Journal of Molecular Catalysis B: Enzymatic 61 (2009) 235–240

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

An active-site mutation enhances the catalytic activity of the yeast *Cryptococcus humicola* D-aspartate oxidase

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ARTICLE INFO

Article history:
Received 11 April 2009
Received in revised form 10 July 2009
Accepted 23 July 2009
Available online 3 August 2009

Keywords:
D-Aspartate oxidase
Cryptococcus humicola
FAD enzyme
Site-directed mutagenesis
Yeast

ABSTRACT

D-Aspartate oxidase (ChDDO) of the yeast *Cryptococcus humicola* has a much higher specificity and reactivity to D-aspartate than those from other sources, making it useful for some applications in science and fine-chemical industries. We constructed a three-dimensional model of ChDDO and compared it with the crystal structure of a yeast D-amino acid oxidase. The comparison showed an arginine residue positioned at 243 of ChDDO that was possibly involved in the substrate recognition. The mutation of R243 to alanine abolished the activity, together with a change in the spectral feature of bound flavin and the loss of inhibitor-binding ability, suggesting its importance in the catalytic activity. R243 was further replaced with M(R243M), K(R243K), D(R243D), or E(R243E). These mutants exhibited different spectral features of bound flavin from that of the wild-type enzyme and, except R243D, displayed considerably or slightly lower DDO activities. R243M and R243E could also oxidize the substrates of DAO, albeit with very low activities. In contrast, R243D exhibited approximately two-fold higher DDO activity than that of the wild type with a high substrate specificity to D-aspartate. Kinetic analysis showed that R243D mutant had a 2.5-fold lower K_m and a 1.6-fold higher k_{cat} for D-aspartate compared to the wild type, resulting in approximately four-fold higher catalytic efficiency.

1. Introduction

D-Aspartate oxidase (DDO; EC 1.4.3.1) is a flavin enzyme that catalyzes the oxidative deamination of acidic D-amino acids to hydrogen peroxide and their corresponding imino acids, which are hydrolyzed non-enzymatically to α-keto acids and ammonia [1]. This enzyme is absolutely specific for acidic D-amino acids and is inactive to both neutral and basic D-amino acids, which are substrates for D-amino acid oxidase (DAO; EC 1.4.3.3). Because they share common catalytic properties and primary sequence, they have been considered to be derived from a single precursor sequence [2]. However, structural differences between their active sites, which possibly account for their different substrate specificity, are still uncertain, because the crystal structure of DDO has not been elucidated and only a limited study has been conducted on the active site.

DDO has been characterized and cloned from various eukaryotic organisms, ranging from yeast to human [3–10]. Of these, the enzyme of the yeast *Cryptococcus humicola* (ChDDO) is the only microbial DDO characterized extensively and cloned so far, and has unique enzymatic properties, such as higher substrate specificity toward D-aspartate, a very high turnover number, and tight binding with the coenzyme flavin [6,9]. These properties distinguish it from other DDOs.

Recently, a significant amount of free D-aspartate has been identified in a variety of organisms [11]. Many studies have indicated that the D-amino acid has important physiological functions in various animals. In many invertebrates and vertebrates, D-aspartate occurs in the central nervous tissues and endocrine glands and is suggested to play important roles in hormone release and synthesis, in the development of nerve system, and in neurotransmission [12–18]. In a bivalve mollusk, the amino acid appeared to be involved in anaerobic energy metabolism [19]. The D-amino acid was also shown to be involved in neurological disorders such as Alzheimer's disease [20]. Thus, an accurate and rapid method for determination and quantification of D-aspartate in biological and food samples has become of great importance.

At present, high-performance liquid chromatography is mainly used to analyze free D-aspartate in biological samples [21]. This method is certainly very sensitive and reliable, but is time-consuming, highly specialized, and expensive. Simple and easily applicable analytical methods are therefore needed. In this sense, development of appropriate methods employing biosensors would be very useful, since they could be simple and easily applicable.

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doi:10.1016/j.molcatb.2009.07.011

Because DDO reaction produces hydrogen peroxide and consumes oxygen, it can be applied in a biosensor for D-aspartate with oxygen or peroxide electrode. Among DDOs examined so far, ChDDO is a promising candidate for the biosensor because of its higher specificity and reactivity to D-aspartate [6,9]. Therefore, the improvement of enzymatic properties, such as catalytic activity and stability, is one of the important issues for the practical application of the enzyme. In addition, the improvements may also contribute to other applications of the enzyme, such as the resolution of racemic mixture and the production of α-keto acid from the D-amino acid in fine-chemical industry.

In this study, we constructed a three-dimensional active-site model of ChDDO and compared it with the active site of a yeast DAO. This comparison showed a unique arginine residue positioned at 243 that was likely to interact with its substrates and to be involved in substrate recognition. We mutated R243 to several other amino acids and characterized the substrate specificity and the kinetic and spectroscopic properties of each mutant. These analyses showed that contrary to our expectation, R243 may be important but not crucial for catalytic activity as well as for substrate specificity. Moreover, one of the mutants containing aspartate in place of R243 showed a remarkably higher catalytic efficiency than that of the wild-type enzyme toward D-aspartate with a high specificity. The mutant could be useful in biotechnological applications, especially for determination of D-aspartate in biological samples.

2. Experimental

2.1. Materials

D-Aspartate was a generous gift from Tanabe Pharmaceutical (Osaka, Japan), and other D-amino acids were purchased from Nacalai Tesque (Kyoto, Japan). Restriction endonucleases and other DNA-modifying enzymes were from Takara Bio (Kyoto, Japan), Toyobo (Osaka, Japan) or New England Biolabs (Beverly, MA, USA). Oligonucleotides were synthesized by Invitrogen (Carlsbad, CA, USA). All other chemicals were of analytical reagent grade and were purchased form Wako Pure Chemical (Osaka, Japan) or Nacalai Tesque.

2.2. Bacterial strains and media

Escherichia coli DH5α was used as the host for DNA manipulations. *E. coli* XL1-Blue MRF' was used as the host for single-strand DNA preparation for sequencing. *E. coli* Rosetta (DE3) was used as the host for protein expression. *E. coli* strains were cultured in Luria–Bertani (LB) or Terrific Broth (TB) media [22]. When necessary, ampicillin, chloramphenicol, or both were added to the media at a final concentration of 100 μg/ml and 34 μg/ml, respectively.

2.3. Site-directed mutagenesis

To express ChDDO as a histidine-tagged fusion protein in *E. coli*, *ChDDO* gene (GenBank accession number AB121230) lacking the stop codon was amplified by PCR using Platinum Pfx DNA polymerase (Invitrogen) with pKSCD2 [9] as a template and the primers CD1p (5′-CACCCATATGCCCCCCT-CCGACCCATCATCG-3′, NdeI site underlined) and DDO-SBp (5′-TCTAAAGCTTCAGCCGTGCACCCCCAGCCCCC-3′, HindIII site underlined). The amplified fragment was digested with NdeI and HindIII and cloned into the same sites of pET25b (+) (Novagen, Madison, WI, USA) to generate pECD25b, which encodes the wild-type ChDDO with six histidine residues at the carboxy terminus.

Site-directed mutagenesis was performed by the megaprimer PCR method [23]. For the mutagenesis, two different plasmid DNA templates were constructed as follows. pKSCD2 was digested by SalI and religated to obtain pKSCD2R, in which *ChDDO* gene is inserted in the opposite orientation of that in pKSCD2. pKSCD2R was then digested with XhoI or PstI and the resulting large fragment was self-ligated to obtain plasmids containing *ChDDO* gene lacking the 5′ XhoI (template 1) or 3′ PstI (template 2) fragment, respectively. In the first PCR reaction, megaprimer was synthesized using a mutagenic primer and DDO-SBp as the 3′ primer and the template 1 under the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min, and a final extension at 68 °C for 5 min. The following mutagenic primers were used (mutated codons are underlined): for R243A, 5′-GAGGGCGGCACGGCTACGTACATTATCC-3′; R243M, 5′-GAGGGCGGCACGATGACGTACATTATCC-3′; R243D, 5′-GAGGGCGGCACGGACACGTACATTATCC-3′; R243E, 5′-GAGGGCGGCACGGAAACGTACATTATCC-3′; R243K, 5′-GAGGGCGGCACGAAAACGTACATTATCC-3′. Second PCR reaction was performed using an aliquot (3 μl) of the first PCR mixture and the template 2 under the following conditions: 94 °C for 3 min, followed by 5 cycles of 94 °C for 1 min, 58 °C for 1 min, 68 °C for 1 min, and a final extension at 68 °C for 5 min, and then DDO-SBp as the 5′ primer and CD1p as the 3′ primer were added to the PCR mixture and the full-length mutated *ChDDO* gene was amplified with the following conditions: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 68 °C for 1 min, and a final extension at 68 °C for 5 min. All the PCR reactions were performed using Platinum Pfx DNA polymerase. The amplified fragment was digested with NdeI and HindIII and cloned into the same sites of pET25b (+). All mutations were confirmed by sequencing both strands using an ALF express II DNA sequencer (Amersham Bioscience, Uppsala, Sweden) with a Thermo Sequenase cycle sequencing kit with 7-deaza-dGTP (Amersham Bioscience).

2.4. Expression and purification of ChDDO mutants in E. coli

E. coli Rosetta (DE3) harboring the wild type or mutant *ChDDO* genes was cultured in 5 ml of LB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol at 30 °C for 12 h with shaking at 160 rpm. Five hundred microliters of the cultures were inoculated into 100 ml of TB medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol and continued growing at 30 °C with reciprocal shaking at 200 rpm until the OD_{600} reached 0.5. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to the culture medium at a final concentration of 1 mM and incubation continued for another 6 h at 30 °C. *E. coli* cells were harvested by centrifugation at 3000 × *g* at 4 °C, washed twice with 50 mM Tris–HCl buffer, pH 8.0, containing 2 mM ethylenediaminetetraacetic acid (EDTA) and kept at −80 °C until use.

All purification procedures were carried out on ice or at 4 °C. The washed cells (approximately 1 g of wet cell weight) were resuspended in lysis buffer [50 mM Tris–HCl buffer, pH 8.0, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and disrupted by sonication. The homogenate was clarified by centrifugation at 20000 × *g* for 30 min, and the resulting supernatant was filtrated with a 0.2 μm membrane filter (Sartrius, Gottingen, Germany). After imidazole was added at a final concentration of 40 mM, the supernatant was then loaded onto a 1 ml Talon metal affinity column (Clontech, PaloAlto, CA) pre-equilibrated with wash buffer [25 mM Tris–HCl, pH 7.0, 0.5 M NaCl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol]. The column was washed with the wash buffer containing 40 mM imidazole and bound proteins were eluted with the wash buffer containing 60 mM imidazole. The eluate was concentrated using an Amicon Ultra-15 concentrator (10 kDa molecular-mass cutoff, Millipore, Bedford, MA, USA). After filtration with a 0.45 μm membrane filter (Sartrius), the concentrated solution was applied onto a Superdex 200HR 10/30 gel filtration column (Amersham Bioscience, Uppsala, Sweden) pre-equilibrated

with 20 mM potassium phosphate buffer, pH 7.0 and run in the same buffer at a flow rate 0.25 ml/min. The fractions containing ChDDO protein were collected and concentrated with an Amicon Ultra-15 concentrator. The homogeneity of the purified proteins was confirmed by SDS-PAGE.

2.5. Enzyme and protein analyses

The oxidase activity toward D-amino acids except D-arginine was assayed by colorimetric measurement of keto acids produced from D-amino acids as described previously [6]. The activity for D-arginine was determined by peroxidase/*o*-dianisidine assay [3]. One unit of activity corresponds to the oxidation of 1 μmol of D-amino acids per min. Protein concentration was determined by the method of Lowry et al. [24] using bovine serum albumin as a standard. SDS-PAGE was performed on 12% polyacrylamide gels according to Laemmli [25] and proteins were stained with Coommasie brilliant blue R-250 solution in a mixture of 50% (v/v) methanol and 10% (v/v) acetic acid. For Western blotting, proteins in the gels were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Immuno-Blot PVDF; Bio-Rad, Hercules, CA, USA) using a semidry trasnsblot apparatus (Transblot SD; Bio-Rad). ChDDO protein was detected with a polyclonal rabbit antiserum raised against ChDDO as the primary antibody and alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) as a second antibody using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium as substrates. Absorption spectra were measured with a UV-2500PC spectrophotometer (Shimadzu, Kyoto, Japan). Wavelength scans (300–550 nm) were carried out at 20 °C with wild type and mutant proteins (0.6 mg/ml) in 20 mM potassium phosphate buffer, pH 7.0, in a 1 cm-path length quartz cuvette. All protein spectra were baseline-corrected by subtracting buffer spectra.

2.6. Computer analysis

A three-dimensional active-site model of ChDDO was generated using SWISS-MODEL protein modeling server (http://swissmodel.expasy.org/) based on the crystal structures of *Rhodotorula gracilis* DAO (Protein Data Bank accession number, 1c0kA) and pig kidney DAO (1kifC), and viewed in DeepView (http://au.expasy.org/spdbv/).

3. Results and discussion

3.1. Comparison of the active site of ChDDO with that of a yeast DAO

To understand the substrate binding of ChDDO, we searched unique amino acid residues in the active site. We first constructed a three-dimensional active-site model of ChDDO and then compared it with the crystal structure of DAO of the yeast *Rhodotorula gracilis* (RgDAO) (Fig. 1). The comparison showed that some of the amino acid residues involved in the substrate binding of RgDAO were found at the structurally equivalent positions in ChDDO. In RgDAO, Y223, Y238, and R285 are hydrogen bonded to the α-carboxyl group and S335 and Q339 are to the α-amino group of amino acid substrates (Fig. 1b) [26]. These amino acid residues were expected to be spatially equivalent to Y245, M260, R306, G344, and Q348, respectively, in ChDDO (Fig. 1a), suggesting their involvement in the substrate binding of ChDDO as shown in the yeast DAO. On the other hand, the amino acid residues located on the βI5-strand-loop-βI6 strand of RgDAO (T212-A222) and the corresponding region of ChDDO (S234-T244) were largely different between them (Fig. 1c). It has been reported that the mutation of M213 on βI5-strand of RgDAO to arginine gave the oxidase activity toward D-aspartate [27]. Thus the difference in these regions seems likely to be implicated in the difference in their substrate specificity. In addition, the corresponding region of porcine kidney DAO contains a loop called "active-site lid" that opens and closes upon substrate/product migration in and out of the active site (Fig. 1c) [28–31], and is also suggested to be involved in substrate specificity [30,32]. We, therefore, postulated a crucial role of the regions in substrate specificity. In this region of ChDDO, an arginine residue, Arg243, was likely to occupy the spatially equivalent position of M213 of RgDAO (Fig. 1). Besides, the residue appeared unique in

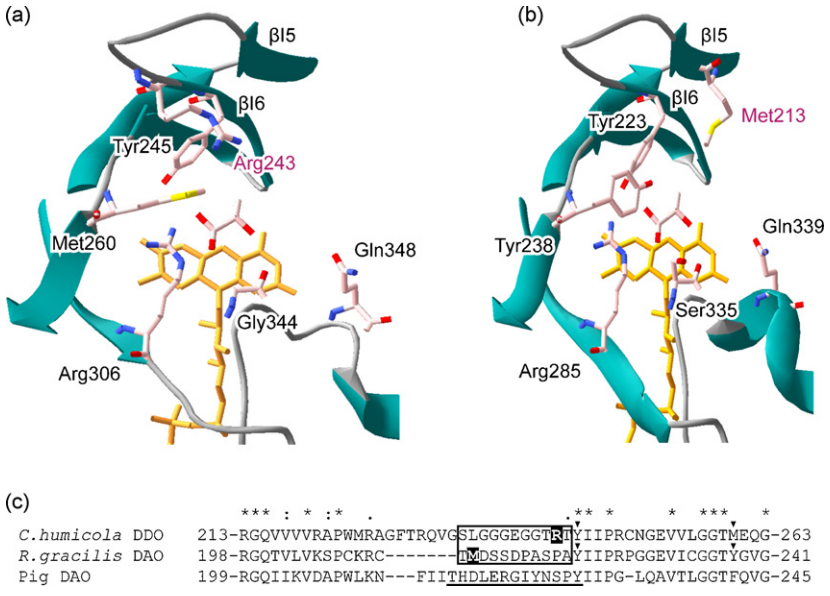

Fig. 1. Comparison of an active-site model of ChDDO (a) with the active site of RgDAO (b). The model of ChDDO was generated by the SWISS-MODEL server and the Swiss-PdB Viewer program using the crystal structures of RgDAO (PDB ID codes 1c0kA) with L-lactate and pig DAO (1kifC) as templates. Atoms of amino acid residues labeled and L-lactate are colored pink for carbon, red for oxygen, blue for nitrogen, and yellow for sulfur. FAD molecules are colored orange. (c) Comparison of amino acid residues located on βI5-strand-loop-βI6-strand [26] overlying the side chain of substrates in ChDDO and RgDAO. A part of the β-strand-loop-β-strand was boxed. R243 of ChDDO and M213 of RgDAO were indicated by reversed letter and the other amino acid residues indicated in the active sites were marked with triangles. The loop called "active-site lid" in pig kidney DAO was underlined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

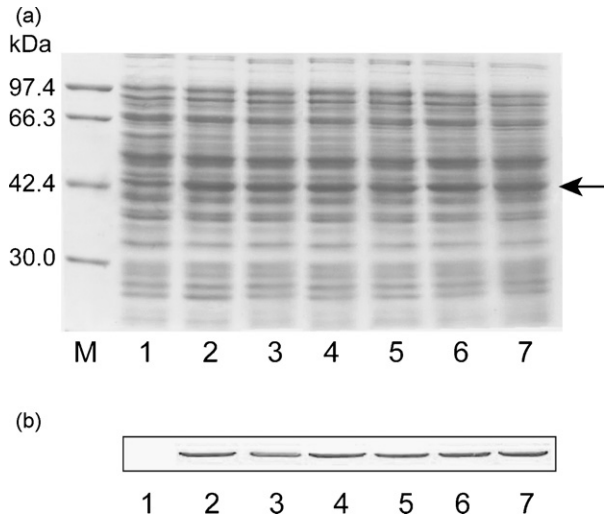

Fig. 2. SDS-PAGE (a) and Western blot (b) analyses of soluble extracts of *E. coli* cells expressing the histidine-tagged wild type or R243 mutants of ChDDO. Lane M: marker proteins; lane 1: empty vector; lane 2: wild type; lane 3: R243A; lane 4: R243M, lane 5: R243E; lane 6: R243D; lane 7: R243K. Five micrograms of each protein was analyzed. The arrow indicates the position of the wild type and mutants expressed.

that it could possibly interact with β-carboxyl group of substrate D-aspartate. We thus focused our attention on R243 and examined its role in substrate selectivity and catalysis by site-directed mutagenesis.

3.2. Characterization of alanine mutant at R243 of ChDDO

We first mutated R243 to alanine and the resulting mutant was expressed in *E. coli* as a fusion protein with histidine-tag. SDS-PAGE and Western blot analyzes of the crude extract showed that R243A mutant was expressed as a soluble protein at a level comparable to the wild-type enzyme at the expected molecular mass (Fig. 2, lanes 2 and 3). However, the extract had only a very low activity with D-aspartate as a substrate. To characterize the mutant more in detail, we purified and characterized the mutant (Fig. 3, lane 2). The wild-type enzyme exhibited 93.7 U/mg of specific activity toward D-aspartate, whereas the mutant showed only 0.09 U/mg and no detectable activity toward other D-amino acids (Table 1). Spectral analysis showed that similar to the wild-type enzyme, the mutant exhibited two typical flavin peaks at 375 nm and 450 nm (Fig. 4a and b). However, the overall intensity of flavin of R243A mutant

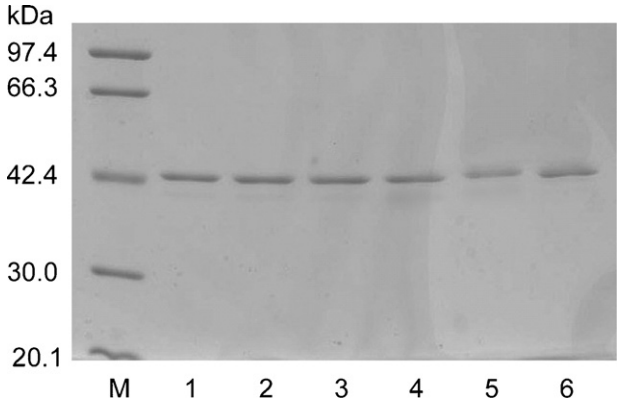

Fig. 3. SDS-PAGE of the purified histidine-tagged wild type and R243 mutants of ChDDO. Lane M: marker proteins; lane 1: wild type; lane 2: R243A; lane 3: R243M, lane 4: R243E; lane 5: R243D; lane 6: R243K. One microgram of each purified protein was analyzed.

Table 1
Oxidase activities of the purified hisitidine-tagged wild type and mutants of ChDDO against various D-amino acids.

Substrate	Specific activity (U/mg protein)[a]					
	Wild type	R243A	R243M	R243D	R243E	R243K
D-Asp	93.7	0.09	86	181	1.9	1.5
NMDA[b]	9.2	–[c]	13.1	18.5	1.0	0.5
D-Glu	1.6	–	–	1.2	0.04	0.04
D-Ala	–	–	5.6	–	0.4	–
D-Arg	–	–	–	–	3.2	–

Data are the mean for three independent determinations.
[a] The enzyme activity was assayed at a substrate concentration of 20 mM.
[b] *N*-Methyl-D-aspartate.
[c] Not detected.

was considerably lower than that of the wild type, implying that FAD attachment to apoenzyme might be decreased. In addition, the two peak intensities were equal each other. From the observation that the semiquinone form of pig kidney DAO exhibited very high absorption maxima around 370 nm [33], a part of bound flavin might be semiquinone form. The addition of malonate, a strong competitive inhibitor, into the wild-type enzyme solution resulted in the peak shift to longer wavelength, caused by the inhibitor binding to the active site, but not into the mutant solution, indicating a loss of inhibitor-binding ability of the mutant. These results showed that the arginine residue, R243, is important for the catalytic activity of ChDDO.

3.3. Expression and purification of various R243 mutants

To examine whether the positive charge of R243 play an important role in the catalytic activity, we further mutated the residue to various amino acids. Considering the effect of charge of side chain, we replaced it to K(R243K) to maintain the positive charge, D(R243D) and E(R243E) to alter it to negative, and M (R243M), which is spatially equivalent residue in RgDAO (Fig. 1), to alter it to neutral. These mutants were expressed in *E. coli* as a fusion protein with histidine-tag. All of the mutants were expressed as soluble proteins at levels similar to that of the wild-type enzyme (Fig. 2, lanes 4–7). The mutants were purified to homogeneity on SDS-PAGE (Fig. 3, lanes 4–7). R243E and R243K mutants exhibited only marginal activity toward D-aspartate (Table 1). On the other hand, R243M had a comparable activity to, and R243D, unexpectedly, exhibited approximately two-fold higher activity than the wild type. Such activity enhancement of DDO by site-directed mutagenesis was also reported on mouse DDO mutated at S308, which is possibly hydrogen bonded to α-amino group of amino acid substrates [34]. The activity of mouse DDO mutant (9.24 U/mg) was still approximately 10- and 19.6-fold lower than those of the wild type and R243D mutant of ChDDO, respectively.

We also tested the catalytic ability of the mutants toward other D-amino acids (Table 1). R243M acted on D-alanine with approximately 6% of the activity of the wild type toward D-aspartate, but abolished on D-glutamate. R243D exhibited activity only toward acidic D-amino acids as well as the wild-type enzyme, but the activity toward *N*-methyl-D-aspartate (NMDA) was twice of that of the wild type. R243E and R243K exhibited markedly reduced activity toward acidic D-amino acids, but R243E gained activity toward D-alanine and D-arginine, albeit at very low levels.

As shown in Fig. 4, spectral analysis showed that all of the mutants displayed two typical flavin peaks. However, except R243K mutant, the peak feature in each mutant was similar to that of the alanine mutant (the two peak intensities were nearly equal each other), whereas the overall intensity of flavin was maintained at the wild-type level (Fig. 4a–e). In R243K, the peak feature was similar to the wild type (the peak intensity at 450 nm was higher than

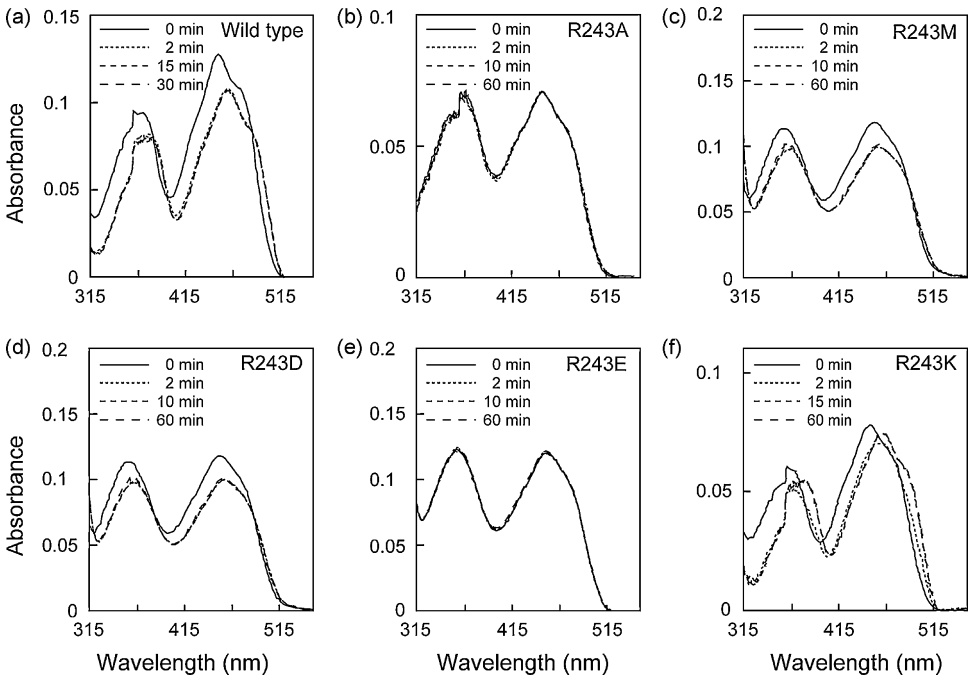

Fig. 4. Visible absorption spectra of the histidine-tagged wild type (a) and R243 mutants (b–f) of ChDDO. The proteins (0.6 mg/ml) in 50 mM potassium phosphate buffer (pH 7.0) were analyzed before and after the addition of malonate solution (pH 7.0) to a final concentration of 50 mM at the indicated times.

that at 375 nm), whereas the intensities were nearly half of those of the wild type (Fig. 4a and f). These results showed that the positive charge in the position at R243 might contribute to maintain the peak feature of flavin spectrum, which indicates the oxidized form of flavin, shown in the wild type. It has been reported that in other flavin enzymes, cholesterol oxidase and xanthine oxidoreductase, an arginine residue in the active-site acts as a "gate" adopting an open conformation and thus appears to control the entrance of oxygen to the active site [35,36]. From these findings with the results in the spectral analysis, R243 might contribute to the oxygen reactivity of bound flavin. In the spectral analysis with malonate, peak shift was observed in R243M and R243D with a similar rate to and in R243K with relatively slower rate than that of the wild type, but not in R243E (Fig. 4).

Taken together, these results demonstrated that the positive charge of R243 was not crucial for the catalytic activity and the substrate specificity to acidic D-amino acids.

3.4. Kinetic properties of R243 mutants

We determined the kinetic parameters of ChDDO mutants with acidic D-amino acids as substrates (Table 2). The mutation of R243 to acidic amino acid residues (R243D and R243E) decreased the K_m value for D-aspartate, suggesting that the mutation of R243 to acidic amino acid residues might cause an increase in binding affinity for D-aspartate, although the properties for D-glutamate and NMDA of R243E could not be determined because of its low activities and no inhibitor binding was indicated in R243E (Fig. 4e). On the other hand, the k_{cat} value of R243D and R243E was much higher and drastically lower than that of the wild type. Consequently, the catalytic efficiency of R243D for D-aspartate was much higher (approximately four-fold) than that of the wild type, whereas that of R243E was considerably lower. The value of the catalytic efficiency of R243D ($1.21 \times 10^6\ s^{-1}\ M^{-1}$) was markedly higher than those of DDOs from other sources: that of porcine, bovine, and human DDOs was 1.49×10^4, 6.1×10^3, and $1.9 \times 10^4\ s^{-1}\ M^{-1}$, respectively [8,37,38]. The higher catalytic efficiency of R243D relative to the wild type was also observed for D-glutamate and NMDA, mainly due to its lower K_m values, particular for D-glutamate. However, the efficiencies were still marginal in comparison to that for D-aspartate. These results showed that R243D mutant have a high catalytic activity toward D-aspartate oxidation together with a high substrate specificity to D-aspartate.

The mutation of R243 to methionine (R243M) or arginine (R243K) increased the K_m value and decreased the k_{cat} value for D-aspartate, thereby decreasing their catalytic efficiency. However, R243M still retained approximately half of the catalytic efficiency of the wild type. In contrast, the efficiency of the mutants was

Table 2
Apparent steady-state kinetic parameters for the histidine-tagged wild type and mutants of ChDDO for acidic D-amino acids.

	D-Aspartate			D-Glutamate			NMDA[a]		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ M^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ M^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}\ M^{-1}$)
Wild type	2.5 ± 0.3	74.5	29 800	106 ± 9.4	2.6	24	27.9 ± 2.4	15.7	562
R243A	13.4 ± 3.6	–[b]	–	–	–	–	–	–	–
R243M	3.8 ± 2.0	62.6	16 500	–	–	–	42.4 ± 7.6	31.0	731
R243D	1.0 ± 0.4	121	121 000	4.6 ± 1.7	0.9	201	10.2 ± 2.3	20.2	1 980
R243E	2.0 ± 0.9	1.3	650	–	–	–	–	–	–
R243K	10.3 ± 2.5	1.4	136	2.8 ± 1.0	0.2	77	30.5 ± 9.4	0.86	28

Data represent the mean ± SD of triplicate determinations.
[a] *N*-Methyl-D-aspartate.
[b] Not determined.

increased for NMDA in R243M and for D-glutamate in R243K relative to the wild type, due to its higher k_{cat} value and its lower k_m value, respectively. The catalytic efficiency of R243K for NMDA was considerably lower compared to the wild type due to its lower k_{cat} value. These results showed that the mutation of R243 with various kinds of amino acid residues remarkably affected the catalytic efficiency.

It was interesting that the mutation of R243 to a similar basic residue, lysine, drastically reduced the catalytic efficiency for D-aspartate and NMDA. In addition, it was also interesting that R243D and R243E exhibited the drastically opposite catalytic efficiency, despite they are only different in the side-chain length of one methylene group. There seems to be therefore no apparent relationship between the catalytic efficiency of the mutants and the net charge, length, or shape of the side chain of amino acid residues introduced. The determination of the three-dimensional structure of R243D mutant in a complex with a substrate analog and a more detailed biochemical analysis of the mutant could reveal the mechanism clearly.

4. Conclusion

In this study, we searched a unique amino acid residue, possibly involved in substrate recognition, in a putative active site of ChDDO by comparing its three-dimensional structure model with the active site of a yeast DAO. From the search, we identified an arginine residue positioned at 243 which was considered to be involved in substrate recognition. We replaced the arginine with several other amino acid residues and analyzed the enzymatic characteristics of the mutants. These mutants demonstrated that the arginine is not crucial for the substrate specificity and the catalytic activity of ChDDO. Almost all the mutants exhibited markedly reduced activity, but one mutant, R243D, in which R243 was replaced with aspartate residue, showed approximately four-fold higher catalytic efficiency toward D-aspartate than that of the wild type with a high substrate specificity to D-aspartate. This hyper active DDO should be valuable for several practical applications, especially for determination of D-aspartate in various biological samples.

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