X-ray structure analysis of a unique D-amino-acid oxidase from the thermophilic fungus *Rasamsonia emersonii* strain YA

Yuya Shimekake,^a Yuki Hirato,^b Rikako Funabashi,^b Sayoko Okazaki,^b Masaru Goto,^b Takehiro Furuichi,^a Hideyuki Suzuki,^a Yoshio Kera^a and Shouji Takahashi^{a*}

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^aDepartment of Bioengineering, Nagaoka University of Technology, 1603-1 Kamitomioka, Nagaoka, Niigata 940-2188, Japan, and ^bDepartment of Biomolecular Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan.

*Correspondence e-mail: shoutaka@vos.nagaokaut.ac.jp

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D-Amino-acid oxidases (DAAOs) catalyze the oxidative deamination of neutral and basic D-amino acids. The DAAO from the thermophilic fungus *Rasamsonia emersonii* strain YA (ReDAAO) has a high thermal stability and a unique broad substrate specificity that includes the acidic D-amino acid D-Glu as well as various neutral and basic D-amino acids. In this study, ReDAAO was crystallized by the hanging-drop vapor-diffusion method and its crystal structure was determined at a resolution of 2.00 Å. The crystal structure of the enzyme revealed that unlike other DAAOs, ReDAAO forms a homotetramer and contains an intramolecular disulfide bond (Cys230–Cys285), suggesting that this disulfide bond is involved in the higher thermal stability of ReDAAO. Moreover, the structure of the active site and its vicinity in ReDAAO indicates that Arg97, Lys99, Lys114 and Ser231 are candidates for recognizing the side chain of D-Glu.

1. Introduction

D-Amino-acid oxidases (DAAOs; EC 1.4.3.3) catalyze the oxidative deamination of basic and neutral D-amino acids. Conversely, acidic D-amino acids are deaminated by D-aspartate oxidase (DDO; EC 1.4.3.1). These enzymes belong to the FAD-dependent oxidoreductase family and share amino-acid sequence homology. Because of its strict stereospecificity, DAAO is useful for various practical applications, such as the determination of D-amino acids (Sacchi *et al.*, 1998), production of 7-aminocephalosporanic acid (7-ACA; Henderson *et al.*, 2008), which is a starting material for antibiotics, and production of α -keto acids (García-García *et al.*, 2008). DAAO has been identified in various organisms ranging from bacteria to humans (Tishkov & Khoronenkova, 2005; Takahashi *et al.*, 2015), and crystal structures of DAAOs from the mesophilic fungus *Rhodotorula gracilis* (RgDAAO; PDB entry 1c0p; Umhau *et al.*, 2000), pig kidney (pkDAAO; PDB entries 1kif and 1ve9; Mattevi *et al.*, 1996; Mizutani *et al.*, 1996) and humans (hDAAO; PDB entry 2du8; Kawazoe *et al.*, 2006) have been determined. In addition to DAAO, the crystallization of pig DDO (Senda *et al.*, 2012) and the crystal structure of a human DDO variant (PDB entry 6rkf; Molla *et al.*, 2020) have been reported.

Rasamsonia emersonii is a thermophilic fungus belonging to the phylum Ascomycota that is used as a source for the isolation of highly thermally stable enzymes (Collins *et al.*, 2007). We recently isolated and characterized the DAAO from

Table 1

Macromolecule-production information.

Source organism	<i>R. emersonii</i> strain YA
DNA source	Synthetic
Expression vector	pET-15b
Expression host	<i>E. coli</i> BL21(DE3) pLysS
Complete amino-acid sequence of the construct produced	MATNNIVVLGAGVSGLTAWLLSKDPSNKI TVAAKHMPGDYDIEYCSPWAGANYLPVG AENSRVGQWERATWPHLRDIAQNHPEAG IHFQDTVYVNRKDGSTTGQWFSELVK PNPWYGVLPNPFRELSKDELPPGIDNAN RFTSVCINTAVYLPWLVGQCRKNGVVFKE RAVFKHVAEAAAHSGQKADLVVNCTG LSSRKLGGVQDNTLLPARGQIVVVRNDP GLMCSISGTDGDDDEVYMMTRAAGGTT ILGGTYQKHNDWSDPDPNLAVRIMKRCI ELCPSLVAPGQIEGLDIIIRHGVGLRPV REDGPRIEKELIDGVVVVHNYGHGGYGY QTSFGCATTAVEVVREALQQQKQRDKA RL

R. emersonii strain YA (ReDAAO; Shimekake *et al.*, 2019). ReDAAO exhibits a high thermal stability; the T_{50} values of ReDAAO, RgDAAO and pkDAAO are 60, 39 and 44°C, respectively (Shimekake *et al.*, 2019; Pollegioni *et al.*, 2004). Moreover, ReDAAO exhibits high activities towards not only various neutral and basic D-amino acids, but also the unusual substrates of DAAO D-Glu and cephalosporin C. These properties are valuable for various practical applications such as the production of 7-ACA and the determination of various D-amino acids, including D-Glu. However, the detailed mechanisms underlying these properties remain unknown because the three-dimensional structure of ReDAAO has not been determined. In this study, in order to obtain insights into these mechanisms, the X-ray crystal structure of ReDAAO was analyzed.

2. Materials and methods

2.1. Macromolecule production

The expression of the *ReDAAO* gene and the purification of the gene product were performed as described previously (Shimekake *et al.*, 2019). Briefly, the ReDAAO cDNA on the pET-15b vector was introduced into *Escherichia coli* BL21(DE3) pLysS cells. The *E. coli* cells were cultivated in LB medium containing 100 µg ml⁻¹ ampicillin and 34 µg ml⁻¹ chloramphenicol at 310 K for 2.5 h. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM and the cells were further cultivated at 310 K for 4 h. The cells were collected and resuspended in 50 mM potassium phosphate buffer pH 8.0. The cells were disrupted by sonication using a UD-201 sonicator (Tomy Seiko, Tokyo, Japan). The supernatant was passed through a 0.45 µm membrane filter (Omnipore; Millipore, Billerica, Massachusetts, USA). Imidazole was added to the supernatant to a final concentration of 10 mM. The supernatant was applied onto a TALON metal-affinity column (Clontech, Mountain View, California, USA). The column was washed once with 50 mM potassium phosphate buffer pH 8.0 containing 300 mM NaCl and 30 mM imidazole, and the bound proteins were eluted

Table 2

Crystallization.

Method	Hanging-drop vapor diffusion
Plate type	24-well plate
Temperature (K)	295
Protein concentration (mg ml ⁻¹)	2.0
Buffer composition of protein solution	50 mM potassium phosphate pH 8.0
Composition of reservoir solution	17.4%(w/v) PEG 1540, 240 mM sodium formate, 120 mM Tris-HCl pH 8.5
Volume and ratio of drop	6 µl (2:1 protein:reservoir)
Volume of reservoir (µl)	100

with the same buffer containing 150 mM imidazole. The purified protein was dialyzed against 50 mM potassium phosphate buffer pH 8.0. The protein concentration was determined using the Bradford method (Bradford, 1976). The homogeneity of the purified protein was confirmed by SDS-PAGE analysis, and the electrophoresis image was analysed using the *ImageJ* software (Schneider *et al.*, 2012). Information pertaining to production of the macromolecule is summarized in Table 1.

2.2. Crystallization

Crystallization experiments were carried out by the hanging-drop vapor-diffusion method at 295 K. The initial screening was performed using the commercially available kits Crystal Screen and PEG/Ion (Hampton Research, USA). ReDAAO solution (2.0 mg ml⁻¹) and reservoir solution were mixed in a drop and equilibrated against 100 µl reservoir solution. The ratio of protein solution to reservoir solution in the drop was 1:1 or 2:1. Initial crystals grew after 12 days in 100 mM Tris-HCl pH 8.5, 180 mM sodium formate, 18%(w/v) PEG 3350. Optimization of this condition led to crystals that were grown by hanging-drop vapor diffusion at 295 K by mixing 4 µl protein solution (2 mg ml⁻¹ in 50 mM potassium phosphate pH 8.0) with 2 µl reservoir solution [120 mM Tris-HCl pH 8.5, 240 mM sodium formate, 17.4%(w/v) PEG 1540] and equilibrating the drop against 100 µl reservoir solution. A single ReDAAO crystal was transferred to cryoprotectant solution [reservoir solution containing 20%(v/v) PEG 400] and flash-cooled. Information pertaining to crystallization is summarized in Table 2.

2.3. Data collection and processing

Diffraction data were collected using a PILATUS3 S6M detector on beamline BL-5A at the Photon Factory (PF), Tsukuba, Japan. The diffraction data were processed using *XDS* (Kabsch, 2010), *POINTLESS* (Evans, 2006, 2011) and *SCALA* (Evans, 2006) from the *CCP4* suite (Winn *et al.*, 2011). Details of the parameters used for data collection and processing are summarized in Table 3.

2.4. Structure solution and refinement

Molecular replacement was performed using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* suite with the structure of RgDAAO (PDB entry 1c0i; Pollegioni *et al.*, 2002)

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL-5A, PF, KEK
Wavelength (Å)	1.000000
Temperature (K)	95
Detector	PILATUS 3S 6M
Crystal-to-detector distance (mm)	336.7
Rotation range per image (°)	0.2
Total rotation range (°)	180
Exposure time per image (s)	2.0
Space group	$P6_1$
a, b, c (Å)	79.13, 79.13, 366.67
α, β, γ (°)	90, 90, 120
Mosaicity (°)	0.18
Resolution range (Å)	45.83–2.00 (2.11–2.00)
Total No. of reflections	885584
No. of unique reflections	87291
Completeness (%)	100 (100)
Multiplicity	10.1 (10.0)
$\langle I/\sigma(I) \rangle$	13.6 (2.2)
$R_{\text{r.i.m.}}$	0.105 (1.178)
Overall B factor from Wilson plot (Å ²)	38.5

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	50.00–2.000 (2.052–2.000)
Completeness (%)	99.980 (99.753)
σ Cutoff	0
No. of reflections, working set	82916
No. of reflections, test set	4229
Final R_{cryst}	0.206 (0.210)
Final R_{free}	0.246 (0.223)
Cruickshank DPI	0.0471
No. of non-H atoms	
Protein	10390
Ligand	212
Water	367
Total	10969
R.m.s. deviations	
Bonds (Å)	0.0081
Angles (°)	1.3215
Average B factors (Å ²)	
Protein	43.052
Ligand	35.177
Water	43.215
Ramachandran plot	
Most favored (%)	98.0
Allowed (%)	2.0

as a search model. Refinement was performed using *REFMAC5* (Vagin *et al.*, 2004) from the *CCP4* suite and *Coot* (Emsley *et al.*, 2010). The determined structure of ReDAAO was illustrated and rendered using the *CCP4mg* software version 2.10.11 (McNicholas *et al.*, 2011). The refinement data are summarized in Table 4.

3. Results and discussion

3.1. Overall structure of ReDAAO

We expressed the *ReDAAO* gene in *E. coli* and purified the gene product as reported previously (Shimekake *et al.*, 2019). SDS-PAGE analysis of the purified protein revealed a single band with an apparent molecular mass of 43 kDa, which

corresponds to the calculated molecular mass of histidine-tagged ReDAAO, showing that the recombinant ReDAAO was highly purified (>95%).

We obtained a hexagonal crystal of ReDAAO using the hanging-drop vapor-diffusion method at 295 K after one week (Fig. 1). The diffraction data were collected and processed to a resolution limit of 2.00 Å. The crystal belonged to space group $P6_1$, with unit-cell parameters $a = b = 79.13$, $c = 366.67$ Å. The asymmetric unit contains four molecules of ReDAAO. The structure was solved by molecular replacement using a monomer of RgDAAO (PDB entry 1c0i; Pollegioni *et al.*, 2002) as a template, as ReDAAO showed the highest amino-acid sequence identity (30.6%) to RgDAAO in a homology search against the PDB using *BLASTP*. The domain and secondary structure of ReDAAO were determined (Figs. 2*a* and 2*b*) by comparison with pkDAAO and RgDAAO (Mattevi *et al.*, 1996; Pollegioni *et al.*, 2002). The constitution of the secondary structure was very similar among these DAAOs. The subunit of ReDAAO is composed of a flavin-binding domain [five α -helices (α F1– α F5) and six β -strands (β F1– β F6)] and an interface domain [two α -helices (α I1 and α I3) and eight β -strands (β I1– β I8)]. The amino-acid residues (Gln101–Lys114) located between β I1 and β I2 were not interpretable because of disorder of the electron density (Figs. 2*a* and 2*b*). The final structure was refined to an R_{cryst} and R_{free} of 20.6% and 24.6%, respectively. The atomic coordinates and structure factors have been deposited in the Protein Data Bank (PDB) with accession code 7ct4 (details are provided in Table 4). The asymmetric unit contains four molecules of ReDAAO. There were no significant differences between the backbone structures of the four molecules in the asymmetric unit, as shown by r.m.s.d. values ranging from 0.30 to 0.46 Å for the superposition of 335–341 common C atoms between each combination of monomer pairs.

Structural analysis using *PDBEPIA* (Krissinel & Henrick, 2007) suggested that ReDAAO forms a homotetramer, which

Figure 1

Crystal of ReDAAO. Crystallization was performed by the hanging-drop vapor-diffusion method using 17.4%(w/v) PEG 1540, 240 mM sodium formate, 120 mM Tris-HCl pH 8.5 at 295 K. The scale bar represents 100 μm.

Figure 2

(a) The secondary, (b) subunit and (c) overall structures of ReDAAO and (d) the difference in the formation of a head-to-tail dimer between ReDAAO and RgDAAO. (a) The constitution of the secondary structure of ReDAAO was determined by comparison with pkDAAO and RgDAAO (Mattevi *et al.*, 1996; Pollegioni *et al.*, 2002) using *PDBFold* (Krissinel & Henrick, 2004) and *ESPrpt* 3.0 (Robert & Gouet, 2014). Arrows indicate β -strands (β). Squiggles represents α -helices (α). The α -helices and β -strands were numbered based on RgDAAO and pkDAAO. The α -helices and β -strands of the flavin-binding domain and the interface domain are abbreviated αF and αI , and βF and βI , respectively. An α -helix corresponding to $\alpha I2$ of RgDAAO and pkDAAO was not found in ReDAAO via the determination of its secondary structure using *ESPrpt* 3.0. The interfaces of subunits A–B, A–C and A–D are enclosed in brown, magenta and green squares, respectively. The unfilled triangles indicate cysteine residues that form a disulfide bond. The filled triangles indicate amino-acid residues that are predicted to interact with the side chain of D-Glu. (b) ReDAAO is shown in cartoon form (color scheme: flavin-binding domain, blue; interface domain, green). The bound FAD and Cys230–Cys285 are shown as spheres (color scheme: FAD, orange; S and other atoms of Cys230–Cys285, yellow and green). (c) The structure is a homotetramer with an FAD in each subunit (cartoon/surface: subunit A, cyan; subunit B, light brown; subunit C, magenta; subunit D, light green). (d) The figures show the differing head-to-tail dimer formation in ReDAAO and RgDAAO. Left, superposition of subunit A of ReDAAO and RgDAAO; center, a head-to-tail dimer composed of the A and B subunits of ReDAAO; right, a head-to-tail dimer composed of the A and B subunits of RgDAAO. ReDAAO and RgDAAO are shown in backbone form. Color scheme: A and B subunits of ReDAAO, cyan (A) and light brown (B); A and B subunits of RgDAAO, blue (A') and gray (B'). The images were illustrated and rendered by *CCP4mg* version 2.10.11 (McNicholas *et al.*, 2011).

Figure 3

Comparison of the active site of (a) ReDAAO and (b) RgDAAO bound to D-Ala (PDB entry 1c0p). ReDAAO and RgDAAO are shown in cartoon form (color scheme: flavin-binding domain, light blue; interface domain, green). The atom color scheme of DAAOs, FAD and D-Ala is as follows: carbon, light blue (flavin-binding domain), green (interface domain), orange (FAD) and pink (D-Ala); nitrogen, blue; oxygen, red; sulfur, yellow. A water molecule is shown as a red sphere. The active-site lid between β I1 and β I2 (Arg97–Pro117) of ReDAAO and the corresponding positions of RgDAAO are shown in gray cartoon form. The interactions between the amino-acid residues of RgDAAO, D-Ala and water are shown as dotted lines. The images were illustrated and rendered by CCP4mg version 2.10.11 (McNicholas *et al.*, 2011).

is consistent with the result obtained by gel-filtration chromatography (Shimekake *et al.*, 2019). RgDAAO and pkDAAO form homodimers in ‘head [the interface domain (I)]-to-tail [the flavin-binding domain (F)]’ and ‘head-to-head’ modes, respectively (Pollegioni *et al.*, 2002; Mattevi *et al.*, 1996). In RgDAAO, a unique loop between β F5 and β F6, which is not present in ReDAAO and pkDAAO, is responsible for the formation of the head-to-tail homodimer (Figs. 2a and 2b). In pkDAAO, the loop between α I3 and β I8 (seven amino-acid residues) is shorter than that in ReDAAO and RgDAAO (15 amino-acid residues), where the long loop might inhibit their head-to-head interactions by steric hindrance (Figs. 2a and 2b). These structural differences in ReDAAO might result in the formation of a novel head-to-tail dimer that differs from that of RgDAAO, and a tetramer that is a dimer of dimers (Figs. 2c and 2d). One subunit in the tetramer interacts with the remaining three subunits. The surface areas of the subunit interfaces are 1215 Å² for subunits A and B, 536 Å² for subunits A and C, and 197 Å² for subunits A and D.

ReDAAO has a much higher thermal stability than other known DAAOs such as RgDAAO and pkDAAO; the T_{50} values (the temperature at which 50% of the initial enzymatic activity is lost after incubation at a protein concentration of 0.1 mg ml⁻¹) of ReDAAO, RgDAAO and pkDAAO are 60, 39 and 44°C, respectively (Shimekake *et al.*, 2019; Pollegioni *et al.*, 2004). In general, various factors contribute to the structural stability of enzymes, such as oligomerization, hydrophobic interactions, hydrogen bonds, salt bridges and disulfide bonds

(Xu *et al.*, 2020; Eijsink *et al.*, 2004; Vieille & Zeikus, 2001; Pucci & Rooman, 2017). We quantified the intrasubunit hydrophobic interactions, hydrogen bonds and salt bridges in ReDAAO, RgDAAO and pkDAAO using the *Protein Interaction Calculator* (Tina *et al.*, 2007). The number of intrasubunit interactions in ReDAAO was lower than those in other DAAOs, suggesting that the number of intrasubunit interactions is not the factor that contributes to the high thermal stability of ReDAAO. It has been reported that oligomerization contributes to the thermal stability of RgDAAO. The decrease in protein concentration and monomerization caused by deletion of the head-to-tail dimerization loop reduced the thermal stability (Betancor *et al.*, 2003; Pollegioni *et al.*, 2003). A *PDBEPIA* analysis suggested that the Δ^iG (the solvation free-energy gain of a subunit upon formation of the interface) of ReDAAO, RgDAAO and pkDAAO is -18.6, -13.6 and -19.3 kcal mol⁻¹, respectively. These values were not significantly different, suggesting that factor(s) other than oligomerization are involved in the higher thermal stability of ReDAAO. Interestingly, an intramolecular disulfide bond that is not present in other DAAOs was identified in the subunit of ReDAAO (between Cys230 and Cys285; Fig. 2b). Therefore, the disulfide bond might be one of the factors that contribute to the higher thermal stability of ReDAAO. We are currently investigating the contribution of the tetrameric structure, which differs from other homodimeric DAAOs, and of the disulfide bond of ReDAAO to its thermal stability via site-directed mutagenesis.

3.2. The active site

In the active site of ReDAAO, the amino-acid residues Tyr244, Tyr260, Arg308, Gly335 and Gln339 are found on the *re* face of FAD (Fig. 3*a*). Such amino-acid residues are also found in the active sites of RgDAAO (Fig. 3*b*), hDAAO and pkDAAO, and are involved in the interaction with the main-chain moiety of the substrates (Umhau *et al.*, 2000; Kawazoe *et al.*, 2006; Mattevi *et al.*, 1996). In DDOs, a basic amino-acid residue located close to the active-site lid, for example Arg216 in human DDO, plays a critical role in the substrate specificity towards acidic D-amino acids by interacting with the carboxyl side chain in the active site (Molla *et al.*, 2020; Katane *et al.*, 2011). ReDAAO recognizes neutral, basic D-amino acids and the acidic D-Glu as substrates, but not D-Asp (Shimekake *et al.*, 2019). In ReDAAO, Arg97 and Lys99, as well as Lys114 (which was not interpreted because of disorder of the electron density), are located at an active-site lid between β I1 and β I2 (Arg97–Pro117). This is not a conserved region in RgDAAO, suggesting that these basic amino-acid residues play an important role in substrate recognition (Figs. 2*a* and 3*a*).

Alternatively, as the substitution of Met213 in RgDAAO with an arginine residue shifted the activity towards D-Asp (Sacchi *et al.*, 2002) and as in ReDAAO Ser231 is located at the spatially corresponding position to Met213 of RgDAAO and Arg216 of human DDO, Ser231 might interact with the carboxyl side chain of D-Glu by a hydrogen bond (Figs. 3*a* and 3*b*). To date, although there are no reports of the involvement of this serine residue in the substrate specificity towards acidic amino acids in DAAO and DDO, Ser8 in glutamate racemase from *Helicobacter pylori* (PDB entry 2jfx; Lundqvist *et al.*, 2007) and Ser380 in glutamate dehydrogenase from *Clostridium symbiosum* (PDB entry 1bgv; Stillman *et al.*, 1993) are involved in substrate binding through a hydrogen bond to the γ -carboxyl group of D-Glu and L-Glu, respectively. In a previous study, we deduced two amino-acid residues (Arg97 and Ser233) that contribute to the activity against D-Glu using the model structure of ReDAAO (Shimekake *et al.*, 2019). The identification of Lys99 (near Arg97), Lys114 and Ser231 (near Ser233) in this study supports the results of previous research. We are currently investigating the importance of the Arg97, Lys99, Lys114 and Ser231 residues via site-directed mutagenesis.

4. Conclusion

In this study, the crystal structure of ReDAAO was determined at a resolution of 2.00 Å. Unlike other DAAOs, ReDAAO forms a homotetramer and contains a disulfide bond (Cys230–Cys285) within the subunit, which might contribute to its higher thermal stability. The amino-acid residues that are conserved in DAAOs and interact with the α -amino and α -carboxy groups of D-amino-acid substrates are found in the active site of ReDAAO. Moreover, the unique amino-acid residues Arg97, Lys99, Lys114 and Ser231 are also found in the active site or its vicinity, and are suggested to be

involved in the binding and recognition of D-Glu via an interaction with the carboxyl side chain.

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