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ORIGINAL ARTICLE

Role of the active site residues arginine-216 and arginine-237 in the substrate specificity of mammalian D-aspartate oxidase

Masumi Katane · Yasuaki Saitoh · Kazuhiro Maeda · Toshihiko Hanai · Masae Sekine · Takemitsu Furuchi · Hiroshi Homma

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Abstract D-Aspartate oxidase (DDO) and D-amino acid oxidase (DAO) are flavin adenine dinucleotide-containing flavoproteins that catalyze the oxidative deamination of D-amino acids. Unlike DAO, which acts on several neutral and basic D-amino acids, DDO is highly specific for acidic D-amino acids. Based on molecular modeling and simulated annealing docking analyses, a recombinant mouse DDO carrying two substitutions (Arg-216 to Leu and Arg-237 to Tyr) was generated (R216L-R237Y variant). This variant and two previously constructed single-point mutants of mouse DDO (R216L and R237Y variants) were characterized to investigate the role of Arg-216 and Arg-237 in the substrate specificity of mouse DDO. The R216L-R237Y and R216L variants acquired a broad specificity for several neutral and basic D-amino acids, and showed a considerable decrease in activity against acidic D-amino acids. The R237Y variant, however, did not show any additional specificity for neutral or basic D-amino acids and its activity against acidic D-amino acids was greatly reduced. The kinetic properties of these variants indicated that the Arg-216 residue is important for the catalytic activity and substrate specificity of mouse DDO. However, Arg-237 is, apparently, only marginally involved in substrate recognition, but is important for catalytic activity. Notably, the substrate specificity of the R216L-R237Y variant differed significantly from that of the R216L variant, suggesting that Arg-237 has subsidiary effects on substrate specificity. Additional experiments using several DDO and DAO inhibitors also suggested the involvement of Arg-216 in the substrate specificity and catalytic activity of mouse DDO and that Arg-237 is possibly involved in substrate recognition by this enzyme. Collectively, these results indicate that Arg-216 and Arg-237 play crucial and subsidiary role(s), respectively, in the substrate specificity of mouse DDO.

Keywords D-Aspartate oxidase · D-Amino acid oxidase · Flavoprotein · Site-directed mutagenesis · Substrate specificity

Abbreviations

DAO	D-Amino acid oxidase
DDO	D-Aspartate oxidase
FAD	Flavin adenine dinucleotide
IC_{50}	50% Inhibitory concentration
IPTG	Isopropyl-β-D-thiogalactopyranoside
NMDA	*N*-Methyl-D-aspartate

Introduction

Of the free D-amino acids that are present in mammals, D-serine (D-Ser) and D-aspartate (D-Asp) are the most studied. D-Ser is found in the mammalian forebrain, where it persists at high concentrations over the lifetime of the animal. It binds to the glycine (Gly)-binding site of the *N*-methyl-D-Asp (NMDA) receptor, a subtype of the L-glutamate (L-Glu) receptor, and potentiates glutamatergic neurotransmission in the central nervous system (Matsui et al. 1995; Mothet et al. 2000). Accordingly, it has been proposed that D-Ser regulates the L-Glu-mediated activation

M. Katane · Y. Saitoh · K. Maeda · T. Hanai · M. Sekine · T. Furuchi · H. Homma (✉)
Laboratory of Biomolecular Science, Department of Pharmaceutical Life Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan
e-mail: hommah@pharm.kitasato-u.ac.jp

of the NMDA receptor by acting as a co-agonist (for reviews see Nishikawa 2005; Scolari and Acosta 2007). In contrast, substantial free D-Asp is found in a wide variety of mammalian tissues, particularly in the central nervous, neuroendocrine, and endocrine systems. Several lines of evidence suggest that D-Asp plays an important role in regulating developmental processes, hormone secretion, and steroidogenesis (for reviews see D'Aniello 2007; Homma 2007).

In mammalian tissues, two types of degradative enzymes that are stereospecific for D-amino acids have been identified: D-amino acid oxidase (DAO, also abbreviated to DAAO, EC 1.4.3.3) and D-Asp oxidase (DDO, also abbreviated to DASPO, EC 1.4.3.1). DAO and DDO are flavin adenine dinucleotide (FAD)-containing flavoproteins that catalyze the oxidative deamination of D-amino acids to generate the corresponding 2-oxo acids, along with hydrogen peroxide and ammonia. DAO displays broad substrate specificity and acts on several neutral and basic D-amino acids, such as D-Ser, D-Ala, and D-Arg. DDO is highly specific for acidic D-amino acids, such as D-Asp, NMDA and D-Glu; none of which are substrates for DAO. DAO and DDO have been found in various organisms and their physiological roles in vivo are now being extensively investigated. Mammalian DAO and DDO reportedly regulate endogenous D-Ser and D-Asp levels, respectively, and mediate the elimination of accumulated exogenous D-amino acids from various organs (for reviews see Katane and Homma 2010; Pollegioni et al. 2007).

Recently, it was shown that the amount of D-Asp in human seminal plasma and spermatozoa is significantly reduced in oligoasthenoteratospermic and/or azoospermic donors compared with normospermic donors (D'Aniello et al. 2005). In human patients undergoing in vitro fertilization, the D-Asp content of the pre-ovulatory follicular fluid is relatively lower in older patients than in younger ones (D'Aniello et al. 2007). This decrease in D-Asp content appears to be related to a decrease in the oocyte quality and fertilization competence. These reports support the physiological significance of DDO in regulating the D-Asp content in vivo. However, little is known about the regulatory mechanisms controlling DDO activity, or the molecular details of its structure–function relationships.

X-ray crystallographic analysis, in vitro mutagenesis, and/or the use of specific enzyme inhibitor(s) are helpful and effective methods for detailed investigation of the structure–function relationships of the enzyme. These approaches have been used to identify several amino acid residues within DAO that are functionally and structurally important (for reviews see Pilone 2000; Tishkov and Khoronenkova 2005). Previously, we cloned a cDNA from mouse kidney encoding DDO (Katane et al. 2007a). Since the three-dimensional (3D) structure of DDO has not been determined, a structural model for mouse DDO was proposed based on the deduced amino acid sequence. In the proposed structure, the side chains of Arg-216, Tyr-223, Arg-237, Arg-278, and Ser-308 were oriented toward the predicted active site binding pocket (Katane et al. 2007a), implicating the involvement of these residues in mouse DDO catalysis. Mutagenesis analysis enabled the identification of Arg-216, Arg-237, and Ser-308 of mouse DDO as functionally important residues (Katane et al. 2007a, 2008). However, the precise roles of these residues in catalysis remain to be clarified.

In the present study, a similar approach was used to investigate the roles of Arg-216 and Arg-237 of mouse DDO in the catalytic process, particularly with respect to substrate specificity. A double-point mutant of mouse DDO (R216L-R237Y variant) was used, in which the Arg-216 and Arg-237 codons were mutated to Leu and Tyr, respectively. This variant, along with previously constructed single-point mutants of mouse DDO (R216L and R237Y variants; Katane et al. 2007a), was characterized. This study reports a crucial role for Arg-216, and a subsidiary role for Arg-237, in the substrate specificity of mouse DDO and provides novel and useful insights into the structure–function relationships of mammalian DDO.

Materials and methods

Chemicals

Ampicillin, D- and L-amino acids, bovine serum albumin, and catalase from *Aspergillus niger* were purchased from Sigma–Aldrich (St Louis, MO, USA). FAD, isopropyl-β-D-thiogalactopyranoside (IPTG), imidazole, 2,4-dinitrophenylhydrazine, benzoate, crotonate, malonate, and *meso*-tartrate were purchased from Wako Pure Chemical Ind. (Osaka, Japan). All other chemicals were of the highest grade available and purchased from commercial sources.

Construction of recombinant protein expression plasmids

The construction of expression plasmids for the N-terminally His-tagged mouse DDO (pRSET-His-mDASPO), N-terminally His-tagged Arg-216-to-Leu (R216L) variant of mouse DDO (pRSET-His-mDASPO-R216L), N-terminally His-tagged Arg-237-to-Tyr (R237Y) variant of mouse DDO (pRSET-His-mDASPO-R237Y), and N-terminally His-tagged human DAO (pRSET-His-hDAO) has been described previously (Katane et al. 2007a, 2010). The expression plasmid for N-terminally His-tagged mouse DAO (pET15-DAO), in which the cDNA fragment corresponding to the entire mouse DAO-coding sequence was

cloned into pET-15b (Novagen, Madison, WI, USA), was kindly provided by Prof. R. Konno (International University of Health and Welfare, Tochigi, Japan).

An R237Y substitution was introduced into pRSET-His-mDASPO-R216L by site-directed mutagenesis using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) to generate an expression plasmid for the N-terminally His-tagged R216L-R237Y variant of mouse DDO (pRSET-His-mDASPO-R216L-R237Y). The following mutagenic oligonucleotides were used (mutated codons are underlined): 5′-GTA ACC CTG GGA GGA ACT TAC CAG AAA GGA GAC TGG AAT CG-3′ and 5′-CGA TTC CAG TCT CCT TTC TGG TAA GTT CCT CCC AGG GTT AC-3′. Introduction of the desired mutations was confirmed by sequencing.

Expression and purification of recombinant proteins

Escherichia coli strain BL21(DE3)pLysS cells were transformed with expression plasmids and cultured at 37°C with shaking in Luria–Bertani medium containing ampicillin (100 μg/mL). Crude extracts were prepared from cells transformed with pRSET-His-mDASPO, -hDAO, and pET15-DAO, as previously described (Katane et al. 2008, 2010). For cells transformed with pRSET-His-mDASPO-R216L, -R237Y, or -R216L-R237Y, the culture was grown to $A_{620} = 0.5$, incubated for an additional 30 min at a reduced temperature (30°C), and maintained at 26°C for an extra 30 min. After adding 0.1 mM IPTG, the culture was further incubated at 26°C for 16 h. Cells were then centrifuged at 10,000 × *g* for 10 min at 4°C, and crude extract was prepared using BugBuster® Protein Extraction Reagent and Lysonase™ Bioprocessing Reagent (Novagen) in the presence of 50 μM FAD and protease inhibitors (Roche Applied Science, Mannheim, Germany), according to the manufacturer's instructions.

All recombinant proteins were purified by affinity chromatography using a chelating column as described below. Crude extracts were applied to a His GraviTrap™ column (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) equilibrated with 20 mM sodium phosphate buffer (pH 7.4) containing 0.5 M NaCl and 10 mM imidazole. The column was then washed with the same buffer, and bound proteins were eluted using a step-wise gradient of 50–500 mM imidazole. Each fraction (2.0–2.5 mL) containing recombinant protein was mixed with 100 μL of 1 mM FAD and dialyzed twice for 3 h at 4°C against 1 L of 10 mM sodium pyrophosphate buffer (pH 8.3) containing 2 mM EDTA, 5 mM 2-mercaptoethanol, and 10% glycerol. The dialyzed fractions were recovered and centrifuged at 10,000 × *g* for 10 min at 4°C to pellet the proteins denatured during dialysis. The supernatants were pooled as purified enzyme and used immediately for enzyme assays or stored frozen at −80°C until use. All recombinant proteins were purified to near-homogeneity when examined by SDS-PAGE. From 1 L of fermentation broth, we obtained the following amounts of purified N-terminally His-tagged proteins: 0.3 mg mouse DDO, 0.3 mg human DAO, 0.4 mg mouse DAO, 0.2 mg R216L variant of mouse DDO, 0.2 mg R237Y variant of mouse DDO, and 0.1 mg R216L-R237Y variant of mouse DDO.

Enzymatic activity assays

The protein concentrations of the purified enzyme preparations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine serum albumin as a standard.

The activity of DDO and DAO was determined using a colorimetric assay for 2-oxo acid production as previously described (Katane et al. 2007b). Briefly, appropriate amounts (0.25–28 μg) of the purified enzymes were added to a reaction mixture of air-saturated 40 mM sodium pyrophosphate buffer (pH 8.3), 23 U *A. niger* catalase, 60 μM FAD, and 20 mM amino acids in a final volume of 150 μL. The mixture was incubated at 37°C for 15 min, and then 10 μL of 100% (w/v) trichloroacetic acid was added to stop the reaction. The 2-oxo acid product was reacted with 2,4-dinitrophenylhydrazine and quantitated by measuring the A_{445} against a blank mixture lacking amino acids. One unit of enzyme activity was defined as the production of 1 μmol 2-oxo acid per min, under the assay conditions described above.

The kinetic parameters of the enzyme reaction were determined under conditions in which the production of 2-oxo acids was linear with incubation time and exhibited Michaelis–Menten-type properties. The maximal velocity (V_{max}) and Michaelis constant (K_m) values for the substrates D-Asp, NMDA, D-Glu, D-Phe, and D-His were determined using different final concentrations (2–40, 1–40, 10–80, 2–40, and 2–40 mM, respectively). The V_{max} and K_m values were calculated according to the method of Wilkinson (1961). The molecular activity (k_{cat}) values were calculated from the V_{max} values and the estimated molecular masses of the recombinant proteins (42,074, 43,631, 40,862, 42,031, 42,081, and 42,038 Da, for N-terminally His-tagged mouse DDO, human DAO, mouse DAO, R216L variant of mouse DDO, R237Y variant of mouse DDO, and R216L-R237Y variant of mouse DDO, respectively).

Results and discussion

Computational modeling and structural comparison of DAO/substrate and DDO/substrate complexes

To determine the preferred substrate for mammalian DAO, the enzymatic activity of purified recombinant human and

mouse DAO against various amino acids was determined. Human and mouse DAO displayed deamination activity against several neutral and basic D-amino acids. In both enzymes, the highest activity was detected when using D-Phe as the substrate (Table 1). None of the recombinant proteins demonstrated activity against any protein L-amino acid or *N*-methyl-L-Asp (data not shown). The 3D X-ray crystallographic structures of porcine and human DAO are known (Kawazoe et al. 2006, 2007; Mattevi et al. 1996; Miura et al. 1997; Mizutani et al. 1996; Todone et al. 1997). In those structures complexed with the DAO substrate analogue *o*-aminobenzoate, the carboxyl group of *o*-aminobenzoate interacts with the side-chain guanidino group of Arg-283 and with the side-chain hydroxyl group of Tyr-228, while the amino group of *o*-aminobenzoate interacts with the backbone carbonyl of Gly-313 (Fig. 1a) (Kawazoe et al. 2007; Miura et al. 1997). Importantly, a loop formed by residues 216–228 has been postulated to act as an "active-site lid" that opens and closes upon substrate/product migration in and out of the active site (Todone et al. 1997). The side chain of Tyr-224 within the "active-site lid" covers the bound ligand (Fig. 1a). This residue is presumably important in the hydrophobic environment of the active site in the closed conformation (Mattevi et al. 1996; Miura et al. 1997; Mizutani et al. 1996).

To gain insight into the binding of a substrate D-amino acid to mammalian DAO, the human DAO/D-Phe complex was modeled using the Lamarckian Genetic Algorithm and AutoDock 4.0 software (Fig. 1b) (Huey et al. 2004, 2007). D-Phe was used as the bound ligand, since recombinant human DAO showed the highest activity against this D-amino acid (Table 1). The 3D X-ray crystallographic structure of human DAO (Protein Data Bank ID: 2E48) (Kawazoe et al. 2007) was used as the docking protein, and a theoretical model of mouse DAO complexed with D-Phe was constructed (Fig. 1c). Since the 3D structure of mouse DAO has not been determined, a structural model for mouse DAO was proposed using the homology modeling method and SWISS-MODEL on the ExPASy web server (http://swissmodel.expasy.org/) (Arnold et al. 2006; Peitsch 1995). The 3D X-ray crystallographic structures of porcine DAO (Protein Data Bank ID: 1KIF; 1DDO) (Mattevi et al. 1996; Todone et al. 1997) were used as template structures. A theoretical model of the mouse DAO/D-Phe complex was subsequently determined using AutoDock 4.0 software (Huey et al. 2004, 2007).

In both the human DAO/D-Phe and mouse DAO/D-Phe complex models (Fig. 1b, c), the orientation of the D-Phe α-carboxyl and α-amino groups in the active sites was similar to that of *o*-aminobenzoate bound to human DAO. In addition, the side chain of D-Phe was oriented toward a space where the side chain of a Leu residue was located (Leu-215 or Leu-213 of human or mouse DAO, respectively). These modes are consistent with those in previously reported structural models of the porcine DAO/D-Leu complex (Miura et al. 1997; Setoyama et al. 2006).

To investigate the binding of a substrate D-amino acid to mammalian DDO, a theoretical model of mouse DDO complexed with D-Asp was constructed (Fig. 1d). Using a mouse DDO structure previously proposed by the authors (Katane et al. 2007a), the annealing docking of D-Asp at the mouse DDO active site was simulated using AutoDock 4.0 software (Huey et al. 2004, 2007). The orientation of the α-carboxyl and α-amino groups of D-Asp in the active site was similar to that of *o*-aminobenzoate and/or D-Phe bound to human and mouse DAOs (Fig. 1d). In addition, the side chain (*β*-carboxyl group) of D-Asp was oriented toward a space where the side chain of Arg-216 was located. These binding modes are consistent with those in the previously reported structural model of the bovine DDO/D-Asp complex (Sacchi et al. 2002). Thus, Arg-216 of mouse DDO is structurally equivalent to Leu-215 or Leu-213 of human or mouse DAO, respectively, and Arg-216 likely interacts with the *β*-carboxyl group of an acidic D-amino acid. Notably, the loop structure corresponding to the "active-site lid" of mammalian DAOs was missing in the proposed structure of mouse DDO, and Arg-237 of mouse DDO was structurally equivalent to Tyr-224 or Tyr-222 of human or mouse DAO, respectively.

Table 1 Enzymatic activity of purified recombinant human and mouse DAO against various D-amino acids

Substrates	Specific activity (U/mg protein)	
	Human DAO	Mouse DAO
D-Asn	1.01 ± 0.08	0.069 ± 0.013
D-Gln	ND	ND
D-Ala	5.13 ± 0.46	3.60 ± 0.74
D-Phe	11.7 ± 0.7	4.19 ± 0.28
D-Met	0.83 ± 0.13	0.16 ± 0.01
D-Val	2.00 ± 0.21	1.98 ± 0.37
D-Leu	4.50 ± 0.31	1.42 ± 0.57
D-Ser	1.49 ± 0.23	0.32 ± 0.04
D-Thr	0.81 ± 0.03	0.085 ± 0.015
D-Trp	5.89 ± 0.29	1.51 ± 0.22
D-Cys	0.39 ± 0.06	0.12 ± 0.03
D-Arg	0.30 ± 0.05	0.083 ± 0.019
D-Lys	ND	ND
D-His	0.74 ± 0.05	0.23 ± 0.05

Data are shown as the mean ± standard deviation of three to four independent assays

ND not detected, 1 U = 1 μmol substrate oxidized per min

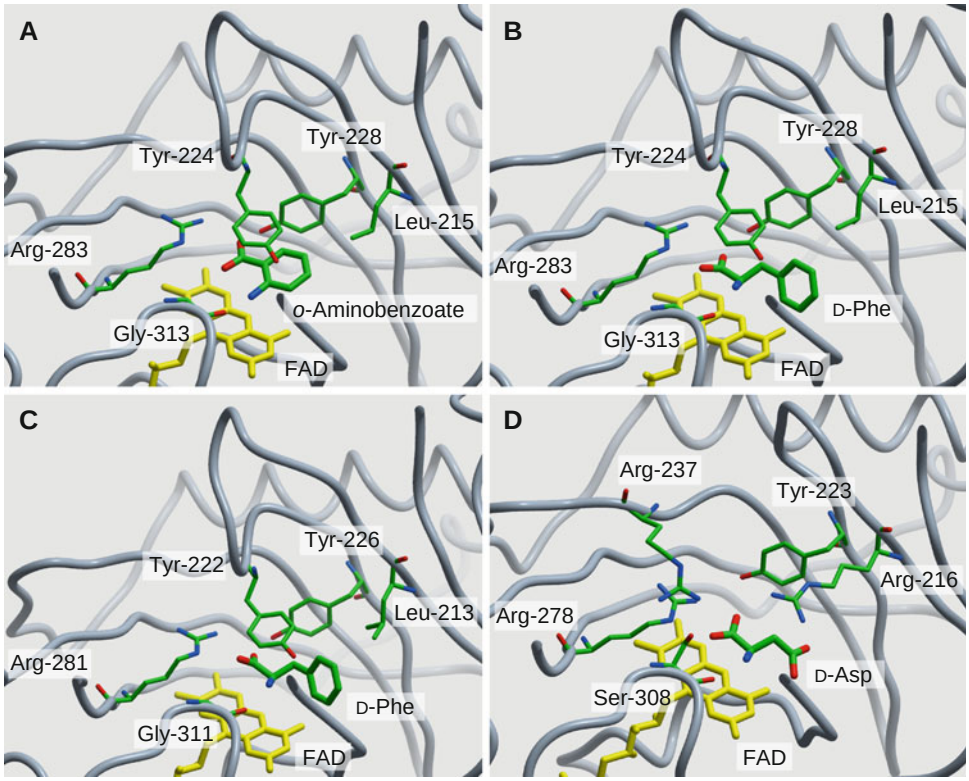

Fig. 1 Structural mapping and comparison of the key catalytic residues in the active sites of mammalian DAO and DDO. The experimentally determined structure of human DAO complexed with *o*-aminobenzoate (Protein Data Bank ID: 2E48) (Kawazoe et al. 2007) is shown in **a**. The proposed structural models of human DAO complexed with D-Phe, mouse DAO complexed with D-Phe, and mouse DDO complexed with D-Asp are shown in **b**, **c**, and **d**, respectively. In these models, the simulated annealing docking of D-Phe or D-Asp at the active site was performed using AutoDock 4.0 software (Huey et al. 2004, 2007) (see text for details). FAD molecules are shown in *yellow*, while bound ligands and side chains of amino acid residues that have been demonstrated, or are believed, to be catalytically important are colored according to the atom type: *green* carbon, *blue* nitrogen, *red* oxygen

Structure-based design of mouse DDO variants

Simulated annealing docking analysis was subsequently used to analyze the roles of two Arg residues of mouse DDO (Arg-216 and Arg-237) in the catalytic process, particularly with respect to substrate specificity. D-Phe was used as the bound ligand and the model with the lowest energy conformation contained this amino acid in an aberrant orientation compared with those observed for human DAO/D-Phe, mouse DAO/D-Phe, or mouse DDO/D-Asp complexes (Fig. 2a). This aberrant orientation is consistent with the observation that the mouse DDO has very low, or undetectable, activity against several neutral and basic D-amino acids.

The mutant mouse DDO carrying R216L and R237Y substitutions (R216L-R237Y variant) used in this study was designed so that its active site was similar to that of mammalian DAO, and so that D-Phe could be bound in the correct orientation at the active site. A theoretical model of the R216L-R237Y variant with D-Phe as the ligand demonstrated that this amino acid docks in the correct orientation, similar to that observed for D-Phe bound to human or mouse DAO, or for D-Asp bound to wild-type mouse DDO (Fig. 2b). The theoretical models were also obtained with two corresponding single-point mutants (R216L and R237Y variants) and D-Phe as the bound ligand. In the active site of the R216L variant (Fig. 2c), D-Phe docks in the correct orientation, similar to that observed for D-Phe in human or mouse DAO, for D-Asp in wild-type mouse DDO, and for D-Phe in the R216L-R237Y variant. In contrast, D-Phe is docked in an aberrant orientation in the R237Y variant (Fig. 2d), similar to the wild-type mouse DDO/D-Phe complex. Collectively, these models predict that Arg-216 in mouse DDO plays crucial role(s) in its substrate specificity, and that this residue is more critical than Arg-237 for substrate recognition by mouse DDO.

Substrate specificity of the mouse DDO variants

In this section, the substrate specificity of the purified recombinant proteins (mouse DDO and its R216L-R237Y, R216L and R237Y variants) was determined using various D-amino acids and their enantiomers as substrates (Table 2). Consistent with our previous report (Katane

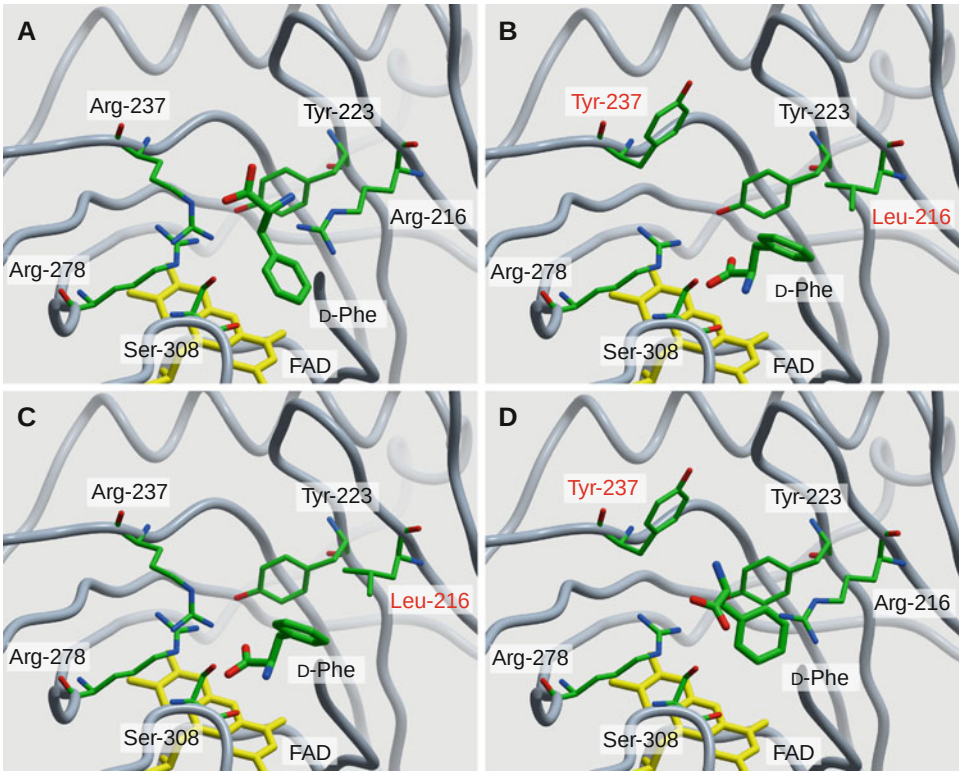

Fig. 2 Structural comparison of the orientation of D-Phe bound to the active sites of mouse DDO and its variant derivatives. The theoretical models of the simulated annealing docking of D-Phe at the active sites of mouse DDO, its R216L-R237Y, R216L, and R237Y variants are shown in **a**, **b**, **c**, and **d**, respectively (see text for details). The color scheme is the same as in Fig. 1

et al. 2007a, 2008), wild-type mouse DDO displayed similar activity levels against D-Asp and NMDA, and relatively low activity levels against D-Glu (Table 2). No activity was detected against any of the neutral or basic D-amino acids examined, with the exception of D-Asn.

The R216L-R237Y variant displayed lower activity against D-Asp and NMDA and higher activity against D-Asn than the wild-type enzyme, and no activity against D-Glu (Table 2). Importantly, this variant acquired an expanded substrate specificity, displaying activity against D-Phe, D-Trp, D-Cys, and D-His. The activity against D-His was significantly higher than that of human or mouse DAO, while activity against D-Phe, D-Trp, and D-Cys was relatively low, or equivalent, to that of mouse DAO (Tables 1, 2).

The R216L variant showed relatively low levels of activity against D-Asp and NMDA compared with the wild-type enzyme, while no activity was detected against D-Glu or D-Asn (Table 2). Interestingly, this variant also showed activity against D-Phe, D-Trp, D-Cys, and D-His, although the activity level was lower than that of the R216L-R237Y variant. The R216L-R237Y and R216L variants showed no

Table 2 Enzymatic activity of purified recombinant mouse DDO and its variant derivatives against various D-amino acids

Substrates	Specific activity (U/mg protein)			
	Wild-type mouse DDO	R216L-R237Y variant	R216L variant	R237Y variant
D-Asp	12.2 ± 0.9	0.36 ± 0.03	0.59 ± 0.03	1.42 ± 0.09
NMDA	13.1 ± 0.6	0.77 ± 0.11	0.69 ± 0.05	1.75 ± 0.11
D-Glu	0.46 ± 0.07	ND	ND	0.13 ± 0.03
D-Asn	0.16 ± 0.02	0.43 ± 0.04	ND	ND
D-Phe	ND	1.37 ± 0.13	0.37 ± 0.03	ND
D-Trp	ND	0.77 ± 0.08	0.098 ± 0.024	ND
D-Cys	ND	0.15 ± 0.09	0.023 ± 0.002	ND
D-His	ND	2.03 ± 0.26	0.29 ± 0.03	ND

Data are shown as the mean ± standard deviation of three independent assays. None of the recombinant proteins showed any activity against D-Gln, D-Ala, D-Met, D-Val, D-Leu, D-Ser, D-Thr, D-Arg and D-Lys

ND not detected, 1 U = 1 μmol substrate oxidized per min

activity against neutral or basic D-amino acids other than D-Phe, D-Trp, D-Cys, and D-His (data not shown). The R237Y variant displayed activity against D-Asp, NMDA, and D-Glu, but at a significantly lower level than that of the wild-type enzyme (Table 2). In contrast to the R216L-R237Y and R216L variants, this variant did not display detectable activity against any of the neutral or basic D-amino acids tested. None of the recombinant proteins showed any activity against protein L-amino acids or *N*-methyl-L-Asp (data not shown).

Taken together, these results indicate that Arg-216 plays critical role(s) in the substrate specificity of mouse DDO, and are consistent with the simulated annealing docking results described above. In addition, and consistent with our previous study (Katane et al. 2007a), Arg-216 and Arg-237 act as key functional residues for its full enzymatic activity.

Characterization of the kinetic properties of the mouse DDO variants

The apparent kinetic parameters (k_{cat} and K_m) of the mouse DDO variants were determined with D-Asp, NMDA and D-Glu as substrates (Table 3), and were compared with those of wild-type mouse DDO. Wild-type mouse DDO showed similar levels of catalytic efficiency (k_{cat}/K_m) for D-Asp and NMDA, but a relatively low efficiency for D-Glu. The k_{cat} values for the R216L-R237Y and R216L variants against D-Asp and NMDA were significantly lower than those of the wild-type enzyme, but the K_m values were higher (Table 3). Therefore, the catalytic efficiency of these variants for D-Asp and NMDA was markedly lower than that of the wild-type enzyme. However, the k_{cat} values for the R237Y variant against D-Asp, NMDA, and D-Glu were significantly lower than those of the wild-type enzyme (Table 3), although the K_m values for this variant against D-Asp, NMDA, or D-Glu were comparable to, or slightly lower than, those of the wild-type enzyme, suggesting that this variant and the wild-type enzyme have relatively similar affinities for acidic D-amino acids.

Next, the k_{cat} and K_m values for the mouse DDO variants were determined using D-Phe and D-His as the substrates and compared with those of human and mouse DAOs (Table 4). The catalytic efficiency (k_{cat}/K_m) of the R216L-R237Y variant for D-Phe was relatively lower than that of the human or mouse DAO. In contrast, the catalytic efficiency of this variant for D-His (447 s^{-1} M^{-1}) was about 2.8 or 45 times higher than that of human or mouse DAO (162 and 10 s^{-1} M^{-1}), respectively. Similarly to the R216L-R237Y variant, the catalytic efficiency of the R216L variant for D-Phe was lower than that of the human or mouse DAO (Table 4), and the catalytic efficiency for D-His (22 s^{-1} M^{-1}) was about 2.2 times higher than that of mouse DAO, while this variant was less efficient at D-His deamination than human DAO.

Table 3 Apparent steady-state kinetic parameters of purified recombinant proteins using several acidic D-amino acids as substrates

Enzymes and substrates	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
Wild-type mouse DDO			
D-Asp	12.2 ± 0.3	8.85 ± 0.60	1,378
NMDA	13.0 ± 0.4	9.73 ± 0.94	1,331
D-Glu	1.60 ± 0.11	79.4 ± 9.6	20
R216L-R237Y variant			
D-Asp	0.53 ± 0.02	83.7 ± 3.3	6
NMDA	0.86 ± 0.03	71.7 ± 3.0	12
R216L variant			
D-Asp	0.91 ± 0.02	22.9 ± 0.9	40
NMDA	1.00 ± 0.02	19.1 ± 0.8	52
R237Y variant			
D-Asp	1.49 ± 0.05	8.90 ± 0.86	167
NMDA	1.85 ± 0.07	7.00 ± 0.87	265
D-Glu	0.36 ± 0.01	80.5 ± 3.7	4

The apparent k_{cat} and K_m values for D-Asp, NMDA, or D-Glu were determined as described in "Materials and methods" and are listed with the standard deviation

Taken together, the results of the kinetic analysis described above suggest that Arg-216 of mouse DDO plays crucial role(s) in both substrate specificity and catalytic activity, while Arg-237 appears to be only marginally involved in the binding affinity for acidic D-amino acids, but is important for catalytic activity. However, the K_m values of the R216L-R237Y variant for D-Asp and NMDA were significantly higher than those of the R216L variant (Table 3), suggesting that the introduction of the R237Y substitution into the R216L variant reduces its binding affinity for acidic D-amino acids. In addition, the K_m values of the R216L-R237Y variant for D-Phe and D-His were significantly lower than those of the R216L variant (Table 4). Thus, the introduction of the R237Y substitution affects the substrate-binding affinity of the R216L variant more appreciably than that of the wild-type enzyme. It is conceivable that the side-chain guanidino group of Arg-216 interacts with the β-carboxyl group of acidic D-amino acids and is essential for the correct substrate orientation within the active site (Fig. 1d). In the R237Y variant, the Arg-216 residue presumably plays a critical role in substrate recognition, and the R237Y substitution may not significantly affect the enzyme interaction with acidic D-amino acids, while in the R216L-R237Y variant, in which the side-chain guanidino group at position 216 is missing and therefore the strict specificity for acidic D-amino acids is decreased, introduction of the R237Y substitution presumably reduces the substrate-binding affinity.

Table 4 Apparent steady-state kinetic parameters of purified recombinant proteins with D-Phe and D-His as substrates

Enzymes and substrates	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
Human DAO[a]			
D-Phe	9.89 ± 0.22	2.59 ± 0.21	3,823
D-His	0.68 ± 0.02	4.18 ± 0.54	162
Mouse DAO			
D-Phe	5.42 ± 0.45	24.2 ± 4.1	224
D-His	0.46 ± 0.01	45.8 ± 1.0	10
R216L-R237Y variant			
D-Phe	1.70 ± 0.05	13.5 ± 0.8	126
D-His	1.50 ± 0.01	3.36 ± 0.10	447
R216L variant			
D-Phe	0.75 ± 0.05	44.4 ± 4.6	17
D-His	0.36 ± 0.01	16.4 ± 1.5	22

The apparent k_{cat} and K_m values for D-Phe and D-His were determined as described in the "Materials and methods" and are listed with the standard deviation

[a] Katane et al. (2010)

Characterization of the sensitivity of the mouse DDO variants to DDO and DAO inhibitors

To further study the role played by Arg-216 and Arg-237 in the substrate specificity of mouse DDO, the effects of *meso*-tartrate and benzoate on the enzymatic activities of the wild-type and variant proteins were examined. Benzoate and *meso*-tartrate reportedly inhibit mammalian DAO and DDO, respectively, by competing with their substrates (De Marco and Crifò 1967; Klein 1960). As expected, *meso*-tartrate dose-dependently inhibited the activity of wild-type mouse DDO against D-Asp, while benzoate had no effect, even at a concentration of 10 mM (Fig. 3a). In contrast, and as expected, benzoate dose-dependently inhibited the activity of both human and mouse DAO against D-Phe, while *meso*-tartrate had no effect, even at a concentration of 10 mM (Fig. 3b, c).

Figure 3d–g indicate that the activity of the R216L-R237Y and R216L variants against D-Asp and D-Phe was dose-dependently inhibited by *meso*-tartrate. Furthermore, the activity of these variants against D-Asp and D-Phe was also dose-dependently inhibited by benzoate, although the sensitivity was significantly less than that of human or mouse DAO. Thus, benzoate sensitivity was increased in the R216L-R237Y and R216L variants compared to the wild-type enzyme, consistent with the expanded substrate specificities of the R216L-R237Y and R216L variants for several neutral and basic D-amino acids (Table 2).

As shown in Fig. 3h, the activity of the R237Y variant against D-Asp was dose-dependently inhibited by *meso*-tartrate more efficiently than that of the wild-type enzyme. In addition, the activity was also inhibited by 10 mM benzoate (a mammalian DAO inhibitor) suggesting the involvement of Arg-237 in substrate recognition. To further investigate the role of Arg-237 in substrate specificity, the effects of malonate and crotonate on the enzymatic activity of the R237Y variant were compared with those of the wild-type enzyme. Malonate and crotonate reportedly inhibit mammalian DDO and DAO, respectively, by substrate competition (Dixon and Kenworthy 1967; Fonda and Anderson 1968). The 50% inhibitory concentration (IC_{50}) values of wild-type mouse DDO and the R237Y variant with *meso*-tartrate, malonate, benzoate, and crotonate as inhibitors are listed in Table 5. The R237Y variant was more sensitive to *meso*-tartrate, benzoate and crotonate, while its sensitivity to malonate was comparable to that of the wild-type enzyme. These results indicate that Arg-237 is possibly involved in substrate recognition by mouse DDO.

The present study demonstrates that the Arg-216 residue plays crucial role(s) in the substrate specificity of mouse DDO. Both Arg-216 and Arg-237 are conserved in all the mammalian DDO that has been cloned to date. The structurally equivalent residue to Arg-216 in mammalian DAOs is Leu or Ile. The simulated annealing docking results of D-Asp in the wild-type mouse DDO active site suggest that the side-chain guanidino group of Arg-216 interacts with the β-carboxyl group of acidic D-amino acids, and is essential for the correct substrate orientation in the active site (Fig. 1d). It is proposed that D-Phe can bind to the mouse DDO active site in the correct orientation in case a side-chain guanidino group is missing at position 216 (Fig. 2). With these results in mind, an R216L substitution was made in mouse DDO that permitted the modification of its substrate specificity. The Arg-216 residue of mouse DDO may function as an essential scaffold for substrate recognition and binding, and therefore is catalytically important for the full enzymatic activity against acidic D-amino acids. This is consistent with a recent report in which the short stretch region (Ile-215–Asn-225) of pig DAO was substituted with the corresponding human DDO short stretch region (Arg-216–Gly-220) (Setoyama et al. 2006). This pig DAO variant acquired activity against D-Asp, together with considerably decreased normal activity against D-Ala and D-Arg.

The present study also suggests that Arg-237 is catalytically important for the full enzymatic activity of mouse DDO against acidic D-amino acids, but is only marginally involved in the substrate-binding affinity. Nevertheless, the introduction of the R237Y substitution into mouse DDO made it more sensitive to several DDO and DAO inhibitors (Fig. 3; Table 5). The additional introduction of the R237Y substitution into the R216L variant increased its catalytic efficiency for D-Phe and D-His (Table 4), and decreased its

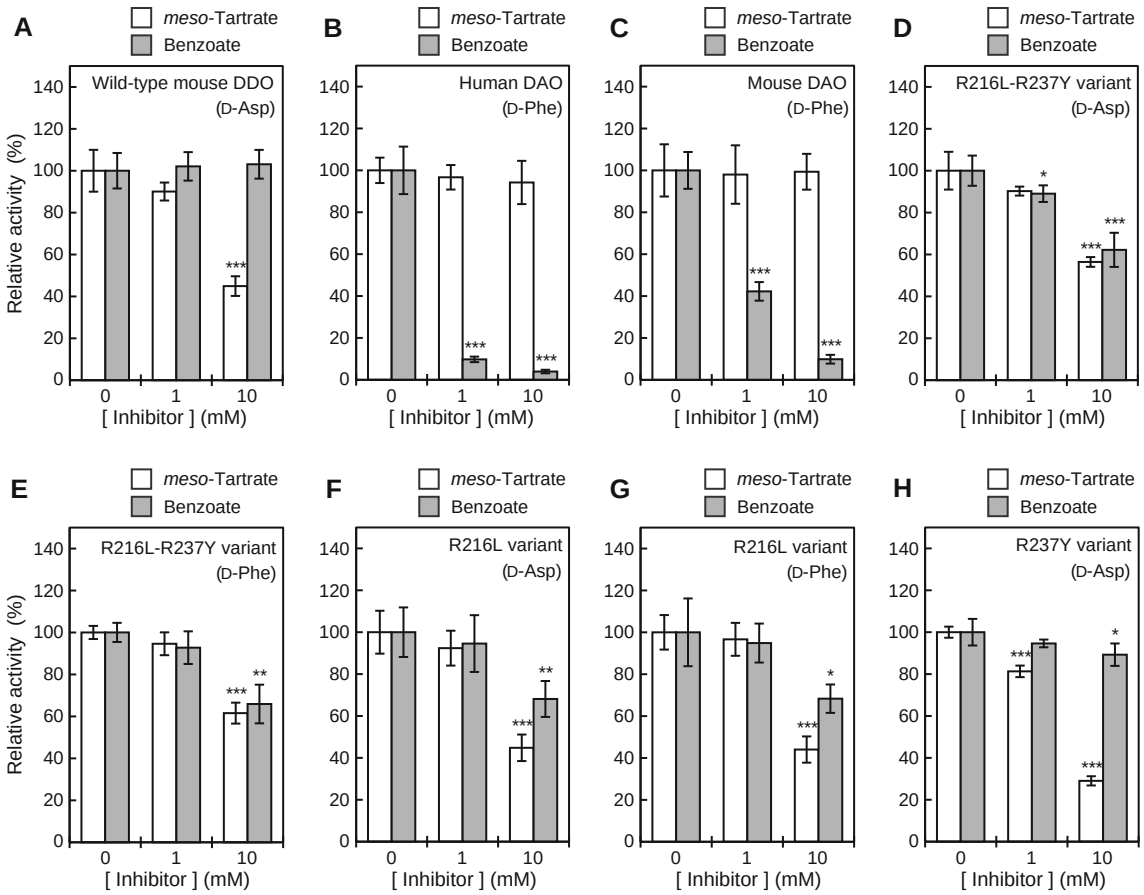

Fig. 3 Effects of inhibitors on the enzymatic activities of recombinant mouse DDO and its variant derivatives. Enzymatic activities were assayed using recombinant mouse DDO (**a**), recombinant human DAO (**b**), recombinant mouse DAO (**c**), the R216L-R237Y variant of mouse DDO (**d** and **e**), the R216L variant of mouse DDO (**f** and **g**), and the R237Y variant of mouse DDO (**h**), in the presence of the indicated concentrations of *meso*-tartrate (*open bar*) and benzoate (*gray bar*). The substrates used were: D-Asp for **a**, **d**, **f** and **h**; and D-Phe for **b**, **c**, **e** and **g**. The activities of the recombinant proteins in the presence of the inhibitors are shown as a percentage of their activities in the absence of inhibitors. Data represent the mean ± standard deviation of three to four independent assays. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Student's *t* test) versus activity in the absence of inhibitor

Table 5 Comparison of IC_{50} values of purified recombinant mouse DDO and the R237Y variant with several monocarboxylic and dicarboxylic acids as inhibitors

Inhibitors	IC_{50} (mM)	
	Wild-type mouse DDO	R237Y variant
meso-Tartrate	8.99 ± 0.41	6.39 ± 0.11***
Malonate	5.31 ± 0.25	5.60 ± 0.14
Benzoate	69.5 ± 6.8	43.3 ± 6.6**
Crotonate	25.3 ± 2.3	19.7 ± 1.5*

Data are shown as the mean ± standard deviation of three independent assays. The substrate used was D-Asp

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Student's *t* test) versus wild-type mouse DDO

catalytic efficiency for D-Asp and NMDA (Table 3). Thus, the Arg-237 residue in mouse DDO is likely to play subsidiary role(s) in substrate specificity, although its precise role(s) in catalysis remains undefined. Additional studies to elucidate the crystal structure of wild-type mouse DDO and its variants are certainly needed.

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