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Effect of site-specific mutagenesis of tyrosine-55, methionine-110 and histidine-217 in porcine kidney D-amino acid oxidase on its catalytic function

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To assess the contributions of Tyr-55, Met-110 and His-217 in porcine kidney D-amino acid oxidase (EC 1.4.3.3, DAO) to its catalytic function, we constructed three mutant cDNAs coding for the enzymes possessing Phe-55, Leu-110 and Leu-217 by site-specific mutagenesis. The mutant and wild type cDNAs could be expressed in vitro with similar efficiency. The three mutant enzymes thus synthesized showed catalytic activities comparable to that of the wild type oxidase. It is concluded that Tyr-55, Met-110 and His-217 are not directly involved in the catalytic function.

D-Amino acid oxidase; Site-specific mutagenesis; Protein engineering; Reaction mechanism; (Porcine kidney)

1. INTRODUCTION

D-amino acid oxidase (EC 1.4.3.3, DAO) is a flavoenzyme that catalyzes the oxidative deamination of D-amino acids. Although extensive studies have been made, its reaction mechanism is not yet fully understood. Recent chemical modification studies, coupled with the determination of the primary structure of porcine kidney DAO [1], have shown that modifications of Tyr-224, Tyr-228 and His-307 [1], Tyr-55 and Lys-204 [1,2], His-217 [1,3], and Met-110 [4] all result in abolishment or drastic decreases of the enzyme activity. However, in view of the difficulty associated with specific and complete modification of a single amino acid residue in a protein, these observations provide only limited information concerning the roles of these residues in the catalytic mechanism. We have recently isolated a full-length cDNA clone for porcine kidney DAO and determined its nucleotide sequence [5]. We have also established an in vitro system for the synthesis of functionally active DAO with the cloned cDNA as template [6]. In this study we attempted to confirm the results of the chemical modification studies by constructing mutant cDNAs by site-specific mutagenesis and by expressing them in vitro. We report here that replacement of Tyr-55 with Phe, Met-110 with Leu, and His-217 with Leu (all in porcine DAO) did not affect the enzyme activity.

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Abbreviations: DAO, D-amino acid oxidase; kbp, kilobase pair

2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (3000–4000 Ci/mmol), [α-^{32}P]dCTP (410 Ci/mmol), the oligonucleotide-directed mutagenesis system and the rabbit reticulocyte lysate were obtained from Amersham; the Riboprobe in vitro transcription system (pSP64 DNA, SP6 RNA polymerase and RNasin) was from Promega Biotech; M13mp19 replicate form DNA from Takara Shuzo Co.; agarose type I from Sigma; restriction endonucleases and sequencing reagents were from Toyobo Co.; nitrocellulose filters from Advantec and Schleicher & Schuell; and guanylyltransferase was from Bethesda Research Laboratories.

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2.2. *Site-specific mutagenesis*

A 1.34 kbp cDNA for porcine DAO was cut out of the DAO cDNA clone [5] with *Sal*I and *Pvu*II and subcloned into the *Sal*I-*Sma*I site of the replicative form DNA of M13mp19. This was used to transform *E. coli* JM103 cells, and the single-stranded DNA was purified from selected transformants, and used for in vitro mutagenesis. Three mutagenic primers designed for desired point mutations (table 1) were synthesized by the phosphoramidite method in an automated DNA synthesizer (Applied Biosystems, Model 380A) and purified by high-performance liquid chromatography on a reverse-phase (C_{18}) column. The cDNAs for mutant DAOs were constructed by the single-primer method of site-specific mutagenesis [7]. The mutated cDNAs were used directly to transform competent *E. coli* JM109 cells.

2.3. *Screening of mutant cDNA*

The oligodeoxynucleotides encoding mutant DAOs were radiolabeled with [γ-^{32}P]ATP and then used for plaque hybridization [8] to select mutant phage DNAs. Plaques exhibiting positive hybridization signals were subjected to sequencing by the dideoxy method [9] to verify that the desired mutations had taken place. Three oligodeoxynucleotides containing the sequences complementary to those of DAO cDNA (5′-CCAGTTCGCCTCCTGTG-3′, 5′-AGTTCTCTGGGAG-TCAG-3′ and 5′-TCCAAGTGTCACTGCCTG-3′) were used as sequence primers to detect the Phe-55, Leu-110 and Leu-217 mutants.

2.4. *Transcription and translation in vitro*

The mutant DAO cDNAs were cut out of the phage vector with *Sal*I and *Eco*RI and ligated to the same site in the polylinker region of pSP64 [10] and the resultant recombinants were used to transform *E. coli* JM109 cells as described previously [6]. The plasmids containing the mutant cDNA inserts were linearized with *Eco*RI. 5 μg each of the linearized template was transcribed with SP6 RNA polymerase. A 5′-terminal cap structure was added to the RNAs thus synthesized with the aid of guanylyltransferase. Translation of the capped RNAs was conducted in a reticulocyte lysate cell-free system as described [6,11].

2.5. *Western blot analysis*

Western blot analysis was used to analyze the translation products as described by Burnette [12]. The products were transferred electrophoretically from an SDS-polyacrylamide gel to a nitrocellulose filter and incubated with polyclonal antiserum raised in a rabbit against purified porcine DAO [6,11] at 1000-fold dilution. The immune complexes were radiographically visualized through binding of [^{125}I]protein A.

2.6. *Enzyme assay*

DAO activity was assayed by determining the production of pyruvate from D-alanine. Pyruvate production was monitored by measuring the rate of decrease in the absorbance at 340 nm in a coupled system containing lactate dehydrogenase. The assay mixture contained 50 μM FAD, 5.9 units/ml of lactate

Table 1

Oligonucleotide-directed mutagenesis of D-amino acid oxidase

Desired mutation	DNA sequence[a]
	55
	Trp Gln Pro Tyr Thr Ser Glu
Tyr-55 (wild type)	5′.... TGG CAG CCC TAC ACC TCT GAG ....3′
	*
Phe-55 (mutant)	5′.....TTC.....3′
	(Phe)
Mutagenic primer	3′-C GTC GGG AAG TGG AGA C-5′
	110
	Trp Lys Asp Met Val Leu Gly
Met-110 (wild type)	5′.... TGG AAA GAC ATG GTC CTG GGA ....3′
	*
Leu-110 (mutant)	5′..... CTG .....3′
	(Leu)
Mutagenic primer	3′-C TTT CTG GAC CAG GAC C-5′
	217
	Ile Ile Thr His Asp Leu Glu
His-217 (wild type)	5′.... ATT ATC ACC CAT GAC CTA GAG ....3′
	*
Leu-217 (mutant)	5′.....CTT.....3′
	(Leu)
Mutagenic primer	3′-A TAG TGG GAA CTG GAT C-5′

[a] The DNA sequence of wild type D-amino acid oxidase cDNA was changed at the positions marked by asterisks to yield the desired mutant cDNAs

dehydrogenase, 0.2% Triton X-100, 50 μM NADH, 37 mM D-alanine, a half of the total translate in the reticulocyte lysate and 50 mM sodium pyrophosphate buffer (pH 8.3) in a total volume of 540 μl. The reaction temperature was 25°C.

2.7. *Purification of porcine kidney DAO*

Porcine kidney DAO, purified by the method of Kubo et al. [13], was further purified by chromatography on hydroxyapatite cellulose [14] and DEAE-Sephadex [15] columns.

3. RESULTS AND DISCUSSION

Since chemical modifications of Tyr-55, Met-110 and His-217 of porcine DAO have been reported to lead to abolishment or extensive inactivation of the enzyme activity [1–4], we attempted to construct three mutant DAOs possessing Phe-55, Leu-110 or Leu-217. For this purpose, DAO cDNA was subjected to oligonucleotide-directed mutagenesis using synthetic 17-mer oligonucleotides (table 1) as mutagenic primers. Each of the 17-mer oligonucleotides was designed to cause a single base substitution. That the desired point mutations had actually taken place was verified by nucleotide sequencing, which also confirmed that the three point mutants were free from contamination by the wild type cDNA.

The mutant cDNAs, cut out of the phage vector, were so ligated into pSP64 that the cDNAs were located downstream from the SP6 promoter. After linearization, the recombinant plasmids were transcribed with SP6 RNA polymerase and the transcripts were capped with guanylyltransferase. The capped RNAs were then translated in a rabbit reticulocyte lysate system. The proteins thus synthesized were analyzed by Western blot analysis. As shown in fig.1, polyclonal anti-porcine DAO serum recognized all the three mutant DAOs (lanes 1–3), wild type DAO synthesized in vitro (lane 4) and DAO purified from porcine kidney (lanes 6–9). The electrophoretic mobilities of the mutant DAOs were the same as that of the wild type enzyme (M_r 38000). Densitometric comparison of the intensities of mutant DAO bands (lanes 1–3) with those of authentic wild type DAO bands of known amounts (lanes 6–9) indicated that about 100 ng each of the mutant proteins was produced from 5 μg each of the capped transcripts. This translation efficiency was similar to that of wild type DAO.

When the catalytic activities of the mutant DAOs synthesized in vitro were measured with D-alanine as substrate, they all showed significant activities, which were strongly inhibited by benzoate, a potent competitive inhibitor of DAO (fig.2). Addition of D-alanine to the reticulocyte lysate did not cause any decrease in the absorbance at 340 nm. As shown in table 2, the specific DAO activities of the wild type and mutant enzymes synthesized in vitro were somewhat variable from one

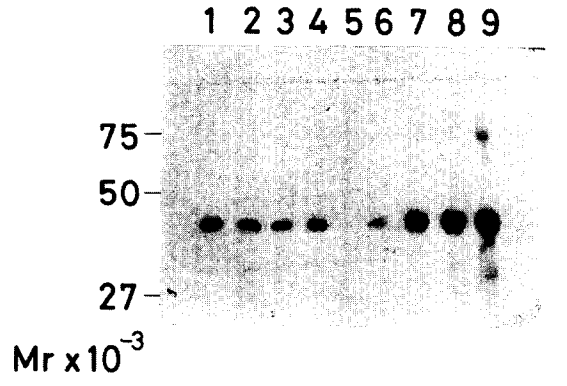

Fig.1. Western blot analysis of the in vitro translated wild type and mutant D-amino acid oxidases. Lanes: 1, Phe-55 mutant; 2, Leu-110 mutant; 3, Leu-217 mutant; 4, wild type DAO; 5–9, the purified porcine kidney DAO (5, 0 ng; 6, 5 ng; 7, 25 ng; 8, 50 ng; 9, 100 ng).

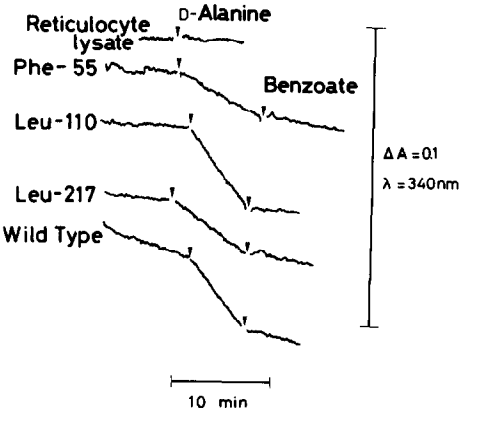

Fig.2. The enzyme reaction of the mutant DAOs and inhibition by benzoate. The time course of D-alanine oxidizing activity was monitored by means of a lactate dehydrogenase coupled assay as described in section 2. D-Alanine (final concentration, 37 mM) was added at the points indicated by the first arrows and sodium benzoate (2 mM) was added at the points indicated by the second arrows.

Table 2

The specific activities of the mutant DAOs

	Specific activity (μmol D-alanine oxidized/min per mg protein)			
	Wild type	Phe-55	Leu-110	Leu-217
Expt 1	9.0	5.5	16.0	3.3
Expt 2	14.0	8.0	12.0	9.6

experiment to another, probably because of the very low amount of proteins synthesized in the in vitro system. Inaccuracy associated with densitometric determination of DAO proteins may also be responsible for the fluctuation of data. In any case, the specific activities of the mutant enzymes were similar to one another and comparable with those of the wild type enzyme synthesized in vitro and purified porcine kidney DAO [16].

From the results described above, it is clear that substitution of Tyr-55 with Phe, Met-110 with Leu or His-217 with Leu in porcine DAO does not affect the catalytic activity significantly, even though previous chemical modification studies have suggested the importance of these three amino acid residues in the catalytic mechanism [1–4]. Tyr-55 has been postulated to play a role in substrate binding through an ion pair with the α-amino group of D-amino acid [2,17]. If this is so, then replacement of this residue by Phe having a nonpolar side chain would result in defective substrate binding and thus lead to a severe impairment of the catalytic activity. The finding that mutant DAO possessing Phe-55 is fully active indicates that this postulate is not tenable. A reaction mechanism involving Met-110 has also been proposed, in which it is thought to participate in the breakdown of the substrate-N_5 flavin adduct to yield a sulfonium salt [4]. Further, His-217 has been suggested to take part directly in the catalysis by facilitating proton transfer or stabilizing the local conformation of the active center through hydrogen bonding [3]. Since substitution of Met-110 or His-217 with Leu has no effect on the enzyme activity, these suggestions have also to be reconsidered. It is to be noted in this connection that in human DAO the 110 position is occupied by Thr instead of Met [18].

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