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Reaction mechanism of flavin dehydrogenation by D-amino acid oxidase

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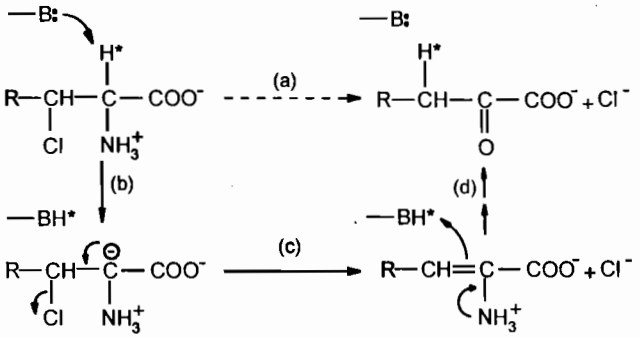
Introduction

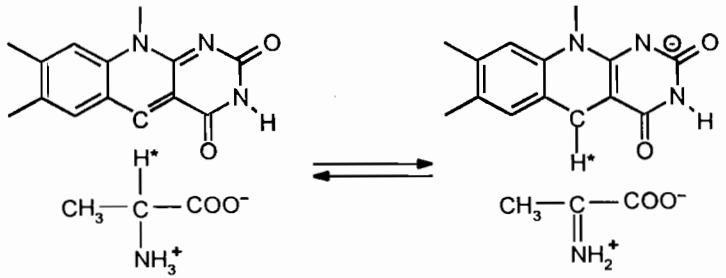
Since its discovery, D-amino acid oxidase (EC 1.4.3.3, DAAO) has received much attention both from the physiological and biochemical points of view. The groups of Massey (USA), Curti (Italy), and Yagi (Japan) were mainly involved in its study over the year (1). In spite of these efforts the basic molecular mechanism underlying the reductive half-reaction, *i.e.* the dehydrogenation step, is still disputed. In the following, we review the salient experiments and recent data on yeast DAAO, which lead, in our opinion, to the establishment of a reasonable chemical mechanism for this reaction.

Early studies

DAAO catalyses the dehydrogenation of D-amino acids to yield α-imino and, upon subsequent hydrolysis, α-keto acids and ammonia. The reduced flavin is then reconverted by oxygen to the oxidised form and gives H_2O_2. DAAO also catalyses the elimination of chloride from β-chloro-amino acids (Scheme 1). The interpretation of this reaction led to the proposal of the so called "carbanion mechanism" (2). This has become a generally accepted mechanism for flavoproteins catalysed dehydrogenation (for a review (1)). This mechanism postulates an active site base that abstracts the



Scheme 1. Mechanism of elimination of halide from the β-position of β-chloro-D-alanine (-B is an active site base) (2).



Scheme 2. Transfer of ^3H from [2-^3H]D-alanine to the C(5) position of DAAO from pig kidney (pkDAAO) reconstituted with 5-deaza-FAD (4).

substrate α-H as H^+ (Scheme 1, b) to form a carbanion. The latter eliminates Cl^- (c) to produce an enamine, that can (partially) incorporate *H into its β-position during its rearrangement to the final products (d). It is essential to note that this mechanism requires an active site base for the generation of the carbanion! The mode of transfer of electrons from the carbanion to the oxidised flavin also has been the object of much dispute, radical steps, or covalent flavin N(5) adducts having been proposed (3). However, in 1975 (4) Hersh and Schuman Jorns challenged this interpretation based on work in which the native DAAO cofactor was substituted with 5-deaza-FAD (Scheme 2). Using labelled substrates ([2-^3H]D-alanine), it was shown that the label was incorporated into the C(5) position of 5-deaza-flavin. This finding can hardly be explained with the carbanion mechanism without important supplementary assumptions, and it cannot proceed via formation of N/C(5) covalent adducts. Instead, the authors proposed a hydride transfer mechanism involving direct addition of label to the flavin position N/C(5).

Information from crystallographic data of mammalian DAAO and molecular modeling

The structure of pig kidney DAAO was recently solved independently by the groups of Mattevi (5) and Mizutani (6) at 2.6-3.0 Å resolution. The picture emerging from the structures of the enzyme complexed with various ligands was unexpected in several important ways. The global arrangement of flavin cofactor, substrate, and functional groups involved in binding is remarkably similar to that found within the class of enzymes dehydrogenating α-OH-carboxylic acids ("LdH class"). This prompted Mattevi et al. (5) to propose a case of convergent evolution. A base involved in acid/base catalysis, such as the essential histidine found in the "LdH class" was not identified in DAAO. On the other hand, a tyrosine (Y228) was found to be in proximity of the substrate-binding site. Its direct involvement in chemical catalysis was ruled out by site-directed mutagenesis (Y228F mutant shows 1 % of the rate of flavin reduction and ~ 40 % of the k_{cat} value of the wild-type pkDAAO) (7). This experiment was interpreted mechanistically in terms of a hydride transfer mechanism (5), who did not address the question of halide elimination directly. Miura et al. (8), on the other hand, have attempted to circumvent the pickle by proposing a mechanism in which the flavin N(5) acts as a base in the abstraction of the substrate αC-H as a H^+.

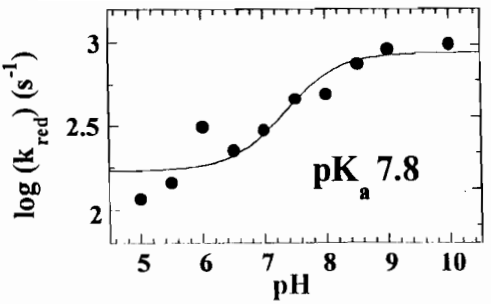


Figure 1. pH dependence of the observed rate of flavin reduction at saturating [D-alanine], obtained from the anaerobic reaction of *Rhodotorula gracilis* DAAO (RgDAAO) in stopped-flow experiments.

Linear Free Energy Relationships

Based on Hammett's postulate we have attempted to verify the occurrence of a carbanionic species in the reductive half-reaction of a yeast DAAO (*Trigonopsis variabilis*, TvDAAO) using *para*-substituted phenylglycines (9). The results are compatible with development of little or no charge in the transition state and suggest that a carbanion mechanism in its classical formulation is improbable.

Effect of pH on DAAO catalysis

As shown in Scheme 1, and depending on the mechanism, three ionizable groups can play a primary role in the dehydrogenation reaction. These are the base required by the carbanion mechanism, the amino acid α-NH_2 group, and the flavin N(1). In an attempt to verify the presence and role of such groups, the reductive half-reaction of RgDAAO with D-alanine as substrate was studied in the range pH 5 to 10.

A role for the ionisation of the reduced flavin in this process is highly improbable, since spectroscopic studies do not provide evidence for a change in ionisation state in this pH range (pK_a for deprotonation of N(1)-H of free reduced FAD is ≈ 6.7). The anionic character of the fully reduced flavin in DAAO has been reported by different authors (see (1)) and was also recently confirmed by resonance Raman spectroscopy of the "purple intermediate" of reduced pkDAAO in complex with iminoacids (10).

The pH dependence of the rate of flavin reduction with D-alanine is depicted in Fig. 1, and shows that it increases with pH reflecting an apparent $pK_a \approx 7.8$. At both high and low pH values the rate has a definite plateau. The one at low pH is apparently inconsistent with the existence of an essential base in RgDAAO. Direct involvement of a base in catalysis would require the observed rate to extrapolate to zero at low pH with unity slope (or some higher integer) in a Dixon plot of pk_{red} *vs.* pH. We assign the $pK_a \approx 7.8$ to the ionisation of the substrate α–NH_2 group in the Michaelis complex. This pK is thus lowered by some 2 units compared to that of free amino acid, assuming no change in rate-limiting step. The other two functional groups present at the active center of RgDAAO, Y223 and Y238 can be excluded from direct measurements (see below).

Isotope effects and their pH dependence

As mentioned, a main point in the mechanistic discussion is the concertedness of the cleavage of the substrate αC-H and αN-H bonds as opposed to the occurrence of distinct intermediates as required by a carbanion mechanism. In a previous study (9),

substrate kinetic isotope effect (KIE) and the solvent KIE were investigated using [2-^{2}H]D-phenylglycine) and at pH 8.3. The finding of a multiplicative double isotope effect ~23 argues for a concerted fission of the two bonds in question in a symmetrical transition state, and it speaks against the occurrence of intermediates such as carbanionic species. In an extension of this work, we have studied the pH dependence of the substrate and solvent deuterium KIEs on the rate of flavin reduction with D-alanine as substrate. The solvent KIE is large at low pH and decreases at high pH ($\approx$ 3.8 and $\approx$ 1.2 at pH 6 and 8, respectively) reflecting an apparent $pK_a = 7.0$ ($\pm$ 0.2). Most likely this apparent pK_a also reflects the ionisation of the substrate α–NH_2 (the reaction proceeding faster with the unprotonated species, compared to the charged one). This also argues against a carbanion mechanism in which a better stabilisation of the transient negative charge by the cationic α–NH_3^+ should be expected.
Proton inventories at pH $\geq$ 7 are linear, indicating that the solvent KIE is due to a single site in the transition state. On the contrary, at pH < 7, where the solvent KIE is larger, the proton inventory is bowl-shaped (Fig. 2). According to Schowen and Schowen (11) this latter behaviour is compatible with the existence of one site (of exchangeable H) in the transition state and one site in the reactant state, while at pH values > 7 the linear fit reflects the fission of a single, exchangeable H-bond in the process. A comparable pH-dependence is also that observed for the primary KIE estimated using [2-^{2}H]D-alanine as substrate. At pH 6, a very large effect $\approx$ 10 is found consistent with a symmetric transition state for the rupture of the αC-H bond, while at pH 8 the KIE is lowered drastically to 1.15 $\pm$ 0.23, a value just above significance. The "double KIE" was measured by comparing the rates of reduction with [2-^{1}H]D-alanine in H_2O to those with [2-^{2}H]D-alanine in D_2O. At pH 6 the double KIE is very large ($\approx$ 43) and reflects a multiplication of the single KIEs (3.4 x 12.6 = 43.1). At pH 8 and 10, both the solvent and primary KIEs are substantially lower, and a distinction between additive and multiplicative effects is not feasible. We thus conclude that at pH < 7 the observed KIEs largely reflect the intrinsic ones, while at high pH this is not the case. In analogy to the situation of TvDAAO (9), also in the present case the evidence is in favour of a concerted reaction (at least at low pH), and against the occurrence of intermediate species.

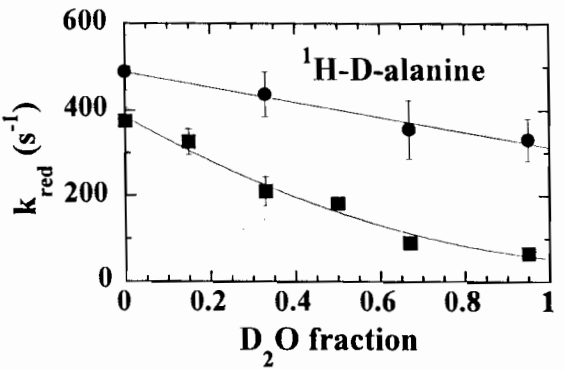


Figure 2. Effect of the D_2O fraction on the rate of flavin reduction using D-alanine at pH 6.0 (■) and pH 8.0 (●).

The three-dimensional structure of the active site in RgDAAO

The structures of oxidised RgDAAO complexed with 3,3,3-trifluoro-D-alanine, L-lactate, and anthranilate, and of reduced RgDAAO with the substrate D-alanine have been solved at 1.2 to1.9 Å resolution (see also Umhau et al., this volume). One of the relevant differences between the active site of RgDAAO and that of pkDAAO is the absence of a loop acting as a "lid" and controlling substrate binding and product release. This gives a rationale for understanding the comparatively higher catalytic efficiency of the yeast oxidase, in which the rate-limiting step is not product release. With the mammalian DAAO, a movement of the 218-226 loop between a "close" and an "open" conformation controls the overall rate of turnover. The interaction of the substrate -COO^- group with R285, Y223, and Y238, serves in ligand fixation. The substrate α-NH_2 group is H-bonded with the S335=O carbonyl and H_2O72, while the substrate side chain is oriented toward the hydrophobic binding pocket of the active site (Fig. 3). The high resolution structure demonstrates that the αC-H bond is in alignment with the extension of the flavin N(5) LUMO orbital. The α-NH_2 group is at 3.4 Å and 3.8 Å from the C(4a) and N(5) flavin positions, respectively. Most importantly, no functional groups are located to function in acid-base catalysis (Fig. 3). Thus, we must conclude that dehydrogenation catalysis in RgDAAO occurs in the absence of functional groups capable of acid-base chemistry. Intriguingly, D-lactate slowly reduces the enzyme ($t_{1/2} \approx 1$ h for reduction at 25 °C with 100 mM D-lactate) *i.e.* it is a substrate of RgDAAO. This is of importance in the context of mechanistic identity between the flavin dependent "LdH class" and that of DAAOs.

On the role of Arg285, Tyr223 and Tyr238 in RgDAAO

The role of these active site residues (see Fig. 3) has been investigated by mutagenesis (Molla et al. and Job et al., this volume). All R285X mutants (X = K, A, Q, D) are competent in catalysis in that they exhibit turnover activity, or by anaerobic reduction of the enzyme flavin by D-alanine. The role of R285 in ligand/substrate binding is

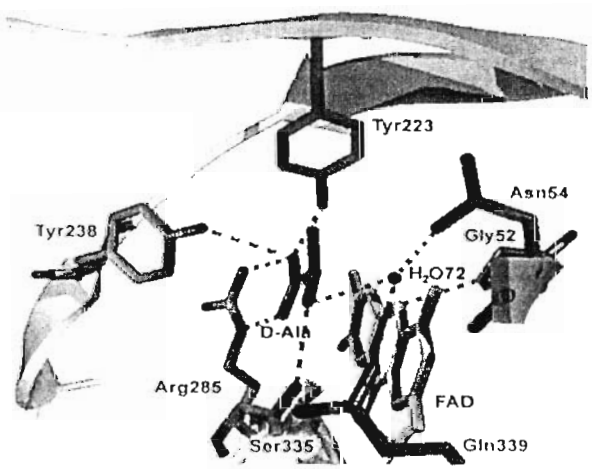


Figure 3. Structure of RgDAAO active site complexed with D-alanine (above the *reduced* flavin *Re*-side). The dashed lines show the hydrogen bonds involved in substrate fixation (this structure is essentially identical with that of oxidised enzyme complexed with L-lactate and CF_3-alanine). For sake of clarity, oxygen has been omitted. We refer to the work by Umhau et al. (this volume) for details on the RgDAAO structure.

evidenced by the ≈ 400-fold increase in the $K_{d,app}$ for D-alanine for the R285K mutant. On the other hand, even a conservative a mutation as R285K profoundly interferes with the rate of flavin reduction ($k_{red} \approx k_{cat}$ and both are decreased ≈ 1000-fold in the mutant), which is attributed to alteration of the orientation of the reactants in the Michaelis complex. The Y223 and Y238 mutants resulted also in catalytically competent enzyme forms. Only Y223S shows a significant (≈ 100-fold) decrease in k_{cat} although the rate of flavin reduction is unchanged. This is essentially due to a decrease in the rate constants for substrate binding and product release from the E_{ox}:IA complex (this latter step is rate-limiting in catalysis for the Y223S mutant). It is thus clear that R285, Y223 and Y238 do not play a role in chemical catalysis.

Studies on the elimination of Cl^- from β-chloro-D-alanine

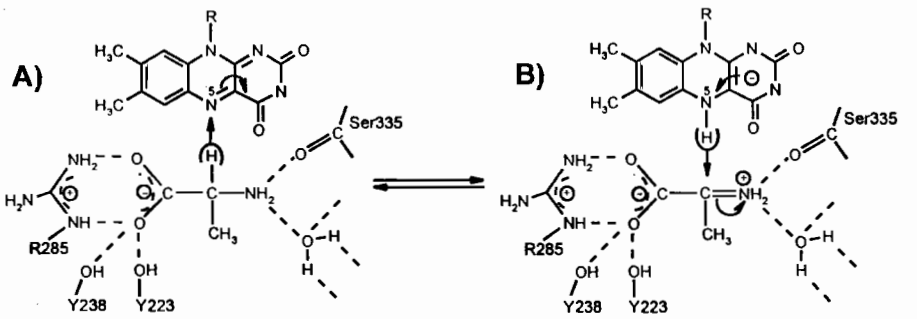
Since the ability of DAAO to catalyse the β-elimination of halide from β-halogenated D-amino acids is an important argument in favour of the carbanion mechanism (Scheme 1), the reaction of RgDAAO with β-chloro-D-alanine has been addressed in some preliminary studies. Like pkDAAO, RgDAAO also eliminates Cl^- from β-Cl-D-alanine. The ratio between chloropyruvate and pyruvate produced, however, is markedly different. With pkDAAO and at 100 % O_2, chloropyruvate constituted ≈ 95 % of the product (2). With RgDAAO under the same conditions the amount of halogenated ketoacid is ≤ 30 % of the total ketoacid while at 0 % and 21 % O_2 essentially only elimination is found. The same product distribution is obtained using the Y223F and Y238F RgDAAO mutants. From this it follows, that elimination does occur in the absence of protein groups potentially capable of H^+ abstraction.

Comments on the electron-proton-electron (EPE) mechanism

The supposed drawback of a hydride mechanism is its apparent irreconcilability with a halide elimination reaction. A dogma underlying the latter is that formation of an intermediate carbanion is mandatory. In an attempt to circumvent this, Miura and his group have proposed a so-called "EPE" mechanism, in which "...two-electrons flow from the amino lone-pair in concert with the α-proton abstraction by N(5) of flavin" (8). While this mechanism might formally explain both elimination and electron transfer to the flavin, we consider it a rather unlikely variant. The oxidised flavin basicity at N(5) is very low (pK > 20), and this would essentially preclude a role in H-abstraction. In addition, the 3D-structure shows an H-bond between N(5) and the backbone NH of G52 (3.04 Å) that would further diminish basicity. On the contrary, this bond it is likely to increase the electrophilic character of N(5) and assist in the uptake of hydride. The EPE would be analogous to a hydride shift, with electrons flowing in the "opposite direction" in the (formal) transfer of a lone pair. The addition of charge in the form of a lone pair from the substrate α–NH to the flavin is conceivable. However, the principle of microscopic reversibility requires the reverse, i.e. the addition to the N of an imine to be viable. In view of the chemistry the latter group, we consider this a rather unlikely event. Also, the generation of a (partial) negative charge contrasts with the lack of charge development suggested by the LFER correlations discussed above (9). The preference of a mechanism such as the EPE only for the scope to rationalise β-elimination thus appears questionable if alternatives exist, as will be pointed out below.

Mechanistic conclusions

The whole body of data reviewed here provides conclusive arguments in favour of a hydride transfer mechanism for the amino acid dehydrogenation catalysed by DAAO. The proposed mechanism appears to be unique in its simplicity and in that it does not require functional groups for carrying out chemical catalysis. As discussed above, the functional groups present at the active site of RgDAAO are involved in substrate binding and orientation in the Michaelis complex. We thus present the mechanism reported in Scheme 3 for the reaction of DAAO. It depicts the Michaelis complex of oxidised DAAO and D-alanine with its uncharged α–NH_2 group at pH > 7, *i.e.* the most active form. In this complex orbital rearrangement coupled with a vibronic mode leads directly to the second (Michaelis) complex consisting of reduced flavin and imino acid (Scheme 3, B). In the latter a negative charge (delocalised on the flavin pyrimidine ring) is balanced by the positive one on the α=NH_2^+ group of the product. The proposed mechanism also concords with the large "multiplicative double KIE" observed at pH 6, in which the αC-H bond and that of the αNH_2^+-H are cleaved concomitantly. In contrast, at pH ≥ 8 only the αC-H bond is cleaved, the rate of the flavin reduction by D-alanine is much faster and the corresponding KIE small. Importantly, D-lactate also is a substrate of RgDAAO, thus allowing a comparison with mechanism of flavin enzymes dehydrogenating lactate. These two classes of enzymes show drastic differences in the presence of functional groups at the active center (12). In flavocytochrome b_2, H290 (in a pair with D180) could abstract the weakly acidic αC-OH of lactate as H^+ (pK_a of αC-OH ≈15), thus initiating the expulsion of hydride. In DAAO thus a function is not necessary since the amino acid binds either in the form suitable for hydride transfer (α–NH_2 at pH > 7.4) or in the α–NH_3^+ form, the required deprotonation is kinetically facile and coupled with hydride transfer. Indeed, the rate of flavin reduction by D-alanine is ≈ seven orders of magnitude faster than by D-lactate, this reflecting approximately the difference in pK_a to be expected for the α-OH *vs.* the α-NH_3^+ groups of the two substrates. From this we propose that dehydrogenation of α-amino acids and of α- hydroxy-acids by flavoproteins indeed proceeds by the same, hydride type mechanism. Halide β-elimination can be rationalised based on a proposal put forward 25 years ago (13).



Scheme 3. Set-up of the active center of RgDAAO for the proposed hydride transfer mechanism (at pH > 7). A) Complex of the oxidized flavin with D-alanine; B) complex of the reduced (anionic) flavin with (cationic) iminopyruvate.

The spectral changes observed during the reaction of pkDAAO and lactate monooxygenase with β-halogenated substrates suggest that the substrate dehydrogenation step precedes the actual elimination reaction. According to this, elimination would originate from the complex of reduced enzyme with imino (or keto) acid. According to this, elimination would not partition at the (intermediate) stage of a carbanion but at the level of reduced E-P complex and would thus be a secondary reaction.

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