor metabolizer (PM). Nucleotide sequence analysis revealed that there were only two nucleotide mutations among them, namely UM with (377G, 2604C), EM with (377G/A, 2604C/T), and PM with (377A, 2604T) (Itoh *et al.*, 2007).

In the current study, we constructed an *E. coli* expression system of two mutant AO cDNAs of Donryu strain rat, Mut-1 with (377G, 2604T) and Mut-2 with (377A, 2604C), and characterized their enzyme activity to directly reveal which of the mutations---377G > A or 2604C > T---is more critical in maintaining catalytic activity.

Materials and Methods

Chemicals and reagents. The (*S*)-enantiomer of RS-8359 and its 2-keto metabolite were supplied by Ube Kosan Co. Ltd. Hydrocortisone, an internal standard of HPLC analysis, was purchased from Sigma Chemical Co. Imidazole was obtained from Wako Pure Chemical Industries, Ltd. All other reagents were of reagent grade.

Animals. Seven-week-old Crj:Donryu strain rats were purchased from Charles River Japan. The animals were housed according to the Guidelines for Animal Experimentation (Tohoku Pharmaceutical University) in cages in rooms with a unidirectional airflow at a controlled temperature (22 ± 2°C), relative humidity (50 ± 10%), and 12-h light/dark cycles (07.00-19.00 h). Tap water was available *ad libitum* and CE2 food (Clea Japan) was available *ad libitum* except for overnight fasting before use.

Enzyme activity assay. (*S*)-RS-8359 (100 μM) was incubated at 37°C for 60 min in a reaction mixture (0.25 ml) consisting of 100 mM phosphate buffer (pH 6.0), 1.0 mM $K_3Fe(CN)_6$, 0.13 mM EDTA, and the expressed protein (5~20 μg/ml, 0.10 ml). The reaction was stopped by the addition of acetonitrile (0.50 ml) containing 0.2 mg/ml of hydrocortisone as an internal standard. The mixture was then centrifuged at 10,000 *g* for 5 min. Aliquots (25 μl) of the supernatant were analyzed for quantification of the oxidation product by reversed-phase HPLC on a YMC ODS A-312 column (6.0 mm i.d. × 150 mm, YMC Co. Ltd.). The mobile phase was composed of acetonitrile/0.5% ammonium acetate (25 : 75); the flow rate was 1.0 ml/min. The HPLC instrument was a Shimadzu model 6A High Performance Liquid Chromatograph System (Shimadzu Seisakusho Co. Ltd.). The peaks were monitored for absorbance at 315 nm; the peak area was calculated on a Chromatopac C-R4A (Shimadzu Seisakusho).

Preparation of anti-rat liver AO antisera. Rabbit anti-rat liver AO antisera used in this study were previously prepared by Sasaki *et al.* (2006). Briefly, the purified AO (1.0 mg protein/ml) from the liver cytosol of Wistar rats was emulsified with an equal volume of Freund's complete adjuvant (Sigma). Each of two rabbits was immunized with 1 ml of immunogen by intradermal injection every 4 weeks. Four months after the first immunization, blood was taken by cardiac puncture and antisera were prepared by a conventional method. Pooled antisera were stored at −80°C until used for Western blot analysis.

Direct sequence analysis of genomic DNA. Genomic DNA was isolated from liver samples with a DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's instructions. Primer sets were designed in reference to the nucleotide sequences of rat (GenBank accession number NW 047816) on introns as follows: 377F:5'-gaggtcttctctccaaattcagtg-3', 377R:5'-ggcatatggtaggtgtccaatt atta-3', 2604F:5'-cttcccaatatcactaaaccagcta-3', 2604R:5'-gcagtgagcc ctttattagatgtta-3'. The PCR reaction was conducted in a 50-μl solution containing 1 × Ex-Taq buffer, 0.2 mM dNTP mix solution, 2.5U Ex-Taq DNA polymerase (TaKaRa Bio Inc.), 2 mM $MgCl_2$, 1 μM each of forward and reverse primer, and 2 μl of the template. PCR amplification was conducted using a PCR thermal cycler MP (TaKaRa) as follows: initial denaturation at 94°C for 5 min, and 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. The PCR products obtained were purified using the Wizard SV Gel and PCR Clean-Up System (Promega Co.). The DNA sequences were determined using a CEQ 8000 Genetic Analysis System (Beckman Coulter Inc.) and a DTCS Quick Start Kit, for which the above-mentioned forward or reverse primer was used as the direct sequence primer.

Construction of expression plasmids for UM, PM, and mutant AO of Donryu strain rats The cDNAs of UM and PM of AO in Donryu strain rats have previously been subcloned in XL-TOPO vector using the TOPO TA Cloning Kit (Invitrogen) in this laboratory (Itoh *et al.*, 2006). The preserved XL-TOPO plasmids of AO were now used to construct the expression system for the two types of polymorphic AO, UM and PM, as follows. The XL-TOPO plasmids (20 μg) were first digested with *Ngo*MIV (New England Biolabs) followed by double digestion with *Acc*65I and *Sal*I (New England Biolabs). pQE-30 Xa vector (20 μg, QIAGEN) was also double digested with the same restriction enzymes, and then treated with Bacterial Alkaline Phosphatase (TaKaRa). The AO cDNA and cleaved pQE-30 Xa vector were purified with Wizard SV Gel and PCR Purification Kit and TE buffer (Promega). The cleaved pQE-30 Xa was ligated with AO cDNA of UM or PM using a TaKaRa DNA Ligation Kit Ver. 2 (TaKaRa) according to the manufacturer's protocol. The resulting plasmids were transfected into *E. coli* XL1-Blue MRF' (TaKaRa), and the insertion of AO cDNA of UM or PM into the plasmids was checked by agarose gel electrophoresis after digestion with *Sac*I for 2 h at 37°C.

The construction strategies for the mutant plasmids of AO in Donryu strain rats are shown in Fig. 1. Both plasmids containing AO cDNA of UM and PM were treated separately with *Hpa*I and then with *Acc*65I for 18 h at 37°C. Small cDNA fragments containing nucleotide mutation at 377 and a large one containing that at 2604 were purified by agarose gel electrophoresis. Ligation was conducted in the same manner as described above in such a way that mutant plasmid Mut-1 had the nucleotide sequences of

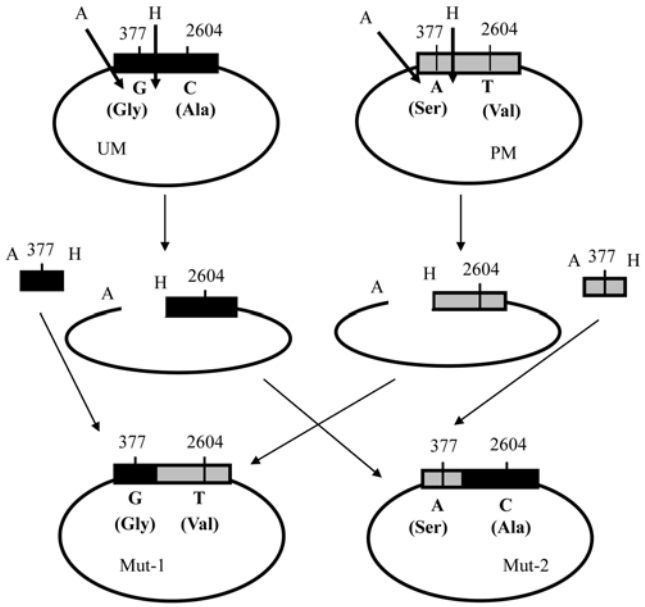

Fig. 1. Construction strategies for mutant plasmids of AO in Donryu strain rats. The chimeric cDNAs, Mut-1 and Mut-2, were constructed by restriction enzyme treatment and ligation in such a way that they had the substituted nucleotide sequences of (377G, 2604T), and (377A, 2604C), respectively. The restriction sites were shown as A for *Acc*65I and H for *Hpa*I, respectively.

(377G, 2604T) and Mut-2 had those of (377A, 2604C). The transfection into *E. coli* XL1-Blue MRF' and the insertion check of each plasmid were performed as described above.

Cell cultures. The four kinds of AO cDNA plasmids purified using Wizard Plus Minipreps DNA Purification Systems were transfected into the expression *E. coli* M15 cells (pREP4, QIAGEN). The control sample was produced by the transfection of the pQE-30 Xa vector. A single colony was cultured at 37°C overnight in 5 ml of LB medium containing 100 μg/ml ampicillin and 25 μg/ml kanamycin. To the overnight culture, 500 ml of LB with 100 μg/ml ampicillin and 25 μg/ml kanamycin were added and cultured at 37°C until absorbance at 600 nm reached 0.5~0.6. Then, IPTG (1 mM) was added to the mixture along with ATP (1 mM), riboflavin (3 μM), and Na_2MoO_4 (50 μM) according to the methods already reported (Huang *et al.*, 1999). Growth of the culture was allowed to continue for 72 h at 22°C. After collection by centrifugation at 7,000 *g* for 10 min at 4°C, the cells were solubilized with QIAexpressionist (QIAGEN) according to the manufacturer's instructions. The soluble proteins were applied to a HisTrap HP Column (GE Healthcare Ltd.) fully pre-equilibrated with 10 mM phosphate buffer (pH 7.4) containing 20 mM imidazole and 0.5 M NaCl (Buffer A). After being washed with 100 mM imidazole in Buffer A, the target enzyme was eluted with 250 mM imidazole in Buffer A.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The expressed enzymes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which was performed using PhastGel gradient 4-15 in PhastGel SDS Buffer Strips (GE Healthcare). An automatic electrophoresis system, PhastSystem (GE Healthcare), was used. Protein bands were stained with Coomassie Brilliant Blue CBB R-350 (GE Healthcare). An HMW Calibration Kit and HMV Native Marker Kit (GE Healthcare) were used for molecular mass standards.

Statistical analysis. The results are expressed as the mean ± SE for the number of experiments. Statistical significance between low and high activity groups was compared by a Student's *t*-test. Values with $p < 0.05$ were considered statistically significant.

Table 1. Analysis of genomic DNA and amino acid substitution of AO in UM, EM, and PM groups of Donryu strain rats

Position of nucleotide or amino acid	UM	EM	PM
Nucleotide 377	G	G/A	A
Amino acid 110	Gly	Gly/Ser	Ser
Nucleotide 2604	C	C/T	T
Amino acid 852	Ala	Ala/Val	Val

The number of rats examined for respective ultrarapid metabolizer (UM), extensive metabolizer (EM), and poor metabolizer (PM) with regard to the AO-catalyzed 2-oxidation activity of (*S*)-RS-8359 was ten for each type. The nucleotide sequences at 377 and 2604 positions were completely consistent within a respective phenotype group. The nucleotide and amino acid was homozygous in the UM and PM rats, but heterozygous in the EM rats.

Results

Genomic DNA analysis. The genomic DNA of livers from the UM, EM and PM Donryu strain rats was analyzed by the direct sequence method to determine whether the nucleotide mutation observed in the cDNA is also present in the genomic DNA. The nucleotide sequences were (377G, 2604C) in the UM group, (377G/A, 2604C/T) in the EM group, and (377A, 2604T) in the PM group, thus being homozygous in the UM and PM groups, but heterozygous in the EM group (Table 1). The results of the genomic DNA were in complete accord with those of the cDNA.

Construction of expression system for naturally existing and mutant AO cDNA. AO cDNAs of UM and PM in Donryu strain rats were introduced into the expression vector pQE-30 Xa. The chimeric cDNA plasmids of Mut-1 having (377G, 2604T) and Mut-2 having (377A, 2604C) were constructed by cleavage of pQE-30 Xa with restriction enzymes *Acc*65I/*Hpa*I followed by cross-ligation of the fragments obtained. The introductions of the objective nucleotides were confirmed by sequence analysis, as shown in Fig. 2.

SDS-PAGE. Each of the constructed plasmids of the UM, PM, Mut-1, and Mut-2 groups was transfected into the expression *E. coli* M15 cells. The solubilized supernatant of the expressed enzymes was analyzed by SDS-PAGE. All of

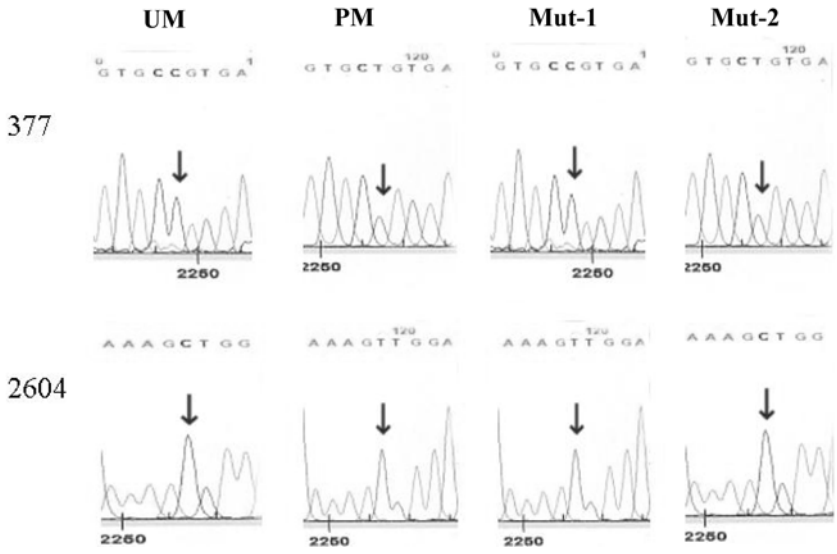

Fig. 2. Sequence analysis at positions 377 and 2604 of AO cDNA from naturally existing UM and PM, and from mutants Mut-1 and Mut-2. The construction strategies used in the current study were shown to result in point mutation at either position 377 or 2604 of UM and PM.

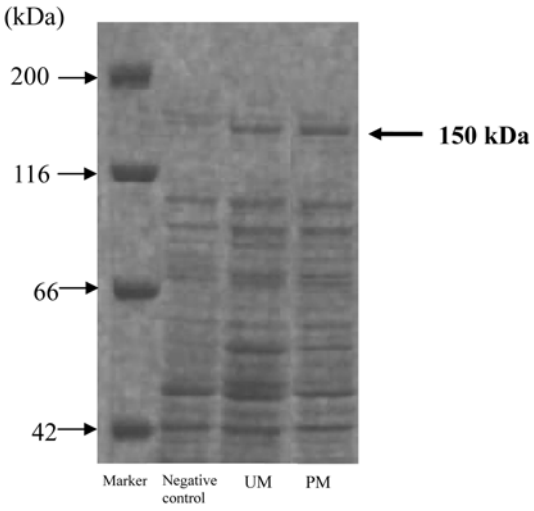

Fig. 3. SDS-PAGE of AO protein expressed in M15 cells. SDS-PAGE was performed using PhastGel gradient 4-15 in PhastGel SDS Buffer Strips; the proteins on the gel were stained with Coomassie Brilliant Blue.

them displayed the target protein at a high concentration that was not detected in the control supernatant sample (Fig. 3).

Analysis of kinetic parameters for expressed enzymes. The AO-catalyzed 2-oxidation activities of (*S*)-RS-8359 by the enzymes expressed in *E. coli* were measured. The kinetic parameters calculated from Hanes-Woolf plots (Fig. 4) are summarized in Table 2. A significant difference of several fold in K_m values was seen between UM and the other groups. The expressed AO enzymes of the UM and PM groups exhibited high and low activity, respectively. Thus, the genetic polymorphism of AO activities in Donryu strain rats observed in liver cytosol was also confirmed in the expression system. Mut-1 and Mut-2 showed almost the same high and low V_{max} values as that of the UM and PM groups, respectively.

Discussion

During the study of rat strain differences in the AO-catalyzed 2-oxidation of RS-8359 (25), which is an MAO-A inhibitor (Yokoyama *et al.*, 1989; Kumagae *et al.*, 1991; Miura *et al.*, 1993; Iwata *et al.*, 1996) and has been developed as an antidepressant (Puchler *et al.*, 1997; Plenker *et al.*, 1997), we were aware of the individual variations in the activity in Donryu strain rats. Analysis of the AO cDNA revealed that the variations were due to genetic polymorphism in that UM possessed the nucleotide mutation of (377G, 2604C) coding for amino acid substitution of (110Gly, 852Ala), EM with (377G/A, 2604C/T) coding for (110Gly/Ser, 852Ala/Val), and PM with (377A, 2604T) coding for (110Ser, 852Val) (Itoh *et al.*, 2007). The same nucleotide mutations were now confirmed to also be present in the genomic DNA. The results suggested that the nucleotide 377G or 2604T is primarily important for maintaining the catalytic activity of AO. The study using the expression system of mutant AO cDNA should be the best method of elucidating which nucleotide mutation plays the most important role in the polymorphism of AO in Donryu strain rats.

Although the expression of catalytically active molybdo-flavoenzymes in prokaryotes has been known to be generally difficult (Garattini *et al.*, 2003), Huang *et al.* (1999) has for the first time presented findings that mouse retinal oxidase (aldehyde oxidase) was expressed in an active form in a prokaryotic system, *E. coli* BL21, in which riboflavin, ATP, and Na_2MoO_4 were added. Based on these findings, we tried

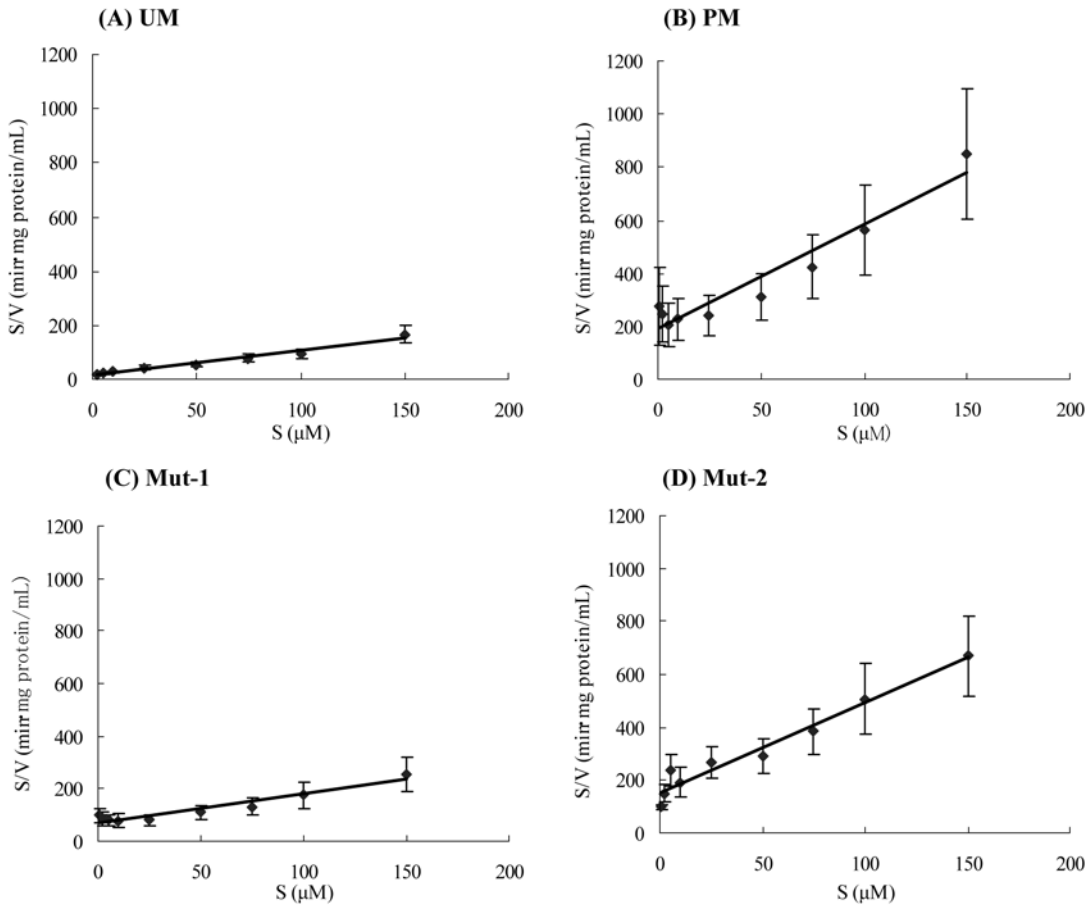

Fig. 4. Hanes-Woolf plots for the AO-catalyzed 2-oxidation of (*S*)-RS-8359 by naturally occurring and expressed AO proteins. A, UM; B, PM; C, Mut-1; D, Mut-2. The 2-oxidation of (*S*)-RS-8359 was determined in three experiments over a substrate concentration range of 1.0 to 150 μM.

to express rat and monkey AO protein in *E. coli* strain M15 that is different from the previously reported strain. As a result, the AO protein was clearly detected on SDS-PAGE/ Western blot analysis, and the expressed monkey AO exhibited a biphasic Eadie-Hofstee profile for the 2-oxidation of (*S*)-RS-8359, but the rat AO did not. The results were in good agreement with those obtained in the liver cytosol (Hoshino *et al.*, 2007). Recently, Yamaguchi *et al.* (2007) has also reported the expression of human xanthine oxidoreductase (XOR) and its mutants in *E. coli* strain JM109.

Table 2. Kinetic parameters for the 2-oxidation of (*S*)-RS-8359 by AO protein expressed in *E. coli* M15 cells

Expression type	K_m (μM)	V_{max} (nmol/min/mg protein)
UM (5)	16.0 ± 2.40	1.26 ± 0.26
Mut-1 (5)	62.4 ± 6.98*	1.20 ± 0.32
Mut-2 (4)	44.3 ± 5.34*	0.36 ± 0.09*
PM (4)	36.4 ± 4.54*	0.32 ± 0.10*

Results are expressed as the mean ± S.E. of four (Mut-2 and PM) or five (UM and Mut-1) determinations. The nucleotide sequence at 377 and 2604 are G and C in the UM group, G and T in the Mut-1 group, A and C in the Mut-2 group, and A and T in the PM group, respectively. *$p < 0.05$ compared with the UM group.

In this report, plasmids Mut-1 with (377G, 2604T) and Mut-2 with (377A, 2604C) were constructed and expressed in *E. coli* M15 as well as the naturally occurring UM with (377G, 2604C) and PM with (377A, 2604T). The expressed UM and PM enzymes showed high and low AO activity, respectively, thus the individual differences observed in liver cytosol were also reproduced in the expression system. Mut-1 and Mut-2 demonstrated almost the same V_{max} values as did those of UM and PM, respectively. The K_m value of UM was one-fourth of those of the other groups and was significantly smaller, although the reason is unclear. However, the difference did not exceed one order of magnitude, suggesting that it might not be large enough to warrant extended discussion. Although the number involved in the ratio in liver cytosolic extracts was only two samples (1 male and 1 female), the ratio between the V_{max} values for the UM as compared to the PM phenotypes was an order of magnitude greater than those found in the *in vitro* expressed proteins. Some *in vitro* and *in vivo* reasons are

imaginable. The ratio calculated from cytosol data (Itoh *et al.*, 2007) might be overestimated by using a crude enzyme that contains a large amount of contaminated proteins. In fact, significantly more AO was expressed in EM rat liver compared to the PM group. The UM group showed a tendency to have a much higher AO concentration than did the PM group (data not shown because the sample consisted of only two rats). If AO protein is purified from both the PM and UM rats and the activity is expressed per mg protein, the discrepancy might be reduced. Additionally, there might be some important posttranslational modification to affect the AO activity of the PM or UM rats *in vivo*. Furthermore, the employment of HisTag for easy and effective purification of the expressed protein might differently affect the expression of the PM and UM cDNA. Regardless of these and other possibilities, the actual reason is still unknown. However, the expression results were at least likely to reflect the cytosolic results qualitatively.

Taken together, the results indicated that the genetic polymorphism of AO activity in Donryu strain rats is possibly caused by the nucleotide mutation of 377G > A, but not by 2604C > T. The 377G > A nucleotide mutation results in the amino acid substitution from a symmetric Gly to an asymmetric Ser at position 110 near the second Fe-S cluster (Wright *et al.*, 1999). The amino acid substitution might provoke a serious conformational change or a posttranslational modification of AO protein to decrease the catalytic activity significantly. It might be helpful to study the physicochemical properties of the proteins expressed by the PM and Mut-2 cDNAs to reveal the mechanistic reasons for the $Gly^{110}Ser$ amino acid substitution affecting the AO structure and its activity. The same genetic reason might apply for the rat strain differences in AO activity. That is one of the subjects we are now investigating to clarify the molecular mechanism of individual differences in AO activity in Donryu rats and strain differences in rats.

In addition to AOX1 and xanthine oxidase, the presence of aldehyde oxidase homolog 1 and 2 (AOH1 and AOH2) has been described as a member of the molybdenum hydroxylases (Terao *et al.*, 2000, 2001; Vila *et al.*, 2004; Kurosaki *et al.*, 2004). The homologs were first known only in mice, but recently were also found in rats. Furthermore, a novel molybdo-flavoenzyme, AOH3, has been identified in both mice and rats. Among the AO isoforms, AOH1 is expressed in liver and shows a similar substrate specificity as that of AOX1, but AOX1 is generally more efficient than AOH1. Although the properties of AOH1 have been well characterized in mice, those of rat AOH1 have not always been clarified in detail other than cDNA cloning. Thus, many properties are still unclear such as substrate specificity, inhibitor susceptibility, molecular mass, tissue distribution, regulation by sex hormone, strain differences, species differences, and so on. Under these conditions, we investigated polymorphic variation in the (*S*)-RS-8359 2-oxidation activity in Donryu strain rats from the viewpoint of nucleotide mutation of AO (Itoh *et al.*, 2007). It was found that AO phenotypes were clearly consistent with nucleotide mutations coding for amino acid substitutions of $Gly^{110}Ser$. However, the presence of AOH1 having similar properties as AOX1 in mice suggests the possibility of participation of AOH1 mutation in the genetic polymorphism seen in Donryu strain rats. This issue should be an interesting subject for future study.

In conclusion, the 377G > A nucleotide mutation was directly proved to be important for the polymorphism of AO in Donryu strain rats by using the expression systems of mutant AO cDNAs.

Acknowledgments We wish to express our thanks to Dr. T. Ikeda, Director of the Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi-Sankyo Co. Ltd., for his kind encouragement.

References

Beedham, C. (1985) Molybdenum hydroxylases as drug-metabolizing enzymes. *Drug Metab. Rev.* **16**, 119-156.

Beedham, C. (1987) Molybdenum hydroxylases: Biological distribution and substrate-inhibitor specificity. *Prog. Med. Chem.* **24**, 85-121.

Beedham, C. (1997) The role of non-P450 enzymes in drug oxidation. *Pharm. World Sci.* **19**, 255-263.

Beedham, C. (1998) Molybdenum hydroxylases; in *Metabolism of Xenobiotics*, Gorrod, J. W., Oeschlager, H., and Caldwell, J. (eds), pp. 51-58, Taylor and Francis, London and New York, USA.

Beedham, C. (2002) Molybdenum hydroxylase; in *Enzyme Systems that Metabolise Drug and Other Xenobiotics*, Ioannides, C. (ed.), pp. 147-187, John Wiley, Chichester, UK.

Beedham, C., Miceli, J. J. and Obach, S. (2003) Ziprasidone metabolism, aldehyde oxidase, and clinical implications. *J. Clin. Psychopharm.* **23**, 229-232.

Glueksohn-Waelsch, S., Greengard, P., Quinn, G. P. and Teicher, L. S. (1967) Genetic variations of an oxidase in mammals. *J. Biol. Chem.* **242**, 1271-1273.

Huang, D. Y., Furukawa, A. and Ichikawa, Y. (1999) Molecular cloning of retinal oxidase/aldehyde oxidase cDNA from rabbit and mouse liver and functional expression of recombinant mouse retinal oxidase cDNA in *Escherichia coli*. *Arch Biochem. Biophys.* **364**, 264-272.

Itoh, K., Yamamura, M., Muramatsu, S., Hoshino, K., Masubuchi, A., Sasaki, T. and Tanaka, Y. (2005) Stereospecific oxidation of (*S*)-enantiomer of RS-8359, a selective and reversible MAO-A inhibitor, by aldehyde oxidase. *Xenobiotica* **35**, 561-573.

Itoh, K., Yamamura, M., Takasaki, W., Sasaki, T., Masubuchi, A. and Tanaka, Y. (2006) Species differences in enantioselective 2-oxidation of RS-8359, a selective and reversible MAO-A inhibitor, and cinchona alkaloids by aldehyde oxidase. *Biopharm. Drug Dispos.* **27**, 1133-1139.

Itoh, K., Masubuchi, A., Sasaki, T., Adachi, M., Watanabe, N., Nagata, K., Yamazoe, Y., Hiratsuka, M., Mizugaki, M. and Tanaka, Y. (2007) Polymorphism of aldehyde oxidase in Donryu rats. *Drug Metab. Dispos.* **35**, 734-739.

Iwata, N., Tonohiro, T., Kozuka, M., Kumagae, Y., Takasaki, W. and Tanaka, Y. (1996) A novel selective and reversible MAO-A inhibitor, RS-8359: its pharmacological properties and

metabolism. *Int. Acad. Biomed. Drug Res.* **11**, 285-286.

Jordan, C. G. M., Rashidi, M. R., Laljee, H., Clarke, S. E., Brown, J. E. and Beedham, C. (1999) Aldehyde oxidase-catalyzed oxidation of methotrexate in the liver of guinea pig, rabbit and man. *J. Pharm. Pharmacol.* **51**, 411-418.

Kitamura, S., Sugihara, K., Nakatani, K., Ohta, S., O'Hara, T., Nimomiya, S., Green, C. E. and Tyson, C. A. (1999a) Variation of hepatic methotrexate 7-hydroxylase activity in animals and humans. *IUBMB Life.* **48**, 607-611.

Kitamura, S., Nakatani, K., Sugihara, K., and Ohta, S. (1999b) Strain differences of the ability to hydroxylate methotrexate in rats. *Com. Biochem. Phys. Part C* **122**, 331-336.

Kitamura, S., Sugihara, K. and Ohta, S. (2006) Drug-metabolizing ability of molybdenum hydroxylases. *Drug Metab. Pharmacokinet.* **21**, 83-98.

Kumagae, Y., Matsui, Y. and Iwata, N. (1991) Deamination of norepinephrine, dopamine, and serotonin by type A monoamine oxidase in discrete regions of the rat brain and inhibition by RS-8359. *Jpn. J. Pharmacol.* **55**, 121-128.

Miura, H., Naoi, M., Nakahara, D., Ohta, T. and Nagatsu, T. (1993) Changes in monoamine levels in mouse brain elicited by forced-swimming stress, and the protective effect of a new monoamine oxidase inhibitor, RS-8359. *J. Neural Transm.* **94**, 175-187.

Plenker, A., Puchler, K. and Volz, H. P. (1997) The effects of RS-8359 on cardiovascular function in healthy subjects and depressed patients. *Int. Clin. Psychopharm.* **12**, S25-S29.

Prakash, C., Kamel, A., Gummerus, J. and Wilner, K. (1997) Metabolism and excretion of a new antipsychotic drug, ziprasidone, in humans. *Drug Metab. Dispos.* **25**, 863-875.

Puchler, K., Schaffler, K. and Plenker, A. (1997) The comparative effects of single and multiple doses of RS-8359, moclobemide and placebo on psychomotor function in healthy subjects. *Int. Clin. Psychopharm.* **12**, 17-23.

Rashidi, M. R., Smith, J. A., Clarke, S. E. and Beedham, C. (1997) *In vitro* oxidation of famciclovir and 6-deoxypenciclovir by aldehyde oxidase from human, guinea pig, rabbit and rat liver. *Drug Metab. Dispos.* **25**, 805-813.

Sasaki, T., Masubuchi, A., Yamamura, M., Watanabe, N., Hiratsuka, M., Mizugaki, M., Itoh, K. and Tanaka, Y. (2006) Rat strain differences in stereospecific 2-oxidation of RS-8359, a reversible and selective MAO-A inhibitor, by aldehyde oxidase. *Biopharm. Drug Dispos.* **27**, 247-255.

Sugihara, K., Kitamura, S. and Tatsumi, K. (1995) Strain differences of liver aldehyde oxidase in rats. *Biochem. Mol. Biol. Int.* **37**, 861-869.

Takasaki, W., Yamamura, M., Nozaki, A., Nitanai, T., Sasahara, K., Itoh, K. and Tanaka, Y. (2005) Stereoselective pharmacokinetics of RS-8359, a selective and reversible MAO-A inhibitor, by species-dependent drug metabolizing enzymes. *Chirality* **17**, 135-141.

Wright, R. M., Clayton, D. A., Riley, M. G., McManaman, J. L. and Repine, J. E. (1999) cDNA cloning, sequencing, and characterization of male and female rat liver aldehyde oxidase (rAOX1). *J. Biol. Chem.* **274**, 3878-3886.

Yamaguchi, Y., Matsumura, T., Ichida, K., Okamoto, K. and Nishino, T. (2007) Human xanthine oxidase changes its substrate specificity to aldehyde oxidase type upon mutation of amino acid residues in the active site: role of active site residues in binding and activation of purine substrate. *J. Biochem.* **141**, 513-524.

Yokoyama, T., Karube, T. and Iwata, N. (1989) Comparative studies of the effects of RS-8359 and safrazine on monoamine oxidase *in vitro* and *in vivo* in mouse brain. *J. Pharm. Pharmacol.* **41**, 32-36.