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Review

Physiological functions of D-amino acid oxidases: from yeast to humans

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Abstract. D-Amino acid oxidase (DAAO) is a FAD-containing flavoenzyme that catalyzes the oxidative deamination of D-isomers of neutral and polar amino acids. This enzymatic activity has been identified in most eukaryotic organisms, the only exception being plants. In the various organisms in which it does occur, DAAO fulfills distinct physiological functions: from a catabolic role in yeast cells, which allows them to grow on D-amino acids as carbon and energy sources, to a regulatory role in the human brain, where it controls the levels of the neuromodulator D-serine. Since 1935, DAAO has been the object of an astonishing number of investigations and has become a model for the dehydrogenase-oxidase class of flavoproteins. Structural and functional studies have suggested that specific physiological functions are implemented through the use of different structural elements that control access to the active site and substrate/product exchange. Current research is attempting to delineate the regulation of DAAO functions in the contest of complex biochemical and physiological networks.

Keywords. D-Amino acids, physiological role, peroxisomal enzyme, neurotransmission, schizophrenia.

Introduction

"Enzymes, as a rule, show extraordinary chiral recognition and use complex steric control to achieve a very high degree of stereospecificity of the reactions they catalyze". This is a quotation from Vincent Massey's speech at the 9th Flavins and Flavoproteins International Symposium, held in Atlanta, Georgia, about 20 years ago [1]. Massey, a pioneer in the development of present concepts of mechanistic flavin reactivity and flavoprotein functionality, pointed at the most puzzling aspect of one of the most interesting class of enzymes found in nature, the dehydrogenase-oxidase flavoproteins. D-Amino acid oxidase (DAAO, EC 1.4.3.3), a FAD-dependent enzyme, catalyzes with a strict stereospecificity the oxidative deamination of D-amino acids to give α-keto acids and ammonia; FAD then reoxidizes on dioxygen (Fig. 1). This flavooxidase was discovered more than 70 years ago by Krebs after he observed that amino acids belonging to the "*d*-series were deaminated much more rapidly than the natural isomerides" by fresh pig kidney and liver homogenates [2]. Since 1935, DAAO has been the focus of an overwhelming mass of research (for a review see [3, 4]) and has become a model for this class of flavoproteins.

All the chemical aspects of the enzyme reactivity were described in detail between 1950 and 1990, using the protein purified from pig kidney (pkDAAO) that for many years represented the only DAAO available in fairly good amounts and in a homogeneous form [5].

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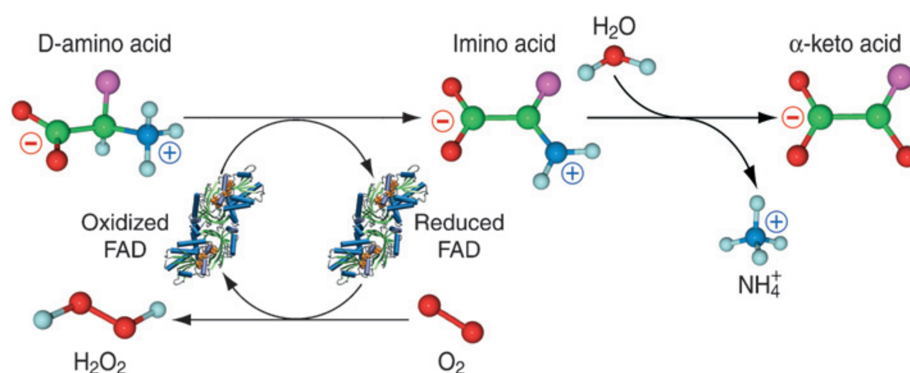

Figure 1. Scheme of the reaction catalyzed by D-amino acid oxidase (DAAO).

Starting in the mid 1980s, other DAAO proteins became available, especially from the microorganisms *Rhodotorula gracilis* (RgDAAO) [6] and *Trigonopsis variabilis* (TvDAAO) [7], which made it possible to carry out detailed biochemical studies [4].
Notwithstanding the impressive amount of data, the mechanism underlying flavin catalysis of DAAO also evoked several contrasting hypotheses in the course of the years: finally, after two groups reported on the 3-D structure of pkDAAO independently of each other in 1996 [8, 9], Mattevi's group was able to provide strong support for a classical hydride mechanism. This hypothesis was then established by the high-resolution structure of RgDAAO [10] together with the results of linear free-energy correlations [11] and from site-directed mutagenesis experiments [12–14]. Presently, even human DAAO (hDAAO), cloned a few years ago [15], has been obtained as a homogeneous protein [16]. The 3-D structure of this human protein was published very recently and shows a substantial similarity to the tertiary structure of pkDAAO [17]. The 'dark side' of this very well-known flavoenzyme is actually its physiological function. The absolute stereospecificity for the D-isomer of the amino acids as substrate has remained completely elusive. DAAO is almost ubiquitous in eukaryotic organisms, from the simplest ones, such as fungi, to fishes and mammals, where its presence was first detected in kidney and liver and is also present in brain [2–4]. In the 1990s, it emerged clearly that the main target of DAAO activity in microorganisms is to make D-amino acids available for cell metabolism and that in vertebrates it may have a detoxifying role, although a number of different and specific roles have been also suggested (Table 1). The demonstration in 1995 that in brain the concentration of D-Ser, a neuromodulator that binds the glycine regulatory site on the NMDA receptors, is reciprocal to that of DAAO represented a milestone in the discovery of DAAO function in mammals [18]. Most recently, a dazzling breakthrough was made when several positional genes involved in the susceptibility to schizophrenia, principally the genes encoding for DAAO and a its putative activator pLG72, were identified by molecular genetics studies [19]. These findings have opened new avenues for research aimed at understanding the pathogenesis of schizophrenia, a severe psychiatric disorder, and have shed light on the crucial role of DAAO in regulating neurotransmission, a most sophisticated and previously unknown role at the top of the evolutionary scale.

Oxidation of D-amino acids in bacteria (D-amino acid dehydrogenase)

In prokaryotic organisms D-amino acids are deaminated by a flavin-dependent dehydrogenase [20]. Bacteria are the major producers of D-Ala and D-Glu in nature since these compounds are fundamental elements of the peptidoglycan layer present in their cell walls. It is therefore not surprising that bacteria possess the biochemical machinery to synthesize and degrade such components. In bacteria, neutral D-amino acids, in particular D-Ala, can be oxidized to the corresponding α-keto acid by a flavoenzyme named D-amino acid dehydrogenase (DAAdH, EC 1.4.99.1). To avoid overproduction of reactive oxygen species that could damage cellular components, D-amino acid oxidation is not coupled to O_2 reduction in bacteria. *E. coli* DAAdH is a heterodimer formed by a 45- and a 55-kDa subunit. The small subunit contains FAD as coenzyme that receives two electrons from the substrate and transfers them to a sulfur-iron center located in the large subunit; the two electrons are eventually transferred to coenzyme Q. DAAdH is considered to be a peripheral membrane protein associated with the bacterial inner cell membrane [20]. DAAdH appears to play two main roles in bacteria. Initially, it allows bacteria to grow using D-amino acids (especially D-Ala) as the sole carbon, nitrogen, and energy source [21, 22]. The expression of

Table 1. Proposed functions for D-amino acid oxidase (DAAO).

Organism		Proposed role	Notes	Reference
Bacteria:	*E. coli*	– Catabolic use of D-amino acids as carbon, nitrogen, and energy source	– This is a FAD-dependent D-amino acid dehydrogenase (45 + 55 kDa)	[22, 23]
		– Prevents local overconcentration of D-amino acids		[25]
	B. subtilis	– Thiamine biosynthesis	– This is a glycine oxidase, also active on some D-amino acids	[27, 28]
Fungi:	*R. Gracilis* *C. boidinii*	– Catabolic use of D-amino acids as carbon, nitrogen, and energy source	These are all inducible enzymes	[43, 44, 46]
		– as well as sulfur source		[33]
	N. crassa	– Protective role against the toxic effects of aromatic D-amino acids		[47]
Invertebrates:				
Mussels (*M. galloprovincialis*)		– Detoxification of polycyclic aromatic hydrocarbons	– The DAAO activity increases in animals after exposure to pollutants	[72, 73]
Insect				
(*D. melanogaster*) (Firefly)		– Biosynthesis of eye pigment – Metabolic cycling of D-luciferin by oxidation of D-thiazoline-4-carboxylate		[75, 76]
Fish (*C. carpio*)		– Elimination of D-amino acids in the intestine, reutilization of D-amino acids in hepatopancreas and kidney	– The enzyme is inducible by feeding carps with D-Ala	[77]
Amphibians		– Oxidative stress to facilitate tadpole tail regression during metamorphosis		[83, 84]
Bird (chicken)		– Facilitate D-Met (as well as other D-amino acids) adsorption in intestine		[85]
Mammals:				
Rat		– Regulation of intracellular levels of oxalate acting on D-thiazolinidine-2-carboxylic acid		[127, 128]
		– Elimination of D-amino acids in kidney	– DAAO could be involved in D-Ser-induced nephrotoxicity	[121, 122, 146]
Mouse		– Modulation of NMDA receptor functionality via D-Ser metabolism	– A number of information have been gathered using mutant mice lacking of DAAO activity	[142, 152]
Human		– Component of an antibacterial system in neutrophilic leukocytes – Elimination of D-amino acids in kidney		[89]
		– Modulation of D-Ser in brain and thus of NMDA receptors functionality	– An hyperactivity of DAAO in the brain (*e.g.*, by pLG72 binding) may result in a local decrease of D-Ser and give a predisposition to schizophrenia	[20]

DAAdH is induced by the presence of D-Ala and even by L-Ala since a catabolic alanine racemase converts the L- into the D-stereoisomer [23]. Second, it prevents local overconcentration of D-amino acids: some D-amino acid analogues have specific inhibitory effects on bacterial growth [24, 25].

Distribution and properties of DAAO in living organisms

A phylogenetic tree reporting the results of a search in all available protein databanks using the BLAST sequence analysis server at NCBI for putative DAAO coding genes is reported in Figure 2. Putative DAAO genes have been identified in all kingdoms; the main exception is represented by plants in which a DAAO was purified only from the alga *Chlorella vulgaris* (its best substrate is D-Leu) [26].

Putative bacterial amino acid oxidases

With the rapid advances in sequencing the whole genome, a number of genes (about 120) coding for putative DAAOs in bacteria have been identified in the last decade. Close inspection of these putative DAAO genes reveals that only 11 genes code for proteins possessing a significant sequence identity with the yeast DAAOs. Interestingly, all 11 putative

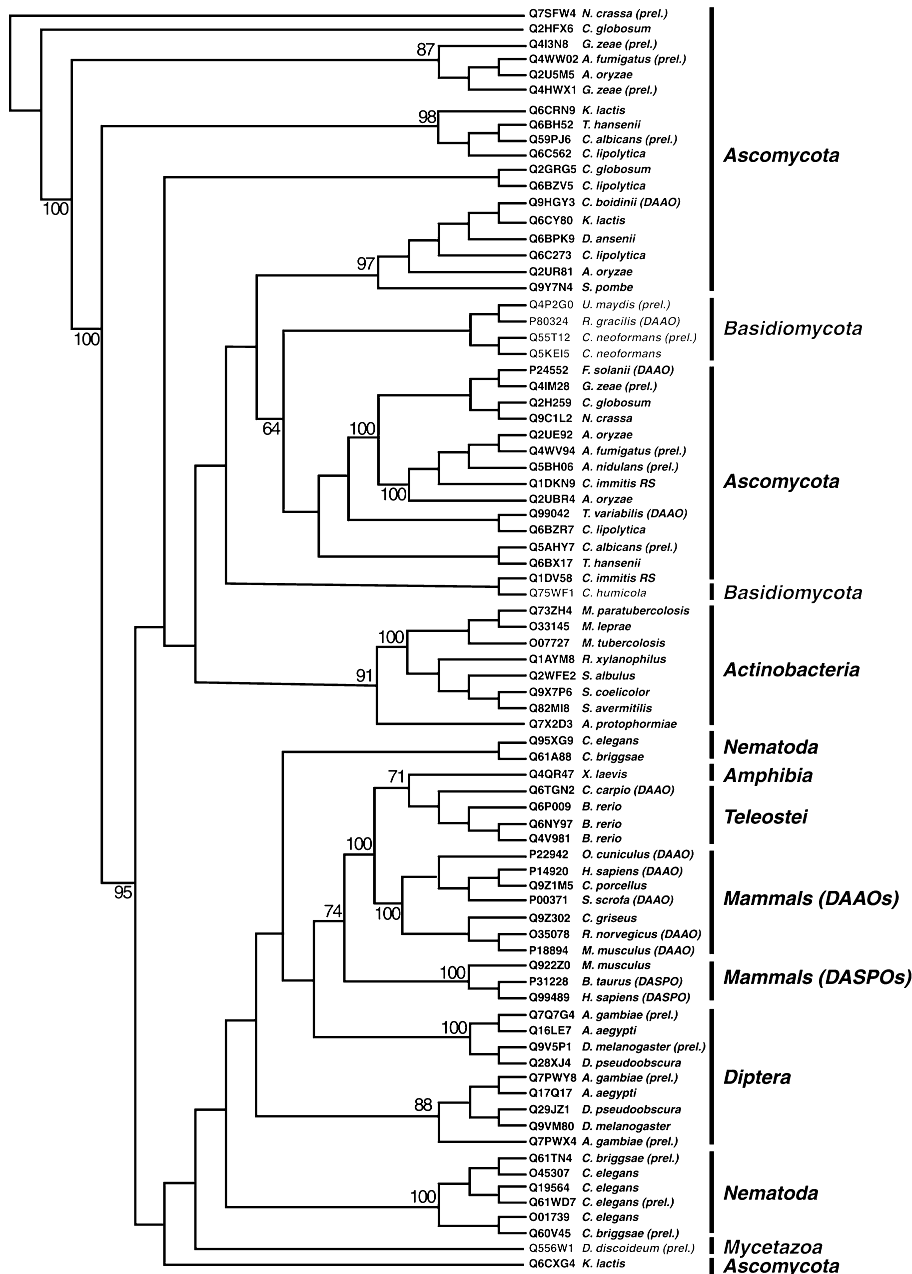

Figure 2. Unrooted phylogenetic tree (UPGMA) of protein sequences similar to *R. gracilis* DAAO from the Swiss-Prot/TrEMBL databases. The tree was built using Phylip software package; bootstrap values (expressed as percent and calculated from a consensus analysis of 1000 independent trees) are shown next to the significant nodes. Prel.: preliminar sequence; DAAO: protein classified as DAAO based on biochemical characterization; DASPO: protein classified as D-aspartate oxidase (DASPO) based on biochemical characterization.

DAAO genes belong to *Actinobacteria* strains, a group of Gram-positive bacteria that share a number of features with fungi and that grow on decomposing vegetable materials. This group of bacteria also comprises some important pathogens, including *Mycobacteria* and *Nocardia*. Although the sequence similarity between these proteins and the fungal DAAOs (ranging from 25 % to 31 % *versus* RgDAAO) points to a clear evolutionary relationship, the degree of similarity is too low to define their substrate specificity and their physiological role(s). For instance, glycine oxidase from *B. subtilis* shares large primary and tertiary structural similarity with DAAO, but its physiological role involves the biosynthesis of thiamine [27, 28].

DAAOs from fungi

Regarding eukaryotic microorganisms, direct evidence for the presence of DAAO activity has been reported for a number of fungi: *Aspergillus niger* [29], *Candida boidinii* [30, 31], *Hansenula polymorpha* [32], *Neurospora crassa* [33], *Fusarium solani* [34], *Trigonopsis variabilis* [7], *Verticillium luteoalbum*, *Fusarium oxysporum*, *Candida parapsilosis* [35, 36], *Candida utilis* [37] (all ascomycota), and *Rhodotorula gracilis* (basidiomycota) [6]. A number of DAAOs

from fungi have been cloned with the aim of obtaining efficient expression systems. DAAO from *F. solani* was cloned and expressed in *E. coli*, reaching ~8 % of the whole soluble proteins [34]. *T. variabilis* DAAO gene was cloned and expressed on *K. lactis* and *S. cerevisiae* up to 150 U/g cell dry weight [38] as well as in *S. pombe* [39]. *R. gracilis* DAAO cDNA was also cloned and overexpressed in *E. coli*, leading to the production of about 2300 U/L of fermentation broth [40, 41].

By analyzing the trEMBL database using the primary sequence of RgDAAO as a query, sequences coding for 47 additional putative DAAOs were identified in the fungi kingdom, particularly in filamentous fungi (moulds) and yeasts (Fig. 2). The pair-wise DAAO sequence identity among fungi ranges from 30 % to 40 %. This analysis also identified DAAO paralogue genes (*e.g.*, D-aspartate oxidase genes), which evolved to play different physiological roles. Unfortunately, because of the lack of direct biochemical characterization and specific sequence patterns to distinguish between such proteins, they cannot be classified.

Induction of DAAO expression in fungi. Given the importance of microbial DAAO in biotechnology (it is exploited in the two-step conversion of cephalosporin C into 7-aminocephalosporanic acid, to detect and quantify D-amino acids, to produce α-keto acids from D-amino acids, and to resolve racemic mixtures of amino acids) [42], a number of studies have been published regarding the expression of these enzymes (both in the native organisms and as heterologous proteins) and their biochemical characterization. As a general rule, DAAOs from fungi are inducible enzymes: expression levels increase when D-amino acids are present in the culture medium and do not depend on the metabolic stress due to low concentration of the primary sources of nitrogen and carbon. For example, DAAO expression in the yeast *R. gracilis* can be enhanced by about 100-fold to reach a level of ~1.0 U/mg protein that corresponds to a fermentation yield of 470 U/L using a medium containing 28 mM D-Ala as the sole carbon and nitrogen source [43, 44]. The induction of DAAO by D-amino acids is not countered by the presence of more readily usable carbon or nitrogen sources such as glucose and/or ammonium salts in the medium but, and contrary to what happens for DAAdH in *E. coli*, L-Ala prevents DAAO induction in *R. gracilis* by inhibiting D-Ala transport into cells [43, 44]. The analysis of the promoter region up to 300 base pairs (bp) upstream of the ATG starting codon of RgDAAO gene failed to identify putative genetically responsive elements [45].

A slightly different means of controlling DAAO induction is active in *C. boidinii*, where the enzyme expression is still triggered by the presence of D-Ala in the medium, but the presence of alternative nitrogen and carbon sources (such as NH_4Cl and oleate) decrease the level of DAAO induction [46]. However, no responsive elements have been identified in the upstream region of the DAAO gene in this organism either [31].

Although all DAAOs from fungi can be induced by D-amino acids, the best compounds are species specific and usually do not coincide with the best substrates of the corresponding purified enzyme. This observation probably reflects differences in the metabolic pathway(s) in which DAAO is involved in different organisms. For instance, in *N. crassa* cells, the DAAO activity is connected to sulfur metabolism: the enzyme is induced by the presence of D-Met as the sole sulfur source in the medium (up to 15 U/mg protein) [31]. Moreover, since aromatic D-amino acids are toxic for *Neurospora* at a relatively low concentration (below the millimolar concentration), DAAO also plays a protective role against the toxic effects of these compounds [47]. Furthermore, even D-amino acid analogues that cannot be metabolized induce DAAO expression; in *T. variabilis* cells, DAAO is induced by the presence of *N*-carbamoyl-D-alanine, with which a 28-fold increase in the production was achieved (*i.e.*, from 180 to 4300 U/L) [48].

DAAOs and peroxisomes. In eukaryotic microorganisms such as fungi, DAAO represents a peroxisomal enzyme. In *R. gracilis* cells, the induction of DAAO activity by D-Ala is accompanied by a moderate increase in organelle size (31 %) and by the biogenesis of new peroxisomes (up to 241 %) [49]. In *Candida boidinii*, DAAO induction also goes hand in hand with peroxisome induction [46]. Surprisingly, it has been reported that DAAO can assemble properly in the cytosol and thus that the catabolic utilization of D-amino acids is also active in the cytosol of *C. boidinii* [31] and *H. polymorpha* cells [32]. In contrast, we recently hypothesized that the folding intermediate identified for RgDAAO possesses the properties (*e.g.*, it lacks the catalytic activity and the characteristic tertiary structure of the native enzyme but has a significant secondary structure and retains flavin binding) proposed for the inactive holoenzyme required for *in vivo* trafficking of DAAO through the peroxisomal membrane [50, 51].

Biochemical properties of fungal DAAOs. The primary structure of biochemically characterized fungal DAAOs ranges from 345 to 368 amino acids, corresponding to a molecular mass of 38 340–40 076 Da per monomer (for *C. boidinii* and *R. gracilis* DAAOs, respectively) (for a comparison of the protein sequences of DAAO from different sources see [4, 52]). DAAOs from ascomycota show a pair-wise sequence

Table 2. Comparison of the main kinetic and binding properties of DAAO from different sources.

	R. gracilis [63, 184]	*T. variabilis* [58, 59, 184]	*C. boidinii* [56]	Carp hepatopancreas [80]	Pig kidney [104, 108, 184]	Human [6]
Kinetic properties						
$k_{cat,app}$ (s^{-1})	85	46	~80[1]	~95[2]	7.3	5.2
$K_{m,app}$ (mM)	1.0	7.0	4.3	0.2	1.7	1.3
Best substrates	D-Val> D-Trp> D-Phe	D-Met > D-Phe >D-Trp	D-Met > D-Ala > D-Ser	D-Ala > D-Val > D-Pro	D-Pro > D-Met > D-Ala	D-Pro > D-Ala > D-Ser
Binding properties						
FAD binding, K_d (M)	2×10^{-8}	n.d. (strong)[3]	n.d. (strong)[3]	n.d. (strong)[3]	2.2×10^{-7}	8×10^{-6}
Benzoate binding, K_d (mM)	0.25	18.8	n.d.	12.5	0.002	0.007

The apparent kinetic parameters were determined on D-alanine as substrate at 25°C and at air saturation (21% oxygen).
[1] For sake of comparison, this value was estimated from the original one determined at 30°C (176 U/mg protein).
[2] For sake of comparison, this value was estimated from the original one determined at 37°C (290 U/mg protein).
[3] No apoprotein was present in the final preparation (the $A_{280nm}/A_{455nm} \leq 11$).

identity ranging from 35% to 40% while, interestingly, the well-characterized DAAO from the basidiomycota *R. gracilis* shows an evolutionary distance, based on the degree of sequence similarity, with other fungal DAAOs (*e.g.*, 27% identity with TvDAAO), which is similar to that with mammalian DAAOs (*e.g.*, 26% identity with pkDAAO). By aligning the primary structures six conserved regions could be identified [52]. Interestingly, the C-terminal region, encompassing only 3 amino acids, represents the PTS1 peroxisomal targeting sequence [Ser-(Lys/His/Arg)-Leu] [53]; its function in protein targeting was demonstrated using a deletion mutant of *C. boidinii* DAAO [31]. In the case of RgDAAO, the presence of three isoforms in the protein purified from the yeast cells was related to removal of the PTS1 sequence [54]: the microheterogeneity stems from the association of two chains differing in the C-terminal tripeptide, giving three different holoenzyme dimers.

The fungal DAAOs have been all purified as holoenzymes, indicating a tight binding of the FAD coenzyme to the apoprotein moiety (the K_d for FAD is 2×10^{-8} M in RgDAAO) [55] (a comparison of the main properties of DAAO purified from different sources is reported in Table 2). Significant differences were reported concerning the quaternary structure of DAAOs from fungi: *C. boidinii* DAAO is monomeric [56] and RgDAAO is a stable dimer [55], while TvDAAO has been reported to be both a dimer [7, 57, 58] and a tetramer [59]. Recently, we demonstrated that the main role of dimerization in RgDAAO is to stabilize the flavin-harboring regions of the structure that increase protein stability and affinity for the cofactor (which is more than 10-fold higher in dimeric wild-type DAAO than in the monomeric mutants of RgDAAO and in the monomeric mammalian enzyme) [4, 50, 51, 60–62].

The specific activity of Rg-, Tv-, and *C. boidinii* DAAOs on D-Ala is quite similar (~180 U/mg protein at 30°C corresponding to a $k_{cat,app}$ of ~80/s at 25°C, Table 2) [56, 58, 63, 64]. The best substrates of RgDAAO are hydrophobic amino acids mostly with bulky side chains (such as D-Val, D-Trp, D-Phe), followed by polar amino acids. Charged (basic and acidic) amino acids are poor substrates [63]. It is noteworthy that, in the active site, RgDAAO also accommodates (and efficiently oxidizes) large substrates such as cephalosporin C and D-naphthylalanine [63, 65]. The substrate specificity pattern of TvDAAO is similar to that of RgDAAO (Table 2), with a preference towards the hydrophobic amino acids and with a slightly higher activity towards polar amino acids (*e.g.*, D-His or D-Asn) than RgDAAO [58, 64, 66]. The ability to efficiently oxidize a large number of D-amino acids differing electrostatically and sterically is related to the physiological role that evolved for DAAO in fungi. This capacity is a selective advantage since it allows these microorganisms to grow under conditions in which classical carbon and/or nitrogen sources are limiting (*e.g.*, on decomposing vegetable materials). The only exception is represented by *C. boidinii* DAAO, which is active on D-Met and, to a lesser extent, on D-Ala and D-Ser (Table 2), but is essentially inactive toward all the other D-amino acids and cephalosporin C [55, 56]. For an extensive comparison of the substrate specificity of DAAOs from different microorganisms (obtained using crude extracts) see [36].

Distribution and properties of DAAO in animals (others than mammals)

DAAO activity has been found in many animal taxa. Regarding its presence in invertebrates and lower vertebrates, a number of morphological studies have detected DAAO activity but the biochemical characterization of the enzyme is frequently lacking.

DAAO activity has been found in crude homogenates of the nematodes *Heligmosomoides polygyrus* and *Panagrellus redivivus*, and its presence has been related to the D-amino acid catabolism in these worms [67]. In mollusks, DAAO activity has been found in the peroxisomes of different tissues of the mussel *Mytilus galloprovincialis* [68], in the terrestrial gastropods *Arion ater* [69] and *Achatina achatina* (giant African snail) [70], and in *Octopus vulgaris* [71]. Studies carried out on *M. galloprovincialis* suggest that DAAO in the digestive gland plays a role in detoxifying the organism of xenobiotic pollutants such as lubricant oil or polycycling aromatic hydrocarbons [72, 73]. In fact, DAAO activity increased in animals after exposure to pollutants. In *A. ater*, the highest DAAO activity (as well as D-aspartate oxidase, DASPO, activity) was found in kidney, digestive gland, intestine, and crop, and its role has been proposed to be related to detoxification or neuromodulatory function [70]. Cytochemical analysis demonstrated DAAO in the non-mineralized electron-dense granules (EDGs) of the hepatopancreatic tubules of the crustacean *Ucides cordatus* [74]; however, the role of DAAO in these organisms is still obscure.

The presence of an abundant population of peroxisomes containing DAAO activity in the fat body of the head of the fruit fly *Drosophila melanogaster* suggested a role of this organelle in the genesis of eye pigments [75]. Furthermore, the ability of DAAO to oxidize thiazolidine carboxylate in conjunction with it being present in the fat body of firefly, which lies near the photocytes, suggested a role in the metabolic cycling of D-luciferin (a D-thiazoline-4-carboxylate) of the firefly [76].

DAAO activity has been found in a wide variety of tissues of various fish species [77]: the highest activities were found in hepatopancreas and kidney. In the common carp (*Cyprinus carpio*) DAAO was localized in peroxisomes [77]. Feeding carps with D-Ala increased DAAO activity in intestine, hepatopancreas, and kidney, whereas no increase was observed in brain. These results suggest that DAAO is an inducible enzyme in fish and that D-Ala represents its physiological substrate. DAAO in the intestine may function to eliminate D-amino acids before absorption, whereas in the hepatopancreas and kidney it could be involved in the reutilization of D- and L-amino acids via the corresponding 2-oxo acids (Table 1) [78]. In fact, among marine fishes, invertebrate-eaters show a higher level of DAAO activity than piscivores: high amounts of D-Ala have been found in many organs of different species of crustaceans and mollusks. D-Pro is the best substrate for DAAO in the crude preparation from kidney of rainbow trout (*Onchorhyncus mykiss*) and from kidney and hepatopancreas of common carp, followed by D-Ala and D-Phe [77].

Recently, the DAAO from common carp hepatopancreas (chDAAO) was cloned, expressed in *E. coli*, and then characterized [79, 80]. The primary structure of chDAAO shows high similarity with DAAO from mammals, especially with regard to the six highly conserved regions as proposed in [52]. All three key residues important for substrate binding and orientation (namely, Tyr224, Tyr228, and Arg283, numbering refers to pkDAAO) are also conserved in chDAAO. DAAO induction by D-Ala is mainly regulated at the transcriptional level, but regulation at the translational or post-translational level may also be active [79]. Purified chDAAO is highly specific for D-amino acids and shows a high catalytic activity (290 U/mg protein at 21 % oxygen saturation and 37°C, see Table 2) on D-Ala as substrate (the most preferred substrate), a value higher than that of DAAO from fungi [80]. Furthermore, temperature and pH stability studies have shown that chDAAO is more stable than the corresponding enzyme from pig kidney [80].

DAAO activity has been found in tadpoles of the anuran amphibians *Alytes obstetricians*, *Xenopus laevis*, *Rana japonica*, *Cynops pyrrhogaster*, *Rana nigromaculata*, and *Rana rugosa* [81–84]. In all these studies, an increase in the DAAO activity coupled with a decrease in catalase activity was observed during metamorphosis. A specific role for DAAO in tadpole tail regression was thus proposed: hydrogen peroxide production by the DAAO activity might contribute to destroying cells in the atrophying tadpole tail (Table 1) [83, 84].

DAAO activity has been found in the small intestine of chickens fed with racemic Met, an amino acid that is frequently included in commercial poultry foodstuff as a supplemental sulfur source: a number of studies have shown that D-Met supports growth in chickens almost as well as the L-isomer ([85] and references therein). DAAO activity in the small intestine comprises about 10 % and 50 % of the activity found in chicken kidney and liver, respectively, a value significantly higher than that determined in rats [85, 86]. These observations suggest that DAAO activity in chicken intestine might facilitate the absorption of D-Met (as well as of other D-amino acids), contributing to its efficient biological utilization (Table 1).

DAAO activity in mammals

The D-amino acids detected in vertebrates have generally been assumed to originate from food, intestinal flora, or spontaneous racemization of L-amino acids during aging. Nevertheless, the enzyme DAAO, which degrades many D-amino acids, is found in a number of vertebrate tissues (see above), and in the past decade it was determined that some D-amino acids are synthesized and used by vertebrates (for an overview on the presence, localization, and role of D-amino acids in mammals see [87]). The presence of DAAO has been observed more or less in each species of the mammalian taxonomic class (see [88] and references therein). The gene coding for DAAO is present in a single copy in the mammalian genome, and this flavoprotein has been detected mainly in kidney, liver, brain, and to a lesser extent in leukocytes [89, 90], small intestine, epididymis [91], and preputial and adrenal glands [92]. Mouse is an exception since the enzyme is not found in the liver [88]. The amino acid sequences of the DAAO from mouse [93], rat [94], hamster [95], guinea pig [96], rabbit [97], pig [98], and human [15, 99] are known. The overall amino acid sequence is well conserved among other mammalian DAAOs (~80 % pair-wise of sequence identity). Although the gene coding for DAAO from a number of mammals has been cloned, only the porcine [100, 101] and the human DAAOs [16, 17] have been efficiently expressed as recombinant protein in *E. coli* and fully characterized.

Porcine DAAO. DAAO from pig kidney is the one that has been most extensively investigated both *in vitro* and *in vivo.* In particular, it was the first DAAO obtained as a homogeneous flavoprotein from an animal source [5], and thus it has been the subject of an impressive number of biochemical, spectroscopic, and kinetic investigations [3, 4] and has become the prototype for the dehydrogenase-oxidase class of flavoproteins [102]. The primary structure of pkDAAO was determined early in the 1980s [103] and, subsequently, the complete cDNA was isolated [98].

In 1996, two groups succeeded in unraveling the crystal structure of pkDAAO [8, 9]. In the crystal, the enzyme is a homodimer containing a molecule of noncovalently bound cofactor per subunit, whereas in solution it exists as a mixture of monomers, dimers, and even higher molecular aggregates in equilibrium, depending mainly upon the protein concentration and the presence of ligands (differing between the apoprotein, the free holoenzyme, or the holoenzyme-benzoate complex) [3]. The monomer-oligomer equilibrium affects the catalytic activity. pkDAAO possesses a low affinity for the natural cofactor: the dissociation constant of the FAD-apoenzyme complex is 2.2×10^{-7} M (Table 2) [104]. The kinetic and catalytic properties of pkDAAO have been thoroughly investigated by a variety of techniques [3, 100, 105–107].

pkDAAO displays a broad substrate specificity, with a preference for D-amino acids bearing hydrophobic side chains up to four carbon-atoms long, followed by those carrying polar and aromatic groups (Table 2) [108]. The enzyme exhibits very low activity towards basic amino acids, and it does not oxidize those with an acidic side chain. The latter are substrates of a specific flavoenzyme DASPO, sharing 50 % sequence identity with DAAO [109, 110].

Tissues expression and distribution. In 1966, pkDAAO was reported to be localized in peroxisomes [111] and since then it has been regarded as a marker enzyme of these organelles. Studies on the tissue distribution of DAAO activity demonstrated that the functional flavoprotein is present in kidney proximal tubules, liver, and in certain regions of the brain [112]. Analysis of the expression of DAAO gene in pig kidney by RNA blot hybridization demonstrated the presence of three mRNA species (occurring because of multiple polyadenylation signal sequences present in the 3'-untranslated region), characterized by different degrees of efficiency in processing RNA [98]. This was also the case in liver but not in brain, where only one mRNA species was detected, at comparatively lower levels. Later on, based on further Northern blot hybridization studies, Fukui and co-workers [113] suggested that the regulatory mechanisms underlying DAAO gene expression in brain may differ from those operating in other tissues.

DAAOs from rodents. DAAOs from rat, mouse, and hamster show a high degree of sequence conservation (~90 % of identity). A peculiar feature of rodent DAAOs is that they all lack the residue at position 27 compared to pkDAAO, while the residue at position 173 is only absent in the mouse protein [95].

Rat DAAO: localization. The presence of DAAO has been reported both in hepatocytes and proximal tubule epithelial cells in the kidney [114, 115]. Peroxisomes have characteristic subcompartments that can be identified by electron microscopy: in the kidney, a single limiting membrane, peripheral tubule inclusions, an electron-dense peripheral matrix, and a central electron-lucent region have been observed. DAAO was the only peroxisomal-specific enzyme confined in the latter subcompartment [116]. Rat DAAO is also present in hepatic peroxisomes as demonstrated by means of enzymatic histochemical and immunohistochemical methods, as well as by electron microscopy [115–117]. Using electron microscopy, DAAO appeared to be restricted to a small area of the hepatic peroxisomal matrix, which was less

electron dense than the surrounding area [118]. Furthermore, this area was negative for various peroxisomal enzymes, and double staining of DAAO and catalase showed separate locations for these two enzymes.

Rat DAAO: mRNA expression/enzyme activity. Two species of DAAO mRNA were identified in rat kidney, liver, and brain, probably generated by alternative splicing [94]. Notably, up to now no evidence for the presence of different DAAO isoforms in various tissues of rat (as well as of pig and humans) was reported that should be related to distinct roles of the enzyme. The activity of the renal DAAO increases during the development of rat kidney coordinately with the H_2O_2-reducing activity of catalase, and reaches a maximum in 14–28-day-old rats [119, 120]. Recent studies indicated that DAAO could be involved in D-Ser-induced nephrotoxicity due to H_2O_2 production in the straight part of the proximal tubules where this D-amino acid is readsorbed (Table 1) [121, 122]. Such a degeneration process occurs only in rat, probably because readsorption and transport of D-Ser are different in rats than in other animals.

The level of DAAO activity in hepatocytes is higher in the periportal area than in the pericentral one, a finding that is in line with the predominance of oxidative processes in this region [123], and that was confirmed by ultrastructural studies demonstrating that rat liver peroxisomes are heterogeneous with respect to the DAAO activity [124]. Early in the 1990s, Frederiks and coworkers [125, 126] determined the kinetic parameters of DAAO in rat liver *in situ:* the enzyme showed a high affinity for D,L-thiazolidine-2-carboxylic acid (the adduct of cysteamine and glyoxylate) in both periportal and pericentral areas of rat liver lobules. This observation prompted the same authors to propose, at least in liver, D-thiazolidine-2-carboxylic acid to be the physiological substrate of DAAO, suggesting a role in the regulation of intracellular levels of oxalate, which in turn influences many metabolic processes (as a part of an intracellular messenger system for various hormones, Table 1) [127, 128].

In the CNS of rats, a regional heterogeneity in DAAO activity distribution has also been observed: specifically, it is more abundant in cerebellum and brainstem than in the forebrain [129]. DAAO activity has also been observed in the gray matter of the medulla and thoracic spinal chord, except for discrete areas, including those involved in autonomic function [130]. Different groups have provided evidence that the enzymatic activity is localized in astrocytes but not in other glial cell types or neurons [131, 132]. A complete map of DAAO in rat brain was more recently obtained by means of an immunocytochemical procedure: this work reported the presence of DAAO in both neuronal and glial cells, albeit at different concentrations [133]. In detail, astrocytes and radial glia are the cell types richest in DAAO; ependymal cells also contain moderate amounts of the enzyme, whereas other glial cells are apparently immunonegative. Regional differences have also been detected: astroglial cells of the caudal brainstem and of the cerebellum are generally more immunoreactive than those in the forebrain. Furthermore, in the radial glia, Golgi-Bergmann cells of the cerebellar cortex and tanycytes bordering the third ventricle and median eminence show distinct immunoreactivity. A possible explanation for the different results obtained using different methodologies is that DAAO protein can be detected by immunohistochemistry even if it is not active. The neuronal immunoreactivity is generally stronger in the hindbrain than in the forebrain, although numerous neuronal subtypes in this region display high staining intensities. The comparison between the reported DAAO distribution and the immunohistochemical map obtained for catalase [134] shows that many brain centers display a matching pattern of immunostaining, while in brain regions such as the globus pallidum cells showing a low amount of DAAO are strongly catalase positive. Taken together, these observations suggest a heterogeneity of the content of peroxisomal enzymes in nervous tissue. Recently, the analysis of the expression of the DAAO gene by RT-PCR in cultured rat pure astrocytes showed higher levels in type-1 than in type-2 astrocyte cultures [135]. In addition, and consistent with histochemical studies (see above), the DAAO expression level was higher in cerebellum than in cerebral cortex cultures. In astrocytes from 2-day-old rats, DAAO activity was only detected in cerebellum, while at 7 days it was also present in cerebral cortex (although at a level only 50% of that present in cerebellum) [136]. Interestingly, catalase was more abundant in cerebral cortex than in cerebellum, an observation that might reflect the *in vivo* maturation of the astrocytic antioxidant function, which may be important in the cerebral cortex. A recent investigation identified a relationship between the DAAO gene expression, as well as that of serine racemase (the enzymes that catalyze degradation and synthesis of D-Ser), and the blockade of NMDA receptors in several brain areas of rats [137].

Mouse DAAO distribution. The DAAO gene was mapped at 65 cM on mouse chromosome 5E3-F [138], in a chromosomal region that is syntenic between human and mouse (several homologues have been mapped in these regions). A high level of DAAO mRNA was identified in the kidney and a lower level was evident in the brain [93]. In contrast with the

expression pattern observed in other animal species, no evidence of the presence of the DAAO mRNA, or of DAAO activity [139], was found in other tissues. Although the enzyme activity has been detected in hepatocytes from mouse fetuses [140], it disappears during the developmental stage and cannot be detected in the liver of the adult animals. During postnatal development, the transcriptional level of DAAO in the kidney and skeletal muscle reached the adult level at day 21, when it was also high in cerebellum [139]. The maximum adult DAAO activity was instead reached at day 49 in cerebellum and kidney (the maximal activity in kidney being 3-fold higher than that determined in cerebellum) but was not detected in skeletal muscle at any developmental stage [139].

In mouse kidney, the hybridization signals for DAAO mRNA are exclusively located in the epithelial cells of the proximal tubule, as already reported for rat. However, in mouse the signals were evenly distributed over the proximal tubules, whereas in rat they were more abundant in specific portions [115, 141].

Investigation of the physiological role of DAAO in mouse brain (the ddY/DAO$^-$ mice system). With the aim of helping to elucidate the physiological role of DAAO, Konno and co-workers [142] established a mutant mouse strain lacking DAAO activity (ddY/DAO$^-$) in the early 1990s. The loss of enzymatic activity is due to a single bp substitution at nucleotide 541, resulting in the mutation of the conserved Arg181 residue in Gly [143]. Immunoblot analysis showed that mutant mice synthesized the protein and integrated it into peroxisomes, albeit as a nonfunctional enzyme [144]. The level of D-Ala increased 60-fold and 110-fold in liver and serum/brain of mutant mice fed with drinking water containing 0.5 % D-Ala, thus suggesting that D-Ala passed the blood-brain barrier quite freely, while the level of DAAO activity did not change [145]. In agreement with the finding that DAAO is involved in D-amino acid metabolism, the ddY/DAO$^-$ mice show a specific renal D-amino aciduria [146]. With mutant mice lacking DAAO activity it was demonstrated that DAAO in kidney is indispensable for the process of chiral inversion of D-Leu [147].

The ddY/DAO$^-$ mouse system has also been used to evaluate the role of DAAO in spatial learning and long-term potentiation (LTP) in the hippocampus. In fact, DAAO metabolizes D-Ser, which could act as an endogenous modulator of the NMDA receptor functionality through binding to the glycine site of the receptor [18, 148]. In the CA1 area of the hippocampus, LTP requires the participation of NMDA receptors; therefore, the increased D-Ser concentration in the mutant mouse brain might enhance NMDA receptor-mediated LTP [149]. Furthermore, learning and memory require long-lasting modification in the strength of specific synaptic connections between neurons; the authors showed that spatial learning in the mutant mice was significantly better than in the wild-type mice [149].

The ddY/DAO$^-$ mutant mice have been also used to characterize the molecular mechanism of schizophrenia (see below). Animals treated with the uncompetitive antagonists of NMDA receptors such as phencyclidine and with MK-801, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]ciclohepten-5,10-imine, have been used as models of the human disease. The blockade of NMDA receptors by these uncompetitive antagonists induced schizophrenic-like behavior, including hyperlocomotion, stereotypy, and ataxia, in rat and mouse, whereas an NMDA-glycine site agonist such as D-Ser and D-Ala compensated for/alleviated these symptoms [150]. These observations provided the basis for the hypothesis that hypofunction of NMDA mediates neurotransmission in schizophrenia [151]. As could be expected, mice lacking DAAO activity displayed a marked diminution of stereotypy and ataxia elicited by MK-801 compared to the wild-type mice, whereas no significant difference in locomotor activity was observed [152]. The findings and the observation that the lack of DAAO activity causes a modest but significant increase in the concentration of D-Ser and D-Ala (as well as L-Glu) in the rostral brain areas prompted the authors to propose that these D-amino acids additively antagonize the MK-801-induced symptoms and that DAAO could play a role in the regulation of NMDA receptors via D-Ser metabolism. Furthermore, the levels of DAAO mRNA in all the brain areas of rats treated with MK-801 significantly increased, thus suggesting a link between DAAO expression and NMDA receptor activity [153].

Human DAAO. In 1969 DAAO was identified within the granule fraction of human neutrophilic leukocytes and proposed to be a component of an antibacterial system (Table 1) [89]. In human polymorphonuclear leukocytes, DAAO was localized on the cell surface and then internalized during phagocytosis [90]. DAAO is capable of producing H_2O_2 within the phagosome, thus supporting the concept that the plasma membrane is involved in peroxide formation in polymorphonuclear leukocytes. cDNA clones encoding DAAO from human kidney were isolated by hybridization with the pig DAAO cDNA: the hDAAO cDNA encodes for a protein of 347 amino acids [15]. The corresponding gene is present as a single copy in the human genome on chromosome 12 (and subsequently assigned by FISH technique to region 12q23–24) [138], comprises 11 exons, and

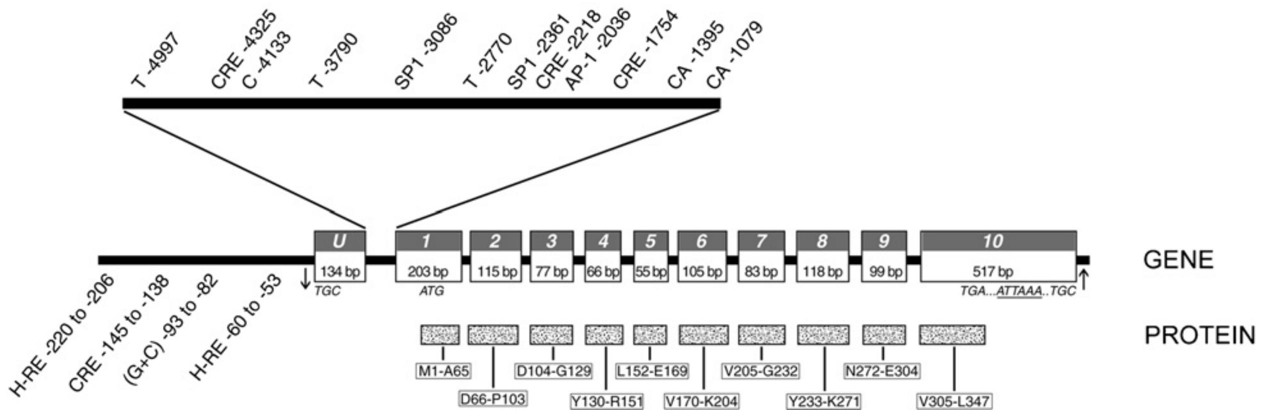

Figure 3. Structure of human DAAO gene constituted by 11 exons and 10 introns. Exon U contains only the 5′-untranslated region. The (↓) and (↑) indicate transcription initiation site and the position of poly(A) attachment, respectively. The polyadenylation signal is underlined. H-RE: hormone-response element like sequence; CRE: cAMP-responsive element; (G+C): (G+C)-rich region (numbers indicate the distance form the transcription initiation site). For sake of clarity, the first intron has been enlarged to better show the internal promoter-like regions (numbers indicate the distance downstream from the translation initiation codon): (T): TATA boxes are located at positions 4997, 3790, and 2770; (C): CAAT box is located at position 4133; AP-1 binding site is located at position 2036; SP-1-like sequences are located at positions 2361 and 3086; cAMP-responsive elements-like sequences are located at position 1754, 2218, and 4325; two sequences of alternating pyrimidine and purine nucleotides $(CA)_{20}$ and $(CA)_{17}$ are located at position 1079–1118 and 1395–1428, respectively.

spans 20 kb (the organization of the DAAO gene is reported in Fig. 3) [99]. The first exon (exon U) contained only the 5′-untranslated region; exon 10 encoded 43 residues and contained 388 bp of the 3′-untranslated region including a polyadenylation signal (ATTAAA) at 18 bp upstream of the poly(A) tail. The identification of an internal promoter-like region in the first intron prompted the same authors to propose a differential regulation for DAAO gene expression [99]. Up to now, and differing from that reported for porcine DAAO [113], no experimental evidence of different mRNA species in various human tissues has been reported. Interestingly, only one single-nucleotide polymorphism (SNP) has been identified that resulted in a nonsynonymous substitution (yielding the G331V replacement).

The expression of hDAAO in a heterologous system such as *E. coli* was initially reported by [154], but only recently have others found it to be expressed in fairly high amounts (≈7 mg/L culture) that were sufficient for detailed characterization [16, 17]. The human enzyme was purified as a stable and active holoenzyme and showed the classical properties of the dehydrogenase-oxidase class of flavoproteins (it reacts quickly with oxygen in the reduced state, stabilizes the anionic red semiquinone, and binds sulfite covalently) and a sequential kinetic mechanism (in which the rate-limiting step is represented by the product release from the reoxidized enzyme, analogously to pkDAAO) [16]. On the other hand, hDAAO possesses some peculiar properties (Table 2): (a) the binding of the FAD cofactor to the human enzyme in the absence of an active site ligand is the weakest among known DAAOs (8×10^{-6} M vs. 2×10^{-7} M and 2×10^{-8} M, for h-, pk- and RgDAAO, respectively); (b) it is the only DAAO apoprotein present in solution as a dimer; (c) hDAAO shows a significantly slower rate of flavin reduction than the pig enzyme.

It was also recently reported the crystal structure of hDAAO in complex with benzoate at 2.5-Å resolution [17]: the overall dimeric structure is similar to the head-to-head structure of pkDAAO, as also would be expected from the 85 % sequence identity between the two enzymes. Noteworthy is that inspection of the 3-D structure of hDAAO does not clarify the mode/site of interaction with the putative modulator pLG72 protein (see below) [19].

hDAAO and diseases (and DAAO clinical use). As reported previously, D-amino acids are present in humans at significant concentrations and fulfill specific biological roles. As an example, D-Ser metabolism (whose local concentration is also controlled by DAAO activity) is relevant for the functionality of NMDA receptor and for the disorders associated with its altered function, such as schizophrenia, ischemia, epilepsy, and neurodegenerative disorders. Thus, D-amino acid concentrations may not only be useful in diagnosis but also provide novel therapeutic targets.

The localization of DAAO (and hydroxyacid oxidase) was investigated by light microscopy in liver and kidney of controls and patients with Zellweger syndrome (an inherited disease characterized by a lack of intact peroxisomes) [126]. Interestingly, no activity of either of these enzymes could be detected in livers and kidneys of Zellweger patients. However, it

cannot be excluded that some enzyme activity is present in the cytoplasm (below the detection limit for histochemical procedures). In fact, about 10 % of the activity found in control subjects was identified in Zellweger patients using biochemical methods [155]. Spinocerebellar ataxia 2 (SCA2) is one form of the neurodegenerative autosomal dominant cerebellar ataxias and has been linked to chromosome 12q. The DS12S105 sequence representing the region of maximal allelic association in the Cuban SCA2 founder effect shows an almost perfect sequence homology match with intron 1 of human DAAO [156]. However, the authors do not clarify how this proposal fits with the substrate specificity of hDAAO.

Very recently it was reported that D-dopa is unidirectionally converted to L-dopa [157]. For this conversion two steps are required: first, oxidation by DAAO and then transamination by dopa transaminase. This finding explains why D-dopa produces pharmacological effects similar to those of L-dopa *in vivo* but with less potency and a slower onset of action.

Concerning the relationships between DAAO and tumors, the use of D-amino acid-containing solutions in cancer patients was reported to improve the nutritional status, prolong the survival effect, and inhibit tumor cell growth [158]. Furthermore, treatment with peroxisome proliferators [such as clofibrate and *bis*-(2-ethylhexyl)phtalate] can cause hepatocellular carcinomas as a result of the persistent production of high amounts of H_2O_2 due to the increase in acyl-CoA oxidase activity [159]. The treatment totally abolished DAAO and polyamine oxidase activities in rat liver, while the activity of xanthine oxidase remained constant and that of catalase increased (because of the larger numbers of peroxisomes) [159]. Because DAAO activity is extremely low in liver tumors (a low level of DAAO activity in AH109 hepatoma cells and Yoshida sarcoma cells was also reported [160]), it has been suggested that the enzyme plays an inhibitory role in cell growth.

Use of DAAO might also promote new strategies in cancer therapy: the formation of H_2O_2 by DAAO activity caused by the administration of neutral D-amino acids can subject tumor cells to oxidative stress and cytotoxicity. This approach has been used to induce cytotoxicity to a C6-glioma tumor cells transfected with a DAAO construct [161] or treated with a polyethylene glycol-conjugated DAAO [162, 163].

From a therapeutic point of view, DAAO can be also used to inhibit neuronal damage by ischemia [164]. In fact, massive stimulation of NMDA receptors was implicated in neuronal damage following stroke because of D-Ser release, and this neuronal damage was markedly inhibited by applying DAAO. The same process may play a role in global ischemia, as caused by perinatal asphyxia.

New insights in the role of DAAO in human brain

A series of investigations demonstrated the presence of high levels of D-Ser in the brain [165], and specifically in astrocytes [18], where it is synthesized by the pyridoxal-5' phosphate-requiring enzyme serine racemase [166]. The distribution pattern of D-Ser overlaps with that of NMDA receptors and its release is evoked by activation of AMPA/kainic acid glutamate receptors on cultured glial cells [167]. D-Ser functions as an agonist at the glycine binding site of NMDA receptors and its release by glial cells plays a regulatory role as a co-agonist necessary for the glutamate activation of NMDA receptors [168, 169]. The mode of release and uptake of D-Ser, the evolutionary framework for the appearance of a glycine site in animals and the metabolic events leading to high levels of D-Ser in brain have been recently reviewed [87, 170, 171].

D-Ser catabolism occurs via the peroxisomal enzyme DAAO, whose ontogenic expression on the hindbrain of mammals is delayed until the postnatal period and absent from the forebrain. DAAO located in the brainstem, medulla, and spinal cord maintains low levels of D-Ser [129, 132]. On the other hand, D-Ser levels are very high in the forebrain and no DAAO activity can be detected. Histochemical, gene expression, and RT-PCR studies revealed that DAAO activity is localized to type-1 astrocytes from cerebellum and, to a lesser extent, to type-2 astrocytes [139, 172], whereas D-Ser is known to be localized to type-2 astrocytes [18].

Recently, a stable rat C6 glial cell line (corresponding to type-1 astrocytes) overexpressing mouse DAAO (C6/DAO) was established [173]. Exposing this cell line to high doses of D-Ser induced H_2O_2 production and apoptosis, an effect that was prevented using DAAO inhibitors such as chlorpromazin (CPZ) or benzoate. These results support the hypothesis that glial DAAO plays a role in the catabolic metabolism of D-Ser. This work also reports that not all DAAO colocalize with catalase in C6/DAO cells (although inhibition of catalase enhanced the D-Ser-induced cell death): this observation indicates there is a substantial inhomogeneity of the peroxisome population/content and also suggests the presence of nonperoxisomal DAAO. This partial colocalization of DAAO and catalase has been confirmed by immunofluorescence using human brain slices and cultured astrocytes (unpublished results).

Recently, an analysis of SNPs and haplotypes found an association between the newly identified primate specific gene G72 with schizophrenia [19]. The yeast two-hybrid system then identified DAAO as a putative pLG72-interacting partner; subsequently, DAAO was itself associated with schizophrenia and combinations of G72/DAAO genotypes had a synergistic effect on disease risk. Both genes are located in chromosomal regions showing evidence for linkage with this disease. Schizophrenia is a neurodevelopmental disorder leading to abnormal synaptic connectivity; in particular, glutamatergic transmission via NMDA receptors may be involved. Almost all the candidate genes related to schizophrenia implicate glutamatergic synapses as a common site of action, and probably influence fundamental processes of neurodevelopmental synaptic plasticity and glutamate neurotransmission [174]. Recently, the D-Ser released by astrocytes was demonstrated to promote the activity of NMDA receptors at synapses in the hypothalamus [175], thus demonstrating that glia "do more than simply provide background for the neuronal melody. Astrocytes actually conduct the orchestra using D-serine as a baton" [176].

When pLG72 is mixed with a 20-fold molar excess with respect to pkDAAO, the activity of the flavoenzyme is enhanced by 3-fold of the basal level [19]. This investigation proposed a model whereby the expression of pLG72 in schizophrenia produces an increase in DAAO activity and a concomitant decrease in D-Ser levels, causing NMDA receptor hypofunction (Fig. 4a). As suggested by [170], the genetic associations are intriguing but the proposed biochemical mechanism does not fit with some observations: (a) the different proposed intracellular localization of pLG72 and hDAAO (Golgi and peroxisomes, respectively) [19]; (b) that D-Ser levels are not different in postmortem schizophrenic brains with respect to controls [177], although more recently a modest reduction in the levels of D-Ser in the serum of schizophrenic patients was reported [178]; and (c) the reported distribution of DAAO in the brains of mammals: schizophrenia involves a deficit in the prefrontal cortex and limbic system, whereas DAAO is located in the cerebellum, brainstem, and spinal cord. The observation that DAAO activity in certain rat brain regions or in certain cells might not correspond with DAAO protein levels indicates that DAAO could be regulated by an inhibitory protein-protein interaction (such as the interaction with pLG72 in schizophrenic patients) or covalent modification in the forebrains of mammals. Recently, we revealed that hDAAO and pLG72 are both expressed in astrocytes of the human cortex where they most likely interact with each other (unpublished results). Furthermore, we demonstrated that *in vitro* the two proteins physically interact (the expression in *E. coli* and characterization of pLG72 has been recently reported [179]). Binding of pLG72 to hDAAO does not significantly affect the catalytic efficiency and FAD-binding ability of hDAAO: the major effect corresponds to a decrease in hDAAO stability when an excess of pLG72 is present (unpublished results). Interestingly, pLG72 binds the cofactor FAD as well as CPZ, an anti-psychotic drug, thus suggesting that pLG72 binding could affect hDAAO properties in a more subtle and complex way than was previously suggested [19]. An alternative modulation of D-Ser level by pLG72 binding to DAAO can thus be proposed (Fig. 4b).

CPZ is on the World Health Organization list of essential drugs and, until recently, it was common practice for anyone with schizophrenia to have been treated with CPZ at some point [180]. CPZ is a dopamine D2 receptor antagonist, which can suppress the positive symptoms of schizophrenia. In the 1950s, Yagi [181] reported that CPZ is a powerful *in vitro* and *in vivo* inhibitor of pkDAAO, acting competitively with the cofactor FAD ($K_i = 2.3 \times 10^{-5}$ M). The inhibitory capacity of DAAO activity of a number of phenothiazine derivatives was in good agreement with their relative clinical efficacy and potency in antipsychotic therapy [182]. For a recent review of the discovery and clinical use of CPZ see [183].

Evolutionary correlations in structure-function relationships of different DAAOs

The 3-D structure of DAAO from three different sources has been elucidated. In 1996, the crystal structure of pkDAAO was resolved [8, 9]; in 2000 the 3-D structure of RgDAAO was determined [10]; and very recently, the crystal structure of human DAAO in complex with benzoate has also been deciphered at 2.4 Å [17]. Although DAAO from these sources share the basic catalytic mechanism, they differ in important aspects such as catalytic efficiency, substrate specificity, oligomeric state, stability, kinetic mechanism, and FAD binding (Table 2). A comparison of the main kinetic properties of Rg-, Tv- and pkDAAOs has been reported [4, 184]. In addition to the higher catalytic efficiency, tightness of FAD binding, and lower thermostability of the fungal enzymes, the profile of the pH dependence of the activity and stability also distinguishes DAAO from fungi from mammalian DAAO.

The structure of the enzyme from pig kidney shows some general properties common to all DAAOs (Fig. 5). Each DAAO monomer is clearly divided

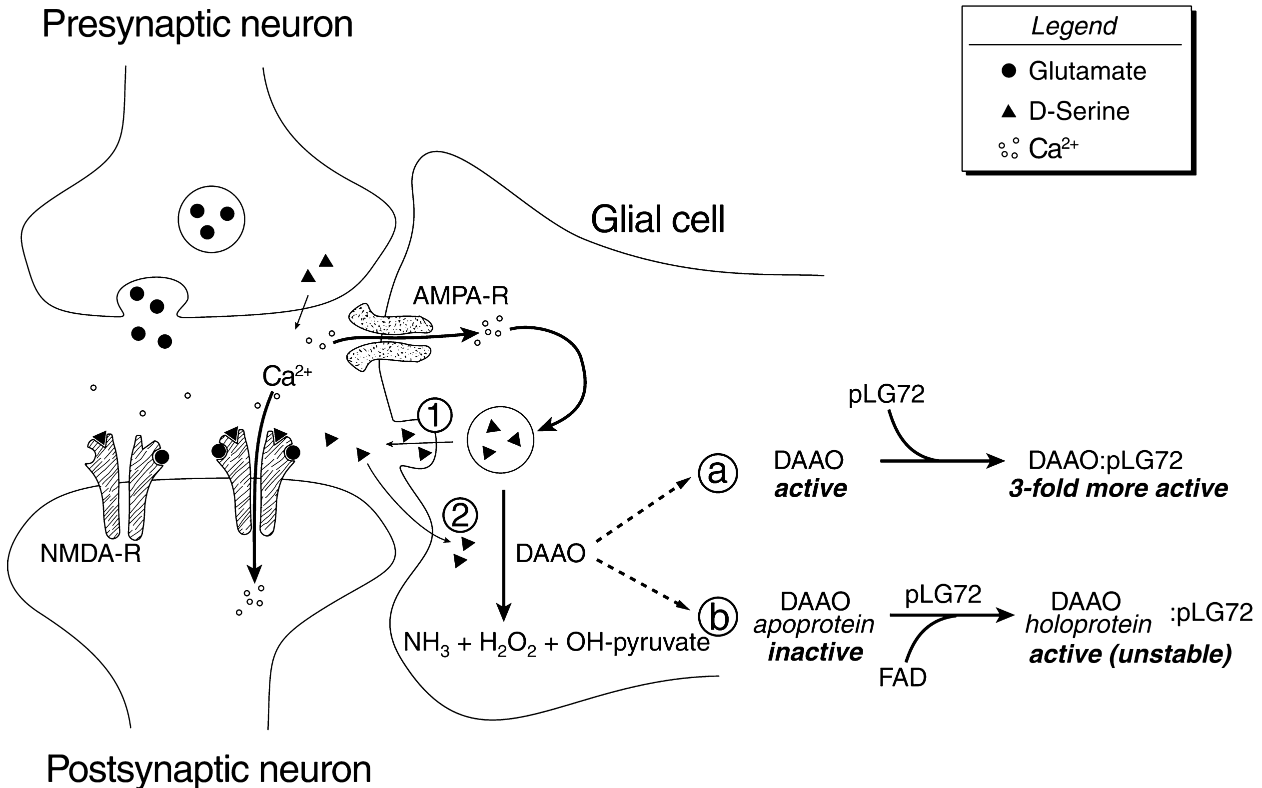

Figure 4. D-Ser biosynthesis and interaction with NMDA receptors at a glutamatergic synapse. Glu is released by the presynaptic neuron into the synaptic space following the depolarization of nerve terminals, yielding the activation of non-NMDA receptors on the membrane of the perisynaptic astrocytes and, subsequently, an increase in cytoplasmic concentration of Ca^{2+}. D-Ser is then released by the glial cells (1), and in the synaptic cleft together with Glu activates NMDA receptors on the membrane of the postsynaptic neuron, leading to the opening of ion channels. The Ca^{2+} entry can induce several intracellular responses, *e.g.*, activate nitric oxide synthase that produces NO; it can diffuse to the neighboring cells where it is proposed to inhibit serine racemase and activates DAAO, which decreases the local concentration of D-Ser [171]. D-Ser is then removed by the synaptic space probably by specific transporters on the membrane of both astrocytes and neurons (2). (a) Proposed mode of involvement of hDAAO in schizophrenia according to [19] in which pLG72 is an activator of hDAAO; (b) alternative proposal in which pLG72 modulates the amount of active DAAO acting on the FAD binding to the apoprotein of hDAAO and on the stability of the holoenzyme. Filled dots, Glu; triangles, D-Ser; open dots, Ca^{2+}.

into two domains, the FAD-binding domain with the typical Rossman fold and the interface domain that forms the contact area with a second monomer in the crystallographic dimer. The FAD cofactor is buried inside the protein, adopts an elongated conformation, and is involved in a number of interactions. In all structures, the C-terminal tripeptide, corresponding to the peroxisomal targeting signal 1 required for targeting to peroxisomes, is not visible in the electron-density map: the flexibility might be important for the interaction with peroxisomal membrane receptors [53].

A few structural characteristics distinguish these three enzymes and might be responsible of their specific functional properties: (a) the monomer-monomer interaction, (b) the active site lid and the substrate specificity, (c) the kinetic properties (the rate-limiting step in catalysis), and (d) FAD binding.

The monomer-monomer interaction

The dimeric structure of pkDAAO and hDAAO shows a head-to-head mode of monomer-monomer interaction, different from the head-to-tail mode of dimerization observed in RgDAAO (Fig. 5a). In pkDAAO (in which interaction between monomers is weak and activity depends upon the oligomeric state of the protein in solution), the contact area represents about 15% of the monomer surface between the interacting subunits, yielding an elongated ellipsoid. Concerning hDAAO, the frequency of substitution at the monomer-monomer interface (10 residues on a total of 30 residues) is higher than the overall substitution frequency (15%), and in addition the electrostatic surface potential differs from the porcine enzyme: at the dimer interface the human enzyme is negatively charged, while pkDAAO is positively charged. This observation could account for the stable oligomeric state of the hDAAO homodimer even in the apoprotein form [16]. On the other hand,

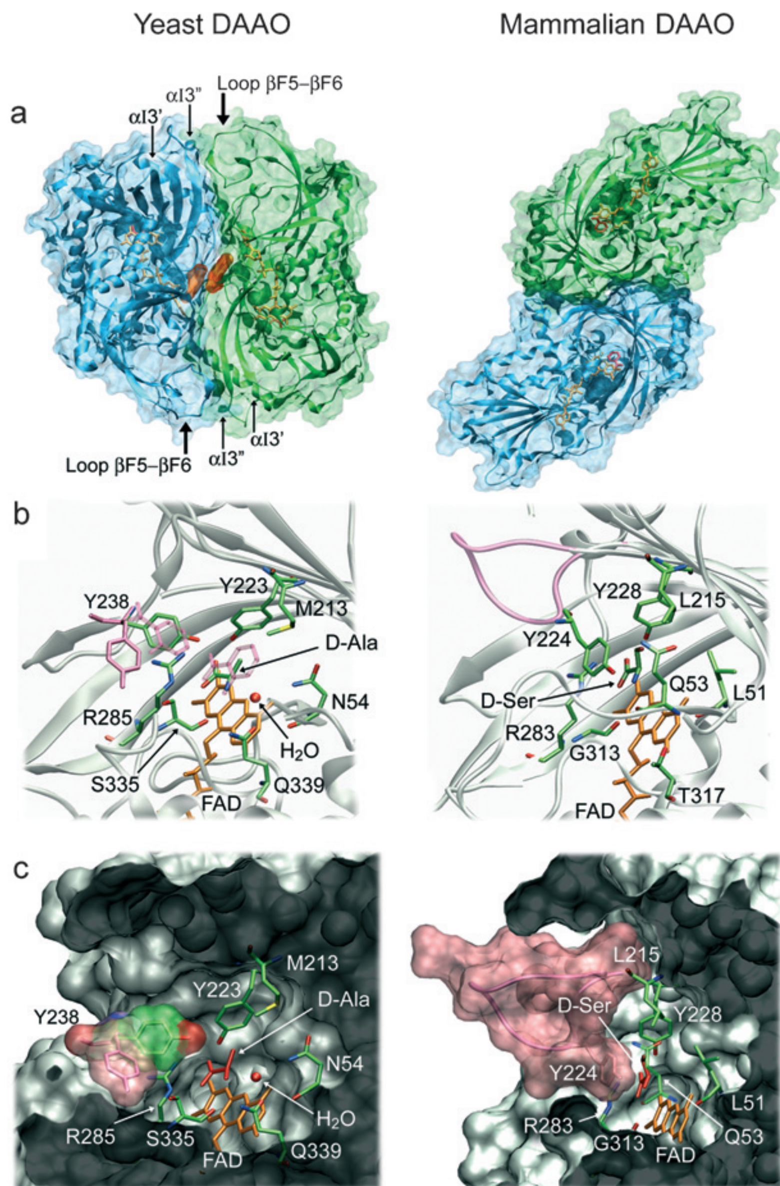

Figure 5. (*a*) Modes of monomer-monomer interaction in *R. gracilis* (head-to-tail, left) vs. pig kidney (head-to-head, right) DAAOs. The thick arrows identify the βF5-βF6 loop and the thin arrows identify the α-helices αI3' and αI3''. W243 at the core of the interface domain in RgDAAO is shown in orange. (*b*) Detail of the active site of yeast and mammalian DAAOs. (*c*) Molecular surface representation of the active site entrance of yeast and mammalian DAAOs, showing a magnified view at the funnel leading to the active site. The structural elements controlling the active site accessibility (the Y238 in RgDAAO and the 216–227 active site lid in hDAAO) have been drawn in pink in (*b*) and (*c*). In green, the alternative position of Y238 in the RgDAAO-D-alanine complex can be seen (closed conformation). Left: RgDAAO in complex with D-Ala (1C0P) and with anthranilate (1C0I); right: hDAAO in complex with D-Ser (2DU8). Notably, pkDAAO shows the same structural properties reported here for hDAAO.

RgDAAO possesses two structural elements responsible for the stable dimeric state in the holoenzyme form (Fig. 5a) [185]: firstly, the electrostatic interactions between positively charged residues belonging to the βF5-βF6 loop of one monomer and negatively charged residues belonging to the α-helices I3′ and I3″ of the other monomer. The presence of this long loop thus results in a completely different mode of dimerization compared to mammalian DAAOs and with a significantly increased monomer-monomer interaction area (3049 $Å^2$ vs. 1512 $Å^2$ for RgDAAO and pkDAAO, respectively). Interestingly, this loop is not conserved in other DAAOs. Secondly, the residues (in particular the conserved residue W243) belonging to the two monomers interact at the mixed interface region. The combination of limited proteolysis [54, 186], site-directed mutagenesis [60–62], and thermodynamic studies [50, 51] showed that the shift from a dimeric to a monomeric form resulted in an RgDAAO enzyme with altered tertiary structure. This interfered with the interaction of the coenzyme and yielded an increased sensitivity to thermal and chemical denaturation, while the catalytic activity was unchanged.

The active site lid and the substrate specificity

Upon inspection of the structure of the pkDAAO-benzoate complex, a loop formed by residues 216–228 was found rendering solvent inaccessible the active site hydrophobic cleft (Fig. 5b and c, right) [8]. The loop could switch during catalysis between the closed conformation and an open state, in which substrate binding and product release take place, respectively.

A subsequent study investigated the structural properties of pkDAAO-imino-Trp complex [187] and found that, besides controlling the active site accessibility, the loop also plays a major role in determining the enzyme substrate specificity. pkDAAO shows an active site formed by hydrophobic residues (Fig. 5b, right): its cavity has a calculated volume of 160 $Å^3$ (220 $Å^3$ in hDAAO), corresponding to the volume occupied by an amino acid with a side chain containing four carbons. As matter of fact, an increase in K_m value is evident for substrates with a side chain longer than four carbon atoms. Although the active site residues are all conserved in hDAAO, a difference is observed at the level of the hydrophobic sequence ^{47}VAAGL^{51} located on the *si*-face of the flavin cofactor [17]. This region shows a different conformation than the one found in a similar portion in the free form of pkDAAO. Therefore, the authors speculated that the conformational variability accounts for the low affinity for the FAD cofactor and for the slower rate of flavin reduction (these assumptions require further investigation).

RgDAAO does not possess an active site lid such as that observed in mammalian DAAOs (Fig. 5b and c, left) [10, 185]. In RgDAAO the function of this loop is partially vicariated by the side chain of Tyr238. This structural difference is the main reason for the differing substrate specificity and kinetics between yeast and mammalian DAAOs: RgDAAO has a low K_m even for bulky substrates such as cephalosporin C or naphthyl-amino acids [63, 65]. Sequence alignment analysis shows that the sequence corresponding to the active site lid of pkDAAO is also lacking in the other fungal DAAOs.

Kinetic properties (the rate-limiting step in catalysis)

Although RgDAAO and pkDAAO are mechanistically similar (for a review see [4, 107, 188]), their catalytic efficiency and kinetic mechanisms are different. The conformational change of the 216–228 loop during access/release of substrate/product is relatively slow and controls the overall turnover rate of the pig flavoenzyme: the product release is rate limiting, leading to a k_{cat} value of 600/min with D-Ala [105] compared to a value of 20 000/min for RgDAAO [189]. The Y238 side chain in RgDAAO presumably shows an open conformation in uncomplexed enzyme to initiate an interaction with substrate, leading it into the active site. A subsequent conformational change of the side chain of Y238 positions the substrate further inside the active site so that this residue can contribute to fixation of the substrate α–COO^- group. This is in agreement with the alternative conformations of Y238 observed in the complex with CF_3-D-Ala and anthranilate (Fig. 5b and c, left) [185], and with the results obtained by site-directed mutagenesis, indicating that the main function of this group is in substrate/product exchange instead of binding [14]. A single side chain can move relatively quickly, the rate of flavin reduction then being the rate-limiting step in RgDAAO catalysis [185, 189].

3-D structure modeling of chDAAO showed that the active site loop found in pkDAAO is also present in DAAO from fish, albeit shorter. The reduced length of the active site loop has been proposed to be the structural element responsible for the low K_m value for the D-amino acid and the high k_{cat} value [80]. Sequence conservation indicates that chDAAO should be structurally similar to pkDAAO, although its catalytic properties more likely resemble those of RgDAAO. This could reflect the primary role of this enzyme in fish in the metabolism of D-amino acids (especially D-Ala) incorporated with diet.

FAD binding

In all DAAOs, the FAD binding domain contains the conserved consensus Rossmann fold βαβ motif (known as the dinucleotide-binding motif), and the isoalloxazine ring is located at the interface of the two domains, with the *re*-face facing the inner part of the substrate binding cavity. In all DAAO proteins, the large majority of the potential FAD hydrogen bonds are with the protein, thus resulting in a tight net of interactions. The flavin N(1) is within H-bond distance with S335(=O) in RgDAAO, while such an interaction is absent in mammalian DAAOs (Fig. 5b). The environment of the O(2) position is substantially different in yeast and mammalian DAAOs: in pkDAAO the partial positive charge of a dipole induced by helix F5 is assumed to favor the stabilization of the negative charge of the reduced flavin, while in RgDAAO such a feature is absent and the O(2) forms two H-bonds with the backbone NH groups of Q339 and Y338 [in pkDAAO the O(2) interacts with T317]. The residues interacting with the pyrophosphate oxygen atoms are better conserved than the other segments: four water molecules form H-bonds with three of the phosphate oxygen atoms in RgDAAO, while in pkDAAO only three water molecules interact with two of the phosphate oxygen atoms. Thus, although different amino acids interact with FAD in yeast and mammalian enzymes, the overall picture is similar in these DAAOs and does not explain the observed differences in flavin binding (K_d ranging from 10 μM in hDAAO to 0.02 μM in RgDAAO).

Conclusions

In conclusion, a comparison of the 3-D structures of yeast and mammalian DAAOs suggests that modulating the structure-function relationships yields enzymes that share the same chemical process but use different kinetic mechanisms for catalysis. In particular, the necessity to regulate activity in mammalian DAAO has caused product release to be limiting, while the necessity of high catalytic activity in the yeast DAAO has given higher accessibility to the active site. This diversity is implemented using different structural elements to control the accessibility to the active site and the substrate/product exchange. Surprisingly, free D-amino acids in the 0.2 – 8 % range relative to the L-amino acids were found in plants [190] but a DAAO activity was never detected. Since D-amino acids were identified in gymnosperms as well as mono- and dicotyledinous angiosperms of major plant families, it has been proposed that free D-amino acids are, in the low percentage range, fundamental constituents of plants and play specific roles not covered by L-amino acids and which still have to be established. Importantly, their deamination does not require DAAO.

A great number of functional and structural investigations on DAAO have been carried out in the course of the years; in these studies the enzyme was considered and examined as an independent and individual entity. Now, DAAO research is moving to a new and more complex, but at the same time more interesting level. Much remains to be done to delineate the functionality and regulation of DAAO as a component of a complex biochemical and physiological network. It is a fascinating but highly difficult task because there are numerous putative roles for DAAO in different organisms and because its involvement in human brain is crucial for various physiological and pathological processes. During evolution, this protein proved to be a highly versatile enzyme able to fulfill very different functions: from the rather simple catabolic role in fungi to tissue-specific and highly specialized roles in mammals.

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