Properties of 130 kDa Subunit of Monkey Aldehyde Oxidase

Tasuku ASAKAWA,^a Kunio ITOH,^a Mayuko ADACHI,^a Kouichi HOSHINO,^a Nobuaki WATANABE,^b and Yori-hisa TANAKA^{*,a}

^aDepartment of Drug Metabolism and Pharmacokinetics, Tohoku Pharmaceutical University; 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan; and ^bDrug Metabolism and Pharmacokinetics Research Laboratories, Daiichi-Sankyo Co., Ltd.; 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan.

Received September 17, 2007; accepted December 10, 2007; published online December 17, 2007

We previously demonstrated the existence of a minor 130 kDa subunit in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blot analysis of monkey liver cytosol and expressed monkey aldehyde oxidase (AO) in *Escherichia coli*. In contrast, the 130 kDa subunit was not observed in rat AO. In the current study, the properties of the 130 kDa subunit were investigated from the viewpoint of species differences in the presence of the subunit and AO activity. Monkey AO with His-tag at the N- and C-terminus were expressed, and were immunoanalyzed with anti-AO and anti-His-tag antisera. The results revealed that the minor 130 kDa subunit was produced by cleavage at the N-terminal side of the 150 kDa subunit. The cleavage point was shown to be located between 188Leu and 189Pro of 150 kDa AO subunit by the Edman degradation method. The two amino acids related to the cleavage are contained in the linkage between the 2Fe-2S and FAD domains in AO of human and monkey, but not in AO of rat and mouse. As a fact, the 130 kDa subunit was observed in AO of human and monkey, but not in AO of rat and mouse, suggesting the two amino acids might be one reason of a species difference in the formation of the 130 kDa subunit. However, the existence of the 130 kDa subunit is not associated with the species differences in AO activity, because the cleavage results in the loss of 2Fe-2S cluster domain essential for exertion of AO activity.

Key words aldehyde oxidase; subunit; *Escherichia coli* expression; monkey

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 molybdopterine cofactor, a flavin-adenine dinucleotide (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. Representative *N*-heterocyclic-containing drugs that serve as substrates for AO are famciclovir, methotrexate, 6-mercaptopurine, and cinchona alkaloids.^{1–6} The oxidation mode by cytosolic AO is a nucleophilic attack at an electron-deficient carbon, which is different from an electrophilic attack at an electron-rich carbon by microsomal cytochrome P450, indicating that AO has complementary substrate specificities with P450 in the metabolism of xenobiotics. In addition, AO can catalyze *in vitro* reduction of a variety of functional groups including sulfoxides, *N*-oxides, azo dyes, and *N*-hydroxycarbonyl substituents in the presence of an appropriate electron donor.⁶ In fact, the atypical antipsychotic drug, ziprasidone, is mostly metabolized to its reductive ring-cleaved *S*-methylidihydroziprasidone by AO in human.^{7,8}

One of characteristics of AO is a marked species difference. For example, Sugihara *et al.*⁹ investigated the enzyme activity in several animal species using a few model substrates with relatively simple chemical structures. Extremely high benzaldehyde oxidase activity was observed in monkey and low activity in guinea pig. 2-Hydroxypyrimidine was oxidized at the highest rate in rabbit and rat and at a low rate in mouse. Monkey showed the highest phthalazine oxidase activity among the animal species studied. *N*¹-methylnicotinamide oxidase activity was highest in rabbit and low in rat and mouse. Preliminary experiments demonstrated that

human AO shows similar substrate specificity to that of monkey. Further, many medicines have been reported to show significant species differences in AO-catalyzed metabolism. They include an ocular hypotensive agent, brimonidine,¹⁰ an antiviral drug, famciclovir and 6-deoxypenciclovir,¹¹ an anti-neoplastic drug, methotrexate,^{12,13} an ultra-short acting hypnotic, zaleplon,¹⁴ and an oral antitumor agent, zebularine.¹⁵ Thus, it is now well established that the variations of AO activity may be dependent on not only animal species, but also on the chemical structures of the substrate. Roughly speaking, AO activity is high in monkey and human, moderate to low in rat and mouse, and deficient in dog.

We have been studying the metabolism of RS-8359, [(±)-4-(4-cyanoanilino)-5,6-dihydro-7-hydroxy-7*H*-cyclopenta-[*d*]-pyrimidine], which is a reversible and selective monoamine oxidase A (MAO-A) inhibitor^{16,17} and has been developed as an anti-depressant.^{18,19} The main metabolic pathway of the compound is the AO-catalyzed 2-oxidation on the pyrimidine ring of the molecule. Similar to many reports on AO, we observed a remarkable species differences,^{20–22} a large strain difference in rat,²³ and an individual difference in Donryu strain rat²⁴ in the 2-oxidation activity. Further, we reported that a minor 130 kDa subunit in addition to a 150 kDa subunit was observed in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/Western blot analysis of monkey and human AO but not in rat AO.²⁵ This minor subunit was suggested to lack the N-terminus by nano-LC Q/TOF MS analysis of AO purified from monkey liver cytosol. Sugihara *et al.*⁹ also demonstrated the existence of 130 kDa subunit in monkey liver AO. The results might suggest the possible relationship between the much higher AO activity in human and monkey than in other animals and the existence of the 130 kDa subunit. One of the best ways to verify this hypothesis would be to know whether

* To whom correspondence should be addressed. e-mail: ytanaka@tohoku-pharm.ac.jp

AO protein including the 130 kDa subunit maintains the catalytic activity. In the current study, we determined the N-terminal amino acid sequences of the 130 kDa subunit, constructed an expression system of AO including the 130 kDa subunit, and then characterized the properties of the expressed AO protein.

MATERIALS AND METHODS

Chemicals and Reagents The (*S*)-enantiomer of RS-8359 and its 2-keto metabolite were supplied by Ube Kosan Co., Ltd. (Yamaguchi, Japan). Hydrocortisone, an internal standard of HPLC analysis, was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Imidazole, sodium molybdic(VI) acid (Na_2MoO_4), and isopropyl- β -D(-)-thiogalactopyranoside (IPTG) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were of reagent grade. Anti-His-tag antiserum was purchased from Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan).

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 $\text{K}_3\text{Fe}(\text{CN})_6$, 0.13 mM EDTA, and the expressed protein (5–20 $\mu\text{g}/\text{ml}$, 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 10000 *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. $\times$ 150 mm, YMC Co., Ltd., Kyoto, Japan). 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., Kyoto, Japan). The peaks were monitored for absorbance at 315 nm; the peak area was calculated on a Chromatopac C-R4A (Shimadzu Seisakusho).

Preparation of Rabbit Anti-monkey AO Antisera Rabbit anti-monkey AO antisera used in this study were previously prepared by Hoshino *et al.*²⁵⁾ Briefly, a necessary amount of monkey aldehyde oxidase was expressed in *E. coli* and purified by HisTrap HP column chromatography. The antibody was prepared by Shibayagi Co., Ltd. (Gunma, Japan). The purified enzyme (1.0 mg protein/ml) 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 injections every 4 weeks. Four months after the first immunization, blood was obtained by cardiac puncture and antisera were prepared by a conventional method. Pooled antisera were stored at –80 °C until use for Western blot analysis.

Construction of Expression System for Monkey AO with His-tag at N- and C-Terminus Monkey AO cDNA has already been subcloned and cryopreserved in pQE-30Xa vector (QIAGEN, Venlo, The Netherlands), which can add His-tag at the N-terminus.²⁵⁾ Another expression system of monkey AO was newly constructed by using pQE-60 vector (QIAGEN), which can add His-tag at the C-terminus. For that, monkey AO cDNA was divided into three fragments of 1.7 kbp and 0.5 kbp, which were synthesized by polymerase

chain reaction (PCR), and 2 kbp, which was prepared from the cryopreserved pQE-30Xa vector. The forward and reverse primers for the synthesis of the 1.7 kbp DNA fragment were ggacactaccatggacaggg with an *Nco*I restriction site and ccaattgggtcttcaggatg, respectively. The reaction was conducted in a 50- μl solution containing 1 $\times$ high fidelity PCR buffer, 200 μM deoxynucleoside triphosphate (dNTP) mix, 1.25 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, U.S.A.), 2 mM MgSO_4 , 0.5 μM of each primer, and 2 μl (0.2 ng) of the template, which was prepared from the cryopreserved pQE-30Xa. PCR amplifications were as follows: initial denaturation at 94 °C for 2 min, and 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 68 °C for 2 min, and a final extension at 68 °C for 3 min. The 0.5 kbp fragment was produced in almost the same manner by simply changing the forward and reverse primers to ggcccgaggttgaaatagactgc and gtttgcatcggatccgacgg with a *Bam*HI restriction site, respectively. The DNA sequences of the PCR products were determined by a CEQ 8000 Analysis System and a DTCS Quick Start Kit (Beckman-Coulter Inc., Fullerton, CA, U.S.A.) according to the method recommended by the manufacturer. Each 1.7 kbp and 0.5 kbp PCR product was treated with *Nco*I/*Pst*I and *Bam*HI/*Pst*I, respectively. All restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA, U.S.A.). The two monkey AO DNA fragments were ligated with a pQE-60 vector that was pretreated with *Nco*I/*Bam*HI. The plasmids were digested with *Pst*I, dephosphorylated, and then ligated with the 20 kbp DNA fragment that was prepared by *Pst*I-treatment of monkey AO full cDNA obtained from the preserved pQE-30Xa vector. The ligation was conducted using TaKaRa DNA Ligation Kit Ver. 2.1 (TaKaRa Bio Inc., Kyoto, Japan) according to the manufacturer's protocol. The insertion of monkey AO cDNA was confirmed by agarose gel electrophoresis of the *Pst*I/*Nco*I treatment product.

Construction of Expression System for Monkey AO Containing 130 kDa Subunit PCR of long nucleotides corresponding to the 130 kDa subunit is subject to result in PCR error. So, the pQE-30Xa plasmids in which complete monkey AO full cDNA had already been introduced were utilized for the construction of the objective expression system. The strategy to connect the two *Hind*III treated fragments is shown in Fig. 1. The forward primer, aggtacccecaaatcgagga, was designed in such a way that the initial amino acid becomes Pro and a restriction site fits to the frame of the pQE-30 Xa vector. The reverse primer was ccaattgggtcttcaggatg which was designed to contain *Pst*I restriction site around the middle of full cDNA. The PCR products were digested with *Acc*65I and *Pst*I, then introduced in pQE-30 Xa vector. After subcloning, the plasmids were treated with *Hind*III and the hydrolyzed fragments were connected with the fragments obtained by *Hind*III treatment of pQE-30 Xa vector with the complete monkey AO full cDNA. The resulting plasmids were transfected into *E. coli* M15 (pREP4, QIAGEN).

Cell Cultures Each AO cDNA plasmid prepared above was transfected into the expression *E. coli* M15 cells (pREP4, QIAGEN). The control sample was produced by transfection of the pQE-30 Xa vector or the pQE-60 vector. A single colony was cultured at 37 °C overnight in 5 ml of LB medium containing 50 $\mu\text{g}/\text{ml}$ ampicillin and 25 $\mu\text{g}/\text{ml}$

Fig. 1. Construction Strategy for AO Containing the 130 kDa Subunit

kanamycin. To the overnight culture, 500 ml of LB with 50 μ g/ml ampicillin and 25 μ g/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 μ M), and Na₂MoO₄ (50 μ M) according to the methods previously reported by Huang *et al.*²⁶⁾ Growth of the culture was allowed to continue for 72 h at 22°C. After collection by centrifugation at 7000 *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. U.K., Buckinghamshire, England) fully pre-equilibrated with 10 mM phosphate buffer (pH 7.4) containing 20 mM imidazole and 0.5 M NaCl. After being washed with 100 mM imidazole in the buffer, the objective enzyme was eluted with 250 mM imidazole in the buffer.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) The expressed enzymes were separated by SDS-PAGE, which was performed using Phast-Gel 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.

RESULTS

Construction of Expression System for Monkey AO with His-tag at the N- and C-Terminus It seems to be a good way to characterize AO with His-tag at the N- and C-terminus by using anti-AO and anti-His-tag antisera in order to determine at which terminus the 150 kDa subunit is cleaved. The expression system of monkey AO with His-tag at the N-terminus has already been constructed.²⁵⁾ In this study, the expression system of another C-terminus His-tag AO subunit was newly constructed. The 150 kDa and 130 kDa subunits were observed in both His-tag AO expression systems by anti-monkey AO antiserum, although the amount

Fig. 2. Western Blot Analysis of Expressed AO Proteins with His-tag at N- and C-Terminus by (a) Anti-monkey AO Antiserum and (b) Anti-His-tag Antiserum

of 130 kDa subunit was much smaller compared to the amount of 150 kDa subunit (Fig. 2a). In contrast, when anti-His-tag antiserum was used, the 150 kDa and 130 kDa subunits were detected in the C-terminus His-tag AO expression system, but only the 150 kDa subunit was seen in the N-terminus His-tag AO expression system (Fig. 2b).

N-Terminal Amino Acid Analysis of 130 kDa Subunit The AO protein containing the 130 kDa subunit was expressed using the pQE-30 Xa vector, which can add His-tag at the N-terminus, and purified with HisTrap HP column chromatography. The proteins on the gel after SDS-PAGE were transferred to a PVDF membrane. The CBB-stained 130 kDa subunit was cut off and its N-terminal amino acids were analyzed by Edman degradation. The N-terminal amino acid of the 130 kDa subunit was 189Pro of 150 kDa subunit (Fig. 3), which is located in the linkage portion between the 2Fe–2S cluster and FAD domains (Fig. 4).

Construction of Expression System for Monkey AO Containing 130 kDa Subunit The construction strategy for cDNA expressing the 130 kDa subunit of monkey AO is illustrated in Fig. 1. The protein expressed in *E. coli* M15 cells was purified by HisTrap HP column chromatography. SDS-PAGE/Western blot analysis of the purified protein using anti-monkey AO antiserum revealed one band with a molecular mass of 130 kDa (Fig. 5), which is the same migrating position as the minor 130 kDa subunit of monkey liver cytosol and the expressed AO proteins.

Activity of Monkey AO Containing 130 kDa Subunit The AO-catalyzed 2-oxidation activity of (*S*)-RS-8359 was measured for the expressed AO containing the 130 kDa subunit. Although the expressed protein of monkey AO full cDNA used as a positive control showed high AO activity, AO containing the 130 kDa subunit had no activity at all (Fig. 6).

DISCUSSION

AO is a homodimer with a subunit molecular mass of about 150 kDa. Each subunit consists of about 20 kDa of 2Fe–2S cluster domain, about 40 kDa of FAD containing domain and about 85 kDa molybdenum cofactor containing domain. Previously, we demonstrated that a minor 130 kDa subunit as well as a major 150 kDa subunit was detected in SDS-PAGE/Western blot analysis of the expressed proteins of

monkey	1:	MDRASELLFYVNGRKVIEKNVDPETMLLPYLRRKKLRLTGTYCGGGGCGACTVMISRYN	60
human	1:	MDRASELLFYVNGRKVIEKNVDPETMLLPYLRRKKLRLTGTYCGGGGCGACTVMISRYN	60
rabbit	1:	MEPAPELLFYVNGRKVVEKQVDPETMLLPYLRRKKLRLTGTYCGGGGCGACTVMISRYN	60
rat	1:	MDPPQ-LLFYVNGQKVVENNVDPETMLLPYLRRKKLRLTGTYCGGGGCGACTVMISRYN	59
mouse	1:	MDPIQ-LLFYVNGQKVVEKNVDPETMLLPYLRRKKLRLTGTYCGGGGCGACTVMISRYN	59
bovine	1:	MEGGSELLFYVNGRKVTEKNVDPETMLLPYLRRKKLRLTGTYCGGGGCGACTVMISRYN	60
monkey	61:	PITNRIHHHPANACLIPICSLYGTAVTTVEGIGSTHTRIHPVQERIAKCHGTQCGFCTPG	120
human	61:	PITKRIHHHPANACLIPICSLYGAAVTTVEGIGSTHTRIHPVQERIAKCHGTQCGFCTPG	120
rabbit	61:	RVTKKIRHYPVNACLTPICSLYGAAVTTVEGIGSTTRHLHPVQERIAKFHGTQCGFCTPG	120
rat	60:	PSTKSIHHHPVNACLTPICSLYGTAVTTVEGIGNTRRLHPVQERIAKCHSTQCGFCTPA	119
mouse	60:	PSTKAIRHHHPVNACLTPICSLHGTAVTTVEGLGNTRRLHPVQERIAKCHGTQCGFCTPG	119
bovine	61:	PITKIRHYPANACLTPICSLYGAAVTTVEGIGSTKTRIHPVQERIAKCHGTQCGFCTPG	120
monkey	121:	MVMSIYTLRRNHPEPTLDQLTDALGGNLCRCTGYRPIIDACKTFCETSGCCQSKENGVC	180
human	121:	MVMSIYTLRRNHPEPTLDQLTDALGGNLCRCTGYRPIIDACKTFCETSGCCQSKENGVC	180
rabbit	121:	MVMSYALLRNHPEPTLDQLADALGGNLCRCTGYRPIIEAYKTFCKTSDCCQNKENGFC	180
rat	120:	RVMSYALLRNHPEPSLDQLTDALGGNLCRCTGYRPIIDACKTFCRASGCCESKENGVC	179
mouse	120:	MVMSYALLRNHPEPTLDQLTDALGGNLCRCTGYRPIIDACKTFCASACCQSKENGVC	179
bovine	121:	MVMSLYTLRRNHPEPTLTQLNDALGGNLCRCTGYRPIINACKTFCETSGCCQSKENGVC	180
monkey	181:	LDQINGLPEFEEGSKTSPKLFEEEEFLPLDPTQELIFPELMMAEKQPQRTVFGSER	240
human	181:	LDQINGLPEFEEGSKTSPKLFEEEEFLPLDPTQELIFPELMMAEKQSQRTRVFGSER	240
rabbit	181:	LDQINGLPEVEEENQTRPNLFSEEEYLPDPTQELIFPELMMAEKQPQRTVFGSER	240
rat	180:	LDQINGSAEFQEGDETSPELFSEKEFQPLDPTQELIFPELMMAEKQPPKTRVFGYNSR	239
mouse	180:	LDQINGLAESQEEDETSPELFSEEEFLPLDPTQELIFPELMMAEKQPPKTRVFGYGER	239
bovine	181:	LDQGMNGLPEFEEGNETSLKLFSEEEFLPLDPTQELIFPELMMAEKKTQKTRIFGSDR	240

Fig. 3. Partial Amino Acid Sequences of AO from Several Animal Species

The bold peptides are the N-terminal amino acid sequences of the 130 kDa component of monkey AO as analyzed by Edman degradation. The gray-shadowed amino acids indicate the sequences lost by cleavage.

Fig. 4. Structural Model of AO and the Cleavage Position Leading to Formation of the 130 kDa Component

Fig. 5. Western Blot Analysis of Expressed AO Protein Containing the 130 kDa Subunit

Fig. 6. AO-Catalyzed 2-Oxidation Activity of (S)-RS-8359 by Expressed AO Protein Containing the 130 kDa Subunit

monkey AO full cDNA.²⁵⁾ Almost the same electrophoretic behavior was observed in the Western blot analysis of AO

purified from monkey liver cytosol. In rat, however, such 130 kDa subunit was observed in neither the *in vitro* expression protein nor in the *in vivo* liver samples. The addition of protease inhibitors during enzyme purification did not affect formation of the 130 kDa subunit. The results suggest that the 130 kDa subunit seems to have already been formed by a proteolytic process after being biosynthesized within the ribosomes in the liver cells of monkey and human, and also in the *E. coli* expression system of monkey AO, but not in rat AO.

The 130 kDa subunit was assumed to be produced by cleavage at the N-terminal side by nano-LC Q/TOF MS analysis of AO purified from monkey liver cytosol.²⁵⁾ To confirm this assumption, two types of AO protein with His-tag at the N- and C-terminus were expressed in *E. coli* by using the pQE-30 and pQE-60 vectors, respectively, and purified with HisTrap HP column chromatography. The N-terminus His-tag AO showed both the major 150 kDa and the minor 130 kDa subunits against anti-AO antiserum, but only the 150 kDa subunit against anti-His-tag antiserum. The C-terminus His-tag AO displayed both the 150 kDa and the 130 kDa subunits against both anti-AO and anti-His-tag antisera. There might be two possible explanations for the production of the 130 kDa subunit. The first possibility is illustrated in Fig. 7, in which AO partially lacks a 20 kDa fragment and exists as a heterodimer of 150 kDa/130 kDa. The heterodimer purified with HisTrap HP column chromatography yielded the 150 kDa and 130 kDa subunits by SDS treatment. The second possibility is given in Fig. 8. As is shown, each of the 2Fe-2S and FAD domains is connected with a MoCo domain by

Fig. 7. Formation Hypothesis of the 130 kDa Component from the 150 kDa/130 kDa Heterodimer or the 130 kDa/130 kDa Homodimer

The expressed proteins were purified by His-Trap HP column chromatography and analyzed by SDS-PAGE/Western blot. The N-terminus His-tag AO showed both the major 150 kDa and the minor 130 kDa proteins against anti-AO antiserum, but only the 150 kDa protein against anti-His-tag antiserum. The C-terminus His-tag AO displayed both the 150 kDa and the 130 kDa proteins against both anti-AO and anti-His-tag antisera.

Fig. 8. Formation Hypothesis of the 130 kDa Component through Bond Cleavage between the 2Fe-2S and FAD Domains in Monkey AO

The results of SDS-PAGE/Western blot analysis were the same as those described in the legend of Fig. 7.

disulfide bonding, but the linkage between the 2Fe-2S and FAD domains, which is maintained in the normal 150 kDa subunit, is absent. Therefore, the subunit exists as a 130+20 kDa protein (apparent 150 kDa). A large amount of the normal dimer along with a small amount of the unusual dimer consisting of the apparent 150 kDa subunit was adsorbed and purified using HisTrap HP column chromatography. SDS-treatment yielded the 150 kDa subunit from the former dimer and the 130 kDa subunit from the latter dimer. Although other explanations might exist, these two seem to clearly describe the results obtained by using the two kinds of His-tag proteins and the two kinds of antisera. We do not have any experimental evidence to conclude which is correct, but it is obvious that the 130 kDa subunit is produced by cleavage at the N-terminus of the 150 kDa subunit.

Determination of the precise cleavage point of the 150 kDa subunit is necessary to clarify the function of the 130 kDa subunit using the expression technique. Edman degradation analysis of the expressed minor 130 kDa subunit revealed that the cleavage point is located between 188Lue and 189Pro of the 150 kDa subunit. These amino acid sequences

were present in AO of human,²⁷⁾ bovine²⁸⁾ and rabbit²⁶⁾ as well as monkey,²⁵⁾ but not in that of rat (188Ser/189Ala)²⁹⁾ and mouse (188Leu/189Ala),³⁰⁾ as shown in Fig. 3. The minor 130 kDa subunit is detected in AO of humans (data not shown), but not in AO of rats and mice. Calzi *et al.*²⁸⁾ reported a single 150 kDa protein band on SDS-PAGE of purified bovine AO, however the minor 130 kDa protein could be observed although a small amount. According to Huang *et al.*,²⁶⁾ the AO purified from rabbit liver cytosol gave a single electrophoretical protein band on SDS-PAGE. The amino acid sequences of guinea pig AO have not been reported and the existence of 130 kDa minor subunit has been controversial.^{9,31)} Thus, it seemed likely that the existence or absence of the 130 kDa minor subunit correlates to the amino acid sequences of 188Lue and 189Pro as seen in human, monkey, rat, mouse, and possibly bovine. However, some factors other than the amino acid sequences might be required for the formation of the 130 kDa subunit in rabbit.

The amino acid sequences of 188Leu and 189Pro are included in the linkage between the 2Fe-2S and FAD domains.²⁹⁾ The cleavage results in the loss of the 2Fe-2S cluster domain essentially required for AO activity. In fact, the expressed AO protein containing the 130 kDa subunit showed no catalytic activity at all. This means that the existence of 130 kDa subunit is obviously not associated with the high AO activity in monkey and human. Previously, a biphasic pattern was shown in Eadie-Hofstee plots for the AO-catalyzed 2-oxidation of (*S*)-RS-8359 that suggested the existence of two AO isoforms or two active sites in a single enzyme.²⁵⁾ The results obtained in this study are likely to rule out the possibility of the existence of two isoforms because the AO protein containing the 130 kDa subunit has no activity. The properties of protease hydrolyzing the peptide bond between 188Leu and 189Pro are as yet unknown, and that will be a future problem.

In conclusion, the 130 kDa AO subunit was shown to be produced by cleavage between 188Leu and 189Pro of the 150 kDa subunit. The existence of the amino acid sequences might be one major reason for the species difference in the formation of the minor 130 kDa subunit. However, the existence of the 130 kDa subunit is not associated with the species differences in AO activity, because the cleavage results in the loss of the 2Fe-2S cluster domain essential for exertion of AO activity.

REFERENCES

- 1) Beedham C., *Drug Metab. Rev.*, **16**, 119–156 (1985).
- 2) Beedham C., *Prog. Med. Chem.*, **24**, 85–121 (1987).
- 3) Beedham C., *Pharm. World Sci.*, **19**, 255–263 (1997).
- 4) Beedham C., "Metabolism of Xenobiotics," ed. by Gorrod J. W., Oeschlager H., Caldwell J., Taylor & Francis, London, 1998, pp. 51–58.
- 5) Beedham C., "Enzyme Systems That Metabolise Drug and Other Xenobiotics," ed. by Ioannides C., John Wiley, London, 2002, pp. 147–187.
- 6) Kitamura S., Sugihara K., Ohta S., *Drug Metab. Pharmacokinet.*, **21**, 83–98 (2006).
- 7) Prakash C., Kamel A., Gummerus J., Wilner K., *Drug Metab. Dispos.*, **25**, 863–875 (1997).
- 8) Beedham C., Miceli J. J., Obach S., *J. Clin. Psychopharmacol.*, **23**, 229–232 (2003).
- 9) Sugihara K., Katsuma Y., Kitamura S., Ohta S., Fujitani M., Shintani H., *Comp. Biochem. Physiol., Part C*, **126**, 53–60 (2000).

- 10) Acheampong A. A., Chien D-S., Lam S., Vekich S., Breau A., Usansky J., Harcourt D., Munk S. A., Nguyen H., Garst M., Tang-Liu D., *Xenobiotica*, **26**, 1035—1055 (1996).
- 11) Rashidi M. R., Smith J. A., Clarke S. E., Beedham C., *Drug Metab. Dispos.*, **25**, 805—813 (1997).
- 12) Kitamura S., Sugihara K., Nakatani K., Ohta S., O'Hara T., Nimomiya S., Green C. E., Tyson C. A., *UBMB Life*, **48**, 607—611 (1999).
- 13) Jordan C. G. M., Rashidi M. R., Laljee H., Clarke S. E., Brown J. E., Beedham C., *J. Pharm. Pharmacol.*, **51**, 411—418 (1999).
- 14) Kawashima K., Hosoi K., Naruke T., Shiba T., Kitamura M., Watabe T., *Drug Metab. Dispos.*, **27**, 422—428 (1999).
- 15) Klecker R. W., Cysyk R. L., Collins J. M., *Bioorg. Med. Chem.*, **14**, 62—66 (2006).
- 16) Yokoyama T., Karube T., Iwata N., *J. Pharm. Pharmacol.*, **41**, 32—36 (1989).
- 17) Miura H., Naoi M., Nakahara D., Ohta T., Nagatsu T., *J. Neural. Transm.*, **94**, 175—187 (1993).
- 18) Plenker A., Puchler K., Volz H. P., *Int. Clin. Psychopharm.*, **12**, S25—S29 (1997).
- 19) Puchler K., Schaffler K., Plenker A., *Int. Clin. Psychopharm.*, **12**, S17—S23 (1997).
- 20) Takasaki W., Yamamura M., Nozaki A., Nitani T., Sasahara K., Itoh K., Tanaka Y., *Chirality*, **17**, 135—141 (2005).
- 21) Itoh K., Yamamura M., Muramatsu S., Hoshino K., Masubuchi A., Sasaki T., Tanaka Y., *Xenobiotica*, **35**, 561—573 (2005).
- 22) Itoh K., Yamamura M., Takasaki W., Sasaki T., Masubuchi A., Tanaka Y., *Biopharm. Drug Dispos.*, **27**, 133—139 (2006).
- 23) Sasaki T., Masubuchi A., Yamamura M., Watanabe N., Hiratsuka M., Mizugaki M., Itoh K., Tanaka Y., *Biopharm. Drug Dispos.*, **27**, 247—255 (2006).
- 24) Itoh K., Masubuchi A., Sasaki T., Adachi M., Watanabe N., Nagata K., Yamazoe Y., Hiratsuka M., Mizugaki M., Tanaka Y., *Drug Metab. Dispos.*, **35**, 734—739 (2007).
- 25) Hoshino K., Itoh K., Masubuchi A., Adachi M., Watanabe N., Kosaka T., Tanaka Y., *Biol. Pharm. Bull.*, **30**, 1191—1198 (2007).
- 26) Huang D. Y., Furukawa A., Ichikawa Y., *Arch. Biochem. Biophys.*, **364**, 264—272 (1999).
- 27) Wright R. M., Vaitaitis G. M., Wilson C. M., Repine T. B., Terada L. S., Repine J. E., *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 10690—10694 (1993).
- 28) Calzi M. L., Raviolo C., Ghibaudi E., deGioia L., Salmons M., Cazaniga G., Kurosaki M., Terao M., Garattini E., *J. Biol. Chem.*, **270**, 31037—31045 (1995).
- 29) Wright R. M., Clayton D. A., Riley M. G., McManaman J. L., Repine J. E., *J. Biol. Chem.*, **274**, 3878—3886 (1999).
- 30) Kurosaki M., Demontis S., Barzago M. M., Garattini E., Terao M., *Biochem. J.*, **341**, 71—80 (1999).
- 31) Yoshihara S., Tatsumi K., *Arch. Biochem. Biophys.*, **242**, 213—224 (1985).