be completely excluded that decreased cell viability could be partly caused by high doses of amino acids, which may be harmful to cultured cells (39), and by D-serine, which inhibits serine palmitoyltransferase, which is responsible for the initial step of sphingolipid biosynthesis (*40*).

To confirm that astroglial DAO activity was involved in the cytotoxic effect of a high dose of D-serine, CPZ as well as sodium benzoate was examined as a DAO inhibitor. CPZ is the most important antipsychotic compound among the large group of phenothiazine derivatives. It is widely used for treating mental and personality disorders, especially schizophrenia, because of its anti-dopaminergic properties. Interestingly, CPZ was also found to inhibit D-amino acid oxidase through competition with its coenzyme, flavin adenine dinucleotide (*23*). As shown in Fig. 5, not only 20 mM sodium benzoate but also 1 μM CPZ significantly protected C6/DAO cells from D-serine. The concentrations of benzoate and CPZ used seem to be puzzling in view of their affinity to the pure enzyme *in vitro* (the K_d for both ligands are in the micromolar range). However, the efficiency of transport or permeation of benzoate and CPZ across the membrane needs to be taken into account. In fact, CPZ is known to be extremely hydrophobic and to exhibit good cell permeability as well as high serum-protein binding. Since CPZ also exhibits inhibitory effects on various other enzymes, the precise mechanisms underlying the effects of these ligands on C6 and C6/DAO cells remain to be elucidated. In addition, loss of recovery of C6 cells with benzoate and CPZ may reflect the catalytic activity of endogenous catalase and the low level of DAO expression in C6 cells. These results show that astroglial DAO is involved in the metabolism of extracellular D-serine.

H_2O_2, one of the major ROS, is a potentially harmful byproduct of normal cellular metabolism that directly affects cellular functions and survival (*41*). The production of H_2O_2 by 9L*daao*17 cells, a stable transformant of 9L glioma cells expressing red yeast DAO, incubated with D-alanine demonstrated oxidative stress is a mediator of cytotoxicity (*42*). Tumor-targeted delivery of polyethylene glycol–conjugated D-amino acid oxidase together with injection of D-proline generated a potent cytotoxic compound, H_2O_2, at the tumor site (*43*). These findings showed that DAO activity and hence the generation of H_2O_2 can be regulated by the exogenous administration of a D-amino acid. As shown in Fig. 3B, we also found that treatment of C6/DAO cells with D-serine resulted in H_2O_2 production. On the other hand, catalase plays an important role in the antioxidant defense of the organism, as do other enzymes such as superoxide dismutase and glutathione peroxidase, by degrading H_2O_2 to H_2O. As shown in Fig. 4B, inhibition of catalase by 3-AT partially enhanced the cell death induced by D-serine treatment in C6 and C6/DAO cells. We consider the following reasons may explain the possibility that H_2O_2 was able to avoid degradation by catalase. One is that H_2O_2 might have come from the remaining cytosolic DAO that was not recruited to peroxisomes. Another possibility is that all DAO was not colocalized with catalase. Indeed, all dots positive for catalase in C6/DAO cells did not overlap with those for DAO (Fig. 3C). Taken together, these results indicated that H_2O_2 is a causative agent of glial cell death induced by D-serine. Since D-serine is present at a micromolar concentration in the normal mammalian brain, the physiological relevance of this cytotoxic effect may seem unclear. However, it is possible that additional cellular stress or up-regulated biosynthesis and accumulation of D-serine in pathological conditions may influence the cytotoxic effect of D-serine, presumably through the production of H_2O_2.

The finding that D-serine is toxic to glial cells quite resembles the phenomenon observed for the rat kidney. It is well known that D-serine is nephrotoxic in rats. Intraperitoneallly injected D-serine damages the cells in the proximal tubules, leading to proteinuria, glucosuria, and aminoaciduria (*44*). D-Amino acid oxidase is considered to be involved in this process. H_2O_2, a product of the D-amino acid oxidase reaction, is also suspected to be a causative agent (*45*). However, this nephrotoxicity is only observed with D-serine. Other D-amino acids, such as D-alanine, D-threonine and D-cysteine, are not nephrotoxic, although D-alanine is a good substrate of D-amino acid oxidase (*46*). These results are quite similar to the findings described in the present paper. The previous studies so far performed have indicated that renal cells become necrotic. However, we demonstrated that a high dose of D-serine causes apoptosis of glial cells, based on the results of the TUNEL assay and the activation of caspase-3 in the culture system.

In conclusion, we have shown that astroglial DAO contributes to the metabolism of extracellular D-serine, by the detection of cytotoxicity of its metabolite, H_2O_2, with a high dose of D-serine. Detailed studies on the D-serine transport system in astroglial cells are also necessary to clarify the mechanism modulating the extracellular level of D-serine in the brain. Moreover, further study on the D-serine–D-amino acid oxidase system in the brain will help overcome diseases related to hyper- and hypofunctions of NMDA receptor–mediated neurotransmission, such as stroke and schizophrenia.

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Functional Roles and Pathophysiology of Brain D-Amino Acid Oxidase

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Introduction

D-Amino acid oxidase (D-amino acid: O_2 oxidoreductase, DAO; EC 1.4.3.3) is a flavoenzyme with noncovalently bound FAD as its prosthetic group, that catalyzes the oxidative deamination of wide range of D-amino acids to the corresponding imino acids, whereas molecular oxygen undergoes reduction to hydrogen peroxide (H_2O_2) (1). The imino acid is nonenzymatically hydrolyzed to α -keto acid and ammonia (Fig. 1). H_2O_2, a type of reactive oxygen species (ROS), is known to damage DNA, proteins and lipids, thereby leading to stress-induced apoptosis (2). In mammals, DAO is found at highest concentration in kidney proximal tubules, liver and granules of neutrophilic leukocyte as well as certain parts of the brain. This enzyme is intracellularly localized in peroxisomes, and DAO is regarded as a characteristic marker enzyme of the peroxisomes. Since the initial characterization of this enzyme, many attempts have been made to clarify the physicochemical properties and reaction mechanisms.

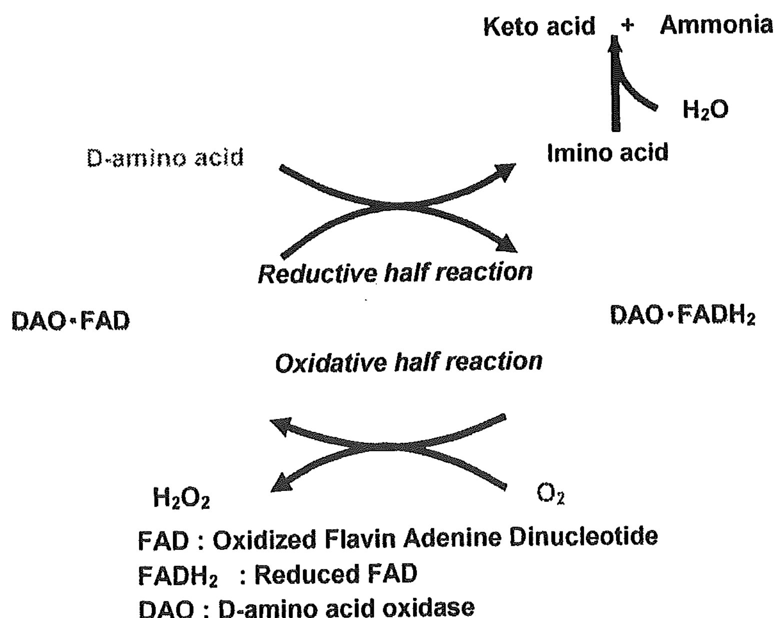

Fig. 1. Enzyme reaction of DAO.

Results and Discussion

In vivo effect of DAO inhibitor

One of the approaches taken for investigating the function of this enzyme was to use an enzyme inhibitor in an *in vivo* study. D-propargylglycine (D-2-amino-4-pentynoic acid, D-PG), an acetylenic substrate for DAO, was oxidatively deaminated by DAO, with accompanying inactivation of the enzyme. The inactivation reaction was demonstrated to be due to a dynamic affinity labeling with D-PG, which caused irreversible inactivation of the enzyme through covalent modification (3). Intraperitoneal injection of D-PG into rats resulted in massive glucosuria, amino aciduria and polyuria. Almost all the amino acids increased in the urine, and Lys, Gly, and Ala were predominant. The symptoms disappeared in a week without leaving any histological changes in the kidney tissue. These observations indicated the disorder in renal proximal tubules, since they contain high level of DAO and uptake glucose and amino acids in the nephron.

In vivo biosynthesis of DAO

We have demonstrated the active biosynthesis of this enzyme in a functionally differentiated cell line, LLC-PK_1, derived from porcine kidney proximal tubules, and also *in vitro* DAO synthesis using pig kidney mRNA and free or membrane-bound polysomes. The intracellular site of synthesis of DAO was concluded to be free polyribosomes, as is the case for other peroxisomal proteins (4).

Molecular biological analysis and in vitro synthesis of DAO

We also determined the primary structures of the porcine (5), human (6), rabbit (7) and mouse (8) kidney DAO mRNAs by molecular cloning and sequence analyses of the respective cloned cDNAs. We then carried out a series of molecular biological studies on the structure-function relationship of DAO for porcine enzyme as well as human enzyme (9-14). We have engineered a human enzyme that contained covalently attached flavin and demonstrated that it exhibited enzymatic properties comparable with those of the wild-type enzyme. An RNA blot hybridization analysis of porcine tissues was performed to show that three mRNA species were expressed in kidney and liver, but only one was detected in brain, indicating the active biosynthesis of this enzyme in brain and the brain-specific regulation of the expression (15) (Fig. 2).

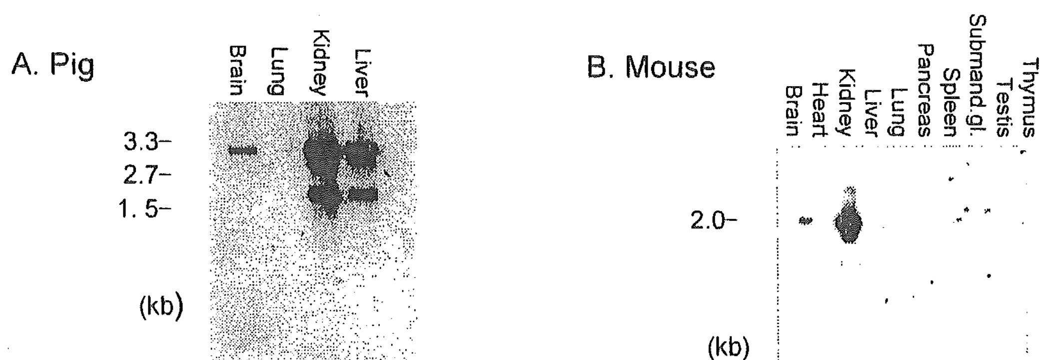

Fig. 2. Tissue specific expression of DAO mRNA.

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Molecular genetics of DAO in human diseases

Moreover, we determined the structural organization of the human DAO gene and found the absence of DAO pseudogenes in human genome (16). We then mapped this gene to human chromosome 12 by chromosome specific hybrids, to within 1-cM of the spinocerebellar ataxia 2 (SCA2) gene locus, suggesting a genetic link between the DAO gene and neurologic disorders (Fig. 3). The microsatellite D12S105 sequence including 342 bp representing the region of maximal allelic association in the Cuban SCA2 founder effect, was subjected to sequence homology analysis at the European Molecular Biology Laboratories database and yielded an almost perfect match with 99.70% similarity with intron 1 of the human D-amino acid oxidase gene. The small sequence differences were the result of length variations in the four primitive repeat motifs contained in this intron. A mutation in DAO gene would fit well with previous hypotheses on the pathomechanism of spinocerebellar degeneration, since oral loading tests with glutamate in such patients have demonstrated a decreased metabolism of amino acids glutamate and aspartate and since accumulation of the excitotoxic neurotransmitter glutamate is known to lead to cerebellar Purkinje neuron death, matching well the morphological changes observed in SCA2. However, the genomic analysis demonstrated the localization of the candidate gene D-amino acid oxidase outside the refined 1-cM region of spinocerebellar ataxia 2 (17).

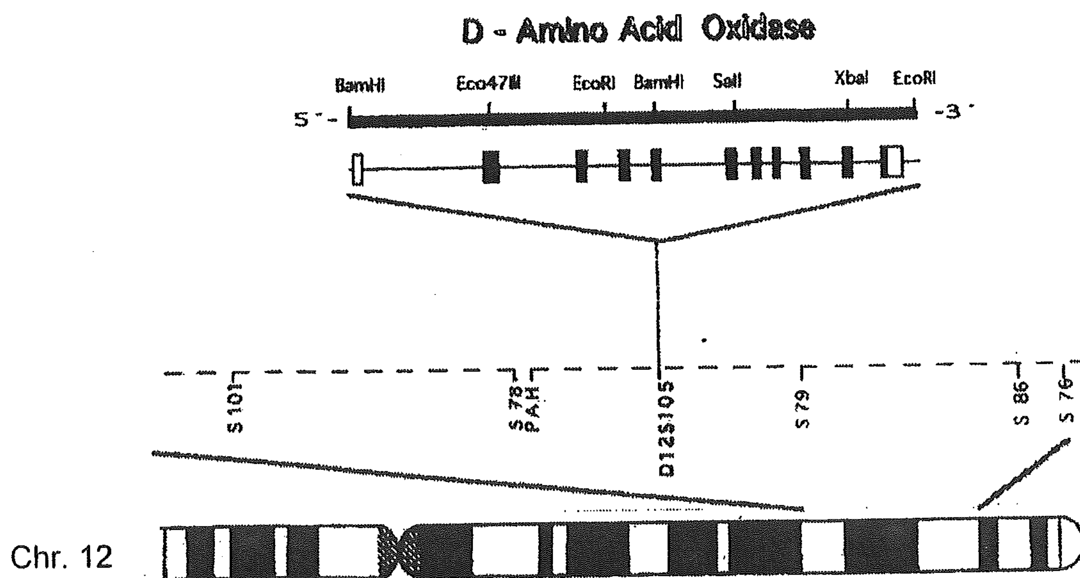

Fig. 3. Chromosomal localization of human DAO gene.

Astroglial expression of DAO

Histochemical studies revealed that DAO activity was located to some types of astrocytes (18). D-serine was found to be localized to type-2 astrocytes in culture, and released by glutamate stimulation (19). We examined the DAO gene expression in cultured rat astrocytes to investigate the physiological role of DAO in the context of free D-serine metabolism. In a study of the regional and cell type-specific expression of DAO in brain, we demonstrated the gene expression of DAO in cultures of type-1 astrocytes from rat cerebellum and cerebral cortex (20). We could establish new method for purified cultures of type-1 and type-2 astrocytes from any brain region. This method utilizes combination of cell type specific separation by shaking and subsequent purification by immunopanning (for type-2) or cytosine arabinoside (Ara-C) treatment (for type-1). Ran-2 antibodies on immunopanning dishes trap and remove contaminated cells (microglia and type-1 astrocytes) from O-2A progenitor population. Ara-C treatment killed dividing O-2A progenitors and microglia, but not type-1 astrocytes. We confirmed high purity of the cultures using immunocytochemical analyses. Type-1 astrocytes are characterized by the expression of glial fibrillary acidic protein (GFAP) and Ran-2, whereas type-2 by GFAP and A2B5 (Fig. 4). We examined the DAO gene expression by RT-PCR, a method with high sensitivity. We could detect the RT-PCR products of predicted size as single band, which was confirmed by direct sequencing of the products. We observed higher DAO expression in type-1 astrocyte cultures from cerebellum than that in type-2 asetrocyte. In addition, the expression level was higher in cerebellum than that in cerebral cortex. Former histochemical studies revealed DAO activities in astrocytes of cerebellum and brain stem. Higher expression of DAO in cultured astrocytes from cerebellum were consistent with the regional specificity of DAO in the histochemical studies. This regional difference was conserved even in cultured astrocytes established from neonates, when DAO activity could not be detected yet.

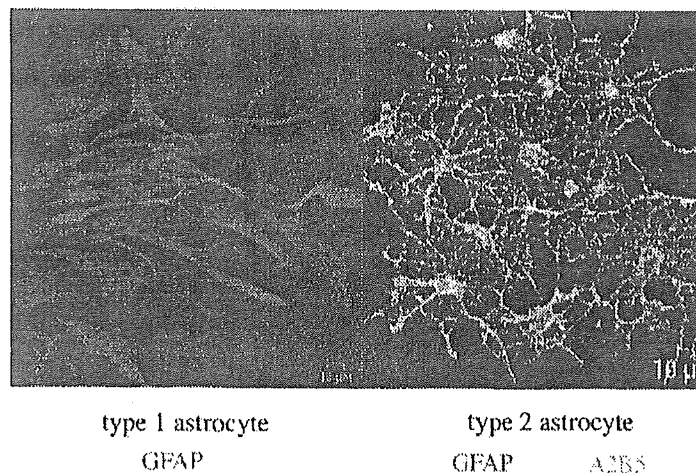

Fig. 4. Cultured type 1 and type 2 astrocytes.

D-serine as allosteric modulator of NMDA receptor

Although the existence of *in vivo* substrate of DAO was not known for many years and D-amino acids did not appear to be intermediates in normal mammalian metabolism, recently it had been demonstrated that substantial amounts of free D-serine are present in mammalian brain. Immunohistochemical analysis revealed that the localization of D-serine parallels that of the N-methyl-D-aspartate (NMDA) receptor (21). D-serine as well as glycine acts as coagonist at glycine site of NMDA type of glutamate receptor, and can modulate neurotransmission (22). The extracellular concentration of endogenous D-serine parallels or is higher than that of glycine in the prefrontal cortex and in the striatum, respectively, as measured by in vivo microdialysis (23).

Several reports indicate that D-serine may play some roles in pathological conditions related to dysfunction of the NMDA receptor. Massive stimulation of NMDA receptors was implicated in neural damage following stroke (24). Elevations of extracellular concentrations of D-serine were observed after transient cerebral ischemia in animal models and drugs that block the glycine site of NMDA receptors prevented stroke-induced damage. Hypofunction of the NMDA receptor has also been implicated in the pathology of schizophrenia. D-serine greatly improved positive, negative and cognitive symptoms in schizophrenic patients (25). Mice expressing only 5 % of normal levels of the NR1 subunit of NMDA receptors displayed behavioral abnormalities related to schizophrenia, including increased motor activity and stereotypy and deficits in social and sexual interaction (26). NMDA receptor antagonists, such as phencyclidine, induced positive, negative and cognitive schizophrenic-like symptoms in healthy volunteers (27).

Effect of D-serine on astrocytes

In search of the physiological role of DAO in the brain, we investigated the metabolism of extracellular D-serine in glial cells. We found that after D-serine treatment, rat primary type-1 astrocytes exhibited increased cell death. In order to enhance the enzyme activity of DAO in the cells, we established stable rat C6 glial cells overexpressing mouse DAO, designated as C6/DAO (Fig. 5). Treatment with a high dose (30 mM) of D-serine led to the production of hydrogen peroxide (H_2O_2) followed by apoptosis in C6/DAO cells.

Fig. 5. DAO expression in C6 and C6/DAO glioma cells.

Protective effect of ebselen against H_2O_2-induced cytotoxicity in C6 glioma cells

The inhibitory effect of H_2O_2 on cell growth of C6 glioma cells was first determined using the MTT assay. The treatment of H_2O_2 in both cell lines showed cytotoxic effect in a dose dependent manner. To confirm that H_2O_2 is implicated in the cell death, we also evaluated the cell viability after treatment of H_2O_2 together with ebselen, which was reported to be related with the removal of H_2O_2. The cytotoxic effects of ebselen itself in C6 and C6/DAO cells were evaluated beforehand by cell growth using MTT assay (Fig 6A). More than 80% of cells were viable at concentrations ranging from 1-6 μM, leading us to consider that the level of cytotoxicity of ebselen within this range of concentrations was negligible. Next, we examined the effect of ebselen treatment (3 and 6 μM) to the D-serine-treated C6 and C6/DAO cells. As shown in Fig 6B, ebselen prevented the cell death induced by D-serine very effectively in C6 cells and also C6/DAO cells at lesser degree. Among amino acids tested, D-serine specifically exhibited a significant cell death-inducing effect. DAO inhibitor, sodium benzoate, partially prevented the death of C6/DAO cells treated with D-serine, indicating the involvement of DAO activity in D-serine metabolism. In addition, 3-amino-1,2,4-triazole, an inhibitor of catalase, enhanced the cytotoxic effect of D-serine.

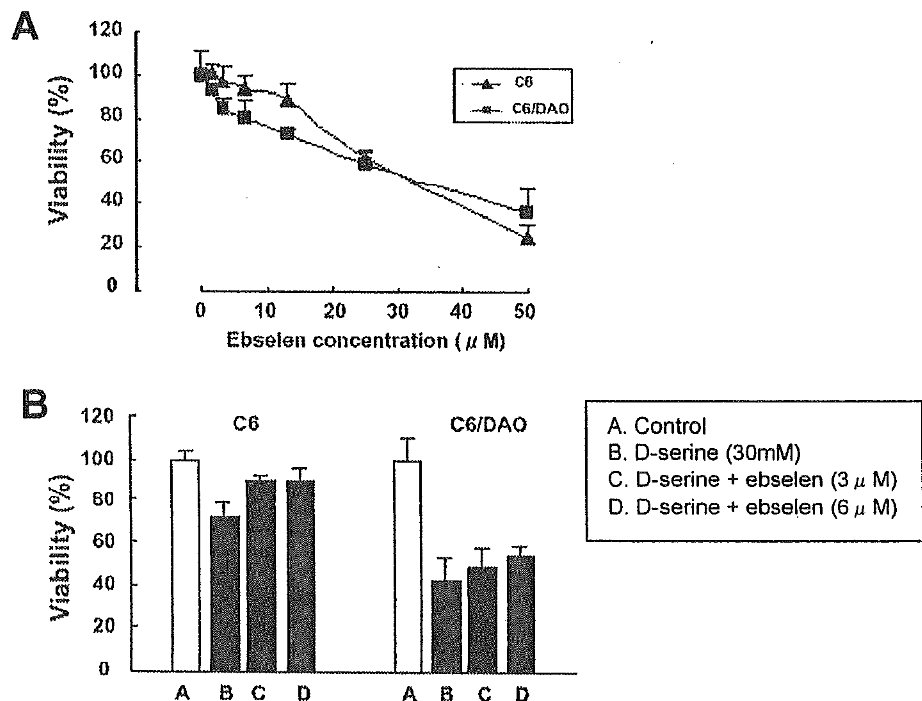

Fig. 6. Effect of ebselen on cell viability.

Schizophrenia and DAO

It is notable that the novel human gene G72 has recently been implicated in schizophrenia and shown to interact with DAO and up-regulate its catalytic activity. DAO was itself associated with schizophrenia, and the combination of G72/DAO genotypes had a synergistic effect on disease risk (28). In this context, it is interesting to note that the inhibitory effect of chlorpromazine, one of the classical antipsychotic drugs, on the activity of DAO through competition with its coenzyme, flavin adenine dinucleotide, was reported back in 1956 by Yagi et al.(29). However, the physiological role of DAO in brain has not been well documented thus far. Given that the distribution of brain DAO is inversely correlated to that of D-serine (19), it is conceivable that DAO in the brain plays a role in the metabolism of D-serine.

Conclusion

We have shown that extracellular D-serine can gain access to intracellular DAO, and that astroglial DAO contributed to the metabolism of extracellular D-serine, leading to a cytotoxic effect at a high dose of D-serine. These results suggest that astroglial DAO is the key enzyme to metabolize extracellular D-serine, a neuromodulate of NMDA receptor.

Acknowledgements

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Astroglial D-amino acid oxidase is the key enzyme to metabolize extracellular D-serine, a neuromodulator of N-methyl-D-aspartate receptor

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D-Amino acid oxidase (DAO) is a flavoenzyme that catalyzes D-amino acids and present in the mammalian kidney, liver and brain. In brain, gene expression of DAO is detected in astrocytes. Among the possible substrates of DAO *in vivo*, D-serine is proposed to be a neuromodulator of the *N*-methyl-D-aspartate (NMDA) receptor. Several reports indicate that D-serine may play a role in pathological conditions related to dysfunction of the NMDA receptor. Massive stimulation of NMDA receptors was implicated in neural damage following stroke. Hypofunction of the NMDA receptor has also been implicated in the pathology of schizophrenia. It is notable that the novel human gene G72 has recently been implicated in schizophrenia and shown to bind with DAO and up-regulate its catalytic activity. DAO was itself associated with schizophrenia, and the combination of G72/DAO genotypes had a synergistic effect on disease risk. In this context, it is interesting to note that the inhibitory effect of chlorpromazine, one of the classical antipsychotic drugs, on the activity of DAO through competition with FAD, was reported back in 1956.

In search of the physiological role of DAO in the brain, we investigated the metabolism of extracellular D-serine in glial cells. We found that after D-serine treatment, rat primary type-1 astrocytes exhibited increased cell death. In order to enhance the enzyme activity of DAO in the cells, we established stable rat C6 glial cells overexpressing mouse DAO, designated as C6/DAO. Treatment with a high dose of D-serine led to the production of hydrogen peroxide (H_2O_2) followed by apoptosis in C6/DAO cells. Among amino acids tested, D-serine specifically exhibited a significant cell death-inducing effect. DAO inhibitors, sodium benzoate and chlorpromazine, partially prevented the death of C6/DAO cells treated with D-serine, indicating the involvement of DAO activity in D-serine metabolism. In addition, 3-amino-1,2,4-triazole, an inhibitor of catalase, enhanced the cytotoxic effect of D-serine. Taken together, we consider that extracellular D-serine can gain access to intracellular DAO, being metabolized to produce H_2O_2. These results suggest that astroglial DAO plays an important role in metabolizing a neuromodulator, D-serine.

Purification and Crystal Structure of Human D-Amino Acid Oxidase

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Introduction

The flavoprotein D-amino acid oxidase (EC 1.4.3.3, DAO) is a peroxisomal enzyme first described by H.A. Krebs in 1935 (1). It contains non-covalently bound FAD as a cofactor and catalyzes the stereospecific oxidative deamination of D-amino acids to the corresponding α-keto acids with the production of ammonia and hydrogen peroxide via imino acid intermediates. DAO exhibits a marked preference for hydrophobic amino acids, while acidic amino acids are independently metabolized by D-aspartate oxidase (EC 1.4.3.1). Although, DAO is present at significantly high concentrations in kidney, as well as liver and brain, the physiological substrate was not clear until the demonstration of high levels of D-serine (D-Ser) in the brain (2), with a distribution pattern similar to that of *N*-methyl-D-aspartate (NMDA) receptors (3). We postulate that DAO, which is considered to be localized to type-1 astrocytes, regulates the glutamate–NMDA neurotransmission through the degradation of D-Ser. On the other hand, as D-Ser biosynthetic enzyme, serine racemase, which directly converts L-Ser to D-Ser, was found to be localized to type-2 astrocytes (4). Furthermore, DAO and its activator G72 were reported to be schizophrenia susceptibility genes (5), suggesting the involvement of the glutamatergic neurotransmission via D-Ser neuromodulation in schizophrenia. Although crystal structures of DAO from pig kidney (6, 7) and yeast *Rhodotorula gracilis* (8) have been determined, the 3D structure of human DAO remained to be elucidated. Here, we purified human recombinant DAO, characterized its kinetic parameters, and determined the crystal structure at 2.4 Å resolution.

Materials and Methods

The cDNA encoding human kidney DAO (9) was amplified using the N-terminal primer 5'–TCC GGC TGC TCA TAT GCG TGT GGT GGT GA–3′ and the C-terminal primer 5'–GCA GCA GTC ACA TAT GTC TTC AGA GGT GG–3′. The PCR product was digested with *Nde*I and ligated with similarly restricted plasmid pET-11b. The ligated product was transformed into *E. coli* DH5α cells. The resulting construct was confirmed by DNA sequencing.

Results and Discussion

Purification and crystallization of recombinant human DAO.

Transformants were grown in terrific broth (1.2 % tryptone, 2.4 % yeast extract, and 0.4 % glycerol) with 0.5 % (w/v) glucose and 50 mg/L ampicillin at 37 °C to an optical density of 0.6 at 600 nm, induced with 0.1 mM IPTG, and grown for an additional 24 h.

Cells were harvested by centrifugation and kept frozen at −80 °C. Unless otherwise stated, all operations were conducted at 4 °C. The recombinant enzyme was purified with a modified procedure from that of pig recombinant DAO (10). The bacterial pellet was suspended in 10 ml/g cell of buffer (17 mM sodium pyrohosphate, pH 8.3, containing 100 μM FAD, 1 mM sodium benzoate, 0.3 mM EDTA, 0.5 mM DTT, and 4.5 μg/ml PMSF). Cells were treated with 1 mg/ml lysozyme for 1 h, followed by a sonication for 30 s, 4 times. The disrupted solution was treated with 1 % (w/v) streptomycin sulfate and the cell debris was removed by centrifugation. The soluble fraction was heated at 59 °C for 3 min and rapidly cooled to below 10 °C in an ice water bath. The denatured proteins were removed by centrifugation, and the supernatant was precipitated with 70 % ammonium sulfate. After dialysis against buffer A (10 mM Tris-HCl, pH 8.0, containing 125 mM KCl, 10 μM FAD, 200 μM sodium benzoate, and 4.5 μg/ml PMSF) followed by centrifugation, the supernatant was applied to a DEAE Sepharose CL-6B column equilibrated with buffer A without adding FAD. Eluant from the DEAE column was fractionated. Yellow fractions, which are supposed to contain the holo–enzyme, were judged by OD_{455}/OD_{280} ratio and SDS–PAGE. The yellow fractions were pooled and precipitated with 70 % ammonium sulfate. After dialysis against buffer B (50 mM sodium phosphate, pH 6.8, containing 10 μM FAD and 200 μM sodium benzoate) and centrifugation, the supernatant was applied to a hydroxylapatite column equilibrated with buffer B without FAD. Eluant from the hydroxylapatite column was fractionated. Yellow fractions, which are supposed to contain the holo–enzyme, were judged by OD_{455}/OD_{280} ratio. The yellow fractions were pooled and precipitated with 70 % ammonium sulfate. The purity of the enzyme was confirmed as a single band on SDS–PAGE. Yield of the purified protein was 5 mg per liter of bacterial culture. Kinetic parameters for various D-amino acids were comparable to those of porcine DAO. The purified enzyme was crystallized by a hanging drop vapor diffusion method (Fig. 1).

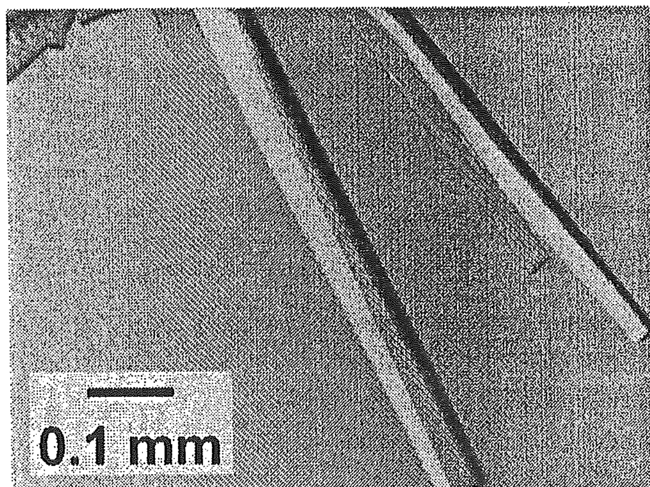

Fig. 1. Crystals of human recombinant DAO.

X-ray data collection and structural determination

The crystals diffracted to 2.8 Å with a conventional X-ray source (Fig. 2), and to 2.4 Å with a synchrotron radiation. The data set was collected at KEK beam line BL5 of the Photon Factory (Tsukuba, Japan) using monochromatized radiation at λ = 1.0 Å and a ADSC Quantum 315 CCD detector. The crystal to detector distance was 350 mm and the scan angle was 1.0 °. The data processing was performed by HKL 2000. The crystals belong to the orthorhombic space group $P2_12_12$ with unit cell dimensions of a = 150 Å, b = 180 Å, c = 50 Å. The asymmetric unit contains two homodimers of 77 kDa. The atomic coordinates for pig DAO–benzoate complex (PDB ID: 1an9) were used as a molecular replacement search model. The search model used consisted of the dimer of the DAO–benzoate complex with FAD, benzoate, and water molecules removed. The program Molrep from the CCP4 suite of software was used to solve the structure. A rotation function was calculated using data from 50.0 to 2.4 Å. The rotation function and the translation function clearly identified the positions of the two dimers. Residues were manually mutated by XTALVIEW. FAD and benzoate were built into the difference electron density using strict non-crystallographic symmetry.

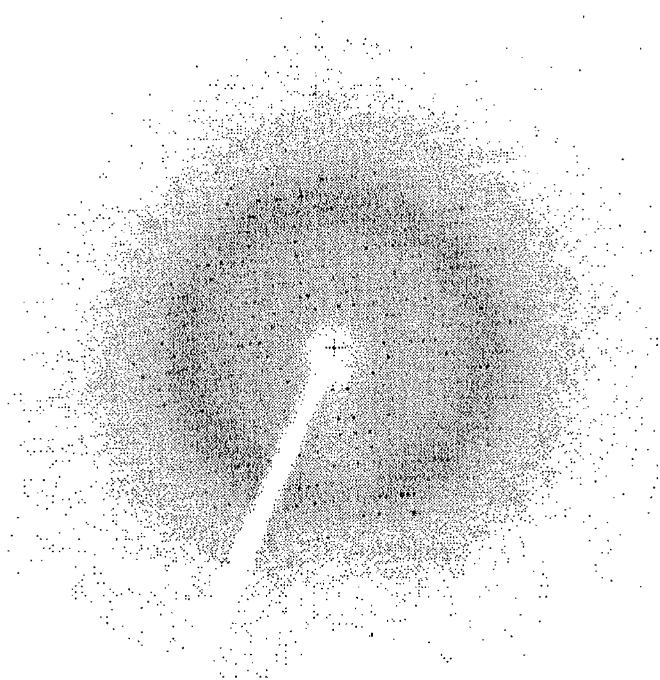

Fig. 2. X–ray data collection of human recombinant DAO.

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Molecular basis of schizophrenia: characterization of human D–amino acid oxidase

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Introduction

D–Amino acid oxidase (EC 1.4.3.3, DAAO) is a model enzyme in the dehydrogenase–oxidase class of flavoproteins. In yeast, DAAO is involved in the metabolic utilization of D–amino acids; in mammals its function is still uncertain. DAAO is able to oxidize D–serine, an allosteric activator of the NMDA–type glutamate receptor in the brain (1, 2). The binding of D–serine to the glycine modulatory site on the NMDA receptor is needed for glutamate to activate the receptor.
In the last years several genes linked to susceptibility to schizophrenia have been discovered. The most intriguing discovery has been a new human gene, G72 on chromosome 13q34 (3). Yeast two–hybrids experiments with pLG72 identified human DAAO (hDAAO) on 12q24 as an interacting partner and functional measurements showed that pLG72 behaves as an *in vitro* activator of pig kidney DAAO (3). In this way, pLG72 could regulate glutamatergic signaling through the NMDA receptor pathway. Moreover there is evidence that D–serine levels are reduced in the brain and the blood of patients affected by schizophrenia, further linking hDAAO with this pathology.
With the ambitious goal to contribute to the understanding of the correlation between modulation of the enzymatic activity in this flavoprotein and its possible role in neurotransmission and schizophrenia susceptibility, we investigated the functional properties of hDAAO.

Materials and Methods

Protein expression and purification.
The recombinant hDAAO is expressed in *E. coli* BL21(DE3) cells using the pET11b expression vector. The best yield was obtained by growing the cells overnight at 37 °C following the induction with 0.6 mM IPTG during the exponential phase of growth, corresponding to a figure of 6.7 mg of hDAAO/liter of fermentation broth. hDAAO is purified using a procedure modified from (4) consisting of ammonium sulfate precipitation at 35 % saturation, followed by a dialysis of the pellet, and anionic exchange chromatography on DEAE Sepharose FF at pH 8.0. Following this procedure, 4.2 mg of hDAAO/liter of fermentation broth with a 85 % purity (estimated by SDS–PAGE) was purified, with an overall purification yield of 60 %. For structural experiments, this latter enzyme sample was further purified by gel–permeation chromatography on a HiLoad Superdex 200 column (using an Akta Chromatographic System).

Determination of the hDAAO oligomeric state.

The oligomeric state of native hDAAO and the binding of pLG72 to hDAAO was followed by gel permeation chromatography on a Superdex 200 column using 20 mM TrisHCl pH 8.5, 150 mM NaCl, 5 % glycerol, 5 mM 2–mercaptoethanol and 40 μM FAD as elution buffer.

Spectroscopy.

Absorbance data were recorded in 20 mM TrisHCl, 5 % (vol/vol) glycerol, and 5 mM 2–mercaptoethanol, pH 8.0. Anaerobic samples were prepared in anaerobic cuvettes by applying 10 cycles of evacuation and flushing with oxygen–free argon.

Kinetic measurements.

The apparent kinetic parameters on D–alanine and D–serine as substrate have been determined at 25 °C and air saturation measuring the oxygen consumption using an Hansatech oxygen electrode. One DAAO unit is defined as the amount of enzyme that converts 1 μmole of D–alanine per minute at 25 °C. The rapid reaction experiments were performed at 25 °C in a stopped–flow BioLogic SFM–300 spectrophotometer equipped with a J&M diode array detector and a thermostat.

For reductive and oxidative half–reaction experiments, the stopped–flow instrument was made anaerobic by overnight equilibration with a concentrated sodium dithionite solution and then was rinsed with argon–equilibrated buffer. Reaction rates were calculated by extracting traces at individual wavelengths (456 and 530 nm) and fitting them to a sum of exponentials equation using Biokine32 (BioLogic).

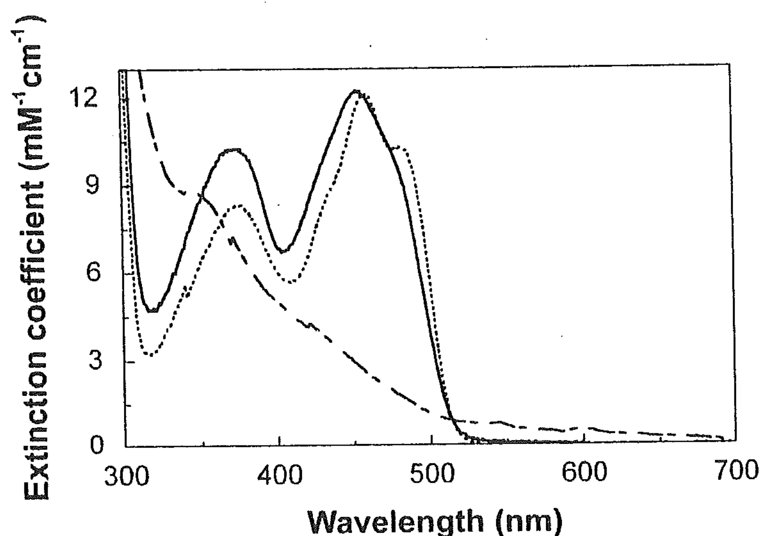

Fig. 1. Absorbance spectrum of hDAAO in the free (——) and benzoate–complex (....) oxidized form and in the reduced form (— — —).

Results and Discussion

Properties of purified hDAAO.

The recombinant protein is isolated as an active homodimeric holoenzyme showing the typical absorbance spectrum of the FAD containing flavoenzymes (absorbance maxima at 454 nm and 372 nm, see Fig. 1). The purified hDAAO is catalytically active as demonstrated by the conversion of the oxidized form of the flavin cofactor to the reduced state following the anaerobic addition of D–alanine (Fig. 1). Upon anaerobic photoreduction, hDAAO produces < 5 % of the anionic semiquinone form of the flavin cofactor, indicating that the 2–electron transfer is highly favored. The midpoint redox potential was determined at pH 8.0 by the equilibration method (5) using indigodisulfonate as indicator: for the two–electron transfer potential an $E_m \approx$ -100 mV was estimated.

The enzyme binds tightly benzoate, a classical DAAO inhibitor, with a K_d of 6.6 μM at pH 8.0, resulting in the classical perturbation of the flavin spectrum reported in Fig. 1.

In solution, and in the 1 mg–24 mg protein/ml concentration range, the native hDAAO is present as a dimeric holoenzyme. The oligomerization state of hDAAO is significantly different from that of the well known pig kidney DAAO: the holoenzyme form of this latter enzyme shows an oligomerization state dependent on protein concentration while the apoprotein is a stable monomer (6).

hDAAO shows a good stability to chemical denaturation: following the 220 nm–CD signal at increasing urea concentrations, a C_m of 4.8 M was determined for the holoenzyme of hDAAO. It also shows a good stability to thermal unfolding: i.e., T_m values of ≈ 52 °C were determined following FAD and protein fluorescence (a value that increases up to 58 °C in the presence of 40 μM exogenous FAD).

FAD binding.

hDAAO contains one molecule of non–covalently bound FAD per protein monomer, which can be easily isolated from the apoprotein by dialysis in the presence of 1 M KBr (with a 70 % yield in terms of protein recovered). This result indicates a weak interaction of the flavin cofactor with the apoprotein moiety: a K_d of $20 \pm 8 \times 10^{-6}$ M was estimated (from titration of the apoprotein with FAD and from activity assays in the absence and in the presence of exogenous FAD). Such a value is significantly higher than the one measured for any other known DAAO (ranging in the 10^{-7}–10^{-8} M range) (6), opening the question about the amount of (active) holoenzyme of hDAAO present *in vivo*.

Interestingly, even the apoprotein form largely elutes as a single peak corresponding to a dimeric state. The apoprotein possesses the secondary structure of the holoenzyme (similar far–UV CD spectrum), while some changes are evident in the near–UV CD spectrum, pointing to alteration of the tertiary structure following FAD elimination.

The holoenzyme can be reconstituted following the incubation of the apoprotein with exogenous FAD: the time course of protein fluorescence is biphasic and the enzyme recovers the full catalytic activity within 1 hour of incubation.

Kinetic properties.

The apparent kinetic parameters on on D–serine, the putative "physiological" substrate of hDAAO, determined at 25 °C and 21 % oxygen saturation indicates that hDAAO possesses a low catalytic efficiency and substrate affinity ($V_{max,app}$ = 3 s^{-1} and $K_{m,app}$ = 7.5 mM). Interestingly, a higher activity was instead determined on D–alanine ($V_{max,app}$ = 5.2 s^{-1} and $K_{m,app}$ = 1.3 mM) and on D–proline ($V_{max,app}$ = 10.2 s^{-1} and $K_{m,app}$ = 8 mM). Even D–aspartate can be oxidized by hDAAO, although the affinity for this substrate is very low ($V_{max,app}$ = 6.7 s^{-1} and $K_{m,app}$

= 2 M). These kinetic parameters are in the same order of magnitude of those determined for pig kidney DAAO, and do not explain how the human enzyme can efficiently recognize and oxidize D–serine *in vivo* (its concentration ranging from 10 to 300 nmol/g wet weight in peripheral tissues and brain, respectively) (7).

The kinetic mechanism of hDAAO was also investigated by the EMTN method using D–alanine and D–serine as substrates. Lineweaver–Burk plots at different substrate concentrations show a set of parallel lines pointing to an apparent ping–pong kinetic mechanism. The steady state coefficients determined confirm the results obtained at fixed oxygen concentration (see Table 1). Interestingly, the K_m for oxygen (0.2 mM–2 mM) largely depends on the D–amino acid used.

Table 1. Comparison of properties of DAAO from different sources (in parenthesis are reported the vaues determined with D–serine as substrate). RgDAAO: DAAO from the yeast *Rhodotorula gracilis*.

	hDAAO	pkDAAO	RgDAAO
Spectral properties			
$\varepsilon_{455\ nm}$ ($M^{-1}cm^{-1}$)	12200	11300	12600
Semiquinone formed (%) pH 8.0	< 5	≥ 90	94[a]
E_m (mV) pH 8.0	−100	−138	−116
Kinetic properties			
k_{cat} (s^{-1})	14.7 (5.7)	10	345
$K_{m,D\text{-}ala}$ (mM)	8.8 (18.0)	2.0	2.6
K_{m,O_2} (mM)	0.16 (2.0)	0.15	2.3
k_{red} (s^{-1})	180 (117)	4000	>335
k_{reox} ($M^{-1}s^{-1}$)	$1x10^4$	$1.7x10^5$	$1.1x10^5$
Structural properties			
Length (amino acids)	347	347	368
Oligomeric state	dimeric (stable)	Isodesmic self–association (depending on [protein])	dimeric (stable)
$K_{d\ FAD}$ (M)	$2x10^{-5}$	$2x10^{-7}$	$2x10^{-8}$

[a] kinetically stabilized.

The reductive half–reaction was investigated under anaerobic conditions: the time course of flavin reduction at 455 nm shows three phases. The first phase corresponds to the true phase of flavin reduction (k_{red} in Table 1), the second phase corresponds to the iminoacid release from the reduced enzyme form ($k_{diss} \approx 0.8\ s^{-1}$), and the third one might correspond to the release of the flavin cofactor ($k \approx 0.02\ s^{-1}$). The observed rate constants of the two latter phases do not depend on the D–amino acid used. The oxidative half reaction was investigated by reacting the re-