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Review

D-Amino acid oxidase: new findings

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Abstract. The most recent research on D-amino acid oxidases and D-amino acid metabolism has revealed new, intriguing properties of the flavoenzyme and enlighted novel biotechnological uses of this catalyst. Concerning the in vivo function of the enzyme, new findings on the physiological role of D-amino acid oxidase point to a detoxifying function of the enzyme in metabolizing exogenous D-amino acids in animals. A novel role in modulating the level of D-serine in brain has also been proposed for the enzyme. At the molecular level, site-directed mutagenesis studies on the pig kidney D-amino acid oxidase and, more recently, on the enzyme from the yeast *Rhodotorula gracilis* indicated that the few conserved residues of the active site do not play a role in acid-base catalysis but rather are involved in substrate interactions. The three-dimensional structure of the enzyme was recently determined from two different sources: at 2.5–3.0 Å resolution for DAAO from pig kidney and at 1.2–1.8 Å resolution for *R. gracilis*. The active site can be clearly depicted: the striking absence of essential residues acting in acid-base catalysis and the mode of substrate orientation into the active site, taken together with the results of free-energy correlation studies, clearly support a hydrid transfer type of mechanism in which the orbital steering between the substrate and the isoalloxazine atoms plays a crucial role during catalysis.

Key words. D-amino acid oxidase; flavin cofactor; physiological role; protein engineering; 3D structure; reaction mechanism; biotechnology.

Introduction

The flavin cofactors play a key role in biological processes. This is illustrated by the continuous discovery of new flavoproteins and the large number of papers on flavoenzymes that have appeared over the years. The flavoprotein D-amino acid oxidase (DAAO, EC 1.4.3.3) was the second flavoenzyme to be discovered. Krebs first detected DAAO activity in tissue specimens [1], and subsequently FAD was identified as its enzyme cofactor [2]. All DAAOs characterized so far contain noncovalently bound FAD as their prosthetic group. They are regarded as model flavooxidase catalysts mainly because they have been so extensively studied. In recent years interest in DAAOs has intensified in view of their widespread occurrence in eukaryotes and the controversy regarding their reaction mechanism(s).

The aim of this paper is to provide an overview of the most recent research on DAAOs to illustrate some of their intriguing properties and to review the gray areas and those which are still a subject of dispute. A detailed review of the mass of information available on both D- and L-amino acid oxidases was published a few years ago, to which the reader is referred as a point of departure [3]. The reaction catalyzed by DAAO proceeds according to the following scheme:

$$RCHNH_2COOH + E\text{-}FAD \rightarrow RC = NHCOOH + E\text{-}FADH_2 \quad (1)$$

$$E\text{-}FADH_2 + O_2 \rightarrow E\text{-}FAD + H_2O_2 \quad (2)$$

$$RC = NHCOOH + H_2O \rightarrow RCOCOOH + NH_3 \quad (3)$$

(eq. 1)

The reductive half-reaction, the only enzymatic step of the whole scheme, dehydrogenates the amino acid to the corresponding imino acid, coupled with the reduction of FAD (reaction 1). FAD reoxidizes spontaneously in the presence of molecular oxygen, which in turn is reduced to hydrogen peroxide (reaction 2). The imino acid then hydrolyzes, nonenzymically, to the α-keto acid and ammonia (reaction 3). In vivo, and in the presence of hydrogen peroxide, a decarboxylation spontaneously occurs which yields the corresponding acid.

Flavoproteins are classified according to their reduced state reactivity with molecular oxygen. DAAO belongs to the large class of dehydrogenases and oxidases of flavoproteins which catalyze the oxidation of amino or α-hydroxyacids and which react very rapidly with O_2 to yield H_2O_2 and the oxidized flavoprotein [4]. The reaction appears to be a simple second-order process [5]. This functional classification of flavoproteins has been refined over the years [6].

The stereospecificity of the reaction is absolute and is restricted to the D-isomers when both D- and L-amino acids are supplied as substrate. DAAO in vitro displays broad substrate specificity, deaminating several neutral and basic D-amino acids; however, the most efficient substrates are amino acids with hydrophobic side chains. D-aspartic acid and D-glutamic acid are not substrates for DAAO, but are oxidatively deaminated by D-aspartate oxidase (DASPO, EC 1.4.3.1). DASPO and DAAO from pig kidney (pkDAAO) share a 50% sequence identity [7, 8]. Like DAAO, DASPO is unable to oxidize L-amino acids. The flavin side reaction in DAAO is also stereospecific. Experiments to reconstitute apo-DAAO from pig kidney with chirally C(5)-labeled 8-OH-5-deaza [5-^{3}H] $FADH_2$ showed that the ^{3}H label is released specifically from the *Re*-side of the flavin ring [9]. This is the same side which is active in DAAO from *Rhodotorula gracilis* [10].

Physiological role of DAAO

DAAO activity is ubiquitous: it is found in numerous eukaryotic organisms, including yeasts, fungi, insects, amphibians, reptiles, birds and mammals [11]. New reports of DAAO activity appear regularly, although very few DAAOs have been isolated and characterized. The enzyme's presence in microorganisms, mainly yeasts [see 3], is related to the well-established ability of yeasts to use D-amino acids for growth [12]. D-amino acids have long been known to play a prominent role in bacterial physiology as constituents of bacterial cell walls (as components of peptidoglycans and teichoic acids); thus, microorganisms and some of their products are likely to provide an important source of dietary D-amino acids (the exogenous source, for example, the source of gut bacteria in mammals, is not significant). However, until recently D-amino acids were thought to be rare under other natural conditions. There are sporadic reports of endogenous D-amino acids in animals, mostly in invertebrates [13], but they were known not to be utilized in ribosome-mediated protein synthesis, although they are present in antibiotic peptides synthesized by microrganisms via a nonribosomal pathway and also in a few neuropeptides present in higher invertebrates, where they are produced in nerve cells by a post translational in-chain epimerization [14]. D-amino acids were only found in appreciable concentrations in higher animals in recent studies, notwithstanding the fact that, in tissues (particularly kidney and liver), DAAO, a peroxisomal enzyme, shows high levels of expression. Furthermore, no racemase that might produce D-amino acids from their L-enantiomers is known to be present in mammals (for a review on the distribution and the utilization of D-amino acids, see [11]) except for a D-Ser racemase that occurs in rat brain (see below) [15, 16].

Thus, the physiological significance of DAAOs remained unclear in higher organisms, and it seemed unlikely that their main role in vivo was to metabolize D-amino acids, as they do so effectively in vitro. However, improved analytical methods greatly facilitated the quest for trace quantities of D-amino acids [17]. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) using highly sensitive chiral columns to separate D- and L-isomers of amino acids enhanced interest in D-amino acid metabolism.

In 1987 Preston used a DAAO enzyme assay method to demonstrate that D-amino acids occur commonly in the tissues of marine invertebrates [18]. The highest concentrations (up to 44 mM) of D-amino acids were found in the polychaete *Glycera dibranchiata* [19]. A specific D-amino acid transport system was also discovered that accumulates and maintains intracellular pools of free D-amino acids. These were proposed to be involved in osmoregulation and as a possible reserve source of L-isomers. Direct evidence was thus provided for the availability of D-amino acids for cell metabolism.

Significant quantities of D-amino acids were later detected in mammalian tissues. For example, D'Aniello et al. [20] showed that in various animals, including humans, endogenous and exogenously administered D-amino acids are oxidized in vivo by DAAO and DASPO in various tissues, particularly in the liver and

the kidney, giving rise to the same compounds as they do in vitro (i.e. NH_3 and H_2O_2). They also observed an inverse correlation between the presence of D-amino acids and the presence of the two oxidases, speculating that if D-amino acids are not metabolized, their accumulation may cause tissue damage. They proposed that the in vivo role of DAAO and DASPO in animals is to metabolize exogenous D-amino acids and to act as detoxifying agents to oxidize endogenous D-amino acids that may have accumulated during aging. There is evidence that D-amino acids are indicators of aging in humans. In long-lived tissues such as dentin, tooth enamel and eye lens a substantial increase in the D/L Asp or D/L hydroxyproline ratios is observed with time [21, 22]. For example, D-Asp accumulates in lens protein at a rate of 0.14% per year. No detectable racemization other than Asp and Ser is usually found in mammalian proteins; nevertheless, the spontaneous racemization of Asp and Ser residues in aging may have deleterious biological effects. Age-related accumulation of D-aspartic acid was detected in the cerebral white matter in humans [23]; this accumulation is probably related to the low or complete lack of turnover of this tissue and may plausibly affect brain functionality. Note, however, that factors other than racemization may increase the proportion of D-amino acids during aging, particularly the consumption of food processed by heat and high pH and which contains increased levels of D-amino acids [24].

Besides acting as a detoxifying agent in the oxidation of exogenous D-amino acids in animals, a novel, intriguing correlation has been established between DAAO and endogenous D-Ser in rat brain. Relatively high concentrations of D-Ser have been detected in the brain of rats, mice, and bulls [25, 26]. It is worthy of note the free D-Ser, measured as the D-to-L ratio, in normal human brain tissue is comparable to that in human brain which has been affected by Alzheimer's (0.086 and 0.099, respectively) [25–27]. The definition of origin and function of free D-Ser in brain tissue and even its possible role in neurotransmission have been extensively studied over the past few years. D-Ser in the brain most probably originates from dietary intake, because the D-amino acid (as its L-isomer) can pass freely through the blood-brain barrier and is then metabolized by DAAO. Another hypothesis is that racemization of L-Ser to D-Ser takes place in the brain itself [28]. This endogenous origin has been lately substantiated by isolation and cloning of a specific D-Ser racemase, a pyridoxal phosphate-dependent enzyme, in rat brain [15, 16]. In 1995 Schell et al. [29] localized D-Ser to multipolar glia in the rat forebrain, using highly specific antibodies. D-Ser appears to be concentrated in gray-matter astrocytes near the *N*-methyl-D-aspartate (NMDA) receptors to which it binds as the endogenous (and selective) ligand at the 'glycine recognition site' of the receptor. The immunolocalization of D-Ser not only correlates with this binding site of NMDA but is inversely correlated to the presence of DAAO activity, i.e. the localization of DAAO is almost the inverse of that of D-Ser. DAAO is, in fact, concentrated in astrocytes of the hindbrain and cerebellum [3, 30]. Thus, recent evidence has demonstrated consistent modulation of NMDA neurotransmission by D-Ser (as a free amino acid or as a part of neuroactive peptides), with a specific role of DAAO in brain function by regulating the level of these compounds [15]. This involvement of D-Ser and DAAO in brain function is also substantiated by the beneficial effects of adding D-Ser to antipsychotics in the treatment of schizophrenia [31]. Through an impaired enzyme metabolism of D-amino acids, DAAO activity can be also involved in some diseases, especially in patients suffering from chronic renal failure, in which the plasma content of D-Phe and D-Tyr is significantly greater than in healthy humans [32, 33].

General properties of DAAO and molecular biology

As noted above, notwithstanding the widespread occurrence of DAAO activity in the tissues of mammals and eukaryotic organisms in general [3], very few DAAO proteins have been isolated in homogeneous form and characterized in detail. The DAAO from pig kidney (pkDAAO) was the first to be obtained as a homogeneous flavoprotein in 1973 [34]. Another 14 years passed before a second purified DAAO, from the yeast *Rhodotorula gracilis* (RgDAAO), became available [35]. This was the first DAAO from a microorganism to be obtained as a resolved flavoprotein. Later, a second microorganism DAAO, isolated from the yeast *Trigonopsis variabilis*, was characterized [36]. An impressive series of mechanistic, kinetic and active-site studies have been performed over the years on these enzymes, and DAAO is now considered to be a model flavin catalyst.

DAAO belongs to a subgroup of the dehydrogenase and oxidase class of flavoproteins which has mechanistic similarities and a series of features in common. All react rapidly with oxygen in reduced form, and all stabilize the red anionic flavin radical via one-electron reduction, the flavin N(5)-sulfite adduct, and the benzoquinoid anionic form of 6- and 8-substituted hydroxy- and mercaptoflavins (these properties have been interpreted to indicate the presence of a positively charged locus of the protein interacting with the N(1) C=O(2) region of the pyrimidine ring of the flavin).

Here, a brief summary of the properties of each of the three purified DAAOs is given.

Structural properties

The active pkDAAO holoenzyme is a monomer of 347 amino acids containing one molecule of noncovalently bound FAD with a molecular mass of 39.6 kDa. The dissociation constant of the FAD-apoenzyme complex is 2.2×10^{-7} [37]. DAAO exists in solution as a mixture of monomer-dimer and even higher molecular aggregates in equilibrium, depending on the enzyme concentration and forms (holoenzyme, apoprotein or benzoate complex, see [3]). The monomer-oligomer association affects the catalytic activity. Its primary sequence contains six regions highly conserved in DAAOs from various sources (and in DASPO) (fig. 1) [38]. Regions I and III are involved in coenzyme binding, region I containing the consensus sequence GXGXXG [39]. Regions II, IV and V contain the highly conserved residues of the active site. The last conserved region is the Ser-Lys/His-Leu terminal sequence, representing the type 1 peroxisomal targeting signal [40]. It is noteworthy that out of the number of essential residues identified in the past by chemical modifications to be in or near the active site of pkDAAO [3] and out of the residues conserved among all known DAAOS, only three residues are present at the active site of pkDAAO, namely Tyr224, Tyr228 and Arg283 [38]. Mammalian DAAOs show a degree of identity (63%), indicating their functional and evolutionary correlation. Taking the three known DAAOs from microorganisms (*R. gracilis*, *T. variabilis* and *Fusarium solanii*, respectively), a lower degree of identity is observed (18%). Considering the degree of identity between just two of them or between a DAAO from yeast and the mammalian protein, a rate of about 30% is reached. In figure 1, the sequences of two other homologous proteins obtained from the genome sequencing of *Streptomyces coelicolor* and *Schizosaccharomyces pombe* are reported for comparison. The sequence homology of these two putative DAAOs with the *R. gracilis* enzyme (30% identity) alone and in the absence of a biochemical characterization of the proteins does not indicate whether the two forms are also functionally similar to DAAO or functionally unrelated. *Rg*DAAO is a homodimeric holoenzyme of 80 kDa, each subunit of ~ 40 kDa of 368 amino acids containing a noncovalently bound FAD molecule (K_d is 2.0×10^{-8} M) [41]. The dimer is a quite stable form, and not dependent on enzyme concentration. In the primary structure of the enzyme the Ser308-Lys321 sequence, a positively charged region is seen as an additional fragment not present in other DAAOs [38] (fig. 1). It contains the region Gly313–Arg318, a sequence easily cleaved by proteases. Limited proteolysis of *Rg*DAAO produces a monomeric, nicked and truncated active enzyme of ~ 38 kDa (containing two polypeptides of ~ 34 and ~ 5 kDa, respectively) [42]. Clearly the cleaved Gly313-Arg318 sequence is essential for the monomer-monomer interaction that gives the stable dimer.

With regard to the molecular mass of DAAO from *T. variabilis* (*Tv*DAAO), a number of discrepancies exist in the literature. Pollegioni et al. [36] purified the enzyme (from Boehringer Mannheim) to homogeneity and reported the enzyme to be a nonglycosylated homodimer: the protein monomer of ~ 38 kDa contained one molecule of noncovalently bound FAD per mole of monomer. This figure fits with a value in SDS-polyacrylamide gel electrophoresis (PAGE) of 39 kDa previously given for the same protein [43], but is far from a molecular mass of 170 kDa (possibly a tetramer) for the native holo- and apoenzyme (no K_d for FAD was calculated) given later by Schräder and Andreesen [44]. These authors also reported the occurrence of higher aggregates related to the protein concentration. As for pkDAAO, the enzyme-specific activity increased at low protein concentration. Interestingly, a single molecular form with an isoelectric point of 5.1 was determined in isoelectrofocusing for *Tv*DAAO [44], whereas both native enzymes from pig kidney and *R. gracilis* are present in two or three isoforms with different isoelectric points (7.0 and 7.2 for pkDAAO and 7.8, 7.4 and 7.2 for *Rg*DAAO). In *Rg*DAAO this microheterogeneity was shown to arise because in the native protein two polypeptide chains, differing for the presence or the absence of the C-terminus SKL-targeting signal, deleted in the latter case by endogenous proteolysis, are present [45]. In isoelectrofocusing, the SKL-deleted mutant gives in fact a single band of 7.2.

Spectral and kinetic properties

DAAOs in the oxidized state have the typical absorbance spectrum of the FAD-containing enzymes with two maxima in the visible region and one in the ultraviolet (UV) region. A typical fluorescence emission related to the presence of the flavin fluorophor can also be observed. Table 1 reports the absorbance and fluorescence values up to which have been determined to date and the figures available for the fully substrate-reduced enzymes (pig kidney, *R. gracilis* and *T. variabilis* DAAO) and for the one electron-reduced semiquinone (anionic) species. As noted above, the stabilization of the red anionic semiquinone and the reactivity with sulfite are the main common features of flavoprotein oxidases [4]. In Table 1 the K_d for sulfite and benzoate are reported. Benzoate represents a well-studied example of a number of carboxylic acids which are competitive inhibitors of mammalian and yeast DAAO and which bind to the enzyme active site, yielding typical absorbance spectrum perturbations ascribed to interactions between the benzene ring of the inhibitor and the isoalloxazine moiety [3].

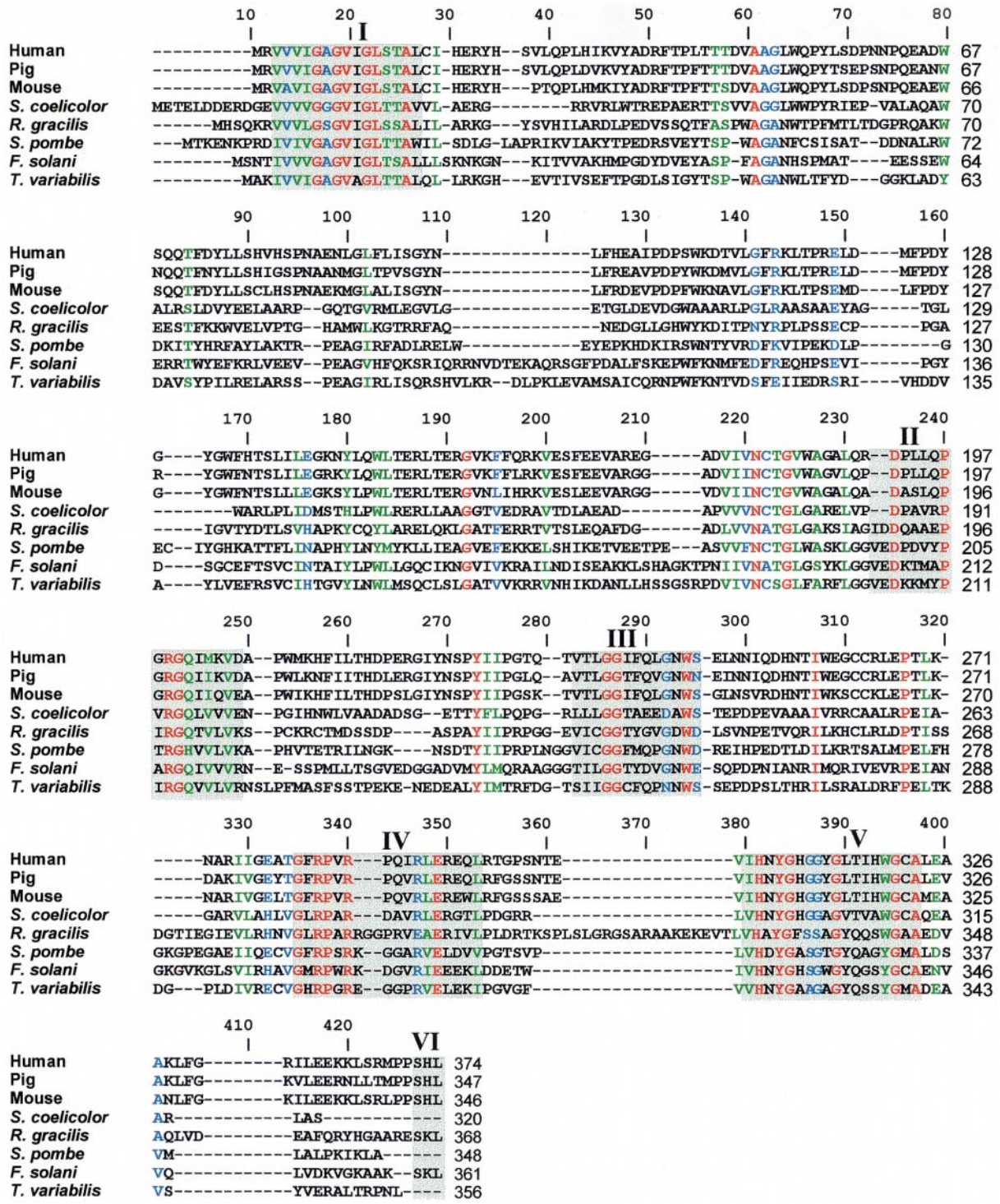


Figure 1. Comparison of the primary structure of DAAOs from significative sources. All listed DAAOs (except those from *S. coelicolor* and from *S. pombe*) have been characterized. Red, identity; blue, high similarity; green, low similarity; black, difference. Total alignment length, 429 amino acids (accession numbers: Human P14920, Pig P00371, Mouse P18894, *S. coelicor* T35265, *R. gracilis* P80324, *S. pombe* T40989, *F. solanii* P24552, *T. variabilis* Q99042).

Table 1. Spectral and binding properties of DAAO.

		Pig kidney DAAO*	*R. gracilis* DAAO†	*T. variabilis* DAAO‡
Abs maxima:	oxidized state	274, 380, 455 nm	274, 368, 455 nm	272, 360, 455 nm
	semiquinone state	–	274, 370, 400, 490 nm	–
	reduced state	355, 415 nm	350 nm	–
Fluorescence maxima		530 nm	355, 530 nm	355, 530 nm
A_{272}/A_{455}		9.5	8.2	8.4
pK_a, N(3)-H		9.4	10.6	10.1
K_d, FAD (M)		2.2×10^{-7}	2×10^{-8}	–
K_d, benzoate (mM)		0.002	0.245	18.8
K_d, sulfite (mM)		3.5	0.11	–

* [37, 3]. † [41, 10]. ‡ [36].

The redox properties of pkDAAO and *Rg*DAAO have been investigated. For pkDAAO, a two-single-electron transfer was established with an E'_0 for the first electron transfer of -0.098 V and -0.204, and of -0.099 and -0.117 V for the second electron transfer for the holoenzyme at pH 8.5 and 7.0, respectively). Benzoate binding modulates these properties, facilitating a two-electron transfer [46]. For the yeast enzyme, a value of -0.05 and -0.196 V, and of -0.044 and -0.131 V were determined for the transfer of the first and the second electrons, at pH 8.5 and 7.0, respectively [L. Pollegioni, personal communication].

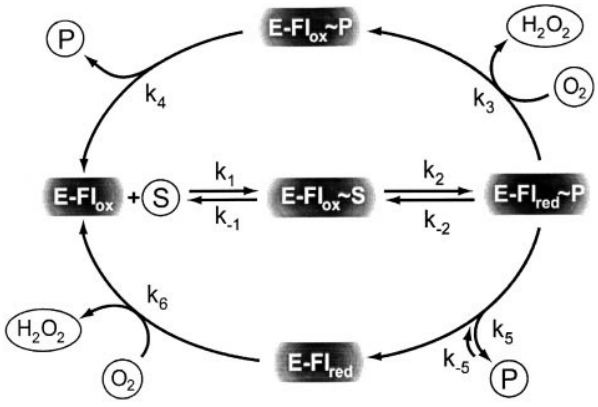
DAAO from pig kidney and those from yeast possess a broad substrate specificity. Except for the acidic D-amino acids D-glutamate and D-aspartate, DAAOs can oxidatively deaminate all the other D-amino acids, although with a different efficiency (expressed as the V_{max}/K_m ratio). In general, the best substrates are the aliphatic and aromatic D-amino acids, whereas polar and basic D-amino acids are very poor substrates. For mammalian DAAO, D-proline and D-methionine are the best substrates [3], whereas for *Rg*DAAO these are D-methionine, D-tryptophan and D-alanine [47]. For *Rg*DAAO an interesting, excellent substrate is cephalosporin C, the natural antibiotic on which the enzyme has a quite high turnover; this antibiotic is also a substrate for other DAAOs, even if with lower turnover. The kinetic mechanism of pkDAAO has been established through steady-state, stopped-flow and kinetic isotope effect measurements. Steady-state experiments suggested a ping-pong mechanism for mammalian DAAO, which only turned out to be true for basic D-amino acids (scheme 1). Actually, the kinetic data, resulting in parallel linear double reciprocal plots, apply for an ordered sequential mechanism in this DAAO, which involves a ternary complex, i.e. this is the limiting case of a ternary complex mechanism in which the bimolecular term Φ_{SO_2} is negligible, the reductive half-reaction being practically irreversible ($k_2 \gg k_{-2}$) (see [3]). The steady-state for this mechanism is described by equations 2, 3 and 4:

$$\frac{E_t}{v} = \Phi_0 + \frac{\Phi_S}{[S]} + \frac{\Phi_{O_2}}{[O_2]} + \frac{\Phi_{SO_2}}{[S][O_2]} \quad \text{(Eq. 2)}$$

$$\frac{E_t}{v} = \frac{k_2 + k_4}{k_2 \cdot k_4} + \frac{k_{-1} + k_2}{k_1 \cdot k_2 \cdot [S]} + \frac{k_2 + k_{-2}}{k_2 \cdot k_3 \cdot [O_2]} + \frac{k_{-1} \cdot k_{-2}}{k_1 \cdot k_2 \cdot k_3 \cdot [S] \cdot [O_2]} \quad \text{(Eq. 3)}$$

$$\Phi_{zero}^{-1} = V_{max} \frac{\Phi_S}{\Phi_{zero}} = k_s \frac{\Phi_{O_2}}{\Phi_{zero}} = k_{O_2} \quad \text{(Eq. 4)}$$

Analogously, the same overall kinetic scheme holds for the *R. gracilis* and *T. variabilis* enzymes [48]. However, whereas in the mammalian enzyme the term k_4, which corresponds to product release, is important in the reaction and thus represents the rate-limiting step, in both yeast enzymes the limiting factor is represented by the reductive step, i.e. $k_4 \gg k_2$ (scheme 1). This different location of the rate-limiting step elicits a quite different catalytic efficiency: k_{cat} is 20700 min^{-1} for *Rg*DAAO and 600 min^{-1} for pkDAAO, with D-alanine as substrate [48]. pH dependence of kinetic parameters has been examined for pkDAAO [3] and for *Rg*DAAO [49]. This point needs further investigations considering also the data from the 3D structures of the two DAAOs.



Scheme 1. Kinetic mechanisms proposed for the catalytic cycle of DAAO. The upper loop represents a sequential mechanism and the lower loop a ping-pong mechanism.

Molecular biology of DAAO

The DAAO gene is present in a single copy in the mammalian genome. The complementary DNA (cDNA) of the complete pig kidney enzyme has been cloned and sequenced [50]. The coding region, an open reading frame of 1041 bp, encodes the 347 amino acids of the enzyme, indicating that proteolytic posttranslational processing is not present. Genes coding for DAAOs from *F. solanii* [51], mouse [52], rabbit [53] and humans [54] have been cloned since, but only the porcine gene has been successfully expressed in *Escherichia coli*.

The cDNA coding for *Rg*DAAO was recently cloned by polymerase chain reaction (PCR), and expressed as holoenzyme in *E. coli*, using the mRNA fraction isolated from *R. gracilis* cells [55]. The coding region consists of 1104 bp encoding a protein whose sequence corresponds to that obtained by Edman degradation of the protein [38]. This cDNA shows an unusual codon usage, with a high preference for G and C, which probably prevented its prior cloning. A higher expression level (up to 8% of total soluble *E. coli* protein) has been more recently obtained by expressing a chimeric recombinant DAAO with six additional amino acid residues at the N-terminus [56]. In 1998 the genomic DAO1 gene encoding *Rg*DAAO was sequenced, and the comparison of cDNA and genomic sequences of DAO1 revealed the presence of five introns [57]. The cDNA gene was overexpressed in *E. coli*, but a large fraction of DAAO protein was recovered in its apoenzyme form. In 1986 Asahi Chemical Ind. KK published in a patent the sequence of 356 amino acids of the primary structure of *Tv*DAAO as derived by the nucleotide sequence of cDNA [58]. The DAO1 gene of *T. variabilis* encoding DAAO was later isolated from genomic clones [59]. In this case the open reading frame of this DAO1 gene was interrupted by only one intron. The cDNA encoding DAO1 was expressed by a yeast expression system in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.

Site-directed mutagenesis studies

Prior to the advent of mutagenesis studies, the topography of pkDAAO, *Rg*DAAO and *Tv*DAAO active site was investigated using specific chemical modifiers and artificial flavins. The extensive studies on this topic, mainly concerning pkDAAO, were reported in [3]; a later few papers appeared on chemical modification of essential residues in *Rg*DAAO and *Tv*DAAO. Histidine was the first putative essential residue considered using specific modifiers. Ramon et al. [60] and Gadda et al. [61] found that an essential histidine is present in *Rg*DAAO: as the first group pointed to a possible involvement of this residue in catalysis, the second group suggested for the residue (most probably His239) an involvement in substrate recognition and specificity with no role in catalysis. A paper reported also on reactive histidine(s) in *Tv*DAAO: in this case, too, a possible involvement of the residue in catalysis was envisaged [62]. As second putative essential residue, cysteine reactivity was examined in *Tv*DAAO [63] and in *Rg*DAAO [64]. For the latter, a single reactive cysteine was identified, clearly involved in FAD binding. As regards *Tv*DAAO, a single functional cysteine (Cys298) was identified; no information was given on the kind of involvement of the residue in catalysis and in coenzyme binding.

Site-directed mutagenesis of pig kidney DAAO

The data reported from early site-directed mutagenesis on the pig kidney enzyme were not in agreement with the results from chemical modification studies [3]. Myano et al. [65] suggested a crucial role of Tyr224 and a secondary role of His307 in the pkDAAO catalytic reaction. The first paper on the mutagenesis of two essential residues (Tyr224 and Tyr228 namely) of the active center of pkDAAO using purified mutant proteins appeared in 1994 [66]. Chemical modification of pkDAAO indicated that both tyrosine residues are in or near the active site [67–69]. Tyr224 and Tyr228 were changed to phenylalanines, and the mutated proteins were expressed in reasonable amounts (as was the wild-type enzyme) in IPTG-induced *E. coli* HB1O1 cells transformed with the pUK-DAAO plasmid, using a cloning procedure devised by Fukui et al. [50] and Miyano et al. [65]. The spectra of the oxidized, light-reduced semiquinone and substrate-reduced forms of Y224F and Y228F were similar to those of wild-type DAAO, and the redox properties are also similar [66]. Both mutant enzymes gave the formation of the flavin N(5)-sulfite adduct. Significantly, it was found that these two mutants stabilized the semiquinone less effectively than the wild-type enzyme. The kinetics of all three recombinant enzymes was studied by steady-state and rapid reaction methods. In steady-state measurements, the turnover numbers of the Y224F mutant with all the substrates used were similar to those of the wild-type enzyme, whereas for the Y228F mutant, turnover numbers were always lower (2.5–8 times). Parallel line Lineweaver-Burk plots were obtained for all mutants, and the results were identical to those with DAAO extracted from pig kidney, where the reductive half-reaction is practically irreversible ($k_2 \gg k_{-2}$) (see scheme 1 and eq. 1) [3]. During anaerobic reduction of the enzyme with D-alanine as substrate, two charge-transfer complex intermediates were subsequently produced. Using [2-^{2}H]D-alanine, an important result was achieved.

The C–H bond of the substrate was broken, and the flavin was reduced in the k_2 step (scheme 1), which is rate controlling. For the wild-type enzyme only the y intercept and the apparent K_d of the double reciprocal plot for the rate of the first phase were affected by deuterium substitution, with a 1.8-fold decrease compared with normal substrate; k_3 and k_4 were unaffected. All these differences between the mutant and wild-type DAAOs demonstrate that both tyrosine residues are in or near the active site and are involved in the reductive half-reaction of the enzyme. These results are in agreement with what was proposed by the chemical modification studies [3].

Note, however, that replacement of these two tyrosines by phenylalanine resulted in fewer marked changes in the catalytic properties than those brought about by chemical modifications. For Tyr228 this was thought to be because the D-propargylglycine used for alkylation also alkylated His307, in addition to Tyr228 [69]. The results of the chemical modification of Tyr224 by *N*-chloro-D-leucine [68] were interpreted as indicating that the role of this residue was to be close to an α-NH_3^+ group of the amino acid backbone and to act as a general acid/base during catalysis. This proposal for the reaction mechanism is not supported by the results of the mutagenesis study on this residue, which indicate, as stated above, that Tyr224 is involved in the reductive half-reaction of the enzyme and probably stabilizes the second charge-transfer intermediate, interacting with the positively charged NH_2^+ group of the imino acid product.

Site-directed mutagenesis of *R. gracilis* DAAO

Following the cloning and expression in *E. coli* of DAAO cDNA from *R. gracilis* [55], the door to site-directed mutagenesis studies was opened to investigate chemical reaction mechanisms of the enzyme so as to understand the important differences between pk and *Rg*DAAO.

Subsequently, on the basis of site-directed mutagenesis of pkDAAO [66] and inspection of its 3D structure [78], studies on mutations to Tyr223, Tyr238 and Arg285 were performed with the intent of throwing light on the reaction mechanism. These three residues are the only residues functionally conserved in the primary structure of DAAOs from pig, *R. gracilis* and *T. variabilis* [38] (see fig. 1). In fact, two tyrosyl residues are present in the active centers of several other flavoenzymes, including glycolate oxidase [70], flavocytochrome b_2 [71] and lactate monooxigenase [72]. In these enzymes a carbanion mechanism has been proposed to be active and the role of active site tyrosines to tune substrate interactions. Further, it was proposed that a basic residue in the vicinity of the flavin N(5) assisted this process by acting as an acid catalyst [6]. However, the mutagenesis studies on pkDAAO mentioned above indicated that no protonated amino group is present in the vicinity; the crystal structure of the pig kidney enzyme (discussed below) confirmed this.

It was initially considered that the Tyr223 of *Rg*DAAO was homologous to the Tyr228 (conserved in all known DAAOs) of the pig kidney enzyme [38]. However, reanalysis indicated that Tyr223 is homologous to Tyr224 of the mammalian enzyme, which is located on the flexible loop that controls substrate interaction and product release; there is no such loop on *Rg*DAAO (fig. 2A and B; fig. 3A and B). Specifically considering the study performed on Y223F and Y223S mutants of *Rg*DAAO overexpressed as holoenzymes in BL21(DE3)pLysS *E. coli* [56], it was found that both are catalytically active in turnover with D-alanine and have spectral properties similar to those of the wild type [73]. Y223F and Y223S enzyme stabilization of the red anionic semiquinone and flavin redox potential was close to that of the wild type as well. The mutants bound the same ligands as the wild-type DAAO, namely the typical inhibitors benzoate and anthranilate as well as trifluoroalanine and sulfite. Interestingly, both mutants are reduced by D-alanine slightly faster than by wild-type enzyme (about 3.5-fold faster than the wild-type for Y223F) (table 2). These findings show conclusively that the hydroxyl group of Tyr223 is not involved in substrate oxidation. The most significant difference observed was that the rate of product dissociation from oxidized enzyme (k_4, see scheme 1) for the oxidized Y223F was more than 17 times slower than for the wild type (the slightly greater rate of reduction compared with that of the wild type concealed changes in the other kinetic constants, since k_2 is rate limiting in catalysis). Thus, the catalytic efficiency $k_{cat}/K_{m\ D\text{-}Ala}$ of Y223F with D-alanine as substrate was $9.8 \times 10^4\ M^{-1}\ s^{-1}$, similar to that of the wild-type enzyme, $1.3 \times 10^5\ M^{-1}\ s^{-1}$ [48] (table 2). For Y223S, a pronounced effect of k_{cat} reduction is observed (table 2). $K_{d\ app.}$ approximates k_2/k_1, as in the case of the other mutant Y223F. Thus, the disturbances in binding as observed in the $K_{d\ app.}$ value from the reductive half-reaction are due to the rate of substrate association, k_1. A 60-fold slower substrate binding is observed for this mutant and an at least 800-fold slower rate of product release compared with the wild type. Thus, the data on Y223F and Y223S mutants of *Rg*DAAO eliminate Tyr223 as an active-site acid-base catalyst and show, instead, that it is crucial for enzyme-substrate and enzyme-product interaction. The aromatic ring is important sterically, functioning to guide the substrate into the correct orientation for efficient catalysis. Mutation of Tyr238 is presently under study: as for Tyr223, the active site Tyr238 does not play a direct role in catalysis but is involved in substrate interaction [L. Pollegioni, personal communication].

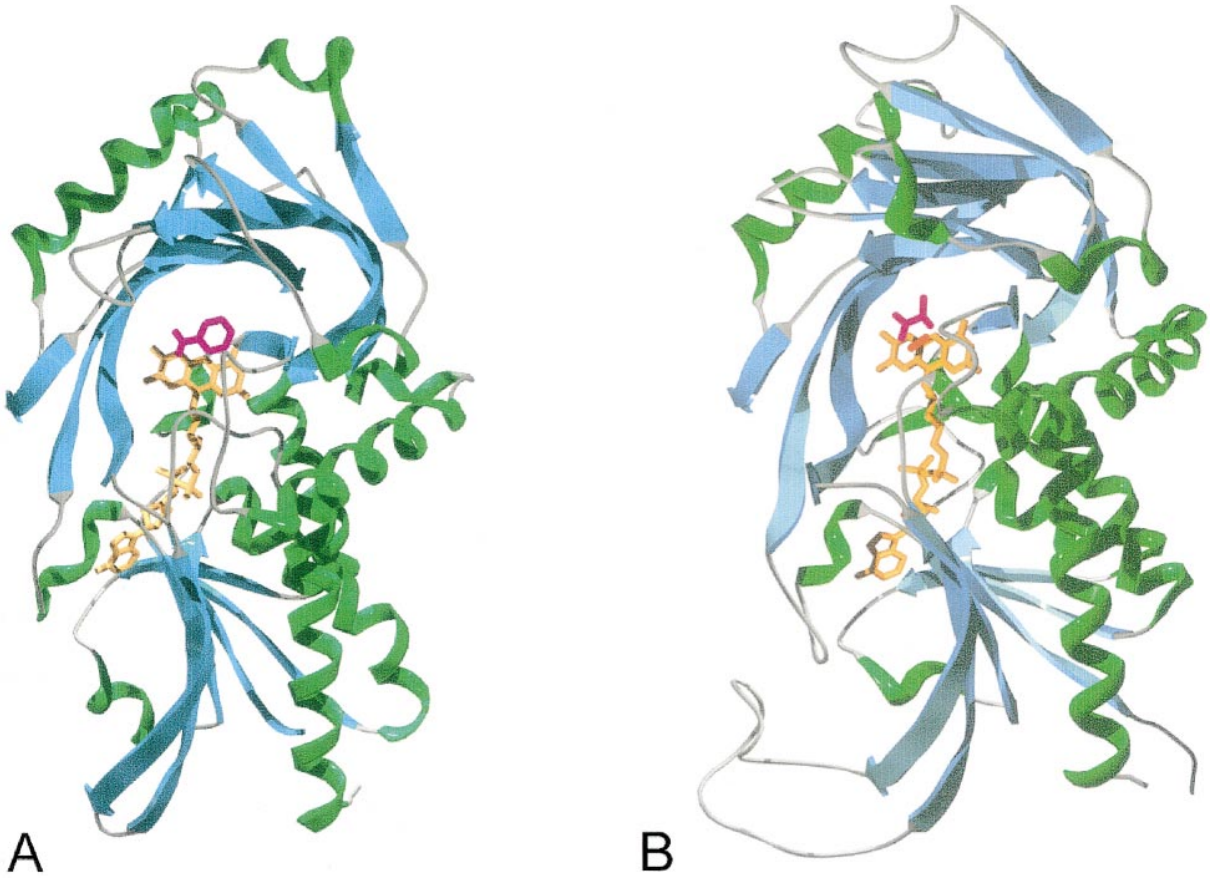


Figure 2. Ribbon representation of DAAO monomer structure. In green, α helices; in blue, β strands; in gray, connecting loops; in yellow, FAD; in purple, ligand. (*A*) pkDAAO in benzoate complex; (*B*) *Rg*DAAO in D-alanine complex.

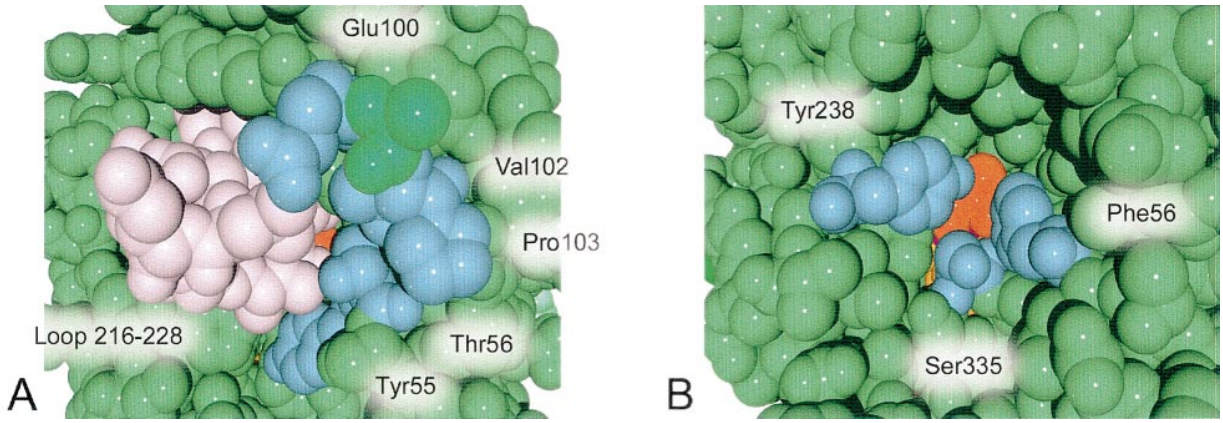


Figure 3. Comparison of the access to the active site in pKDAAO (*A*) and in *Rg*DAAO (*B*). In *A* the loop of 11 residues controlling the access is clearly seen (in pink), as in *B* only Tyr238 (in green) is involved in this function. In blue, residues lining the active site.

Turning now to consider Arg285, a conserved residue in DAAO, we note that the presence of an active-site arginine involved in catalysis was inferred from chemical modification studies [3]. The residue was identified by phenylglyoxal modification and unambiguously identified in the amino acid sequence of *Rg*DAAO in 1994 [74]. Most authors consider that the guanidinium group of this amino acid functions either as a positively charged group interacting with the negatively charged N(1)–C(2)=O locus of the flavin or as a basic residue ion-pairing with the substrate carboxylate [75, 76]. Arg285 was mutated to lysine, glutamine, aspartate and alanine. All four mutants had spectral properties similar to wild-type DAAO and were catalytically competent; however, they were inefficient in stabilizing the flavin semiquinone [77]. Loss of the guanidinium group of Arg285 drastically reduced the binding of several carboxylic acids, including benzoate, and also altered the redox properties of the enzyme, with little effect on catalysis. The catalytic properties of R285A and R285K were measured by the enzyme turnover method; R285Q and R285D were too slow to be examined. There was a large decrease (about 500-fold) in turnover numbers and an increase (about 1000-fold) K_m for D-alanine, whereas the rate of flavin reduction k_2 was very close to k_{cat} and was still the rate-limiting step of the reaction (table 2).

These results show that most probably Arg285 does not function as a base for the carbanion mechanism and suggest that, at difference with pkDAAO, its role is to interact electrostatically with the flavin at its negatively charged N(1)–C(2)=O locus when the enzyme is in the free form. The large reductions in reaction velocity (k_{cat} and k_{red}) for these mutants compared with the wild-type enzyme indicate changes in reaction trajectories, suggesting that Arg285 could play a role in orienting substrate and cofactor during catalysis [77].

3D structure of DAAO and flavin dehydrogenation mechanism

In 1996 two groups independently succeeded in solving the problem of the crystal structure of pkDAAO [78, 79]. The drawing of the structures (fig. 2A) is basically the same. The structure of the mammalian protein was identified by Mattevi et al. as a complex of the competitive inhibitor benzoate with a single isomorphous replacement (eightfold on average) at a maximum resolution limit of 2.6 Å. The enzyme is a dimer comprising 2×347 amino acids and a molecule of noncovalently bound FAD per subunit. Each subunit has two domains with an overall topology similar to that of *p*-hydroxybenzoate hydroxylase (PHBH) [80]. The FAD-binding domain (residues 1–63, 141–183, 294–339) has the dinucleotide binding fold typical of several flavoenzymes [39]; the interface domain (residues 64–140, 184–293) contains an eight-stranded, mixed β sheet. The interface domain forms the contact area (about 15% of the monomer surface) between the tightly interacting subunits yelding an elongated elipsoid with 'head-to-head' contacts. The presence of benzoate shows that it is the active site. The inhibitor binds parallel to the flavin ring on the *Re* face of the cofactor (3.4 Å between them) [78]. The carboxylate group of benzoate is juxtaposed to the flavin C(6) atom by a salt bridge with the side chain of Arg283 and an H bond with Tyr228, in agreement with chemical modification studies [68, 74, 75]. In addition to these strong polar contacts, benzoate interacts with the side chain of Tyr224 (fig. 2A). Due to such extensive interactions, none of the benzoate atoms is accessible to solvent. Moreover, the N(3), O(4) and N(5) atoms are H-bonded to the main chain of residues 49–51. The N(1)–C(2)=O locus of the flavin interacts with the positive charge of the helix F5 dipole and not with a positively charged residue of the protein, as previously postulated. The flavin ring, positioned at the interface between the two domains of the subunit, is not accessible to the solvent, and in this structure is essentially planar. This structure of pkDAAO shows an active site formed by hydrophobic residues: its cavity has a calculated volume of 160 Å^3, roughly corresponding to the volume occupied by an amino acid with a four-carbon-atom side chain. In fact, pkDAAO shows K_m values for substrates with a side chain longer than four carbon atoms much

Table 2. Steady state coefficients and reductive half-reaction rate constants for wild-type, Y223S, Y223F, R285K and R285A *Rg*DAAOs with D-alanine as substrate, at pH 8.5 and 25 °C.

	Steady state			Reductive half-reaction	
	k_{cat} (s^{-1})	K_{AA} (mM)	K_{O_2} (mM)	k_2 (s^{-1})	$K_{d,app}$ (mM)
Wild-type	350 ($\approx k_2$)	2.6	2.3	340	2.8
Y223S*	4.2	6.0	0.15	1400	710
Y223F*	210	2.2	1.4	1220	23
R285K†	0.8	800	0.06	0.6	1000
R285A†	0.05	310	0.08	0.035	200

* [73]. † [77].

higher than that for D-Ala. The enzyme stereospecificity for the D-isomer is related to the unfavorable contacts of the α-amino group of a L-amino acid with the C(5)–C(6) atoms of the flavin. Interestingly, the limited volume of the active-site cavity is apparently related to the presence of a loop or 'lid' (residues 216–228) which covers the ligand and blocks the access to the active site (fig. 3A). The loop in the absence of the ligand may switch from the 'closed' conformation to an 'open' one to allow substrate binding and product release. The 3D analysis of reduced DAAO in a complex with the reaction product imino tryptophan (iTrp) confirms the structural role of the 216–228 loop in controlling active-site accessibility and plasticity and in controlling catalysis by increasing hydrophobicity of the cavity in 'closed' conformation [81]. The shielding of the active site is thought to enhance the efficiency of the hydride transfer reaction in amino acid dehydrogenation, supporting the direct transfer of the substrate α-hydrogen to the flavin in the reductive half-reaction, as envisaged in the work of Mattevi et al. [78]. The 3D pkDAAO structures show that at the active site there are no side chains properly positioned to act as general base, thus ruling out the possibility of a carboanion reaction mechanism (the resolution of the structures does not in any event allow identification of the αC–H in the electron-density map). The notion of a classical hydride mechanism is supported by linear-free energy correlations studies on *T. variabilis* DAAO; the results clearly indicate that no significant charge develops in the transition state of the reaction [82]. The architecture of pkDAAO catalytic site shows a close similarity to that of flavocytochrome b_2, even if they have an opposite substrate stereospecificity: benzoate is located on the *Re* side of the flavin in DAAO and pyruvate on the *Si* side in flavocytochrome b_2 [83]. DAAO and flavocytochrome b_2 represent examples of convergent molecular evolution toward a common, but enantiomeric, catalytic group arrangement [78].

The group of Mizutani worked on a recombinant form of pkDAAO expressed in *E. coli* [79, 84]. The crystallographic structure was also determined to be of the complex form with benzoate, using the multiple isomorphous replacement (MIR) method. Resolution was at 3.0 Å, with a final R factor of 21.0%. The general array of the protein structure was as depicted by the group of Mattevi, confirming the importance of three residues at the active site: Arg283 and Tyr228, which interact with the carboxylate groups of the pseudosubstrate, and Tyr224, with a possible role in catalysis. In this 3D structure no protein-positive charge or α-helix dipole is seen near N(1) of flavin. As for the previous case, a very important feature of this DAAO crystal structure is that the postulated protein base required for abstracting the substrate α-proton (in a catalytic carboanion mechanism) is not found at the enzyme-active center.

In a subsequent paper the same group [85] identified the crystal structure of the complex of recombinant pkDAAO with the substrate analog *o*-aminobenzoate (OAB). When OAB binds to DAAO, distinct spectral changes are observed, and a very broad absorption band in the long-wavelength region beyond 700 nm appears [86]. OAB faces the *Re* face of the FAD from one side and the phenol ring of Tyr224 from the other. The presence of the amino group in the OAB molecule causes an interaction by hydrogen bonding with the backbone carbonyl oxygen of Gly313 (with a distance between the two groups of 2.6 Å). This strong interaction, absent in the DAAO-benzoate complex, shifts the OAB phenol ring away from the flavin and also weakens the ionic interaction between the OAB carboxylate and the guanidino group of Arg283 with respect to the DAAO-benzoate complex. According to molecular modeling of the pkDAAO-D-leucine complex, the authors propose an electron-proton-electron mechanism as an alternative to the anionic mechanism, which is in contrast to the mechanistic interpretation of structural data given by Mattevi et al. [78]. The 'EPE' mechanism proceeds via one-electron processes with an intervening proton transfer, yielding the anionic form of the reduced flavin and the imino acid with cationic imino nitrogen. In the ionic mechanism two electrons flow from the amino lone pair in concert with the α-proton abstraction by N(5) of the flavin.

In the past year a set of X-ray data at very high resolution were collected on *Rg*DAAO that shed light on the mode of reaction mechanism of the enzyme in relation to the active site. The structures of monomeric

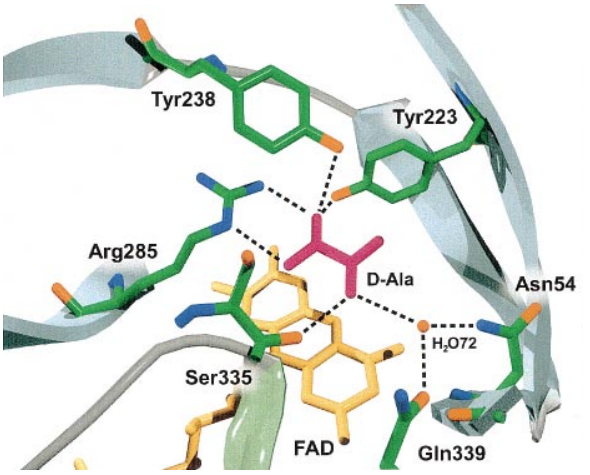
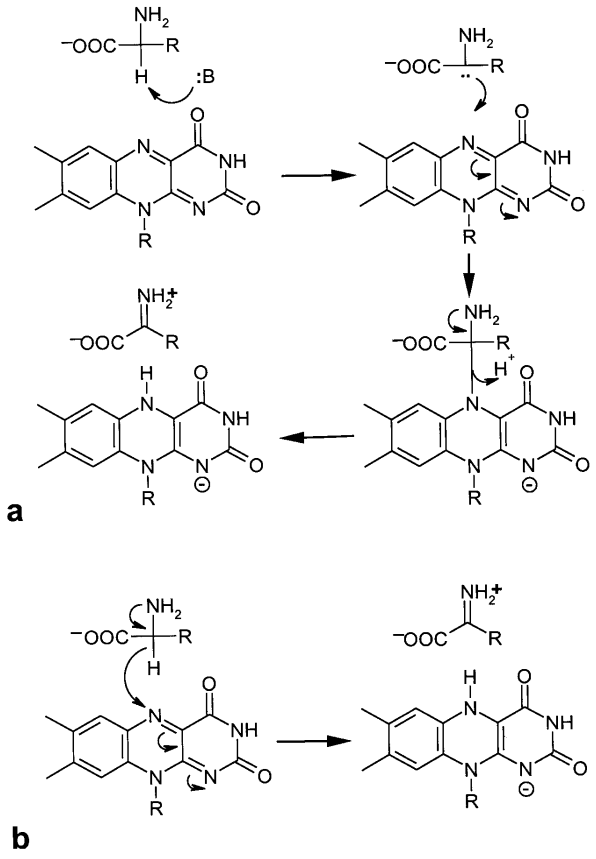


Figure 4. Active site region of *Rg*DAAO in complex with D-alanine at 1.2-Å resolution. Hydrogen bonds between the ligand and active site residues are shown as dotted lines.



Scheme 2. Reaction mechanisms of DAAO. (*a*) Carbanion mechanism; (*b*) α-CH hydride transfer mechanism.

*Rg*DAAO reduced with D-alanine and of the oxidized form complexed with 3,3,3-trifluoro-D-alanine (D-CF_3-Ala), anthranilate and lactate were determined and refined at 1.20, 1.72, 1.46 and 1.90 Å resolution, respectively [87]. Crystals of the enzyme in its complex with the substrate D-alanine are colorless and show that the flavin is reduced to a major extent. This species arises from the exchange of the product iminopyruvate with the substrate and corresponds to the Michaelis complex (fig. 4). The crystal diffracted to 1.20 Å, to date the highest resolution obtained for a protein of this size. With this electron density, detailed observation of the molecular conformation and clear identification of a biatomic species with partial occupancy on the *Re* side of an alloxazine ring and a dioxygen molecule (either O_2 or most probably H_2O_2) were possible: no covalent bond with the flavin was observed, as there was no distortion of the flavin plane (fig. 2B and fig. 4). The observation of a planar flavin structure contrasts with the proposal of Todone et al. [81], based on the structure of a nonplanar covalent flavin-N(5)-adduct obtained photochemically, that pkDAAO is able to bind a highly distorted cofactor. A proposed mode of dimerization is a 'head-to-tail' interaction of two monomers, yielding a spherical dimer. Experimental evidences are given by light scattering and gel filtration chromatography measurements and by the presence of a 21-residue loop connecting two β sheets near the N-terminus (fig. 2B), which can be cleaved off by limited proteolysis, producing a monomeric protein [42, 87]. As a major difference from pkDAAO, in the *Rg*DAAO structure the 'lid' which limits active site access in the mammalian protein is absent. This portion of the protein is not conserved in the primary structure of *Rg*DAAO, and only Tyr238 is modulating active-site accessibility (fig. 3B). This observation is consistent with the fact that *Rg*DAAO has a low K_m even for bulky substrates such as the β-lactam antibiotic cephalosporin C. Moreover, this is the structural rationale for the very high turnover of *Rg*DAAO, since according to kinetic mechanisms, the rate-limiting step is represented by the reductive step and not by the product release from oxidized enzyme, as for pkDAAO [48]. This fact probably stems from an evolutionary drive to obtain a higher catalytic efficiency and a broader substrate specificity. CF_3-Ala, structurally similar to alanine, is a substrate analog, which for thermodynamic reasons does not react with the enzyme, its Michaelis complex-forming behavior resembling that of D-alanine ($K_d \approx 6$ mM, at pH 8.5 and 25 °C vs. a $K_d \approx 3$ mM at pH 8.5 and 25 °C) [73, 49]. L-lactate was also found to bind to *Rg*DAAO, and the structure of its complex with the enzyme gave information complementary to that from the pseudoenantiomeric D-alanine.

The mode of binding of the three ligands follows the general arrangement observed for pkDAAO, flavocytochrome b_2 and glycolate oxidase: the ligand carboxylate function is bound via an electrostatic interaction to the guanidinium group of Arg285 and forms H bonds with the hydroxylate groups of Tyr223 and Tyr238 (in pkDAAO only Tyr228, corresponding to Tyr223 of *Rg*DAAO, forms an H bond to the substrate carboxylate oxygen) [78]. The substrate α-NH_2 is H-bonded symmetrically with Ser335=O and H_2O72, both probably being acceptors (fig. 4). The ligand side chain is oriented toward the hydrophobic binding pocket. The mode of binding of D-alanine and of D-CF_3-Ala is of primary importance for mechanistic interpretation: the orientations of D-alanine and of D-CF_3-Ala precisely form the configuration required for efficient hydride transfer to the N(5) position of the flavin (see scheme 2). This goes along with the striking observation that at the active site no functional groups that might act as acid-base catalyst are present, i.e. the catalytic event involving the rupture of two covalent bonds (substrate αC–H and NH–H) occurs in the absence of residues capable of acid-base catalysis. This observation excludes a classical carbanion mechanism that strictly requires a base not present, in this case at the active center for H^+ abstraction, and points to the role of

orbital steering and optimal substrate alignment in this type of catalysis.

Biotechnology of DAAO

DAAO from yeast is one of the very few flavoproteins to have been exploited in enzyme technology. A recent review summarizes the biotechnological applications of *Rg*DAAO [88]. The characteristics rendering this possible are high turnover number, stable FAD binding and the fact that molecules other than D-amino acids are good substrates for the enzyme. For example, *Rg*DAAO efficiently deaminates cephalosporin C to 7-(5-oxoadipoamido)cephalosporanic acid (k_{cat} is 44200 min^{-1} at saturating oxygen concentration, 37 °C and pH 8.5), which then decarboxylates spontaneously to glutaryl-7-cephalosporanic acid. This reaction is part of the two-step biocatalytic route (DAAO plus an acylase) from cephalosporin C to 7-amino-cephalosporanic acid (7-ACA), the latter being a key intermediate in the production of semisynthetic cephalosporins (world market 2.5×10^9 USD in 1997). This use was first proposed in the early 1970s when only pkDAAO, an enzyme not suited for its low turnover for industrial applications, was available [89]. Later conversion of cephalosporin C was attempted using immobilized DAAO from *T. variabilis* (the source cited most in the technical literature) and *R. glutinis* [90, 91]. None of these attempts were successful; either the immobilized system was insufficiently stable or conversion yields were low. A more successful process is reported using *Rg*DAAO immobilized on commercial ion-exchange resins [92]. The conversion to produce the glutaryl derivative has at pH 7.5 a 90% yield after 0.5–3 h, which could be brought to 95% by adding H_2O_2 to the reaction solution. A drawback of the system is the presence of ketoadipyl derivatives which remain as impurities in the final product. In the industrial and patented applications of DAAO for antibiotics production, the enzyme from *T. variabilis* is the one mainly employed, as indicated by the number of patents [e.g. 93–95]; few research papers in the literature deal with this issue. An interesting recent report on *Tv*DAAO is the engineering of a His-tagged protein, its overexpression in *E. coli* and its purification in a single step by using a tailor-made metal chelate support containing cobalt ligands [96]. A laboratory-scale process for producing glutaryl-7-ACA from cephalosporin C using Affi-Gel 10-coupled RgDAAO with high conversion efficiency was reported in 1995 [97]. A 2.5- to 12-fold reduction in the apparent K_m was found for all substrates tested. No exogenous H_2O_2 was required to shift the reaction equilibrium toward the decarboxylated product glutaryl-7-ACA because of the high rate of reoxidation of the reduced enzyme, nor was exogenous FAD necessary. The bioconversion yield at pH 8.5 reaches nearly 100% after 90 min of reaction with productivity, as determined by HPLC, of 54 g of glutaryl-7-ACA/day per milligram of enzyme. No side products were detected. The same Affi-Gel 10-coupled DAAO, coimmobilized with catalase, was used in a laboratory process to produce α-keto acids (2-oxo-acids) from D-amino acids or racemic mixtures of D and L forms [98]. *Rg*DAAO has been used in a novel approach to gene-directed enzyme prodrug therapy (GDEPT), in which D-alanine is employed as substrate to generate H_2O_2, a reactive oxygen species (ROS). H_2O_2 is a membrane-permeable metabolite known to damage biopolymers, and its cytoxicity is exploited in a number of antineoplastic therapies. The strategy is to express a xenobiotic enzyme in tumor cells that can convert a nontoxic prodrug into a cytotoxic metabolite, overcoming the systemic toxicity of chemotherapy. Xanthine oxidase and glucose oxidase have been experimented with in this way [99–101]. However, both suffer from the disadvantage that substrate availability cannot be controlled; hence, ROS production cannot be controlled. The use of DAAO was proposed by Ben-Yoseph and Ross in 1994 [101], since production of H_2O_2 by DAAO in malignant cells can be regulated by exogenous administration of D-amino acids. *Rg*DAAO is the most suited for this application, not only because of its high turnover number, but also because constitutive expression of the enzyme is predicted not to be cytotoxic due to the absence of endogenous substrates [102]. *Rg*DAAO cDNA was truncated to remove the carboxy-terminal peroxisomal targeting sequence (SKL) [45], so the enzyme was targeted to cytoplasm. A line of 9L glioma cells was stably transfected with the construct (9LDAAO17) and found to synthesize active *Rg*DAAO. The cells were exposed to increasing concentrations of D-Ala for 24 h with the result that there was a dose- and time-dependent cytotoxicity with an IC_{50} of 2.6 ± 0.7 mM [103]. At these concentrations D-alanine was nontoxic to parental 9L cells. Concentration-dependent stimulation of the pentose phosphate pathway activity (absent in untransfected 9L cells) was also measured. The results demonstrate that *Rg*DAAO expression in tumor cells confers sensitivity to D-alanine and to oxidative stress, the latter mediating the cytoxicity. This in vitro GDEPT appears to have several advantages over other commonly investigated GDEPTs [102, 103]. An efficient in vivo transfer of this experimental approach is not yet demonstrated. Finally, we note that the absolute stereospecificity of the DAAO reaction has rendered it useful as an industrial scale biosensor for the detection and quantification of D-amino acids in biological samples. Presence of the D-isomer of amino acids or the occurrence of racemization can decrease the nutritional value of food products and raises concerns about safety, although it is a matter of controversy as to whether D-amino acids are toxic. The D-alanine content

can be considered as an indicator of the presence of bacterial contaminants in milk [104]. A biosensor for the analysis of D-amino acids represents an attractive device that can overcome the time-consuming methods for enantiomeric separation of amino acids. The attempt to use pkDAAO immobilized on microelectrodes resulted in very low amperometric currents (about 100-fold smaller than those obtained with glucose oxidase in the same system) [105]. Coimmobilization in carbon paste electrodes of pkDAAO and peroxidase to reduce the H_2O_2 produced by DAAO has also been attempted [106]. Riklin et al. [107] reported a system in which the enzyme-electrode contacts are improved by modification of cofactors. They reconstituted apo-pkDAAO with ferrocene-tethered FAD cofactor and a direct electrical communication between the electrode and the reconstituted DAAO was demonstrated. Recently, a sensitive biosensor was formed [108] by adsorbing *Rg*DAAO (1.5 U) onto the graphite electrode of a stirred flow electrochemical cell [109, 110]. The response is linear in the 0.2–3 mM concentration range, with a detection limit of about 0.15 mM and a good reproducibility. The chemical species revealed by the electrochemical transducer was identified as H_2O_2. Only a very small amount of enzyme is adsorbed on the working electrode (about 10 μg of protein), and the addition of exogenous FAD is not required.

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