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Structural and Mechanistic Studies on D-Amino Acid Oxidase·Substrate Complex: Implications of the Crystal Structure of Enzyme·Substrate Analog Complex[1]

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As an extension of our recent X-ray crystallographic determination of the tertiary structure of D-amino acid oxidase (DAO) [Mizutani, H. *et al.* (1996) *J. Biochem.* 120, 14-17], we solved the crystal structure of the complex of DAO with a substrate analog, *o*-aminobenzoate (OAB). The alignment between flavin and OAB in the crystal structure of the complex is consistent with charge-transfer interaction through the overlap between the highest occupied molecular orbital of OAB and the lowest unoccupied molecular orbital of flavin. Starting with the atomic coordinates of this complex as the initial model, we carried out molecular mechanics simulation for the DAO-D-leucine complex and thus obtained a model for the enzyme-substrate complex. According to the enzyme-substrate complex model, the α-proton is pointed toward N(5) of flavin while the lone-pair of the substrate amino group can approach C(4a) of flavin within an interacting distance. This model as well as DAO-OAB complex enables the evaluation of the substrate-flavin interaction prior to electron transfer from the substrate to flavin and provides two possible mechanisms for the reductive-half reaction of DAO, *i.e.*, the electron-proton-electron transfer mechanism and the ionic mechanism.

Key words: D-amino acid oxidase, enzyme-substrate complex, flavoenzyme, molecular mechanics, tertiary structure.

Among the known flavoenzymes, D-amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] (DAO) is one of the most extensively investigated. This stems from its long history since its discovery by Hans Krebs in 1935 as the first mammalian flavoenzyme and as the first FAD enzyme (*1, 2*) and from its abundance and wide distribution in the biological kingdom, ranging from microbes to mammals. Thus DAO can be regarded as a prototype or a representative of flavoenzymes in terms of the number of reports published and the scope of the underlying disciplines [see a recent review (*3*) for comprehensive coverage].

DAO catalyzes the dehydrogenation of a D-amino acid, producing an imino acid which is subsequently hydrolyzed nonenzymically to an α-ketoacid and ammonia. The reduced enzyme is reoxidized by molecular oxygen, generating hydrogen peroxide (Scheme 1). The reaction mechanism which came first to general attention is the "carbanion mechanism" (*4, 5*), which is characterized by its initial step, *i.e.*, α-proton abstraction by a protein base, giving rise to the substrate carbanion which subsequently forms a covalent linkage with flavin at the C(4a) position. We have pointed out that the α-proton abstraction by a protein base to generate the substrate carbanion is energetically unfavorable, and we proposed an alternative "concerted mechanism" (*6-8*) which can circumvent the energy barrier encountered in the α-proton abstraction step in the "carbanion mechanism." It has not been settled, however, which of the mechanisms is correct, or whether there is still another alternative, mainly due to the lack of direct evidence in favor of either of the mechanisms and particularly due to the lack of the precise three-dimensional structure of DAO. The three-dimensional structure of DAO at atomic resolution has recently been solved by us (*9, 10*) and independently by another group (*11*) and the active site structure is finally known.

Various substrate analogs and competitive inhibitors for DAO have been explored by several research groups and their interactions with the enzyme have provided valuable information, both dynamic and static. Among the substrate analogs studied in detail, aminobenzoates constitute a unique group in the sense that they carry both amino and carboxyl groups within each molecule, as does the substrate

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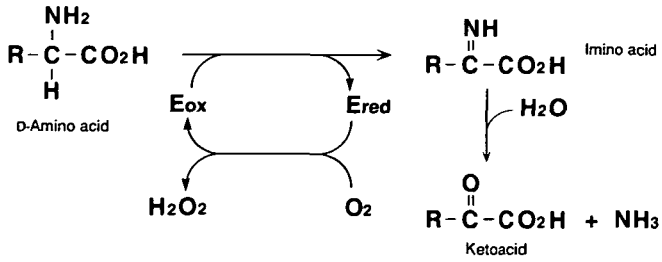
Abbreviations: DAO, D-amino acid oxidase; EPE, electron-proton-electron; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; OAB, *o*-aminobenzoate.



Scheme 1

D-amino acid. The complexes of *o*-, *m*-, and *p*-aminobenzoates exhibit characteristic spectral properties (*12*). Very distinctive spectral changes are observed when *o*-aminobenzoate (OAB) is bound to DAO; the first absorption band around 450 nm is shifted to a shorter wavelength concurrent with a decrease in intensity both in the first and the second absorption bands and with the appearance of a very broad absorption band in the long-wavelength region extending beyond 700 nm. The latter broad band was proposed to arise from the charge-transfer interaction between OAB and flavin (*12*) and the charge-transfer nature of the interaction in DAO-OAB complex was proved by resonance Raman spectroscopy with excitation within the absorption band in question (*13*). Among the three aminobenzoates, OAB is the best substrate analog in terms of the geometrical proximity between the amino and carboxyl groups and the DAO-OAB complex can be regarded as an excellent analog of the enzyme-substrate complex. In this context, the peculiarity of the spectral properties and charge-transfer interaction of the DAO-OAB complex should be viewed in relation to the enzyme-substrate complex.

We report herein the crystal structure of the DAO-OAB complex and the mode of charge-transfer interaction in this complex. We also present the structure of the DAO-substrate complex model obtained by molecular mechanics simulation using the atomic coordinates of the DAO-OAB structure. Furthermore, we discuss the reaction mechanism for the reductive-half reaction, *i.e.*, substrate-oxidation/flavin-reduction step, of DAO based on the structure of the DAO-substrate complex model.

MATERIALS AND METHODS

DAO was prepared as described previously (*9*) from a culture of *Escherichia coli* which had been transformed with a vector containing the DAO cDNA. *o*-Aminobenzoate (OAB) was purchased from Wako Pure Chemicals. Other chemicals were of the highest grade available from commercial sources and were used as supplied.

Crystals of the DAO-OAB complex were obtained by the soaking method as described below. The crystals of DAO-benzoate complex prepared as reported previously (*9*) were immersed in a buffer, which consisted of 200 mM sodium acetate, 100 mM sodium citrate pH 6.3, 30% polyethylene glycol, and 5 mM D-alanine, until the yellow crystals became colorless. The colorless crystals were then allowed to stand in the buffer which contained all the above ingredients except D-alanine. By this procedure the crystals were freed of benzoate. Finally, the crystals were soaked in a buffer which comprised 200 mM sodium acetate, 100 mM sodium citrate pH 6.3, 30% polyethylene glycol, and 10 mM OAB. Within an hour the crystals acquired the greenish yellow color characteristic of the DAO-OAB complex.

TABLE I. **Data collection and refinement.**

Space group	$P2_12_12_1$ No. 19
Cell constant	
a (Å)	109.31
b (Å)	92.58
c (Å)	71.32
Resolution range (Å)	10–2.5
Observed reflections	66,408
Unique reflections [$>2\sigma(F)$]	22,424 (21,755)
Completeness (%)	87.5
R_{merge} (%)	5.8
Number of protein atoms	5,458
Number of water atoms	112
Number of heterogen atoms	126
Residues not well defined in electron density	341-347 741-747
$R1$[a] (%)	20.1
R_{Free}[b] (%)	26.0
rms deviations from ideal geometry	
Bond length (Å)	0.007
Bond angles (deg.)	1.52
Improper torsion angles (deg.)	1.56
Average *B* values (Å^2)	
Protein main-chain	20
Protein side-chain	22
Ligand FAD	14
Ligand *o*-aminobenzoate	19

[a] $R=\Sigma||F_{obs}|-|F_{calc}||/\Sigma|F_{obs}|$; $|F_{obs}|$ and $|F_{calc}|$ are observed and calculated structure factor amplitudes, respectively. [b] The free *R* was calculated using 10% randomly selected reflections.

X-ray diffraction data for the DAO-OAB complex were collected to 2.5 Å resolution using an X-ray beam of wavelength 1.0 Å at 10°C on Fuji Imaging Plates with a screenless Weissenberg camera (*14*) at the BL6A station, Photon Factory, KEK, Tsukuba. Data were evaluated, scaled and merged using the programs DENZO and SCALEPACK (*15*).

DAO-OAB complex crystals were isomorphous to DAO-benzoate complex with two subunits in an asymmetric unit related by a non-crystallographic twofold axis. The refinement of the DAO-OAB complex structure was initiated using the coordinates of DAO-benzoate complex determined at 2.5 Å resolution (refinement of the initial structure (*10*), Mizutani, H. *et al.*, unpublished results), with benzoate and water molecules removed as an initial refinement model. The complex was refined by simulated annealing followed by some rounds of least-squares refinement using XPLOR (*16*). After each round of refinement, manual refitting of each current model to the electron density was done against $2F_o-F_c$ omit difference map using the program O (*17*). The density of OAB was clearly visible in the active-site region. Water molecules were picked up on the basis of the peak heights and distance criteria from the difference map, and further inspected against F_o-F_c difference Fourier maps. The water molecules whose thermal factors were above 80.0 Å^2 after refinement were removed from the list. The final model lacks the C-terminal seven amino acid residues, for which no convincing electron density was obtained. Analysis of the stereochemistry with PROCHECK (*18*) showed that 98.8% of the backbone torsional angles fall within the additional allowed regions of

the Ramachandran plot and the remaining 1.2% are in the generously allowed region. The mean positional error of the atoms from a Luzzati plot (*19*) was 0.28 Å. Details of data collection and refinement are given in Table I. The atomic coordinates of the DAO-OAB complex have been deposited in the Protein Data Bank, Brookhaven (accession number 1an9).

The molecular orbital calculation of OAB was carried out by the extended Hückel method with the program provided by Nishimoto *et al.* (*20*). Molecular mechanics simulation for modeling the DAO-D-leucine complex was done by the energy minimization method using XPLOR (*16*) on the basis of the atomic coordinates for the DAO-OAB complex.

RESULTS

The Crystal Structure of DAO-OAB Complex—The overall dimeric structure (not shown) and the folding pattern of each subunit (Fig. 1) of DAO-OAB complex are similar to those of DAO-benzoate complex (*10*). The active site of the enzyme is located in the boundary region of the two domains, *i.e.*, the α/β domain and the pseudobarrel domain (*10*). OAB faces the *re*-face of the flavin ring from one side and the phenol ring of Tyr 224 from the other (Fig. 2). OAB binds to the active site of DAO in a slightly but distinctly different manner as compared with the binding mode of benzoate at the active site (*10*). The benzoate moiety of OAB is shifted away from the flavin ring, apparently to accommodate the amino group. The carboxylate group of OAB makes an ion-pair with the guanidino group of Arg283 and a hydrogen bond with the hydroxyl group of Tyr228, just as in the DAO-benzoate complex (*10*). The interatomic distances between the carboxyl oxygens and guanidino nitrogens are 3.3 and 3.1 Å (Fig. 3) and that between hydroxyl oxygen of Tyr228 and carboxyl oxygen is 2.8 Å. The corresponding distances between Arg283 and benzoate in DAO-benzoate complex are 3.0 and 2.8 Å. Thus, the interaction between the carboxylate of

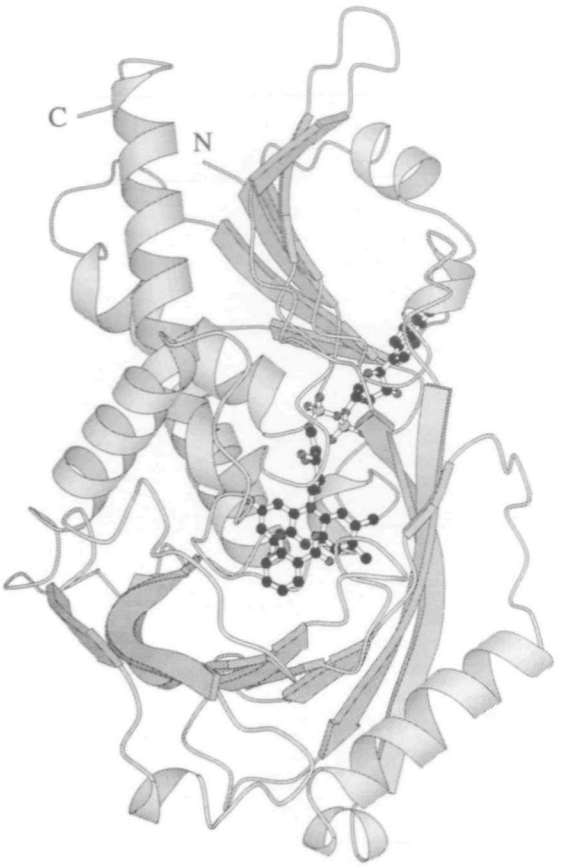


Fig. 1. **View of one subunit of DAO perpendicular to the flavin ring.** FAD and OAB are shown as ball-and-stick models. The upper half is the α/β domain and the lower one is the pseudobarrel domain. OAB bound at the domain interface is parallel to the flavin ring of FAD with N-C(2) of OAB lying just above C(4)=O of the flavin ring.

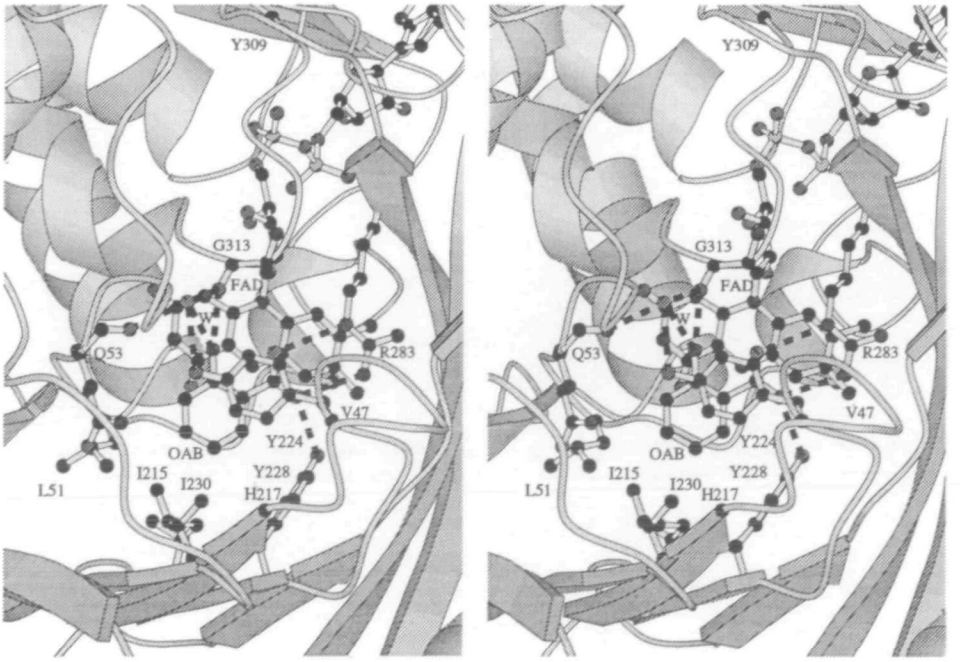


Fig. 2. **A close-up stereo view of the active site in DAO-OAB complex from the same direction as that in Fig. 1.** The flavin, OAB and the active site residues interacting with OAB are shown as ball-and-stick models with the important hydrogen bonds shown by dotted lines. The active site pocket is surrounded by the pseudo β-barrel and the loop from 217 to 228 in the side of the pseudobarrel domain, the inter-domain loop from 47 to 53, and the loop from 309 to 315 in the side of the α/β domain.

OAB and guanidino group of Arg283 is weaker than the corresponding interaction between benzoate and Arg283. The amino group makes a hydrogen bond with the backbone carbonyl oxygen of Gly313, which is located within the loop connected to the N-terminal side of an α-helix. The distance between the carbonyl oxygen of Gly313 and the amino nitrogen of OAB is 2.6 Å. This interaction, which is absent in the DAO-benzoate complex, shifts the molecule slightly away from the flavin ring. Due to the shift of the benzoate moiety, the interaction between the carboxylate and guanidino groups becomes weaker for OAB than for benzoate. A water molecule is found within hydrogen-bonding distances from the main-chain carbonyls of Gln53 and Gly313 and from Tyr224 OH; the interatomic distances from water oxygen to the carbonyl oxygens of Gln53 and Gly313 are 2.7 and 2.6 Å, respectively. The corresponding water molecule has been found in DAO-benzoate complex [*11* and refinement of the initial structure (*10*), Mizutani, H. *et al.*, unpublished results]. The parallel planes of the OAB skeleton and flavin overlap with each other at N-C(2) of OAB with C(4)=O of flavin and probably at carboxyl carbon of OAB with N(5) of flavin [the numbering of the flavin ring system is illustrated in (I)]. The interatomic distances from flavin N(5) to carboxyl carbon and the carboxyl oxygen of OAB are 3.3 and 3.5 Å, respectively; the interaction of flavin N(5) with OAB carboxyl carbon is more likely than that with OAB carboxyl oxygen, even though the interactions of flavin N(5) with OAB carboxyl carbon and with OAB carboxyl oxygen are equally possible from a simple inspection of Fig. 2. These interactions are consistent with the charge-transfer interaction between the highest occupied molecular orbital (HOMO) of OAB and the lowest unoccupied molecular orbital (LUMO) of flavin (*21*). Figure 3 illustrates the mode of overlap between the molecular orbitals of the two counterparts. It should be noted that the overlap is adequate with respect to the

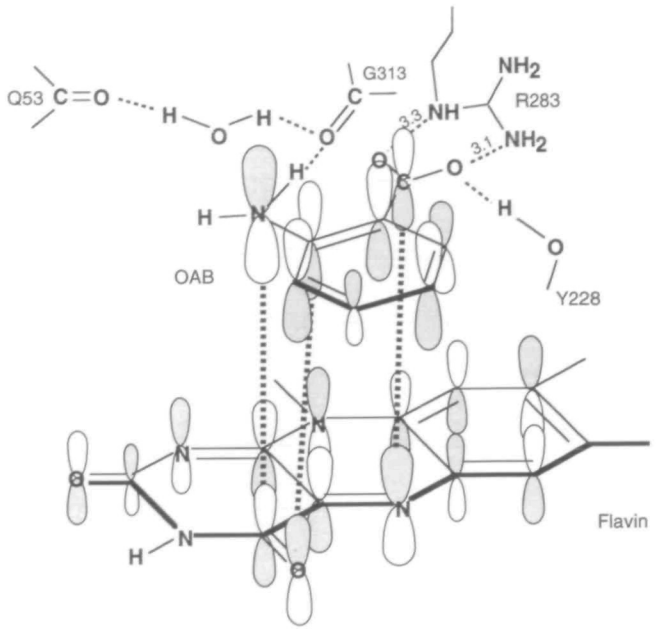
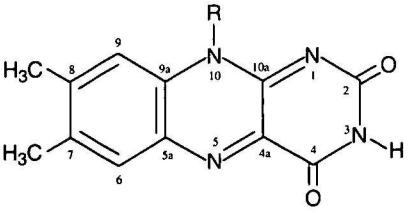


Fig. 3. **Charge-transfer interaction mode between HOMO of OAB and LUMO of flavin (*21*) based on the structure of the OAB-binding site.** Thick dotted lines indicate the overlap between HOMO of OAB and LUMO of flavin (*21*). Thin dashed lines indicate hydrogen bondings and ionic interactions.



(I) (II)

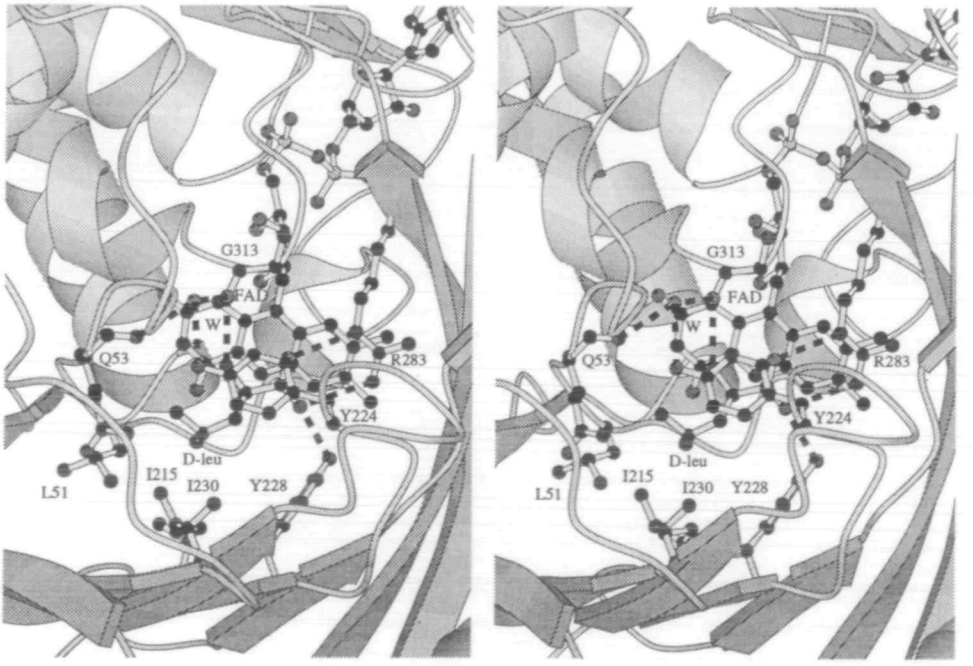


Fig. 4. **A close-up stereo view of the D-leucine-binding region in the DAO-D-leucine complex model obtained by molecular mechanics simulation from the same direction as that in Figs. 1 and 2.** The flavin, D-leucine as a substrate and the active site residues interacting with D-leucine are shown as ball-and-stick models with the important hydrogen bonds shown by dotted lines.

symmetry and the magnitude of the atomic orbitals at the respective overlapping positions. This charge-transfer interaction together with the hydrogen bond of the amino group of OAB with Gly313 carbonyl shifts the position of the benzoate moiety away from the flavin ring at the expense of weakening of the ionic interaction between the carboxyl group of OAB and the guanidino group of Arg283. An important feature of the DAO-OAB crystal structure is that a strong protein base suitable for abstracting the substrate α-proton is not found within the active site; the presence of such a residue is prerequisite for the mechanisms of DAO proposed to date. This finding rules out "the carbanion mechanism" (*4, 5*) and at least requires a major revision for "the concerted mechanism" (*6-8*).

Molecular Modeling of DAO-D-Leucine Complex—The model of DAO in the complex with the substrate, D-leucine, was designed utilizing the active-site structure of DAO-OAB complex and the interactions between OAB and the active site residues; D-leucine is one of the best substrates of DAO (*22*). The carboxylate group of OAB makes a salt bridge and a hydrogen bond with Arg283 and Tyr228, respectively. The amino group makes a hydrogen bond with the backbone C=O group of Gly313 and the phenyl moiety of OAB interacts with the side chains of hydrophobic residues. The D-alanine moiety of D-leucine as a substrate can be unequivocally fitted in the active site of DAO by reproducing the interactions between OAB and the active site residues described above. However, the orientation of the isopropyl group was somewhat ambiguous due to the rotational freedom around the C_α-C_β and C_β-C_γ bonds, and six conformational isomers of D-leucine were taken into consideration as candidate structures for the substrate. Six complex models thus obtained were optimized by the energy minimization method using XPLOR (*16*). The amino group of D-leucine was set to the neutral state, as will be discussed later. The active site model of DAO-D-leucine complex with the lowest conformational energy is shown in Fig. 4. The D-alanine moiety in these six complex models was found to assume the same orientation. The carboxylate group of D-leucine is recognized by the guanidino group and hydroxyl group of Arg283 and Tyr228, respectively, as in the DAO-benzoate and DAO-OAB complexes. As observed in DAO-OAB complex, the amino group of D-leucine is recognized by Gly313 C=O through a hydrogen bond. The water molecule corresponding to the one found in the OAB-binding region (Figs. 2 and 3) was found to make hydrogen bonds with Gly313 C=O, Gln53 C=O (main chain), and Tyr224 OH. The hydrophobic side chain of D-leucine fits in the pocket surrounded by hydrophobic amino acid residues. Very important characteristics are unveiled in this model with regard to the electron transfer from the substrate to flavin and the fate of the substrate α-hydrogen. Namely, the α-hydrogen of D-leucine is directed toward N(5) of flavin. The lone-pair orbital of the amino nitrogen, which is available, since the amino group is in the neutral form (see "DISCUSSION" for the ionic state of the substrate amino group), can come within the interacting distance from C(4*a*) of flavin, though the direction of the lone pair orbital cannot be definitely established in this model. The details of this interacting mode can be better envisioned in the illustration of Fig. 5. That the α-hydrogen is near flavin N(5) bears special significance in relation to the finding that no candidate residue for the α-proton abstraction is found within the active site, either in the DAO-benzoate and DAO-OAB complexes or the DAO-D-leucine complex model. The overlap between the lone-pair orbital of the substrate amino group and the C(4*a*) atomic orbital would not conflict with the "concerted mechanism" (*6-8*) which proceeds *via* electron flow from the lone-pair of the amino nitrogen.

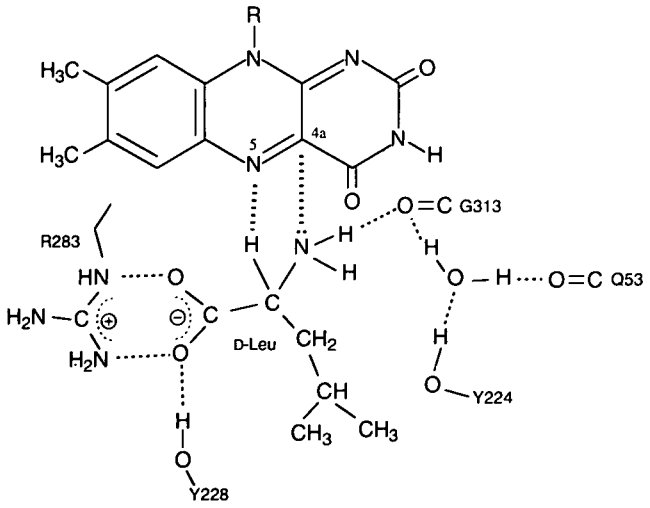


Fig. 5. **Illustration of DAO-D-leucine complex model of Fig. 4 with emphasis on the orientation of the α-hydrogen and of the lone-pair orbital of the amino group with respect to flavin.** Thick dotted lines indicate proximities between α-hydrogen of D-leucine and N(5) of flavin and between the lone-pair of amino nitrogen of D-leucine and C(4*a*) of flavin. Thin dashed lines represent hydrogen bondings.

DISCUSSION

The detailed structure of the OAB-binding site clearly demonstrates the binding mode at the amino and carboxylate groups of OAB. Moreover, the molecular mechanics simulation utilizing the DAO-OAB complex as the initial model has revealed unique features of the flavin-protein-substrate network (Figs. 4 and 5). One of our main goals in the present study was to understand the molecular mechanism of the DAO reaction. The results obtained herein are particularly valuable for this purpose.

Several requirements must be met by any proposed mechanism. These concern the fate of the substrate α-hydrogen and the ionic form of the substrate bound in the active site prior to electron transfer.

As to the fate of the α-hydrogen of the substrate, we conclude that the α-hydrogen is removed from the substrate as a proton on the following grounds. In the reductive half-reaction, the C_α-H bond is cleaved to generate an imino acid and the mode of the cleavage can be either homolytic or heterolytic. In heterolytic cleavage, the α-hydrogen is removed as a proton or a hydride. There are three possibilities, therefore, for the mode of the α-hydrogen removal, *i.e.*, as a hydrogen radical (homolytic cleavage), a proton (heterolytic cleavage), or a hydride ion (heterolytic cleavage). Among these possibilities, the α-hydrogen removal as a proton is the most likely on the ground that in the reaction of DAO with β-chloro-D-alanine DAO catalyzes elimination of HCl in addition to normal

oxidation, forming pyruvate and chloropyruvate as the products of elimination and oxidation, respectively (*23-25*), because this can only be achieved by the removal of the α-hydrogen as a proton (*25-28*). With regard to the fate of the substrate α-proton, Hersh and Jorns have obtained critical information in their experiments with DAO reconstituted with 5-deazaFAD (*29*). In the reductive-half reaction of deazaFAD-DAO with α-tritium-labeled substrates, the tritium is retained at C(5) of 5-deazaFAD, implying that the substrate α-hydrogen is retained at N(5) of FAD during the reductive-half reaction with native DAO. DeazaFAD turned out to be an excellent FAD derivative for deducing the fate of α-hydrogen, since N(5)-H of reduced FAD undergoes rapid exchange with solvent water, making the elucidation of the origin of N(5)-hydrogen difficult, whereas C(5)-H of reduced deazaFAD does not exchange with the bulk water within the experimental time scale. On the basis of the foregoing arguments, we have the following requirements for the fate of the substrate α-hydrogen: (1) the mechanism must explain both oxidation and elimination reactions, (2) the substrate α-hydrogen must be removed as a proton, and (3) the substrate α-hydrogen must be retained at N(5) of FAD in the reductive half reaction. Consequently, we conclude that N(5) of flavin serves as a base for abstracting the α-proton from the substrate.

In considering the reaction mechanism of DAO, precise knowledge of the enzyme-substrate complex is of critical importance. This includes not only the mutual orientation of the substrate, amino acid residues in direct contact with the substrate, and the flavin moiety of FAD, but also the ionic state of the substrate D-amino acid. With regard to the ionic state of the carboxyl group of substrates bound to the active site, resonance Raman studies by Nishina *et al.* have shown unequivocally that the carboxyl group of substrate analogs bound to DAO is in the anionic carboxylate form irrespective of the oxidation-reduction state of flavin, *i.e.*, oxidized, semiquinoid or fully reduced form (*30-32*). It is known that some straight-chain carboxylic acids are competitive inhibitors of DAO (*33*) but that alkyl amines with the same alkyl chains do not inhibit the DAO reaction (*22*). These observations imply that the active site involves a carboxylate-binding site, but does not involve a definite binding site for a cationic amino group. Another experimental result concerning the ionic state of the substrate amino group in the enzyme-substrate complex has come from the work of Nishina *et al.* (*34*), who demonstrated that the pK_a value of flavin N(3)-H is lowered from 9.2 to 8.0 when zwitterionic trigonelline (II) binds at the active site of DAO. This unique phenomenon demonstrates that binding of trigonelline at the active site induces a tendency to release a proton; releasing a proton from flavin creates a negative charge to accommodate the positive charge of trigonelline. This is because there is no amino acid residue that would create a negative charge to stabilize the positive charge of bound trigonelline. The only way to accommodate the positive charge is to release a proton from nearby flavin N(3)-H, since zwitterionic trigonelline can not release a proton to become neutral at the quaternary nitrogen. When the substrate zwitterionic D-amino acid with its cationic amino group ($-NH_3^+$) comes into the active site, the ammonium group will lose a proton to become neutral ($-NH_2$) fitting better in the hydrophobic environment around the amino group; creating a negative charge at flavin N(3) to fit the $-NH_3^+$ group would be unfavorable within the hydrophobic pocket. Thus, the neutral form for the amino group of the substrate is favored when it is bound in the active site. These experimental observations and the interpretations thereof are in agreement with the active-site structure of DAO; the anionic carboxylate group of the competitive inhibitor, benzoate or OAB, makes a salt-bridge with the cationic guanidino group of Arg283 and a hydrogen bond with the hydroxyl group of Tyr228 (Fig. 3), whereas there is no negative charge within the active site

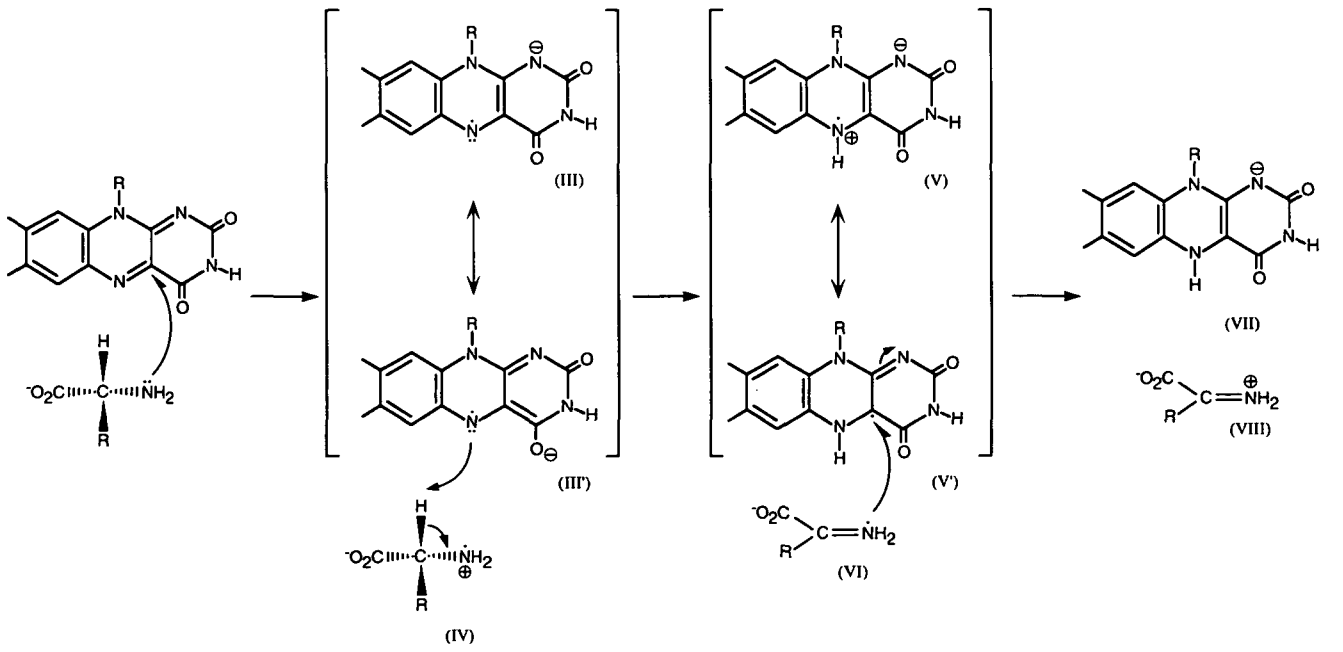


Fig. 6. **Electron-proton-electron transfer mechanism for the reductive-half reaction of DAO.**

that would stabilize the positive charge of a cationic amino group. We conclude, therefore that the substrate is bound at the active site with the carboxyl group in the anionic state and the amino group in the neutral state.

Taking the foregoing arguments into consideration, there are two possible mechanisms shown below for the reductive-half reaction of DAO; which of the two is correct remains to be established.

Electron-Proton-Electron Transfer Mechanism—This mechanism is in essence one-electron processes with an intervening proton transfer (Fig. 6). The initial step in the substrate oxidation is the one-electron transfer from the lone-pair of substrate amino nitrogen to flavin at C(4*a*), generating the flavin anion radical (III)/(III′) and the substrate radical cationic at the amino nitrogen (IV). The N(5) of the flavin anionic radical then abstracts the substrate α-proton leading to the neutral flavin radical (V)/(V′) and the imino acid radical with neutral imino nitrogen (VI). Subsequent one-electron transfer from the imino acid radical to the flavin neutral radical completes the reductive-half reaction, yielding the anionic form of reduced flavin (VII) and the imino acid with cationic imino nitrogen (VIII). The final product is known as "purple intermediate" and its identity as the complex between the anionic form of reduced flavin (VII) and imino acid (VIII) cationic at the imino nitrogen and anionic at the carboxyl group has been proved by resonance Raman (*30*) and NMR (*35*) spectroscopy. Overall, the reaction proceeds *via* electron-proton-electron (EPE) transfer, and accordingly we call this mechanism the "EPE mechanism." The activation energy for the initial one-electron transfer from the substrate amino lone-pair is substantially lowered by the overlap between the lone-pair of the amino group and the C(4*a*)-atomic orbital of LUMO of flavin (Fig. 5). The pK_a value for the α-proton of the radical (IV) can be assumed to be sufficiently low. Relevant to this estimation is the pK_a value of around neutral for the methyl-hydrogen of *N,N*-dimethylaniline cationic amine radical (*36*). The pK_a value of the N(5)-hydrogen of flavin semiquinone (V)/(V′) is known to be around 8 (*37*). Therefore, the activation energy for the deprotonation step should be sufficiently low if the pK_a values for the radicals (IV and V/V′) are taken into account. The EPE mechanism depicted above reconciles the behavior of the substrate α-hydrogen as a proton and the retention of the α-hydrogen at flavin N(5). It is known that a zwitterionic ligand stabilizes the anionic flavin radical of DAO (*32, 38*) and that an anionic ligand (*e.g.*, benzoate) transforms the anionic flavin radical into the neutral flavin radical of DAO (*39*). These observations support the reaction sequence (III)/(III′)+(IV)→(V)/(V′)+(VI) in the EPE mechanism. Namely, the anionic flavin radical (III)/(III′) is stabilized by the zwitterionic ligand (IV) and the proton transfer from (IV) to (III)/(III′) converts the ligand into the anionic form (VI), which in turn stabilizes the neutral flavin radical (V)/(V′). Both anionic (III)/(III′) and neutral (V)/(V′) flavin radicals can be expressed in the canonical forms shown in Fig. 6. Though it is not clear at this point which of the canonical forms for each radical species (III or III′; V or V′) prevails, the ones with anionic charge at N(1), *i.e.*, (III) and (V) in Fig. 6, may be stabilized by the α-helix dipole located in the vicinity of N(1) of flavin (Figs. 2 and 4).

Ionic Mechanisms Involving a Covalent Bond between Substrate and Flavin—An alternative mechanism compatible with the DAO-substrate complex model (Figs. 4 and 5) and with the requirements outlined in the foregoing section is presented in Fig. 7. This mechanism is characterized by two-electron flow from the amino lone-pair in concert with

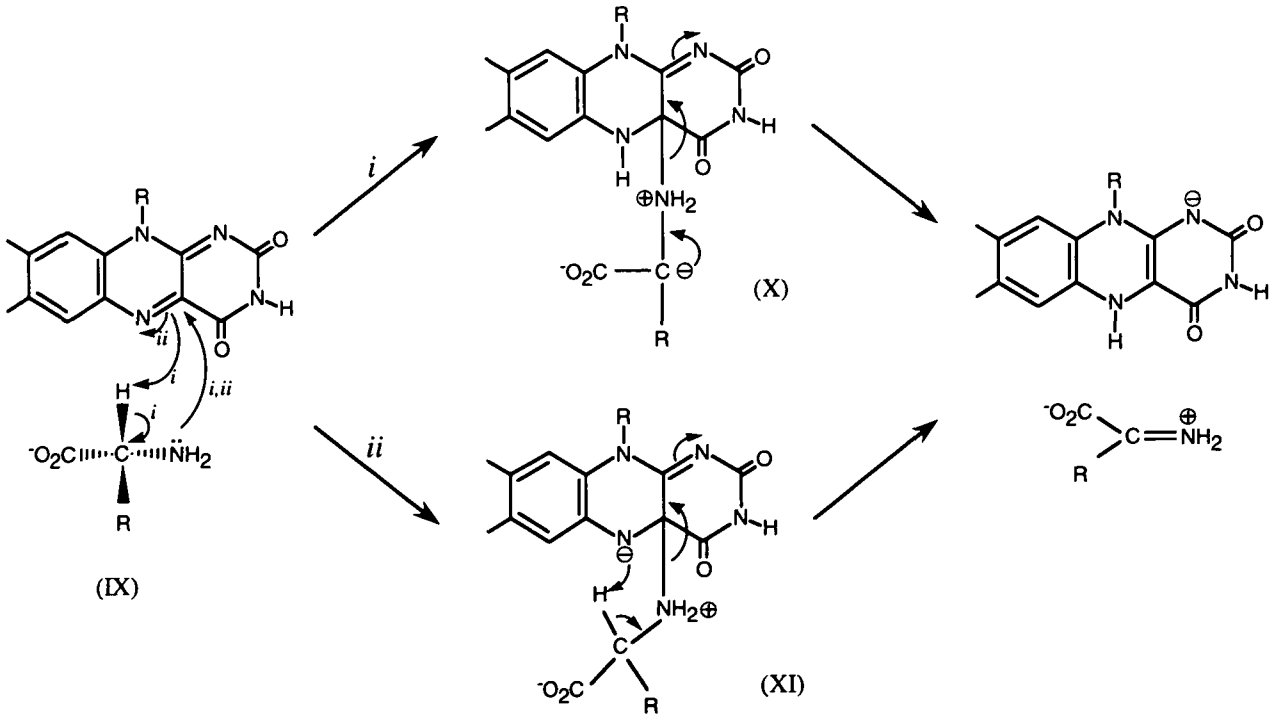


Fig. 7. **Ionic mechanism for the reductive-half reaction of DAO.**

the α-proton abstraction by N(5) of flavin. There are two possible pathways (*i* and *ii*) after the initial covalent-bond formation (IX), the difference between the two being in the anionic covalent intermediates, one with a carbanionic intermediate (X) and the other with a nitrogen anion intermediate (XI). This mechanism is analogous to our "concerted mechanism," albeit flavin N(5) rather than a protein base acts as the base to abstract the substrate α-proton. The activation energy for the initial step (IX) will be lowered by the overlap between the lone-pair orbital of the amino group and the atomic orbital of the flavin LUMO at N(5) (Fig. 5). The apparent difficulty associated with this mechanism lies in the instability of the anionic intermediates (X and XI). It is not possible at present, however, to assess the stability or instability of these intermediates, nor can we understand how these intermediates would be stabilized within the enzyme active site.

We still lack experimental evidence in favor of either of the two mechanisms put forward in the present report. Mattevi *et al.* proposed in their report on the crystal structure of DAO (*11*) a "direct hydride transfer mechanism" by which a hydride ion derived from the substrate α-hydrogen is transferred to flavin N(5) with bound water acting as a base to abstract a proton from the cationic amino group. Pollegioni *et al.* more recently reinforced this mechanism for the microbial DAO on the basis of the structure/linear free energy relationship (*40*). Though this "direct hydride transfer mechanism" is not totally in conflict with the overall active-site structure, we do not favor this mechanism because (1) the substrate α-hydrogen should behave as a proton during the reductive-half reaction, (2) the lone-pair electrons of the neutral amino group are more mobile for electron transfer to flavin than the electron pair on a hydride ion of the substrate α-hydrogen from a chemical point of view. It should also be noted that either of the mechanisms proposed herein can successfully explain the dual catalysis of DAO, *i.e.*, oxidation and elimination of β-halo-substituted D-amino acids (*23-28*). However, the "direct hydride transfer mechanism" (*11, 40*) does not seem able to explain this dual catalysis.

The mechanistic consideration of DAO catalysis has reached a new stage now that we have a precise active-site structure of this enzyme at hand. The final elucidation of the reaction mechanism at the sub-molecular level must still await further theoretical elaboration based on the structural background. We are currently in the process of solving the crystal structures of various intermediary states of DAO and of complexes with different ligands as well as the structures of various mutants at critical amino acid residues. DAO shares certain common features in catalysis with other families of flavoenzyme oxidases, such as the one including glycolate oxidase and lactate oxidase, which are known to have some common structural characteristics with DAO (*11, 41*). The reaction mechanism for DAO discussed here, therefore, may well apply to those other oxidases as well. An understanding of the catalytic mechanism of DAO at the sub-molecular level should accordingly lead to an understanding of not only these enzymes, but also flavoenzyme oxidases in general.

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