A conserved aspartate is essential for FAD binding and catalysis in the D-amino acid oxidase from *Trigonopsis variabilis*

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Abstract To evaluate the possible contribution of Asp^{206} of *Trigonopsis variabilis* D-amino acid oxidase (DAO) to its flavin adenine dinucleotide (FAD) binding and catalytic function, six mutant enzymes were constructed by site-directed mutagenesis. Western immunoblot analysis revealed that a protein with an apparent molecular mass of about 39.2 kDa was present in the cell-free extracts of wild-type and mutant strains. Replacement of Asp^{206} with Leu, Gly, and Asn resulted in the loss of DAO activity and characteristic absorption spectrum for flavoenzyme, while the other mutant DAOs, Asp^{206}Glu, Asp^{206}Ser, and Asp^{206}Ala, exhibited a similar spectral profile to that of wild-type enzyme and retained about 6–90% of the enzyme activity. These results suggested that Asp^{206} of *T. variabilis* DAO might play an important role in the binding of FAD.

Key words: D-Amino acid oxidase; Site-directed mutagenesis; Flavin adenine dinucleotide binding; *Trigonopsis variabilis*

1. Introduction

D-Amino acid oxidase (DAO, EC 1.4.3.3) is a flavoenzyme that catalyzes the oxidation of D-amino acids to their corresponding keto acids [1]. The oxidative reaction is coupled to the reduction of an obligatory flavin adenine dinucleotide (FAD) cofactor and the reduced prosthetic group is reoxidized by molecular oxygen with the release of hydrogen peroxide [2]. The enzyme shows an ubiquitous distribution among eukaryotic cells ranging from yeasts to mammals [3–8]. In addition, DAO activity has been found in prokaryotes [9–11] despite the lack of its physiological significance. The detection of significant quantities of D-amino acids in various mammalian tissues suggests that DAO is not a vestigial enzyme [8].

Trigonopsis variabilis DAO is a dimeric enzyme that consists of two identical subunits and needs one FAD molecule per subunit for its function [12]. To date, the nucleotide sequences of cDNA encoding DAO have been determined in *T. variabilis* [13], *Fusarium solani* [14], *Rhodotorula gracilis* [15], *Rhodotorula toruloides* [16] and a variety of animal cells [17–20]. Sequence analysis of DAOs reveals that there are six conserved regions (designated as regions I, II, III, IV, V and VI) among the DAOs and regions I and III are indicated to be essential for FAD binding [15]. In the present study, the interaction between an aspartate residue in region II of *T. variabilis* DAO and the coenzyme FAD was investigated by site-directed mutagenesis.

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2. Materials and methods

2.1. Strains, vectors, media and reagents

T. variabilis CCRC 21509 was obtained from the Culture Collection and Research Center, Hsinchu, Taiwan. *Escherichia coli* strains used were JM109 [21], CJ236 [22] and MV1190 [21]. The vectors used were pQE30 (Qiagen, CA, USA) and M13mp19 (Bio-Rad, CA, USA). Yeast was grown in a YCBA medium (0.67% yeast carbon base and 0.2% D,L-alanine) at 30°C with shaking at 150 rpm on a rotary shaker. Recombinant *E. coli* strains were cultivated at 37°C in Luria-Bertani (LB) broth containing 100 μg/ml ampicillin. Oligonucleotides (Table 1) were obtained from Quality Systems (Taipei, Taiwan). Restriction enzymes were purchased from Boehringer Mannheim (Mannheim, Germany) and Promega (WI, USA). Deep Vent DNA polymerase was obtained from New England Biolabs (MA, USA). All other chemicals were of the highest purity available.

2.2. Recombinant DNA methods

DNA isolation, agarose gel electrophoresis and transformation of *E. coli* were carried out as described by Sambrook et al. [23]. Restriction endonucleases and other DNA modifying enzymes were used in accordance with the manufacturers' instructions. DNA sequencing was performed by the dideoxy method [24] with a Sequenase R version 2 sequencing kit (United States Biochemical, OH, USA).

2.3. Construction of expression plasmid

T. variabilis was cultivated in 1000 ml of YCBA medium for 24 h. Cells were harvested by centrifugation and homogenized with 2 g of glass beads in 25 mM sodium citrate buffer (pH 7.0) containing 4 M guanidium thiocyanate and 0.5% *N*-lauroyl sarcosine. The total RNA was isolated from the cell extract according to the method of Logemann et al. [25]. The *dao* cDNA was prepared from poly(A) RNA with a SuperScript Preamplification kit (BRL, MD, USA) followed by a PCR amplification. PCR mixtures (100 μl) contained 20 μl of cDNA sample, 200 μM of each deoxynucleotide triphosphate, 10 μl of a 10× reaction buffer (New England Biolabs, MA, USA), 10 pmol of each primer and 0.5 U of Deep Vent DNA polymerase (New England Biolabs). Samples were amplified with a DNA thermal cycler (model 9600; Perkin-Elmer, CO, USA) under the following conditions: initial denaturation at 94°C for 1.5 min, followed by 30 cycles of denaturation at 94°C for 3 min, annealing at 55°C for 1 min and extension at 74°C for 2 min. The PCR product was digested with *Bam*HI and *Hin*dIII and subcloned into the corresponding sites of pET21b or pQE30 vectors to generate pET21b-DAO and pQE30-DAO, respectively.

2.4. Purification of the recombinant proteins

A single colony from LB plate was inoculated in 100 ml of LB medium containing 100 μg/ml ampicillin in 500-ml flasks. Cells were grown at 28°C until OD_{600} was 1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 1 mM and the cultivation was continued for 5 h. The cells were harvested by centrifugation at 9000×*g* for 10 min and the pellets were washed once with binding buffer (5 mM imidazole, 0.5 M NaCl and 20 mM Tris-HCl; pH 7.9), resuspended in 3 ml of the above buffer and disrupted by sonication. Cell debris was removed by centrifugation (12 000×*g*) and the resulting extracts were used for protein purification. Plasmid pQE30-DAO has an IPTG-regulated T5 promoter, followed by the multiple cloning sites with a six-histidine tag. Under the induction of IPTG, the six-histidine tag and the inserted gene were expressed as a

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fusion protein and it could be purified by affinity chromatography with His·Bind resin (Novagen, WI, USA).

2.5. Site-directed mutagenesis

For site-directed mutagenesis, the *dao* gene on a *Bam*HI-*Hin*dIII fragment from pQE30-DAO was cloned into phage M13mp19 to generate pM19-DAO. The uridinylated DNA template of pM19-DAO was prepared in *E. coli* CJ236 and oligonucleotide-directed mutagenesis was performed according to Kunkel [26] using the 'Muta-Gene M13 in vitro Mutagenesis' kit from Bio-Rad. *E. coli* MV1190 was used as the recipient strain for in vivo screening of the transformants on glucose-minimal medium. Single-stranded DNA from the mutant clones was prepared and subjected to dideoxy sequencing to confirm the successful mutations.

2.6. Enzyme assay

DAO activity was measured spectrophotometrically according to the procedure described by Lee et al. [27]. One unit of DAO activity was defined as the amount of enzyme releasing 1 nmol of pyruvate equivalent per min. Specific activity is expressed as units per milligram of protein. Protein content was estimated by the method of Bradford [28] and bovine serum albumin was used as a standard. The K_m (Michaelis constant) and k_{cat} (number of substrate molecules transformed per second per molecule of enzyme) values for DAO were estimated from Lineweaver-Burk plots.

2.7. Electrophoresis and immunobloting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially as described by Laemmli [29]. Proteins were electrophoretically transferred to Immobilon-P transfer membrane (Millipore, MA, USA) from SDS-PAGE in a Bio-Rad Transblot Cell with a transfer buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine and 20% methanol. The proteins were detected on Immobilon-P membrane with polyclonal anti-DAO antibody and alkaline phosphatase-conjugated antibody. Non-fat milk powder (5%) in Tris-buffered saline was used as a blocking agent.

3. Results and discussion

For the expression of *T. variabilis dao* gene, oligonucleotides DAO-1 and DAO-2 (Table 1) were used for gene-specific amplification of double-stranded *dao* cDNA. The amplified fragment was cloned downstream from the IPTG-inducible T7 promoter of plasmid pET21b to give plasmid pET21b-DAO. *E. coli* BL21(DE3) harboring pET21b-DAO was assayed for DAO activity. A very low level of enzyme activity was detected in the extract of IPTG-induced transformant. This low level of expression for DAO was thought to result from the formation of large amounts of inclusion bodies in *E. coli* (data not shown). Alternatively, the PCR-amplified fragment was cloned into *Bam*HI and *Hin*dIII sites of pQE30 to obtain pQE30-DAO. The constructed plasmid was transformed into *E. coli* JM109. Transformants were randomly picked for sequence confirmation of the *dao* gene in the plasmids and one transformant was chosen for expression analysis. When crude extracts from strain JM109 harboring pQE30-DAO were analyzed by SDS-PAGE, a protein band exhibiting an apparent molecular mass of 39.2 kDa was detected (Fig. 1A). Western blot analysis confirmed the presence of the recombinant DAO in the *E. coli* crude extracts (Fig. 1B). These results suggested that the translation of *dao* gene was assumed to be initiating at the correct AUG codon. A DAO specific activity of 18.61 U/mg was obtained in the crude extract of *E. coli* JM109 harboring pQE30-DAO (Table 2).

Table 1
Oligonucleotide primers used in this study

Primer for	Sequence
PCR	
DAO-1	5′-GCT**GGATCC**ATGGCTAAAATCGTTGTTATTGG-3′ *Bam*HI
DAO-2	5′-CAT**AAGCTT**CTAAAGGTTTGGACGAGTAAGAG-3′ *Hin*dIII
Site-directed mutagenesis	
Asp^{206}Ala	5′-AGGCGTCGAG**GCC**AAGAAGA-3′
Asp^{206}Glu	5′-AGGCGTCGAG**GAA**AAGAAGA-3′
Asp^{206}Leu	5′-AGGCGTCGAG**CTG**AAGAAGA-3′
Asp^{206}Asn	5′-AGGCGTCGAG**AAC**AAGAAGA-3′
Asp^{206}Gly	5′-AGGCGTCGAG**GGT**AAGAAGA-3′
Asp^{206}Ser	5′-AGGCGTCGAG**TCC**AAGAAGA-3′

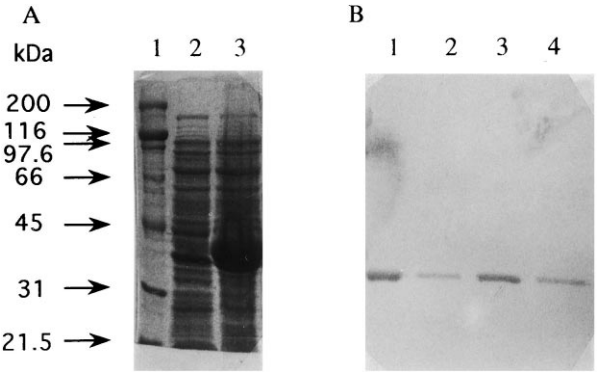

Fig. 1. SDS-PAGE (A) and Western blot (B) analyses of the wild-type and mutant DAOs. A: Lane 1: protein size marker; lane 2: total proteins prepared from *E. coli* JM109 (pQE30-DAO) without IPTG induction; lane 3: total proteins prepared from *E. coli* JM109 (pQE30-DAO) with IPTG induction. B: Lane 1: wild-type DAO; lane 2: Asp^{206}Ala; lane 3: Asp^{206}Glu; lane 4: Asp^{206}Leu.

Random mutagenesis of the *dao* gene was performed using the procedure of Cadwell and Joyce [30]. Of the transformants, one mutant enzyme was found to display a rather striking change in the absorbance spectrum as compared with that of the wild-type enzyme. Sequence analysis of the mutant gene revealed that double changes (A□G and C□T) occurred at positions 652 and 653, respectively, of the *dao* gene, indicating the replacement of Asp^{206} with Gly. In order to assess the role of Asp^{206} in *T. variabilis* DAO, six site-directed mutants were constructed: Asp^{206}Ala, Asp^{206}Leu, Asp^{206}Gly, Asp^{206}Glu, Asp^{206}Asn and Asp^{206}Ser. Expression of the six DAO mutants was detected by Western blotting (Fig. 1B). Specific activity was determined to be 16.71 U/mg for Asp^{206}Glu and 6.41 U/mg for Asp^{206}Ser (Table 2). Less than 6% of the specific activity was retained for the other mutant DAOs. These results suggested that Asp^{206} plays an essential role for the DAO activity. The kinetic parameters K_m and k_{cat} obtained with Asp^{206}Ala, Asp^{206}Glu and Asp^{206}Ser are given in Table 2. As compared with the wild-type DAO, those mutations caused 2–4.4-fold increase in K_m and 59–97% decrease in k_{cat} values. The values of k_{cat}/K_m for Asp^{206}Glu, Asp^{206}Ala, and Asp^{206}Ser are about 6/1000–1/5th of that of the wild-type enzyme, indicating that substitution of amino acid residue at position 206 significantly influences the DAO-substrate interaction.

Spectral analysis is one of the well-known approaches for the observation of FAD-dependent enzymes [31–33]. In order to obtain information about the DAO-bound FAD, the spectral properties of the wild-type and mutant enzymes were investigated (Fig. 2). The wild-type DAO exhibited a typical

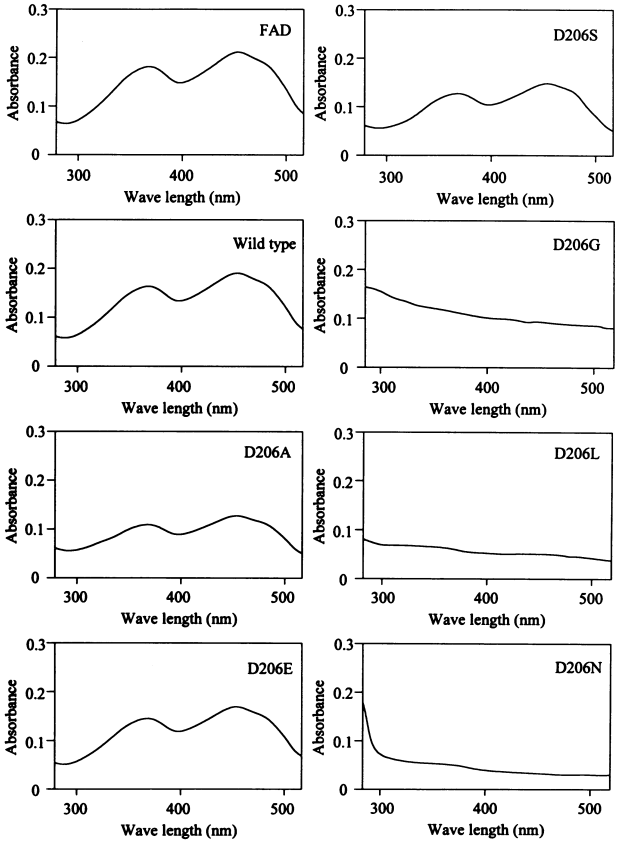

Fig. 2. Spectral profiles of free FAD, wild-type and mutant DAOs. FAD and enzymes were dissolved in 20 mM Tris-HCl buffer (pH 7.9) and approximately 0.1 mg of enzyme per ml was used for analysis. Spectrophotometric analysis was done with a Beckman DU 600 at wavelength ranging from 300 to 500 nm.

absorption spectrum of flavoprotein with two peaks at 380 and 450 nm as that of the free FAD. Asp^{206}Glu displayed a similar spectral profile to that of the wild-type enzyme. Although the spectra of Asp^{206}Ala and Asp^{206}Ser still exhibited two absorption peaks as that of the wild-type enzyme, the absorbance at 380 and 450 nm was greatly reduced. For the three other mutants (Asp^{206}Gly, Asp^{206}Leu and Asp^{206}Asn), the FAD-dependent peaks disappeared in the absorbance spectra. These results indicated that the incorrect interaction between the mutant enzymes and FAD could significantly decrease the attachment of FAD to apoenzyme, which led to a defect in the catalytic function of DAO.

Aspartate residues are often found on the surface of globular proteins or are partially buried with the hydroxyl group available for intermolecular hydrogen bonding [34]. It is noted that Asp^{206} of *T. variabilis* DAO in region II is highly conserved among different DAOs (Fig. 3). Examples of aspartate that play a role in catalytic activity can be illustrated by glycolate oxidase in which it is postulated to contribute a part of charge in positioning the lysine residue close the N(1)–C(2)=O locus of the flavin through a water molecule [35]. Another example is that the oxygen atoms in the side chain of Asp^{35} of sarcosine oxidase and Glu^{33} of lipoamide dehydrogenase could both interact with the ribose ring of FAD and form two hydrogen bonds [33,36]. The loss of the FAD characteristic spectra of the three DAO mutants (Asp^{206}Asn, Asp^{206}Leu and Asp^{206}Gly) indicated that the oxygen atom in the carboxyl group of the Asp^{206} side chain was required for FAD-apoenzyme binding. As shown in Fig. 2, Asp^{206}Glu had a similar absorption spectrum for flavoenzyme as that of wild-type DAO. Since the side chains of Asp and Glu differ by one methylene group, the results further supported that the carboxyl group of amino acid residue is involved in FAD binding. About 34% of the DAO activity was retained in Asp^{206}Ser and the mutant enzyme showed a decrease in absorbance intensity for flavoenzyme. The results implicated that the hydroxyl group of serine residue is less likely to interact with FAD as compared with the carboxyl group of aspartate residue. The Asp^{206}Ala enzyme had detectable but extremely low specific activity (1.04 U/mg of protein), which was only 5.6% of the specific activity for the unaltered DAO. The small amount of activity recorded for the Asp^{206}Ala enzyme is unexplained; however, it is possible that replacement with alanine may have increased the accessibility of FAD to other residues, which provided hydrogen bonds in place of Asp^{206}.

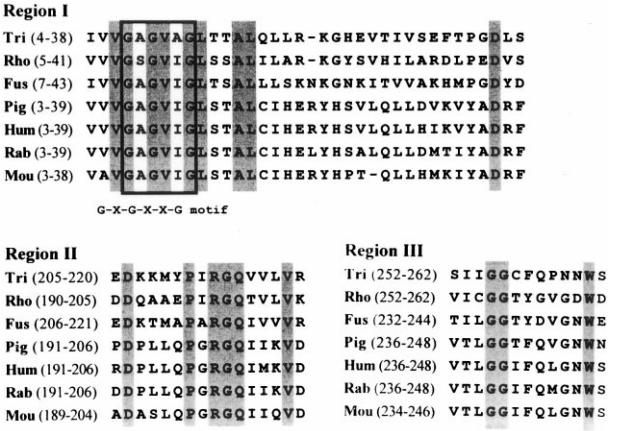

Fig. 3. The proposed FAD-binding domains of DAOs. Amino acid residues are indicated by single letters. Conserved amino acid residues are in gray boxes. Tri: *Trigonopsis variabilis* DAO; Rho: *Rhodotorula gracilis* DAO; Fus: *Fusarium solanii* DAO; Pig: pig DAO; Hum: human DAO; Rab: rabbit DAO; Mou: mouse DAO.

Table 2
Catalytic properties of wild-type and mutant DAOs

Enzyme	Specific activities (U/mg)	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
Wild type	18.61	0.34	4.63	73.43
Asp^{206}Glu	16.71	0.14	9.36	14.95
Asp^{206}Ser	6.41	0.06	20.52	2.92
Asp^{206}Ala	1.04	0.01	18.12	0.44
Asp^{206}Gly	0.00	–[a]	–	–
Asp^{206}Leu	0.00	–	–	–
Asp^{206}Asn	0.00	–	–	–

[a]–, Not determined.

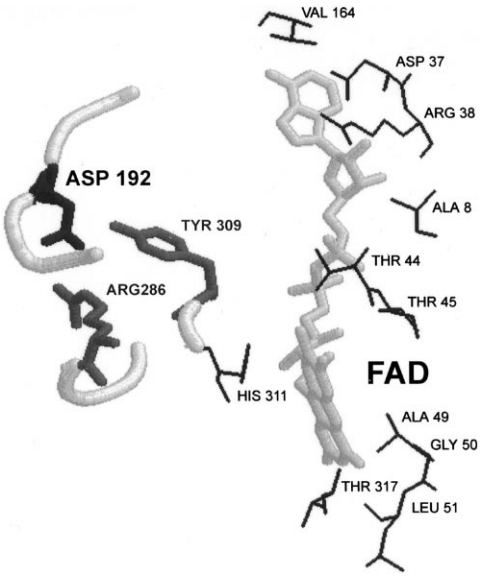

Fig. 4. RasMol diagram of the FAD binding cavity and Asp^{192} in the pig kidney DAO crystal structure.

Taken together, these results suggested that the oxygen atom in the carboxyl group of the Asp^{206} side chain was involved in FAD-apoenzyme binding. Although the three-dimensional structure of *T. variabilis* DAO is not known, a homologous protein, pig kidney DAO, has been determined [2]. Sequence alignment of these two DAOs indicates that this specific aspartate residue corresponds to Asp^{192} of pig kidney DAO, which is located on a loop 190–195 between an α-helix (αF4) and a β-strand (β14) (Fig. 4). Based on this structure, it is not likely that Asp^{192} can directly participate in binding with FAD. Instead, the hydroxyl group of Tyr^{309} in a loop 309–311 and the guanidinyl group of Arg^{286} in another loop 286–289 are in close proximity to the oxygen atoms of the Asp^{192} residue. Firm interactions between Asp^{192} and these two residues may possibly take place and thus yield a stable conformation essential for FAD binding. Indeed, His^{311}, one of the proposed residues involved in FAD binding, is found on the loop 309–311. Since *T. variabilis* DAO is homologous to pig kidney DAO and since all these three residues (Asp^{192}, Arg^{286} and Tyr^{309} of pig kidney DAO) are well conserved among different DAOs, it is speculated that Asp^{206} of *T. variabilis* DAO may play a similar role as that of Asp^{192} of pig kidney DAO.

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