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Structure–function relationships in human D-amino acid oxidase variants corresponding to known SNPs☆

Pamela Cappelletti [a,b], Luciano Piubelli [a,b], Giulia Murtas [a], Laura Caldinelli [a,b], Mattia Valentino [b,c], Gianluca Molla [a,b], Loredano Pollegioni [a,b], Silvia Sacchi [a,b,*]

[a] Dipartimento di Biotecnologie e Scienze della Vita, Università degli Studi dell'Insubria, via J. H. Dunant 3, 21100 Varese, Italy
[b] The Protein Factory, Politecnico di Milano, ICMR–CNR, Università degli Studi dell'Insubria, via Mancinelli 7, 20131 Milano, Italy
[c] CNR, Istituto di Chimica del Riconoscimento Molecolare, Sezione Adolfo Quilico, via M. Bianchi 9, 20131 Milano, Italy

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ABSTRACT

In the brain, D-amino acid oxidase plays a key role in modulating the N-methyl-D-aspartate receptor (NMDAR) activation state, catalyzing the stereospecific degradation of the coagonist D-serine. A relationship between D-serine signaling deregulation, NMDAR dysfunction, and CNS diseases is presumed. Notably, the R199W substitution in human DAAO (hDAAO) was associated with familial amyotrophic lateral sclerosis (ALS), and further coding substitutions, i.e., R199Q and W209R, were also deposited in the single nucleotide polymorphism database. Here, we investigated the biochemical properties of these different hDAAO variants. The W209R hDAAO variant shows an improved D-serine degradation ability (higher activity and affinity for the cofactor FAD) and produces a greater decrease in cellular D/(D + L) serine ratio than the wild-type counterpart when expressed in U87 cells. The production of H_2O_2 as result of excessive D-serine degradation by this hDAAO variant may represent the factor affecting cell viability after stable transfection. The R199W/Q substitution in hDAAO altered the protein conformation and enzymatic activity was lost under conditions resembling the cellular ones: this resulted in an abnormal increase in cellular D-serine levels. Altogether, these results indicate that substitutions that affect hDAAO functionality directly impact on D-serine cellular levels (at least in the model cell system used). The pathological effect of the expression of the R199W hDAAO, as observed in familial ALS, originates from both protein instability and a decrease in kinetic efficiency: the increase in synaptic D-serine may be mainly responsible for the neurotoxic effect. This information is expected to drive future targeted treatments.

1. Introduction

The peroxisomal FAD-dependent enzyme D-amino acid oxidase (DAAO, EC 1.4.3.3) catalyzes the stereospecific oxidative deamination of D-amino acids to give the corresponding α-keto acids and ammonia, coupled with the reduction of FAD. The cofactor then reoxidizes on molecular oxygen yielding hydrogen peroxide [1,2]. In the brain, DAAO has been involved in the selective degradation of D-serine, an "atypical" signaling molecule which, in addition to glutamate, modulates the activation state of N-methyl-D-aspartate subtype of glutamate receptors (NMDARs) by acting as the main endogenous coagonist in different brain areas. D-Serine is synthesized from L-serine via an isomerization reaction catalyzed by the PLP-dependent enzyme serine racemase (SR, EC 5.1.1.18) [3], an enzyme also shown to catalyze the α,β-elimination of water from L- or D-serine to yield pyruvate and ammonia [4,5]. SR is predominantly expressed in neurons, which release D-serine upon membrane depolarization [6,7], while, differently from what was previously assumed, it is barely detectable in astrocytes [6,8], the cells containing the highest amount of D-serine. A recent model of D-serine dynamics, the "serine shuttle model" (which is still being defined) suggested that D-serine is predominantly synthesized and released by neurons and subsequently taken up by astrocytes for storage and activity-dependent release [9]. Considering the low levels of SR in astrocytes, the proposed model indirectly assigns to DAAO a key role in regulating D-serine concentration in these cells. Thus, we can assume

Abbreviations: ALS, amyotrophic lateral sclerosis; CBIO, 6-chloro-benzo(*d*)isoxazol-3-ol; CD, circular dichroism; CNS, central nervous system; CPZ, chlorpromazine; DAAO, D-amino acid oxidase (EC 1.4.3.3); EYFP, enhanced yellow fluorescent protein; hDAAO, human D-amino acid oxidase; NLS, N-lauroylsarcosine; NMDAR, N-methyl-D-aspartate subtype of glutamate receptor; SR, serine racemase (EC 5.1.1.18); SNP, single nucleotide polymorphism

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* Corresponding author at: Dipartimento di Biotecnologie e Scienze della Vita, Università degli Studi dell'Insubria, via J. H. Dunant 3, 21100 Varese, Italy.
E-mail address: silvia.sacchi@uninsubria.it (S. Sacchi).

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that the flavoenzyme activity, through the regulation of D-serine levels, may affect the NMDAR-dependent physiological functions (including brain development, synaptic transmission, and long-term synaptic plasticity) involved in key cognitive abilities, such as learning and memory [10,11]. Notably, human DAAO (hDAAO) has been the object of intensive structural and functional studies [12–15] that highlighted its peculiar biochemical properties as compared to all known DAAOs: it shows a weak FAD binding, a stable homodimeric state, and the specific regulation by pLG72 binding [12,16,17].

Several studies indicate that a dysregulation in D-serine signaling is implicated in the NMDAR dysfunctions observed in pathological conditions. In particular, D-serine and SR levels are greatly increased in the spinal cord of patients affected by familial and sporadic amyotrophic lateral sclerosis (ALS) as well as in Alzheimer's disease, while they are downregulated in schizophrenia, see [18,19] and references therein. On the other hand, increased levels of DAAO expression and activity have been observed in schizophrenic individuals [20–22]. It has been also reported that a coding mutation in hDAAO associated with familial ALS (the R199W substitution) strongly affects the enzyme activity, promotes the accumulation of ubiquitinated protein aggregates, and causes primary motor neuron death [23,24]. Further single nucleotide polymorphisms (SNPs) resulting in nonsynonymous substitutions and also discrepancies in the deposited protein sequences encoded by the hDAAO gene have been reported.

We consider that it is very important to understand the correlation between the modulation of hDAAO enzymatic activity and its possible role in human CNS pathologies, therefore we recently investigated the effect of the SNP rs4262766 resulting in G331V replacement, and of the D31H and R279A substitutions [25]. Characterization of the biochemical properties of recombinant enzymes and cellular studies based on their expression in U87 human glioblastoma cells prompted us to propose that, in vivo, the latter substitutions could affect cellular D-serine concentration and its release at synapses and thus might be relevant for schizophrenia susceptibility.

With the same aim, we investigate in this paper the effect of the R199W substitution and of two other reported SNPs (rs200850756 and rs111347906 resulting in the R199Q and W209R replacements, respectively) on recombinant hDAAO biochemical properties. The latter two SNPs were confirmed but currently are not associated with human diseases, although the R199Q substitution involves the same hDAAO residue mutated in familial ALS. Arg199 lies close to FAD and substrate binding sites, while Trp209 is located at the monomer's interface (Fig. 1). Here, we discuss the significance of the expression of the different protein variants and of the related aberrant hDAAO functionality in pathological conditions.

2. Materials and methods

2.1. Preparation and expression of hDAAO variants in Escherichia coli

Mutagenesis reactions on the pET11b-His-hDAAO expression plasmid [12] were performed using the QuickChange site-directed mutagenesis kit (Stratagene, Santa Clara, CA, USA). The best production yields for the hDAAO variants were obtained by growing the BL21(DE3) Star *E. coli* cells overnight at 37 °C followed by further growth under the conditions reported in Suppl. Table 1. The recombinant His-tagged hDAAO variants were purified by HiTrap chelating chromatography (GE Healthcare, Uppsala, Sweden) as reported in [26]. The final preparation of the different hDAAO variants was stored in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 40 μM FAD, and 5 mM 2-mercaptoethanol.

hDAAO apoproteins were prepared by dialysis of 2 mg protein/mL solution vs. 50 mM sodium pyrophosphate, pH 8.3, 1 M KBr, and 1 mM EDTA for 48 or 72 h (R199W/Q and W209R hDAAOs variants, respectively) at 4 °C. The final apoprotein preparations were stored in 50 mM sodium pyrophosphate, pH 8.3, 5% glycerol, and 5 mM 2-mercaptoethanol.

pLG72 was prepared as reported in [27] and stored in 20 mM Tris–HCl, pH 8.5, 100 mM NaCl, 5% glycerol, 5 mM 2-mercaptoethanol, and 0.1% N-lauroylsarcosine (NLS).

2.2. Kinetic measurements

The standard assay for hDAAO activity was performed by employing an oxygen electrode at air saturation (0.253 mM O_2) at 25 °C, using 28 mM D-alanine as substrate in 75 mM sodium pyrophosphate, pH 8.5, containing 40 μM FAD [12]. One hDAAO unit is defined as the amount of enzyme that converts 1 μmol of D-amino acid per minute at 25 °C. The kinetic parameters were determined with the same assay, employing increasing D-alanine or D-serine concentrations in the 50–1200 mM D-alanine and 500–1850 mM D-serine range for R199W/Q hDAAO variants and in the 0.625–200 mM D-alanine and 5–750 mM D-serine range for W209R variants. The initial reaction rates at different substrate concentrations were used to calculate the apparent kinetic parameters ($k_{cat,app}$ and $K_{m,app}$) according to a Michaelis–Menten equation using the Kaleidagraph software (Synergy Software, Reading, PA, USA).

The rapid reaction measurements and the turnover experiments of W209R hDAAO were performed in 50 mM sodium pyrophosphate buffer, pH 8.3, containing 1% glycerol, at 25 °C in a stopped-flow BioLogic SFM-300 spectrophotometer equipped with a J&M diode array detector [12]: following the enzyme-substrate mixing, the concentration of free FAD was 1.4 μM. To investigate the reductive and oxidative half-reactions, the stopped-flow instrument was made anaerobic by equilibrating it overnight with a sodium dithionite solution and then rinsing with argon-equilibrated buffer [28]. For reductive half-reaction experiments, ~15 μM enzyme solution was anaerobically mixed with different concentrations of D-alanine (1–7.5 mM range, in 50 mM sodium pyrophosphate, pH 8.3) in the stopped-flow instrument. The oxidative half-reaction from the free reduced enzyme was investigated by reacting ~15 μM enzyme (in 50 mM sodium pyrophosphate, pH 8.3, 100 mM glucose, 2% glycerol, and 5 mM 2-mercaptoethanol, 0.1 μM glucose oxidase), which was reduced with a 5-fold excess of D-alanine under anaerobic conditions, with buffer solutions (50 mM sodium pyrophosphate, pH 8.3, 100 mM glucose) equilibrated at increasing oxygen concentrations (range: 1.25–50%). For reoxidation from the complex of the reduced enzyme with the imino acid, both the reduced enzyme and the reoxidation buffer contained 400 mM ammonium chloride and 20 mM pyruvate. The O_2-free samples were prepared in anaerobic cuvettes by applying ten cycles of evacuation/flushing with O_2-free argon [29]. Reaction rates were calculated by extracting traces at individual wavelengths (450 and 530 nm) and fitting them to a sum of exponential equations using the program Biokine32 (Bio-Logic Sci. Instr., Grenoble, France) [12,28,30].

2.3. Spectral analysis

UV-visible spectral analysis was performed at 15 °C in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10% glycerol, 40 μM FAD, and 5 mM 2-mercaptoethanol. Protein concentration of the purified enzymes was determined using the extinction coefficient at 450 nm (12.5 mM^{-1} cm^{-1}) [12].

Dissociation constants for ligands were determined spectrophotometrically by adding small volumes of concentrated stock solution of the ligands to samples containing ~10 μM enzyme and calculated from the change in absorbance at 493 nm for benzoate and at 496–499 nm for CBIO [12,31].

Circular dichroism (CD) spectra were recorded in 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 5% glycerol, and 40 μM FAD (for the holoenzyme form) by using a Jasco J-815 spectropolarimeter and analyzed by means

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