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Journal of Molecular Catalysis B: Enzymatic 12 (2001) 115–119

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Remarkable decrease in D-amino acid oxidase activity in the kidney of both prone and resistant strains of senescence-accelerated mouse

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Received 16 September 1999; received in revised form 10 November 1999; accepted 11 November 1999

Abstract

D-Amino acid oxidase (DAO) activity was remarkably low in the kidney of both prone P1 and resistant R1 strains of the senescence-accelerated mouse (SAM) compared with that of AKR/J mouse, in spite of the presence of a nearly equal amount of protein as demonstrated by Western blot analysis. A nucleotide sequence analysis of DAO-cDNA from SAMP1/Yag kidney indicated that the 541st guanine was replaced by adenine, resulting in the change of the 181st amino acid from glycine to arginine. The results of reverse transcription-polymerase chain reaction-restriction fragment length polymorphism analysis indicate the presence of this mutation in all SAM strains examined (P1, P2, P3 and R5). © 2001 Published by Elsevier Science B.V.

Keywords: D-Amino acid oxidase; Senescence-accelerated mouse; Point mutation

1. Introduction

D-Amino acid oxidase (DAO; EC 1.4.3.3) is a flavoprotein with flavin adenine dinucleotide (FAD) as its prosthetic group, and catalyzes the oxidative deamination of D-amino acids. Even though the amino acid sequence of this enzyme is conserved among mammalian species, its physiological role in higher animals still remains unclear. Mutant mice of the ddY strain lacking DAO activity were shown to secrete D-alanine into their urine [1], and to have a point mutation in the open reading frame (ORF) of the DAO gene [2].

It has been shown that racemization of L-amino acids, especially L-aspartic acid and L-serine, occurs in neuritic plaque amyloid of Alzheimer's disease [3]. From these findings, it might be expected that the accumulation of D-amino acids has some relation to ageing in the animal body.

The senescence-accelerated mouse (SAM) is a murine model of ageing developed by Takeda et al. [4] from the AKR/J mouse strain. From this mouse,

Abbreviations: DAO, D-amino acid oxidase; FAD, flavin adenine dinucleotide; ORF, open reading frame; SAM, senescence-accelerated mouse; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; RFLP, restriction fragment length polymorphism

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1381-1177/01/$ – see front matter © 2001 Published by Elsevier Science B.V.
PII: S1381-1177(00)00211-3

senescence-prone (P series) and senescence-resistant (R-series) series were established. SAMP1 mice display various senescence-related signs such as systemic amyloidosis, impaired immune responsiveness, hair loss, increased lordokyphosis, and periophthalmic lesions. In our preliminary study, SAM exhibited remarkably decreased DAO activity. This finding led us to investigate DAO structure in SAM. In this paper, we demonstrate the occurrence of a point mutation in the DAO ORF of the kidney of all the strains of SAM examined.

2. Materials and methods

2.1. Materials

Materials used and their sources were as follows: Prestained sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) Standards Low Range, from Bio-Rad Laboratories, Hercules, CA; PolyScreen PVDF Transfer Membrane, from NEN Life Science Products, Boston, MA; TRIZOL Total RNA Isolation Reagent, from Life Technologies, Gaithersburg, MD; Synthetic oligo nucleotide primers, from Greiner Japan, Tokyo; RNA PCR Kit (AMV) Ver. 1.1, Ligation Kit, and various restriction enzymes, from Takara, Kyoto; Original TA Cloning Kit (pCR^{TM}II), and *Escherichia coli* INVαF′ (competent cell), from Invitrogen, San Diego, CA; QIAprep Spin Miniprep Kit, from Qiagen Inc., Chatsworth, CA; Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP, from Amersham Life Science, Backinghamshire, UK. All other reagents were purchased from Nakarai Tesque, Inc., Kyoto. SAMP1/Yag and SAMR1/Yag were maintained in the Institute of Applied Biochemistry and SAMP2, P3, and R5, in Kyoto University. Mice of ICR, ddY, and BALB/c strains were purchased from Japan SLC Co., Hamamatsu.

2.2. Assay for DAO activity

DAO activity was assayed using D-alanine (20 mM) as a substrate in 20 mM pyrophosphate buffer (pH 8.3) including 5 μM FAD at 37°C. The pyruvic acid produced was measured by the dinitrophenylhydrazine method [5]. One unit of enzyme was defined as the amount required to oxidize 1 μmol of D-alanine per minute at 37°C. Protein concentration was determined by the method of Markwell et al. [6].

2.3. Electrophoresis

SDS-PAGE was carried out according to the method of Laemmli [7]. After SDS-PAGE, protein bands were electro-transferred to PVDF membranes. Western blot analysis was carried out according to the method of Towbin et al. [8] with polyclonal antibody against hog kidney DAO raised in a rabbit.

2.4. Cloning of DAO-cDNA of SAMP1 and nucleotide sequencing

For cDNA cloning of DAO from the kidney of SAMP1/Yag, the reverse transcription polymerase chain reaction (RT-PCR) method was employed, and PCR primers 5′-GGTTATTTTTCTCCCGACAC-3′ (forward) and 5′-AAGGGGTTGGGGTGTCGTCT-3′ (reverse) were synthesized based on the nucleotide sequence of mouse DAO as reported by Tada et al. [9]. Template RNA was prepared from the kidney (132 mg wet weight) of SAMP1/Yag (10-month-old) and the obtained RNA (1 μg) was subjected to RT reaction, according to the supplier's instructions. The PCR mixture contained the cDNA, 1 μM each primer, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 1.25 units of *Taq* DNA polymerase and 200 μM each of dATP, dCTP, dGTP, and dTTP, in a total volume of 50 μl. PCR was carried out in a GeneAmp PCR System model-9600 (Perkin Elmer, Foster City, CA). The first PCR cycle consisted of a denaturation step (94°C, 2 min), an annealing step (60°C, 30 s), and an elongation step (72°C, 1 min). In the subsequent 30 cycles, the duration of the denaturation was 30 s; the annealing, 30 s; and the elongation, 1.5 min. For the final step, the duration of the elongation was 5 min. Wild type DAO-cDNA was cloned from the kidney of BALB/c as described above.

The amplified DNA was ligated into pCR^{TM}II, and the resulting plasmid was transfected into *E. coli* INVαF′. These plasmids were purified and sequenced by the dideoxy-mediated chain-termination method using a DNA sequencer (LI-COR, Lincoln, NE).

Table 1
DAO activities in mouse kidneys

Mouse	Specific activity (mU/mg protein)
AKR/J	3.10–4.10
SAMP1/Yag	0–0.20
SAMR1/Yag	0–0.48
ICR	2.08–2.60
ddY	1.45–1.92

3. Results and discussion

3.1. Expression of DAO in the kidney of SAM and AKR/J mice

To investigate whether DAO was expressed in SAM, we carried out DAO activity assays and Western blot analysis on crude extracts of SAM kidney and those of other strains. The specific activity of the AKR/J mouse kidney enzyme was 3.10–4.10 mU/mg protein, whereas that of both SAMP1/Yag and R1/Yag was extremely low, as shown in Table 1. However, Western blot analysis showed that immunoreactive proteins of about 38 kDa, which corresponds to the molecular mass of DAO protein, were expressed at nearly the same level in the kidney of both the AKR/J and the SAM mouse strain (Fig. 1).

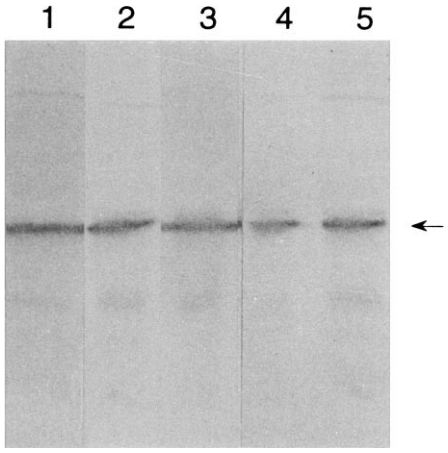

Fig. 1. Western blot analysis of kidney DAO of various mouse strains. The supernatant obtained after 5000 g centrifugation of mouse kidney homogenate (protein, 10 μg) was subjected to 10% SDS-PAGE, and transblotted to PVDF membrane. Lane 1, AKR/J (2-month-old); lane 2, SAMP1/Yag (2-month-old); lane 3, ICR mouse (4-month-old); lane 4, SAMP1/Yag (14-month-old); lane 5, SAMR1/Yag (14-month-old). Arrow indicates the position of DAO protein.

3.2. Cloning and sequencing of SAM kidney DAO

Using PCR primers (listed in Section 2.4) prepared on the basis of the BALB/c mouse DAO-cDNA nucleotide sequence, we carried out RT-PCR on the total RNA of the SAMP1/Yag kidney. A band of about 1.2 kb, corresponding to the DAO-cDNA of BALB/c, was detected by agarose gel electrophoresis of the PCR products. This band was subcloned in pCR^{TM}II, and we obtained 24 positive clones. Among them, six samples were randomly selected and sequenced. These clones had the same ORF as that of BALB/c except that the guanine at the 541st position was replaced by adenine, resulting in the change of the 181st amino acid residue from glycine to arginine.

3.3. RT-PCR restriction fragment length polymorphism (RT-PCR-RFLP)

To determine, whether this mutation occurred generally in various SAM strains, we performed RT-PCR-RFLP analysis. In this case, a *Van*91I restriction site was created at nucleotide positions 539–549 of the DAO ORF due to the mutation. Using the primers 5′-GGAATTCCATATGCGCGTGGCCGTGAT-3′ (forward) and 5′-CGGGATCCTCAGAGGTGGGATGGA-3′ (reverse), we employed the step-down PCR method to reduce the background. The first three PCR cycles consisted of a denaturation step (94°C, 30 s), an annealing step (58°C, 30 s), and an elongation step (72°C, 1 min). The following PCR cycles consisted of the same steps except that the annealing temperature was gradually decreased from 56°C to 50°C over three cycles. In the subsequent 20 cycles, the duration of the denaturation step was 30 s; annealing, 30 s; and elongation, 1 min. For the final step, the duration of the elongation step was 5 min.

In this experiment, RNA fractions of the kidneys of SAMP1/Yag, SAMP2, P3, and R5 mice were tested. For comparison, the RNA of the kidney of an AKR/J mouse was also subjected to RT-PCR-RFLP. The products obtained from RT-PCR were of nearly the same quantity, and their size corresponded to that of DAO-cDNA (Fig. 2, lanes 1–5). Accordingly, we concluded that DAO mRNA was expressed at the

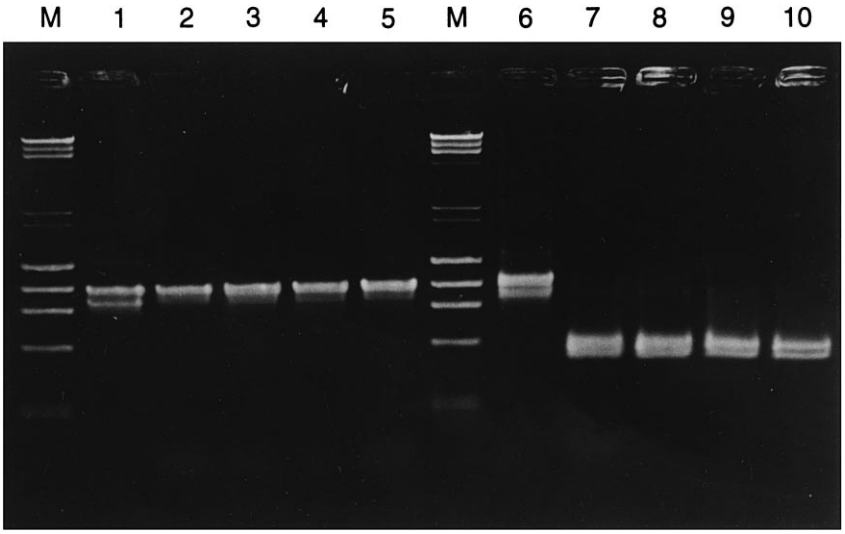

Fig. 2. RT-PCR-RFLP analysis of expression of kidney DAO of various strains of SAM. The RT-PCR products from the total RNA of the kidneys were digested with *Van*91I endonuclease and subjected to a 2% agarose gel electrophoresis. Lanes 1 to 5, RT-PCR products; lanes 6 to 10, *Van*91I digests of RT-PCR products. Lane M, A double-stranded DNA markers (a mixture of λ-*Hin*d III and ϕX174-*Hae* III digest). Lanes 1 and 6, AKR/J; lanes 2 and 7, SAMP1/Yag; lanes 3 and 8, SAMP2; lanes 4 and 9, SAMP3; lanes 5 and 10, SAMR5.

same level in all the mouse kidneys examined. All the RT-PCR samples from the SAM kidney were digested into two fragments by *Van*91I (Fig. 2, lanes 7–10), whereas the sample from the AKR/J mouse was not (Fig. 2, lane 6).

3.4. Occurrence of the point mutation at the genomic level

To confirm that the mutation at the 541st base is also present at the genomic level, we conducted step-down PCR with 5′-GCAAGAGGAGTGGATGTGAT-3′ (forward) and 5′-TGGGTGAGGATGAAGTGTTT-3′ (reverse), using the genomic DNA of BALB/c mouse kidney and that of SAMP1/Yag as templates. A 948 base pair amplification product was obtained, which included 811 base pairs of deduced introns. Sequence analysis indicates that the guanine to adenine mutation at the 541st base pair was present at the genomic level.

In the present study, we demonstrated that in SAM the activity of kidney DAO markedly decreased and this decrease was brought out by a nucleotide mutation. There was no difference between the expression level of DAO protein in AKR/J and SAM strains. RT-PCR-RFLP analysis made clear that this mutation is present in the kidneys of all the SAM strains examined. This indicates that the mutation occurred during the establishment of the SAM strain from the AKR/J mouse. The same mutation was also found in the ddY mouse [2].

In relation to this mutation in SAM, Sato et al. [10] reported that DAO activity in the cerebral cortex of SAMP8 (specific activity, 0.34 ± 0.04) did not differ from that of age-matched SAMR1 (0.33 ± 0.05). These values are similar to those obtained in the present study. DAO activity decreased remarkably in both senescence-prone and senescence-resistant strains due to one point mutation. The present result seems to deny the possibility of a direct relationship between the reduction in the activity of DAO and ageing, because of the similar decrease in the activity of DAO in the senescence-resistant and senescence-prone strains of SAM. However, this point still remains to be clarified by future investigation.

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