The role of a conserved histidine residue, His324, in *Trigonopsis variabilis* D-amino acid oxidase

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Abstract

To investigate the functional role of an invariant histidine residue in *Trigonopsis variabilis* D-amino acid oxidase (DAAO), a set of mutant enzymes with replacement of the histidine residue at position 324 was constructed and their enzymatic properties were examined. Wild-type and mutant enzymes have been purified to homogeneity using the His-bound column and the molecular masses were determined to be 39.2 kDa. Western blot analysis revealed that the in vivo synthesized mutant enzymes are immuno-identical with that of the wild-type DAAO. The His324Asn and His324Gln mutants displayed comparable enzymatic activity to that of the wild-type enzyme, while the other mutant DAAOs showed markedly decreased or no detectable activity. The mutants, His324/Asn/Gln/Ala/Tyr/Glu, exhibited 38–181% increase in K_m and a 2–10-fold reduction in k_{cat}/K_m. Based on the crystal structure of a homologous protein, pig kidney DAAO, it is suggested that His324 might play a structural role for proper catalytic function of *T. variabilis* DAAO. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

D-Amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the oxidative deamination of free D-amino acids to produce the corresponding α-oxo acids and ammonia with a concomitant reduction of molecular oxygen to hydrogen peroxide [1]. DAAOs have been reported in a wide variety of organisms, including animals and microorganisms [2]. The potential uses of this enzyme in the production of α-keto acids, in biosensors, and in the conversion of cephalosporin C to 7-glutarylcephalosporanic have spurred studies of catalytic mechanisms [3,4]. Although it is considered to be a marker enzyme for peroxisomes, the physiological function of the enzyme remains unknown.

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The detection of significant amounts of DAAO in various mammalian tissues suggests that the enzyme may be involved in removing the D-amino acids derived from either exogenous or endogenous sources [5].

The primary structures of DAAOs from *Trigonopsis variabilis* [6], *Fusarium solani* [7], *Rhodotorula gracilis* [8], *Rhodosporidium toruloides* [9] and animal cells [10] have been determined. Although six highly conserved regions within the DAAOs have been postulated to play important roles in FAD binding (regions I and III), in active topology (regions II, IV and V), and in peroxisomal targeting (region VI) [8], a conserved Asp206 in region II of *T. variabilis* DAAO might involve in FAD binding [11]. Sequence comparison reveals that residues Tyr228 and His307 (corresponding to Tyr243 and His324 of *T. variabilis* DAAO), proposed to be in the active site of pig kidney DAAO by chemical modification studies [12,13], are conserved in the *T. variabilis* DAAO sequence. In addition, site-directed mutagenesis [14] and atomic structure of enzyme-inhibitor complex [15] have confirmed Tyr228 as one of the catalytic residues. His307 of pig kidney DAAO is known to be important for enzymatic activity since the His307Leu mutant has a 10-fold reduced preference for D-alanine [16]. This histidine residue is located at region V and is highly conserved in all DAAOs (Fig. 1). To examine the role of this conserved residue, mutations at position 324 of *T. variabilis* DAAO were performed and kinetic parameters of its mutants were studied.

2. Materials and methods

2.1. Bacterial strains and plasmids

Escherichia coli JM109 [*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)] [17] was used for plasmid propagation. For site-directed mutagenesis, *E. coli* strains CJ236 [F⁻ *dut-1 ung-1 thi-1 relA1*/pCJ105 (*cm*ʳ)] [18], MV1190 [Δ(*lac-proAB*) *rpsL thi endA spcB15 hsdR4* Δ(*srl-recA*)306::Tn*10*(*tet*ʳ) F⁻(*traD36 proAB*⁺ *lacI*ᑫ *lacZ*ΔM15)] [17] and the M13 helper phage R408 were used. The plasmids used were pM19-DAO and pQE30-DAO [11].

Table 1
Oligonucleotide primers used in site-directed mutagenesis studies

Enzyme	Mutagenic primer
Wild-type	5′-GGCTTTGTTGTC**CAT**AACTATGG-3′
His324Leu	5′-GGCTTTGTTGTC**CTT**AACTATGG-3′
His324Arg	5′-GGCTTTGTTGTC**CGT**AACTATGG-3′
His324Tyr	5′-GGCTTTGTTGTC**TAT**AACTATGG-3′
His324Glu	5′-GGCTTTGTTGTC**GAG**AACTATGG-3′
His324Ala	5′-CTTTGTTGTC**GCC**AACTATG-3′
His324Lys	5′-CTTTGTTGTC**AAG**AACTATG-3′
His324Gln	5′-CTTTGTTGTC**CAA**AACTATG-3′
His324Asn	5′-CTTTGTTGTC**AAC**AACTATG-3′

2.2. Recombinant DNA methods

Transformation of *E. coli* and other routine DNA manipulations were carried out according to the procedures described by Sambrook et al. [19] or as specified by the manufacturers. The uridinylated DNA of pM19-DAO was prepared in *E. coli* CJ236 and used as a template for in vitro mutagenesis. Site-directed mutagenesis was performed by the method of Kunkel [18] using the Muta-Gene phagemid in vitro Mutagenesis kit from Bio-Rad Laboratories (Hercules, CA, USA). The mutagenic primers used are shown in Table 1 and the designed DAAO mutations are shown in bold. Mutations of DAAO were verified by DNA sequencing.

2.3. Protein expression and purification

E. coli transformants were grown in Luria-Bertani medium supplemented with 100 μg ml⁻¹ ampicillin at 28°C up to an absorbance of 1.0 at 600 nm, then isopropyl-β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. After 5 h induction, cells were harvested by centrifugation at $9000 \times g$ for 10 min and the pellets were resuspended in 20 mM Tris-HCl buffer (pH 7.9) containing 5 mM imidazole and 0.5 M NaCl and disrupted by sonication. The cell-free extracts were applied on a His-bound resin column and chromatography was performed according to the manufacturer's instructions (Novagen Inc., Madison, WI, USA).

2.4. Electrophoresis and Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electro-

phoresis (SDS-PAGE) was performed in a vertical minigel system (Mini-Protean II System; Bio-Rad, Hercules, CA, USA) with a 10% denaturing polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred onto an Immobilon-P transfer membrane (Millipore Corp., Bedford, MA, USA) with a Bio-Rad Transblot Cell. DAAO bands were identified with polyclonal anti-DAAO antibodies and thereafter reacted with commercial alkaline phosphatase-conjugated antibodies.

2.5. Enzymatic determinations

The activity of wild-type and mutant DAAOs was determined by a spectrophotometric method [20]. Reaction was carried out at 37°C in 100 mM potassium phosphate buffer (pH 8.0) containing 10 mM D-alanine and 400 U of bovine liver catalase. One unit of DAAO activity represents the amount of enzyme producing 1 nmol of pyruvate per minute under the assay conditions. Protein concentration was determined with the dye binding procedure [21] using bovine serum albumin as the standard. Specific activity is expressed as units per milligram of protein. Values of k_{cat} and K_m were calculated by fitting the initial rates as a function of substrate concentration to the Michaelis-Menten equation using Grafit software (Sigma Chemical Co., St. Louis, MO, USA).

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Trv  317- P G V G F V V H N Y G A A G A G Y Q S S Y G M A T
Fus  320- D D E T W I V H N Y G H S G W G Y Q G S Y G C A E
Rhg  322- E K E V T L V H A Y G F S S A G Y Q Q S W G A A E
Pig  300- S S N T E V I H N Y G H G G Y G L T I H W G C A L
Hum  300- P S N T E V I H N Y G H G G Y G L T I H W G C A L
Rab  300- P S K T E V I H N Y G H G G Y G L T I H W G C A L
Mou  298- S S S A E V I H N Y G H G G Y G L T I H W G C A M
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Fig. 1. Sequence comparison of DAAOs surrounding the conserved His324 residue. Amino acid residues, expressed in one-letter codes, are numbered from the translational methionine of each enzyme. Strictly conserved residues are in gray boxes and the conserved histidine is marked by an asterisk. Trv, *T. variabilis* DAAO; Fus, *F. solani* DAAO; Rhg, *R. gracilis*; Pig, pig kidney DAAO; Hum, human DAAO; Rab, rabbit DAAO; Mou, mouse DAAO.

3. Results and discussion

To investigate the functional role of the highly conserved amino acid residue, His324, in *T. variabilis*, eight mutant enzymes were constructed by site-directed mutagenesis. The gene of the mutant enzyme was cloned into *Bam*HI and *Hin*dIII sites of pQE30 to obtain pQE30-DAAO. The constructed plasmid was transformed into *E. coli* JM109. Transformants were randomly picked for sequence confirmation of the *daao* gene in the plasmids and one transformant was chosen for expression analysis. Wild-type and mutant enzymes purified from IPTG-induced *E. coli* cells exhibited an apparent protein band with a molecular mass of approximately 39.2 kDa (Fig. 2A). As shown in Fig. 2B, polyclonal anti-*T. variabilis* DAAO recognized the wild-type enzyme (lane 1) and all the mutant
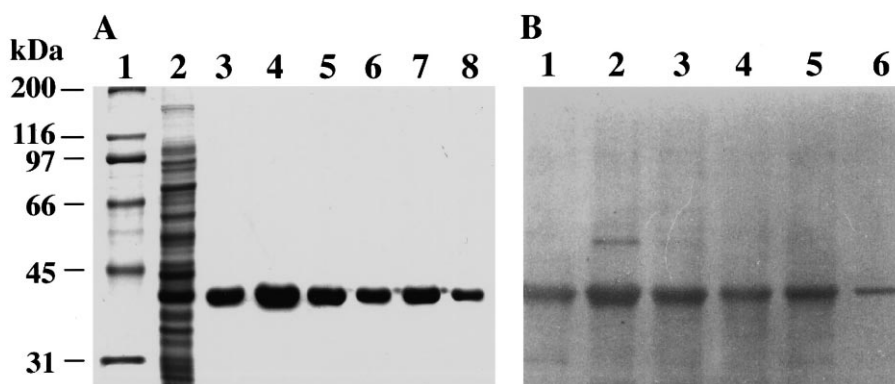

Fig. 2. Analysis of wild-type and mutant DAAOs by SDS-PAGE (A) and Western blot (B). A: Lanes: 1, protein size marker; 2, total protein prepared from *E. coli* JM109 (pQE30-DAAO) with IPTG induction; 3, wild-type DAAO; 4, His324Leu mutant; 5, His324Arg mutant; 6, His324Tyr mutant; 7, His324Glu mutant; 8, His324Ala mutant. B: Lanes: 1, wild-type DAAO; 2, His324Leu mutant; 3, His324Arg mutant; 4, His324Tyr mutant; 5, His324Glu mutant; 6, His324Ala mutant.
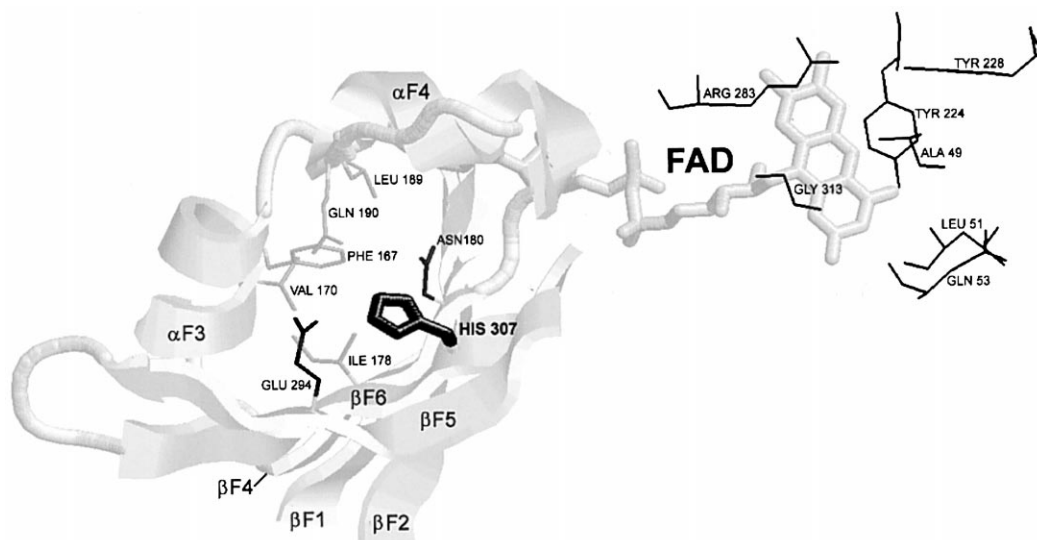
Fig. 3. RasMol diagram of the FAD binding cavity and the environment of His307 in the pig kidney DAAO crystal structure.

DAAOs (lanes 2–6). The electrophoretic mobilities of the mutant DAAOs were the same as that of the wild-type enzyme.

Replacements of His324 with asparagine or glutamine (His324Asn or His324Gln) resulted in a slight decrease ($>75\%$ of wild-type) in enzymatic activity (Table 2), while substitutions of histidine with leucine, arginine and lysine (His324Leu, His324Arg and His324Lys), respectively, caused a dramatic loss of DAAO activity. With D-alanine as a substrate, the K_m values increase by 38–156% for His324Asn, His324Gln and His324Ala mutants. The k_{cat} value increases by 26% for His324Ala, while it decreases by 39–76% for His324Asn, His324Gln, His324Ala and His324Tyr. However, the mutant enzymes,

His324Leu/Arg/Lys, did not have detectable activity for the determination of kinetic parameters. It is noteworthy that those catalytic efficiencies (k_{cat}/K_m) for the mutant DAAOs were reduced by 2–10-fold, as compared to the wild-type enzyme (Table 2). These observations suggest that the histidine residue at position 324 of *T. variabilis* DAAO appears to be important for enzymatic activity.

Previous studies using the chemical modification approach indicate that two histidine residues, His217 [22,23] and His307 [12], of pig kidney DAAO are located near the active center. Although His217 has been suggested to participate in the catalysis by facilitating proton transfer or stabilizing the local conformation of the active center through hy-

Table 2
Specific activity and kinetic parameters of wild-type and mutant DAAOs

Enzyme	Specific activity (U mg^{-1})	K_m (mM)	k_{cat} (min^{-1})	K_{cat}/K_m (min^{-1} M^{-1})
Wild-type	12.07	4.63	0.34	73.43
His324Asn	10.40	8.97	0.23	25.64
His324Gln	9.12	6.38	0.17	26.64
His324Ala	5.04	11.84	0.43	36.32
His324Tyr	6.43	10.12	0.13	12.85
His324Glu	5.12	13.01	0.08	6.67
His324Leu	0.00	ND[a]	ND	ND
His324Arg	0.00	ND	ND	ND
His324Lys	0.00	ND	ND	ND

[a]ND, not determined.

drogen bonding [24], the possibility of extensive involvement has been ruled out by a mutation study [25]. On the other hand, the mutation of the other histidine residue, His307, to leucine abolishes enzymatic activity [14]. Mutation of the corresponding histidine residue (His324) of *T. variabilis* DAAO to leucine showed a similar decrease of enzyme activity (Table 2). In addition, three DAAO mutants (His324Leu, His324Arg and His324Lys) were found to lose their typical FAD spectra (data not shown), implying that His324 might be important for FAD-apoenzyme binding. Based on the crystal structure of a homologous protein, pig kidney DAAO [24], it was found that His324 of *T. variabilis* DAAO corresponds to His307 of pig kidney DAAO, located on a β strand (βF6) of the FAD binding domain. The side chain of His307 is embedded in a core built up by the β-sheets and two helices (αF3 and αF4) with loops connecting them. As shown in Fig. 3, several conserved hydrophobic side chains are found in this pocket, including Phe167, Val170, Ile178 and Leu189. In addition, side chains of two non-hydrophobic and well-conserved residues (Glu294 and Asn180) are near the imidazole ring of His307 and may interact with each other. Taken together, hydrophobic forces contributed from hydrophobic side chains and interactions between Asn180, Glu294 and His307 in this pocket may produce a stable conformation essential for the binding of FAD, thereby supporting the catalytic activity. Replacement of the corresponding His with Asn and Gln might be still sterically allowable for interacting with Asn180 or Glu294, but with weaker affinity. Indeed, we found that these two mutants had $>75\%$ relative activity (Table 2). Two other mutants, His324Tyr and His324Glu, had less relative activity (53.3% and 42.4%, respectively), probably because they could only interact with either Glu294 or Asn180. When His327 was substituted with a hydrophobic residue, it was found that His324Ala had higher activity than His324Leu. A possible interpretation is that the more bulky hydrophobic side chain in Leu is unfavorable at this location. The least active mutants characterized in the site-directed mutagenesis results were His324Arg and His324Lys. It is likely that the long side chain of Lys and Arg causes steric hindrance in this pocket, resulting in an unstable conformation or a different conformation that could not support FAD binding.

In summary, sequence alignment showed that His324 of *T. variabilis* DAAO has been conserved throughout the evolution of DAAOs due to its structural importance. Eight mutations created by substitutions of His324 with polar, non-polar, charged or aromatic acids were made, and the results revealed that this residue could not be replaced without affecting the attachment of FAD to apoenzyme. Our data support the essential structural role of this specific histidine residue in the DAAO. Atomic structure determination is in progress, which will help to clarify the three-dimensional structure of FAD binding in *T. variabilis* DAAO.

Acknowledgements

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