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Porcine kidney D-amino acid oxidase: the three-dimensional structure and its catalytic mechanism based on the enzyme–substrate complex model

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Abstract

The three-dimensional structure of porcine kidney D-amino acid oxidase (DAO), an FAD-dependent oxidase, has been solved by X-ray crystallography. The overall structure is a dimer, subunits of which are correlated by a non-crystallographic two-fold axis. Each subunit comprises two domains, 'αβ domain' and 'pseudo-barrel domain'. The coenzyme FAD is in an elongated conformation and is bound at the *N*-terminal βαβ dinucleotide binding motif. The active site is located in the boundary region between the two domains. The crystal structure of DAO in complex with a substrate analog, *o*-aminobenzoate, was also solved and is used for modeling the DAO-D-leucine complex, i.e. Michaelis complex, by means of molecular mechanics simulation. The Michaelis-complex model provided structural information leading to two alternative hypothetical mechanisms for the reductive half-reaction of DAO. These two hypotheses characterize themselves by electron transfer from the lone-pair orbital of the substrate amino nitrogen to flavin C(4a) and by proton transfer from the substrate α-position to flavin N(5) which acts as a catalytic base.

Keywords: D-amino acid oxidase; Three-dimensional structure; Michaelis-complex model; Reductive half-reaction; Catalytic mechanism

1. Introduction

D-Amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), E.C.1.4.3.3] (DAO), an FAD-dependent flavoenzyme, occurs widely among the biological kingdom from microbes to mammals and in numbers of organs and tissues. Ever since DAO was discovered in animal tissues by Krebs [1], it has served as subjects for enzymological, biophysical, and medical investigations (see recent review [2]). Porcine kidney DAO is the most extensively studied among those found in various species and is regarded as the prototype of flavin-dependent oxidases. The primary structure has been reported on the bases of amino acid sequence and the nucleotide sequence of cDNA [3,4].

The physiological role of DAO had remained elusive, since D-amino acids had long been considered 'foreign' to organisms, particularly to higher animals. But due to the improvement in the detection method

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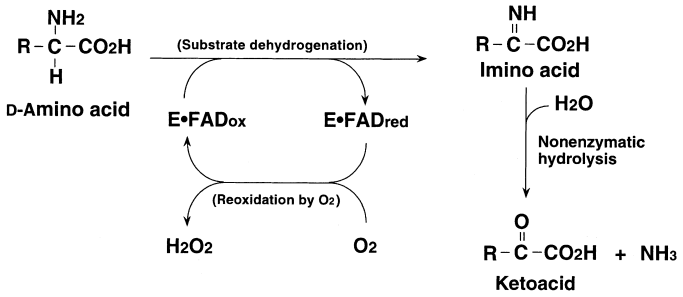


Scheme 1. The reaction catalyzed by D-amino acid oxidase (DAO).

of distinguishing enantiomeric amino acids, it has now been established that D-amino acids do occur in various organisms including mammals and that some of them are no longer 'foreign' to organisms. Moreover, certain D-amino acids play critical roles in regulating neural activities in vivo. In view of the growing interests toward the physiological functions of D-amino acids, the role of DAO which oxidatively decomposes a wide variety of D-amino acids is likewise considered important in regulating the levels of those D-amino acids related to the neural activity [5,6].

DAO catalyzes oxidative deamination of a variety of D-amino acids (Scheme 1). When a substrate binds at the active site of the enzyme, it becomes dehydrogenated to an imino acid by enzyme-bound FAD which becomes reduced. Imino acid then is released from the active site and hydrolyzed nonenzymatically to ketoacid and ammonia. Reduced FAD is reoxidized by molecular oxygen, thereby closing the catalytic cycle.

Despite its long history of enzymological as well as physico-chemical studies, the detailed molecular events during the catalytic sequence are not totally free of ambiguity, mainly due to the lack of the active-site structure at atomic resolution until very recently we solved it by X-ray crystallography [7]. Quite independently, identical crystal structure was published elsewhere by another group [8]. The present paper summarizes the recently solved structure of the active site of DAO and the catalytic mechanism based on the active-site geometry and particularly on the alignment of the substrate with respect to flavin ring system deduced from the crystal structure of DAO in complex with a substrate analog, *o*-aminobenzoate (OAB).

2. Three-dimensional structure of DAO

The tertiary structure of DAO was solved by X-ray crystallography [7]. Shown in Fig. 1 is the crystal structure of DAO expressed by *Escherichia coli* to which DAO cDNA had been introduced. DAO in Fig. 1 is complexed with a competitive inhibitor, OAB [9] and is in a dimer with a non-crystallographic two-fold axis. This overall dimeric structure is consistent with the basic dimeric unit in the self-association mode [10]. Each subunit consists of two domains, 'αβ domain' and 'pseudo-barrel domain' (Fig. 2); the former is characterized by an α/β structure with an open twisted β-sheet of six β-strands, all of which are parallel with one another except the terminal β-strand, and four α-helices, while the latter forms a distorted β-barrel structure with two twisted β-sheets, one antiparallel β-sheet with five strands and the other with three strands and two parallel β-strands at the junction of the two sheets. The active site is found in the region between the two domains; both flavin ring and OAB are found within this pocket and the wall of the distorted barrel seems to refine the size of the active-site pocket. The coenzyme FAD is in an elongated conformation and is recognized by a typical βαβ dinucleotide binding motif at the *N*-terminal region, with the positive end of the α-helix dipole stabilizing the negative charge of the pyrophosphate moiety (Fig. 3). OAB is located above the *re*-face of the flavin ring system, while the *si*-face is covered by the hydrophobic stretch V47-A48-A49-G50 blocking access of a substrate on the *si*-face. This provides the direct structural proof for the *re*-face specificity of DAO-catalyzed reaction which had been postulated by kinetic analysis [11].

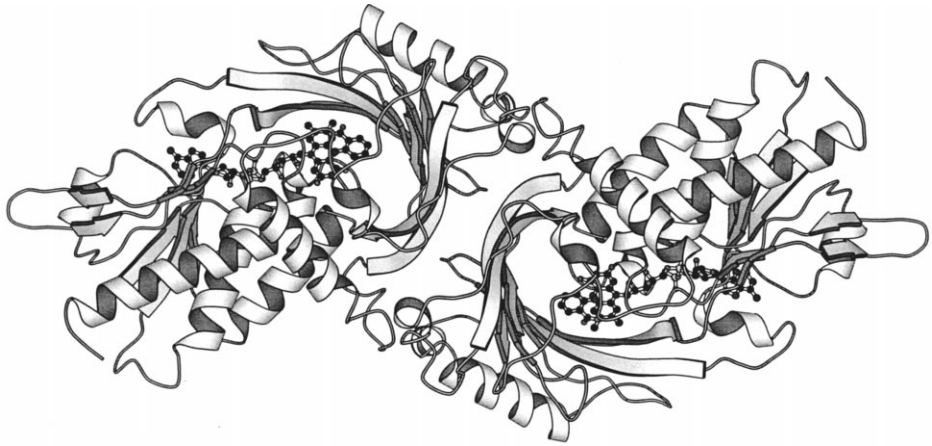


Fig. 1. The overall dimeric structure of D-amino acid oxidase-*o*-aminobenzoate complex. The figure was constructed by a program Molscript [25]. The two subunits are correlated by a non-crystallographic two-fold axis running vertically.

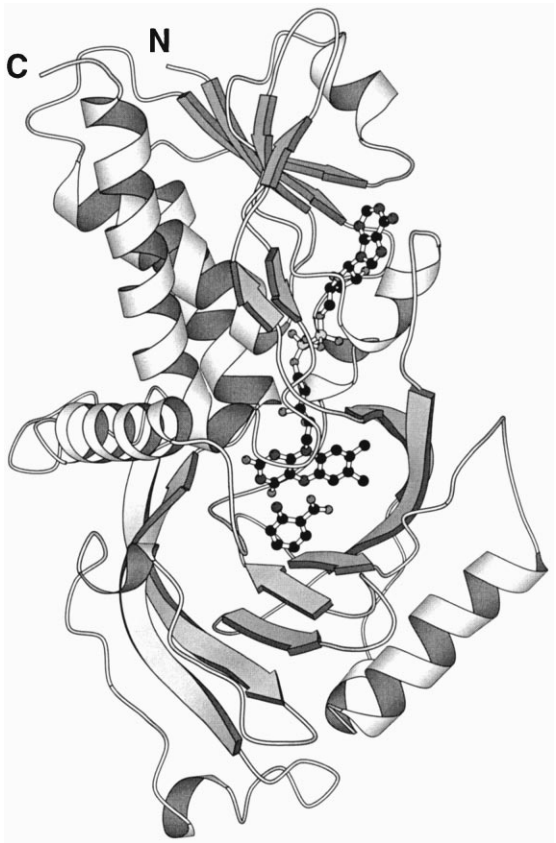


Fig. 2. The monomer structure of D-amino acid oxidase-*o*-aminobenzoate complex. The figure was constructed by a program Molscript [25].

3. Active-site structure of DAO

The isoalloxazine ring system forms a unique hydrogen-bonding network with amino acid residues of the protein moiety as shown in Fig. 4. It is known that each flavoenzyme possesses a characteristic hydrogen-bonding network between the isoalloxazine ring and the protein moiety and that the hydrogen-bonding network associated with flavin is

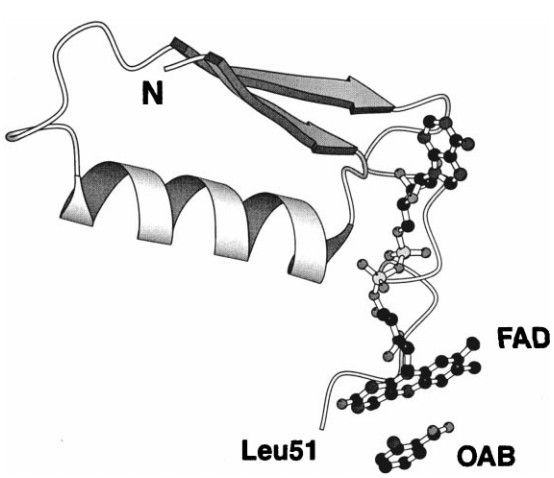


Fig. 3. The FAD-binding site characterized by a typical bab dinucleotide binding motif. The *si*-face of the flavin ring is covered by a hydrophobic stretch V47-G50. *o*-Aminobenzoate is above the *re*-face.

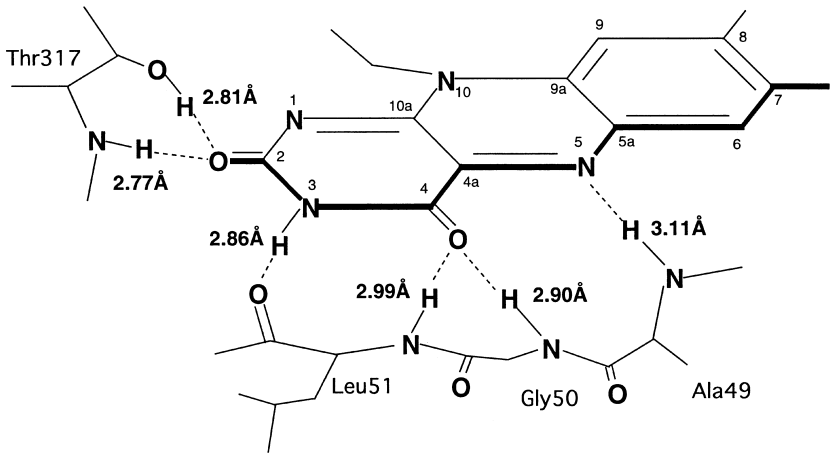


Fig. 4. The hydrogen-bonding network between flavin and the protein moiety.

one of the important factors that fine-tune the reactivity of the flavin ring system necessary for the specificity of each flavoenzyme as deduced from the frontier molecular orbital calculations [12,13]. The active-site structure is shown in Fig. 5. The competitive inhibitor OAB is found above the *re*-face of flavin as described in the preceding section and is sandwiched between the flavin ring and the phenol ring of Tyr 224, the three rings being almost parallel with one another. The carboxylate of OAB makes a salt bridge with the guanidino group of Arg 283 and a hydrogen bond with the hydroxyl of Tyr 228. The amino group of OAB is hydrogen bonded with the backbone carbonyl of Gly 313 which also forms a hydrogen bond with a bound water molecule which is further stabilized by two hydrogen bonds with Gln 53 back-bone carbonyl and with Tyr 224 hydroxyl. DAO–OAB complex exhibits a broad absorption band in the long wavelength region extending beyond 700 nm. This characteristic band has been proposed to derive from charge-transfer from OAB to flavin [14] and the charge-transfer interaction has been proved by resonance Raman spectroscopy with excitation in the long-wavelength band [15]. The alignment between OAB and flavin is in such a way that C(2)–N of OAB overlaps with O=C(4) of flavin and the carboxyl carbon of OAB overlaps with flavin N(5). The charge-transfer interaction is generally attained by the overlap between the highest occupied molecular orbital (HOMO) of an electron donor and the lowest unoccupied molecular orbital (LUMO) of an electron acceptor. In view of the oxidant nature of oxidized flavin, flavin should serve as the electron acceptor, hence OAB as the electron donor, for the

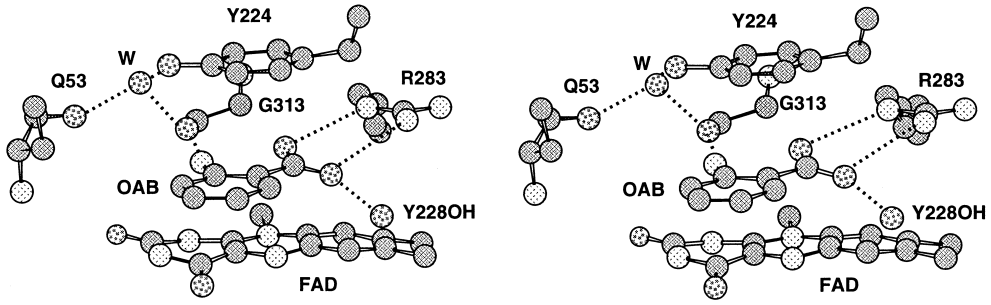


Fig. 5. A stereo view of the *o*-aminobenzoate-binding site.

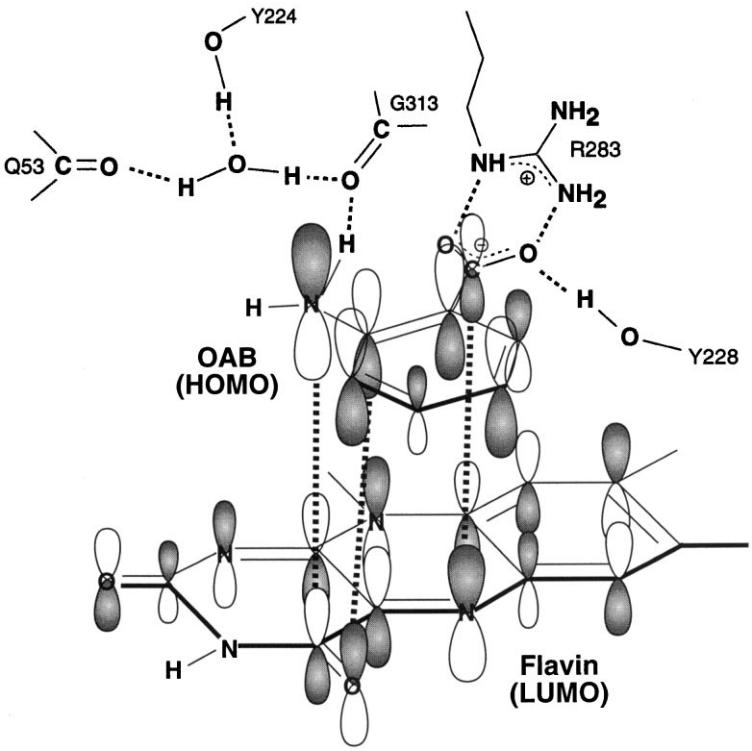


Fig. 6. Illustration of the *o*-aminobenzoate binding mode with emphasis on the charge-transfer interaction between HOMO of OAB and LUMO of flavin [26].

charge-transfer interaction in the DAO–OAB complex. The alignment of OAB with respect to flavin indeed guarantees the interaction between HOMO of OAB and LUMO of flavin (Fig. 6). The overlap is optimum in terms of the orbital symmetry and sizes of the atomic orbitals at the overlapping points. OAB is not only a competitive inhibitor but is also an excellent substrate analog on the bases of the following. OAB has both amino and carboxyl groups just like a substrate D-amino acid, the amino and carboxyl groups are close together as in a substrate and it is not oxidized by DAO but forms a charge-transfer complex with flavin and can thus be interpreted as being 'partially oxidized' in the form of charge transfer. We regard, therefore, DAO–OAB complex as the transition-state analog or Michaelis-complex analog and scrutiny of the OAB-binding should offer valuable information on the mechanism of the reaction between a substrate and the flavin moiety of FAD.

4. Construction of a DAO-substrate complex model

Since OAB is an excellent substrate analog as described above, the DAO–OAB complex can be regarded as a transition-state analog for the DAO-catalyzed oxidation of a D-amino acid and should serve as a starting model for simulating the DAO-substrate complex prior to electron transfer. We, therefore, replaced a substrate, D-leucine, into the OAB binding pocket and the structure was optimized by molecular mechanics simulation and DAO-D-leucine Michaelis complex was thus modeled [9]. The D-leucine binding mode in this Michaelis-complex model is shown in Fig.7. The anionic carboxylate group of D-leucine forms a salt-bridge with the cationic guanidino group of Arg 283 and a hydrogen bond with Tyr 228 hydroxyl, as in DAO-benzoate complex [7] or DAO–OAB complex [9]. However, the amino group

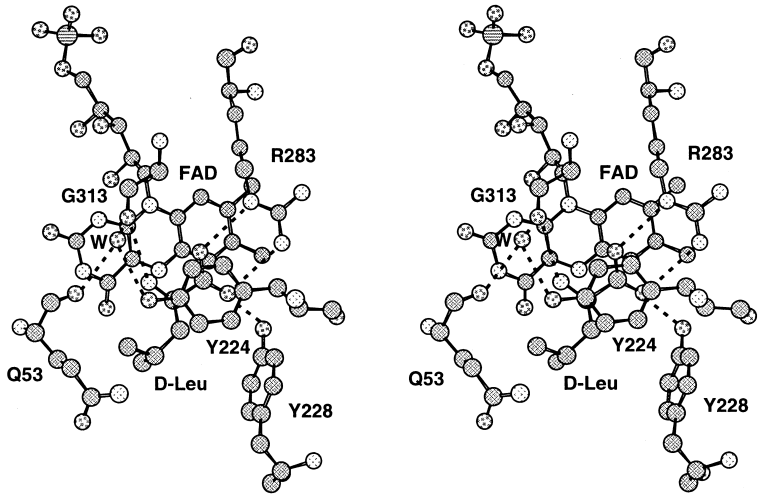


Fig. 7. A stereo view of the D-leucine-binding site of the DAO-D-leucine complex which was modeled by molecular mechanics simulation based on the DAO–*o*-aminobenzoate complex structure.

does not form a salt-bridge but exits as a neutral form; no negative charge is found within the active site. Moreover, the neutral amino group fits well within the hydrophobic environment surrounding the amino group. Consistent with this notion is the finding that zwitterionic ligand trigonelline lowers the pK_a value of flavin N(3)–H from 9.2 to 8.0 [16]. Trigonelline cannot release a proton to become monoanionic to accommodate itself into the active site but, instead, flavin tends to release a proton from N(3)–H to accommodate the zwitterionic ligand. The amino group is hydrogen-bonded with the back-bone carbonyl of Gly 313 as in DAO–OAB complex (Fig. 5). As the result of this hydrogen bond and the neutral nature of the amino group, the lone pair orbital of the amino nitrogen is oriented toward C(4a) of flavin. Another important point in the alignment between D-leucine and flavin is that the α-hydrogen can approach the lone pair of flavin N(5). The D-leucine binding mode in the Michaelis-complex model with emphasis on the points discussed above is illustrated in Fig. 8.

5. The reaction mechanism of reductive half-reaction

Reactions catalyzed by flavoenzyme generally consists of two half-reactions, i.e. reductive and oxidative half-reactions. The reductive half-reaction refers to substrate oxidation at the expense of flavin reduction as the result of electron transfer from a substrate to flavin, while in the oxidative half-reaction reduced flavin is reoxidized by another substrate or an electron acceptor which is molecular oxygen in the case of DAO. The structure of the D-leucine binding mode in the Michaelis-complex model offers several critical clues for elucidating the reductive half-reaction of DAO.

Before the advent of the active-site structure of DAO, the reductive half-reaction was explained mainly as two alternative mechanisms. One is known as the ‘carbanion mechanism’, while the other as the ‘concerted mechanism’. The former characterizes itself in the formation of a substrate carbanion intermediate as the result of α-proton abstraction by a protein catalytic base [17]. The latter describes the reductive half-reaction as a concerted process of α-proton abstraction by a protein catalytic base and electron transfer from the amino nitrogen to flavin [18]. Either of the two mechanisms presupposes an amino acid residue acting as a base to abstract substrate α-proton. However, the active-site structure of DAO revealed by X-ray crystallography clearly contradicts these mechanisms, since no candidate amino acid residue for the α-proton-abstracting catalytic base is found within the active-site. Particularly, no

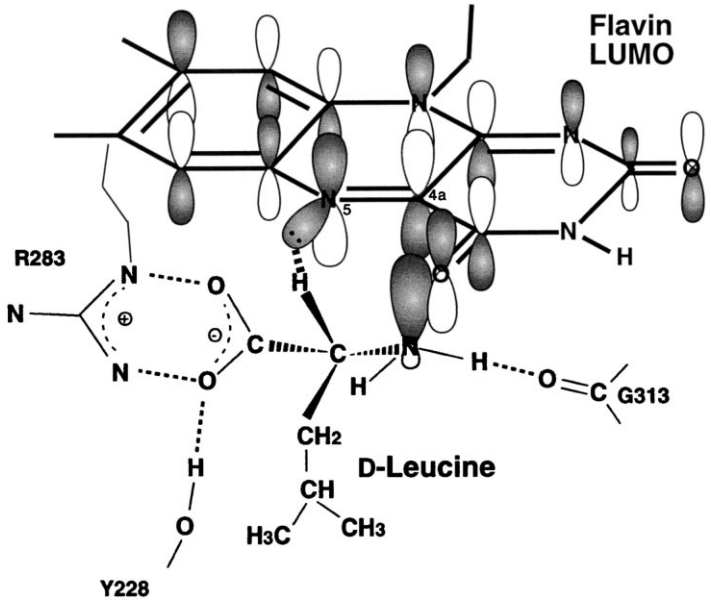


Fig. 8. Illustration of the D-leucine-binding mode with emphasis on the overlap between the lone-pair orbital of amino nitrogen of D-leucine and flavin LUMO at C(4a) and on the proximity of substrate α-hydrogen toward flavin N(5).

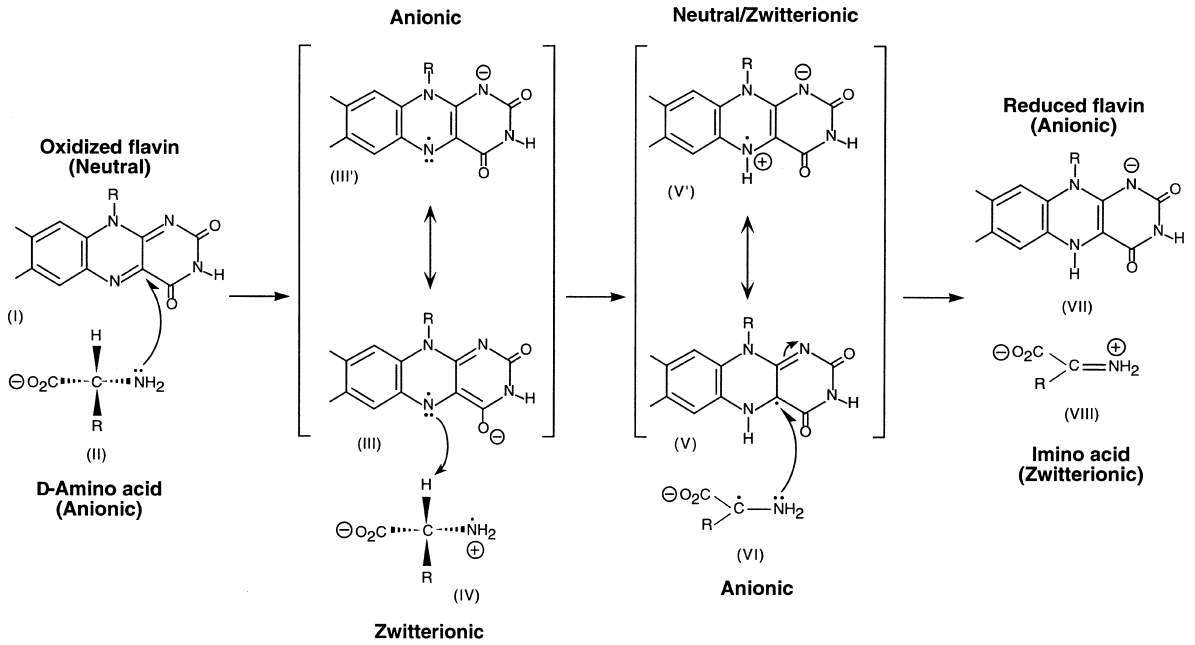
candidate amino acid residue exists in the vicinity of α-hydrogen of D-leucine in the DAO-D-leucine complex model illustrated in Fig. 7. An entirely new mechanism should, therefore, be constructed on the bases of the Michaelis-complex model (Figs. 7 and 8) and accumulation of physico-chemical and enzymological experimental results.

In the DAO-catalyzed reductive half-reaction, the Cα–H bond is cleaved and α-hydrogen is removed as the result (Scheme 1). In discussing the details of the reaction scheme, it is of fundamental importance to know whether the α-hydrogen is removed as a proton, a hydrogen atom or a hydride. As to the fate of the substrate α-hydrogen, a unique reaction of DAO with β-chloro-D-alanine deserves a remark. When β-chloro-D-alanine is allowed to react with DAO, it undergoes elimination of hydrogen chloride in addition to normal oxidation yielding pyruvate and chloropyruvate, respectively [19–21]. In the elimination reaction, substrate α-hydrogen behaves as a proton; hydrogen atom removal or hydride removal cannot successfully explain the elimination of hydrogen chloride from β-chloro-D-alanine. Since there is only one substrate binding-site, this dual elimination–oxidation reaction should proceed at the identical binding-site. It is, therefore, concluded that the substrate α-hydrogen behaves as a proton in the normal oxidation, i.e. reductive half-reaction of DAO, as well as in the elimination reaction.

This behavior of α-hydrogen as a proton together with the Michaelis-complex model depicted in Fig. 8 can be integrated into the reaction processes of electron transfer from lone pair of the substrate amino nitrogen to flavin C(4a) and transfer of the substrate α-proton to flavin N(5), which, instead of an amino acid residue, acts as a proton-abstracting catalytic base. We have, thus, proposed two possible mechanisms, i.e. the ‘electron–proton–electron mechanism’ and the ‘ionic mechanism’ for the reductive half-reaction of DAO [9].

5.1. Electron–proton–electron mechanism

The electron–proton–electron transfer mechanism is outlined in Scheme 2 and comprises two single-electron transfer processes intervened by a proton transfer. The initial step involves a single electron transfer from amino nitrogen of the substrate (II)

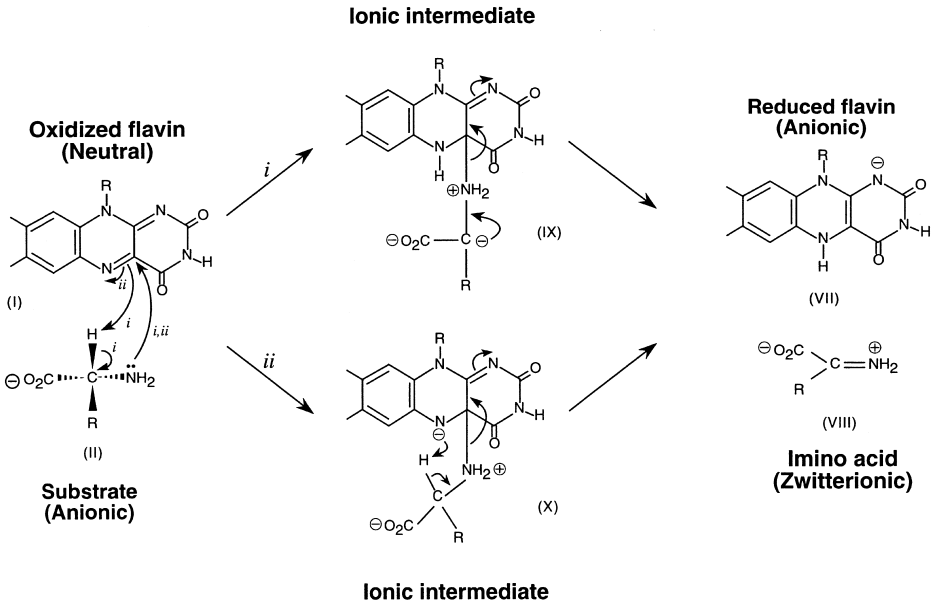


Scheme 2. Electron–proton–electron transfer mechanism for the reductive half-reaction of DAO.

to neutral oxidized flavin (I) yielding anionic flavin radical (III/III′) and a substrate intermediate radical (IV) with an unpaired electron residing at the cationic nitrogen. Flavin N(5) then acts as a catalytic base to abstract the substrate α-proton, generating neutral/zwitterionic flavin radical (V/V′) and an anionic substrate radical species (VI). Finally, second single electron transfer occurs from the substrate intermediate radical (VI) to flavin radical (V/V′), resulting in the formation of the complex between anionic reduced flavin and zwitterionic imino acid. The final species is known as the 'purple intermediate' whose identity as a charge-transfer complex between anionic reduced flavin and zwitterionic imino acid has been demonstrated by resonance Raman and NMR spectroscopy [22,23]. The activation energy for the initial electron transfer is expected to be sufficiently low owing to the overlap between lone-pair orbital of the substrate amino nitrogen and flavin LUMO at C(4a). The pK_a of the α-proton in the radical species (IV) can be reduced considerably due to the cationic amino radical moiety. The pK_a of the flavin radical (V/V′) is known to be in the neutral pH region so that the proton transfer step should proceed without difficulty. The final electron transfer from (IV) to (V/V′) is expected to be feasible in view of the proximity of the amino nitrogen to flavin C(4a) (Figs. 7 and 8). In the substrate/flavin binding region, the sole electrostatic center on the protein moiety is the cationic guanidino group of Arg 283 (Figs. 5 and 7). To maintain the electrostatic neutrality within the active site, either flavin or the substrate/intermediate/product is required to be anionic while the other is neutral or zwitterionic. This requirement indeed is fulfilled during the whole reaction processes of Scheme 2.

5.2. Ionic mechanism

Another possible mechanism for the reductive half-reaction is depicted in Scheme 3, which distinguishes itself in the ionic intermediates (IX and X). In the pathway (i), a covalent bond between substrate nitrogen and flavin C(4a) is formed in concert with α-proton abstraction by flavin N(5) leading to the ionic intermediate (IX). Subsequent two-electron transfer from the substrate moiety to flavin produces



Scheme 3. Ionic mechanism for the reductive half-reaction of DAO.

the ‘purple intermediate’, i.e. the complex between anionic reduced flavin (VII) and zwitterionic imino acid (VIII). The pathway (ii) proceeds via the ionic intermediate (X) which results from the covalent bond formation between the substrate amino nitrogen and flavin C(4a). Two-electrons are then transferred from the substrate moiety to the flavin ring in concert with α-proton abstraction by flavin N(5) ending up with the ‘purple intermediate’ consisting of anionic reduced flavin (VII) and zwitterionic imino acid (VII). The initial steps (i and ii) can be justified by the overlap between the lone-pair orbital of the substrate amino nitrogen and flavin LUMO at C(4a) as well as by the proximity between substrate α-proton and the lone-pair orbital of flavin N(5) (Fig. 8). As in Scheme 2, the electrostatic-neutrality requirement is maintained throughout the entire process of Scheme 3.

We have proposed two possible reaction mechanisms for the reductive half-reaction as described above. However, we still do not have direct experimental evidence which unequivocally distinguishes one from the other. On the other hand, based on three-dimensional active-site structure of DAO, Mattevi et al. [8] and Pollegioni et al. [24] have proposed yet another mechanism, ‘direct hydride transfer mechanism’, according to which the substrate α-hydrogen is transferred as a hydride ion to flavin N(5). We do not favor their ‘direct hydride transfer mechanism’ mainly on the ground that the substrate α-hydrogen behaves as a proton as discussed above. We must admit that even the long-sought-for active-site structure cannot provide the final answer to the controversial reaction mechanism of the historical enzyme DAO. We are in the process of refining the DAO reaction mechanism of reductive as well as oxidative half-reactions and expect to find a final answer in the very near future.

Acknowledgements

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