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Structure–function relationships in human D-aspartate oxidase: characterisation of variants corresponding to known single nucleotide polymorphisms

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ABSTRACT

D-Aspartate oxidase (DDO) is a degradative enzyme that is stereospecific for the acidic amino acid D-aspartate, an endogenous agonist of the *N*-methyl-D-aspartate (NMDA) receptor. Dysregulation of NMDA receptor-mediated neurotransmission has been implicated in the onset of various neuropsychiatric disorders including schizophrenia and in chronic pain. Thus, appropriate regulation of the amount of D-aspartate is believed to be important for maintaining proper neural activity in the nervous system. Herein, the effects of the non-synonymous single nucleotide polymorphisms (SNPs) R216Q and S308N on several properties of human DDO were examined. Analysis of the purified recombinant enzyme showed that the R216Q and S308N substitutions reduce enzyme activity towards acidic D-amino acids, decrease the binding affinity for the coenzyme flavin adenine dinucleotide and decrease the temperature stability. Consistent with these findings, further experiments using cultured mammalian cells revealed elevated D-aspartate in cultures of R216Q and S308N cells compared with cells expressing wild-type DDO. Furthermore, accumulation of several amino acids other than D-aspartate also differed between these cultures. Thus, expression of *DDO* genes carrying the R216Q or S308N SNP substitutions may increase the D-aspartate content in humans and alter homeostasis of several other amino acids. This work may aid in understanding the correlation between DDO activity and the risk of onset of NMDA receptor-related diseases.

1. Introduction

A variety of D-amino acids are present in living organisms that play important roles in physiological functions [1–5]. Among the free D-amino acids found in mammals, D-aspartate (D-Asp; all proteinogenic L-amino acids and their enantiomers are described using the three-letter code in this article) plays a critical role in the central nervous system, as well as the neuroendocrine and endocrine systems. Several lines of evidence suggest that D-Asp plays an important role in regulating developmental processes, hormone secretion and steroidogenesis [6–8]. The amount of D-Asp in human seminal plasma and spermatozoa is significantly lower in oligoasthenoteratospermic and azoospermic donors than normospermic donors [9]. Furthermore, in female patients undergoing *in vitro* fertilisation, the D-Asp content of pre-ovulatory follicular fluid is lower in older patients than younger patients [10]. This decrease in D-Asp content appears to reflect a reduction in oocyte quality and fertilisation competence. Overall, current evidence suggests that D-Asp may be involved in the pathophysiology of infertility. D-Asp is also known to stimulate the *N*-methyl-D-aspartate (NMDA) receptor (a subtype of the L-Glu receptor family) by acting as an agonist that binds to the L-Glu-binding site on the NR2 subunit of the receptor [11–13]. Recent studies suggest that D-Asp acts as a signalling molecule in the nervous and neuroendocrine systems, at least in part, by binding to the NMDA receptor, and plays an important role in regulating brain functions [7,8,14]. This was supported by recent reports demonstrating that D-Asp levels in the prefrontal cortex and/or striatum of post-mortem brains of schizophrenic patients are significantly lower than those of non-psychiatrically ill individuals [15,16].

In mammalian tissues, three types of degradative enzymes have been identified that are stereospecific for D-amino acids, namely, D-Asp

Abbreviations: DAO, D-amino acid oxidase; DDO, D-aspartate oxidase; FAD, flavin adenine dinucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; NMDA, *N*-methyl-D-aspartate; OPA, *o*-phthalaldehyde; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; SNP, single nucleotide polymorphism

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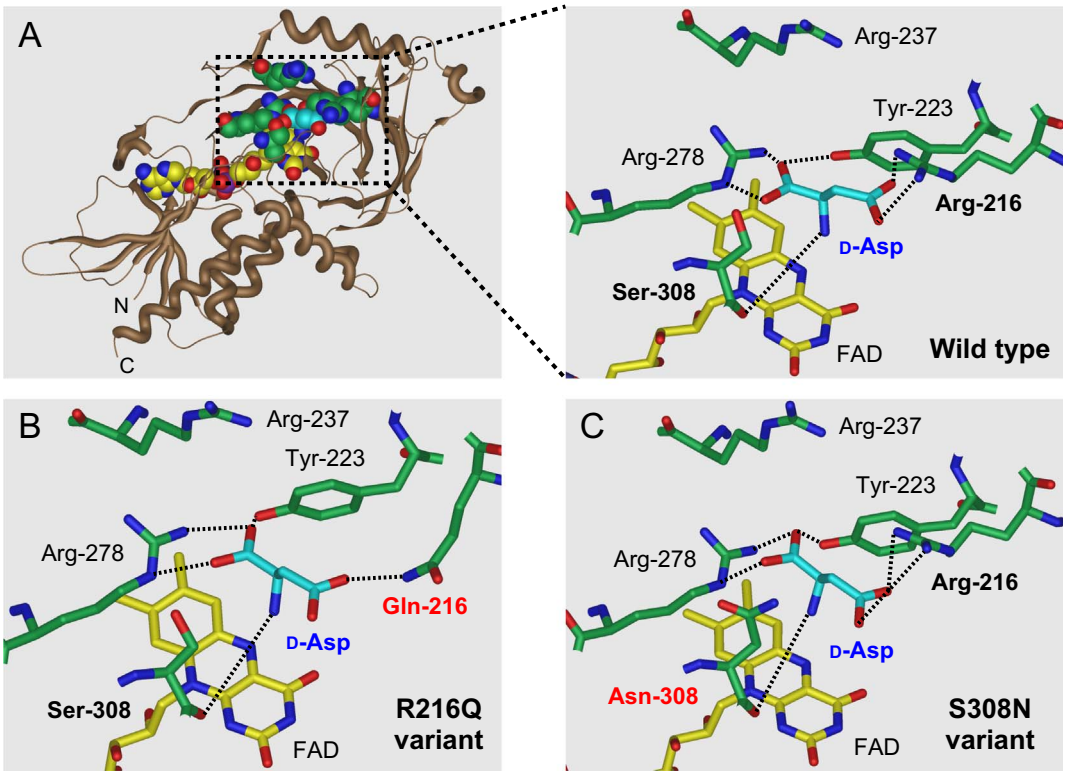

Fig. 1. Structural models of human D-Asp oxidase (DDO) complexed with D-Asp. The substrate D-Asp was docked into the active site of wild-type human DDO (A) and the Arg-216-to-Gln (R216Q) (B) and Ser-308-to-Asn (S308N) (C) variants by simulated annealing (see main text for details). Flavin adenine dinucleotide (FAD) cofactor molecules are also shown. Carbon atoms in D-Asp, FAD and the side chains of amino acid residues are coloured cyan, yellow and green, respectively. Nitrogen atoms are coloured blue, and oxygen atoms are red. Black dotted lines denote possible hydrogen bonds.

oxidase (DDO, also abbreviated as DASPO; EC 1.4.3.1), D-amino acid oxidase (DAO, also abbreviated as DAAO; EC 1.4.3.3) and D-Glu cyclase (EC 4.2.1.48) [1,17,18]. Among them, DDO is the only catabolic enzyme that acts on D-Asp. This enzyme is a flavin adenine dinucleotide (FAD)-containing flavoprotein that catalyses the oxidative deamination of D-amino acids to generate 2-oxo acids along with hydrogen peroxide and ammonia. DDO is highly specific for acidic D-amino acids such as D-Asp and D-Glu, none of which are substrates of DAO, while D-Glu cyclase acts on D-Glu, but not D-Asp. DDO has been identified in various organisms, and its physiological roles *in vivo* are receiving much attention. In mammals, DDO activity is highest in the kidney, followed by the liver and brain, while activity is low in other peripheral tissues. DDO localises to the peroxisome [19,20] where catalase degrades toxic hydrogen peroxide, one of the enzymatic reaction products. Mammalian DDO is presumed to regulate the levels of endogenous and exogenous D-Asp in various organs [17], but its physiological roles *in vivo* remain to be fully clarified.

Reduced levels of D-Asp in the nervous system and the resulting dysfunction of NMDA receptor-mediated neurotransmission are thought to occur during onset of various mental disorders including schizophrenia [7,8,14–16]. Hence, a substance capable of increasing D-Asp levels and activating NMDA receptor function may provide a novel foundation for the development of antipsychotic drugs. In this regard, it was recently reported that olanzapine, a commonly used second-generation antipsychotic drug, inhibits DDO activity and the chronic olanzapine administration increases extracellular D-Asp levels in the prefrontal cortex of freely moving mice, suggesting novel therapeutic strategies for treating neuropsychiatric disorders associated to cortical NMDA receptor hypofunction [21]. Thus, one way to increase the level of D-Asp is to prevent its metabolic degradation by DDO. Indeed, DDO-deficient mice have elevated concentrations of D-Asp in several brain regions and exhibit specific behaviours suggestive of potential antidepressant and antischizophrenic activities [22,23]. In addition, *DDO* mRNA expression and/or DDO activity are increased in the prefrontal cortex of post-mortem brains of schizophrenic patients compared with the brains of non-psychiatrically ill individuals [16,24], consistent with the lower D-Asp levels in this tissue described above [15,16].

NMDA receptor-induced hyperexcitability at the spinal cord dorsal horn is thought to result in central sensitization during the initiation and progression of chronic pain [25]. It was recently reported that the nocifensive responses during the second phase in formalin tests are significantly higher in DDO-deficient mice than wild-type mice [26], suggesting excess amounts of D-Asp may be involved in the onset and/or exacerbation of chronic pain. Thus, it appears that appropriate regulation of the amount of D-Asp by DDO in distinct tissues and cell types is extremely important for maintaining proper neural activity. However, little is known about the regulatory mechanisms controlling human DDO activity or the molecular details of its structure–function relationships.

X-ray crystallographic analysis, *in vitro* mutagenesis and/or the use of specific enzyme inhibitor(s) are typical approaches for studying enzyme structure–function relationships, and several amino acid residues within human DAO that are functionally and structurally important have been identified [18,27–32]. The three-dimensional (3D) structure of human DDO has not yet been reported, although some enzymatic and structural properties of this enzyme have been investigated [33–37]. We previously proposed structural models for human, mouse and rat DDO enzymes based on their deduced amino acid sequences [36]. In the proposed structures, the side chains of Arg-216, Tyr-223, Arg-237, Arg-278 and Ser-308, which are well-conserved in mammalian DDOs, are oriented toward the predicted active site binding pocket, implicating them in DDO catalysis. Furthermore, it was also demonstrated by mutagenesis that Arg-216, Arg-237 and Ser-308 are functionally important residues in the mouse enzyme [38–40]. Consistent with these findings, determination of a theoretical model of the human DDO/D-Asp complex showed that the aforementioned residues are located close to the

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