BIOORGANIC CHEMISTRY 16, 97–110 (1988)

The Reaction Mechanism of D-Amino Acid Oxidase: Concerted or Not Concerted?

RETSU MIURA AND YOSHIHIRO MIYAKE

Department of Biochemistry, National Cardiovascular Center Research Institute, Suita, Osaka 565, Japan

Received May 27, 1987

It seems widely accepted that the reductive half reaction of D-amino acid oxidase is initiated by the carbanion which is generated as the result of the α-proton abstraction from the substrate D-amino acid by a protein nucleophile. However, this process is energetically unfavorable even in the enzyme–substrate complex irrespective of how short the lifetime of the carbanion might be. To circumvent this dilemma the authors have put forward an alternative mechanism, according to which the α-proton abstraction by the protein nucleophile is coupled in a concerted manner with the electron transfer from the amino nitrogen of the substrate to the oxidized flavin without intermediary generation of the carbanion as a discrete entity. The present review describes the background and the rationales for the concerted mechanism with specific emphasis on the flavin–substrate (intermediate) charge-transfer interactions.

I. INTRODUCTION

The variety of reaction catalyzed by flavoenzymes has long enchanted biochemists and chemists as well. This versatility of the flavin coenzymes originates in the peculiar molecular structure of the flavin nucleus. It possesses, in addition to the redox center, several functional groups which are capable of interacting with solvent water, solutes, or protein moiety, and it can take three redox states, i.e., oxidized, one-electron reduced, and two-electron reduced states, each of which can exist in different ionic forms (Scheme 1). This versatility is so modulated in each particular flavoenzyme that specificity of the reaction develops out of the broad reactive potentiality. Because of this specificity, details of the reaction mechanism in one particular flavoenzyme or in a chemical model may not always apply to other flavoenzymes. In this review we are concerned with the reaction of a particular FAD enzyme, D-amino acid oxidase [D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3] (DAO) from porcine kidney cortex.

Ever since the discovery of DAO by Krebs in animal liver and kidney as early as 1935 (*1, 2*), DAO has been a subject of extensive investigation in various aspects. Nevertheless, it is not totally free of ambiguity in the essence of its reaction as well as in the physiological function. The enigmatic characteristic of DAO with regard to its physiological function is its wide distribution among living organisms ranging from microbes to mammals with strict specificity to D-amino

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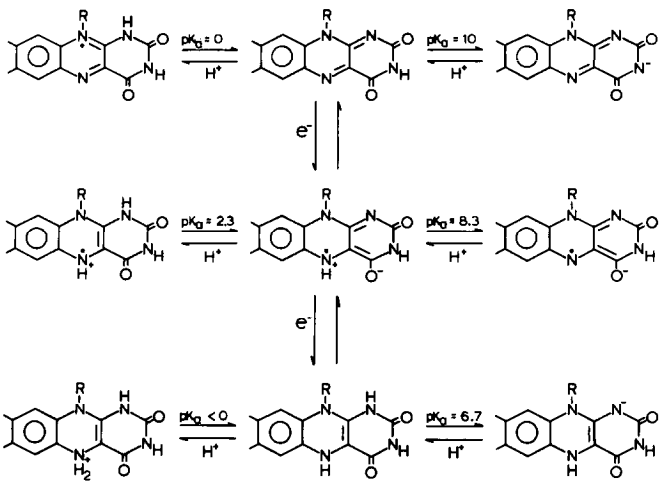
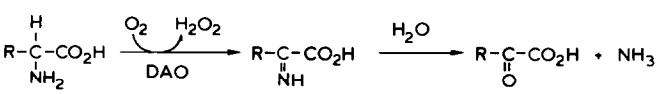


SCHEME 1

acids against the L-isomers but yet with broad organ specificity and broad substrate specificity among the D-amino acids, which are "foreign" to living organisms ((*3*) and references cited therein). In connection with the physiological function of DAO, a new hypothesis has been introduced by Hamilton and his co-workers (*4, 5*), according to whom the physiological substrate is the adduct of cysteamine and glyoxylate. In the present review, however, we discuss the reaction of DAO with D-amino acids as substrates.

DAO contains one molecule of non-covalently bound FAD per subunit of 39,000 da ((*6, 7*) and references cited therein) with known primary sequence (*6*). The nucleotide sequence of the cDNA clone of porcine kidney DAO has recently been determined (*8*), and the amino acid sequence deduced from the nucleotide sequence was found to be identical to that reported previously (*6*).

The overall reaction of D-amino acids catalyzed by DAO can be expressed in Eq. [1]. The imino acid formed is hydrolyzed nonenzymically to a keto acid and ammonia. The conversion of a D-amino acid into an imino acid is the primary concern in this review. This reaction is coupled to the redox counterpart, reduction of the flavin in DAO. Thus, the oxidized flavin oxidizes, or dehydrogenates, a D-amino acid to the imino acid and becomes reduced. This step is termed as the reductive half reaction. The reduced flavin in turn is reoxidized by molecular oxygen (oxidative half reaction), thereby closing the catalytic cycle.

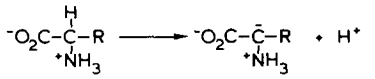


[1]

We focus our attention here to the first half (the reductive half reaction) of the redox cycle catalyzed by DAO; problems underlying the dehydrogenation of a D-amino acid are treated in detail and a mechanism is presented.

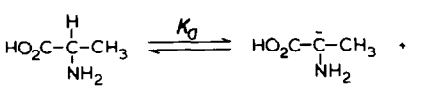
II. ABSTRACTION OF THE α-PROTON FROM A D-AMINO ACID

It seems to have acquired general consensus that the very initial step in the DAO-dependent oxidation of a D-amino acid is the formation of a carbanion as the result of α-proton abstraction from the D-amino acid (e.g., (9) for review) (Eq. [2]).



(I) [2]

However, this apparently established step needs deliberate consideration. The ionic form of the carbanion (**I**), where negative charges are localized adjacent to each other, should be very unstable. Moreover, even if the positive and negative charges in (**I**) are neutralized by amino acid residues of the protein moiety, the pK_a of the α-proton is substantially high such that the generation of the carbanion is energetically unfavorable. Although experimental evaluation of the pK_a of the α-proton in D-alanine has not been available thus far, we can make a rough estimation by comparing pK_a values of related compounds. Note that D-alanine is one of the best substrates of DAO. The pK_a values of acetone and ethyl acetate (α-proton) are 20 and 24.5, respectively, at 25°C (*10*). The pK_a value for the α-proton of acetamide is expected to be greater than 25 since the α-proton is assumed to be less acidic than the amide proton for which the pK_a value was reported to be 25 at 25°C (*10*). It is reasonable, therefore, to estimate the pK_a value of the α-proton of D-alanine to be greater than 20 by at least a few pK_a units. This evaluation is for the nonionic neutral form of D-alanine. If the positive protonated amino group and the negative unprotonated carboxylate group are neutralized by salt bridges with amino acid residues of the protein, the pK_a should correspond to that of the nonionic form (**II** in Eq. [3]).



(II) (III) [3]

This high pK_a value for the α-proton makes the equilibrium (Eq. [3]) in favor of the protonated state (**II**) at a neutral pH, resulting in a high activation energy for the deprotonation. Suppose the pK_a of the α-proton is 22; the activation energy for deprotonation should be greater than 125 kJ mol^{-1} at 25°C, the free energy difference between (**II**) and (**III**), as calculated from $\Delta G^{\circ} = -RT\ln K_a$, K_a being the equilibrium constant in Eq. [3]. The activation energy $\Delta G^{\neq}$ should be inevitably greater than ΔG° for the equilibrium (Fig. 1). Note that 1 pK_a unit is equivalent to 5.7 kJ mol^{-1} at 25°C. The activation energy for the DAO-dependent oxidation of D-alanine was reported to be 17.6 kcal mol^{-1} (73.6 kJ mol^{-1}) (*11*), remarkably smaller than that estimated above. If the enzymic reaction proceeds via the carbanion intermediate, the activation energy of the carbanion formation should be lowered by more than 50 kJ mol^{-1} by the enzyme; this would be equivalent to lowering the pK_a of the α-proton by more than 9 pK_a units. In the carbanion

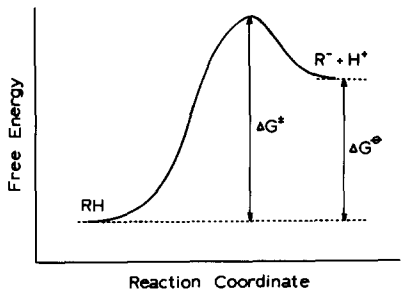


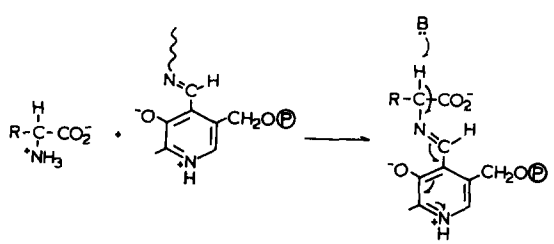
FIG. 1. Energy profile in the α-deprotonation. RH and R^- represent a D-amino acid and the α-deprotonated carbanion, respectively.

mechanism this energy imbalance would have to be compensated by protein–D-alanine binding, no matter how short the lifetime of the carbanion might be. The energy of ion-pair formation at the carboxylate and amino groups is already included in the pK_a estimation, since the foregoing calculation is based on the neutral nonionic form of D-alanine. The strain energy which would be derived from protein–D-alanine binding would not suffice the energy gap in question; the strain energy of cyclopentane is 5.4 kJ mol^{-1} per carbon atom relative to strain-free cyclohexane (*12*). These problems with regard to the pK_a of the α-proton, as discussed above, can be easily understood in comparison with pyridoxal phosphate-dependent amino transferase reactions, in which the pK_a of the α-proton is substantially decreased through the extended conjugation from the Schiff base aldimine to the pyridinium electron sink (e.g., *13, 14*) (Scheme 2).

III. CONCERTED REACTION MECHANISM

In search of the answers to the questions introduced in the preceding chapter, we performed a reverse reaction of DAO, starting with chloropyruvate, ammonia, and the oxidized form of DAO (*15, 16*). This reaction was supposed to be the reversal of the forward reaction of DAO with β-chloro-D-alanine.

The forward reaction with β-chloro-D-alanine provides the first example that DAO catalyzes elimination of HCl in addition to the normal oxidation, yielding pyruvate and chloropyruvate as the products of elimination and oxidation, respec-



SCHEME 2

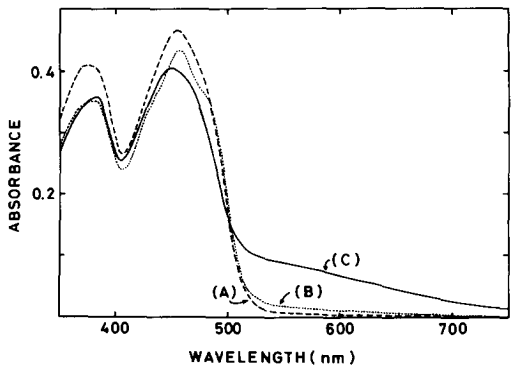


FIG. 2. The reverse reaction of DAO with chloropyruvate and ammonia (*15*, *16*). Absorption spectra of DAO (41 μM) before (broken line), immediately after (dotted line), and 30 min after (solid line) the addition of chloropyruvate (4 mM) and ammonium sulfate (100 mM).

tively (*17–20*). Walsh and his co-workers explained these results and those of related DAO reactions as the intervention of a discrete cabanion intermediate (**IV**) which is formed by the α-proton abstraction in the initial step of the DAO reaction (*20–23*).

$$^{-}O_2C-\overset{\overset{+}{N}H_3}{\overset{|}{\overset{-}{C}}}-CH_2Cl$$

(IV)

The reverse reaction of DAO with chloropyruvate and ammonia proceeded with gradual formation of a species characterized with a broad absorption band in the long wavelength region (Fig. 2, solid line) (*16*). This spectral species was identical to the reaction intermediate observed in the forward reaction with β-chloro-D-alanine (Fig. 3, solid line) (*19*). This species was assigned to the charge–transfer complex of oxidized DAO and α-imino-β-chloropropionate (Scheme 3, **VII**); the identity of this species to (**VII**) was later confirmed by resonance Raman spectroscopy (*24*). Specific reduction of flavin in this intermediate (**VII**) completed the reverse reaction yielding β-chloroalanine as the only product, contrary to what the carbanion theory predicts. If the forward reaction proceeds by way of the carbanion intermediate (**IV**), the microscopic reversibility would require the for-

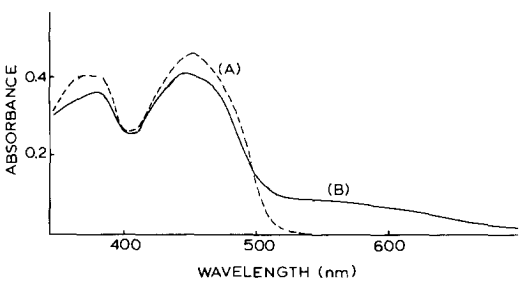
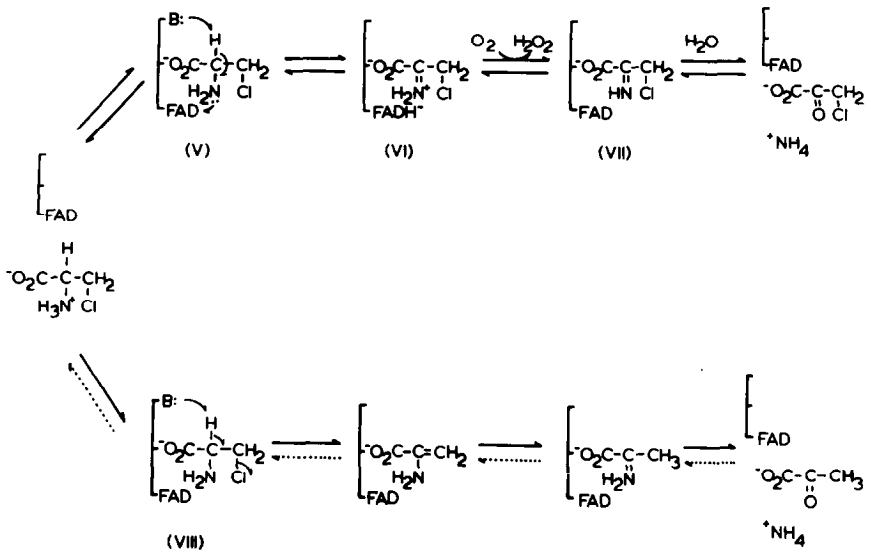


FIG. 3. The aerobic reaction of DAO with β-chloro-D-alanine (*17–19*). Absorption spectra of DAO (41 μM) before (broken line) and after (solid line) aerobic incubation with β-chloro-D-alanine (32 mM).

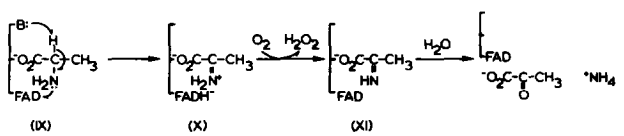


SCHEME 3

mation of the carbanion (**IV**), which would generate the elimination product, pyruvate, in addition to β-chloroalanine. We interpreted the results as the supporting evidence for the concerted reaction as depicted in Scheme 3 (*16*). In this concerted mechanism, the abstraction of the α-proton is concerted with the electron flow from the substrate to flavin (step **V**) and with chloride elimination (step **VIII**). This hypothesis can be extended to the DAO reaction with D-alanine (Scheme 4). The reaction should proceed without generation of the carbanion, thus circumventing the great potential barrier discussed in the preceding section (Fig. 1).

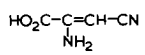
IV. REEVALUATION OF THE CONCERTED REACTION MECHANISM

The concerted theory just introduced as above distinguishes itself in the very initial step (Scheme 4, **IX**) where the electrons are pushed from a protein nucleophile to the α-proton, and by way of the lone pair of the amino nitrogen, to the oxidized flavin which pulls the electrons from the amino nitrogen. The "push" from the nucleophile and the "pull" from flavin are coupled via the substrate in a concerted fashion. The driving force for the pull from flavin can be derived from the capability of the oxidized flavin of forming charge–transfer complexes with



SCHEME 4

various amino compounds. The charge–transfer complexes are known to be unique characteristics of flavoproteins and their importance in the catalysis has been well documented (*25*). The best known amino compounds that form charge–transfer complexes with oxidized DAO are *o*-, *m*-, and *p*-aminobenzoates (*26–28*), which are also known to be potent competitive inhibitors. Of the three isomers, the *o*-isomer exhibits the most prominent spectral properties, i.e., the strongest charge–transfer absorption band in the long wavelength region (*26*), a substantial blue shift in the first absorption band (*26*), and the strongest rotational strength for the charge–transfer band (*27*). These characteristic features of *o*-aminobenzoate can be correlated to its close resemblance to the substrate D-amino acid in terms of the mutual orientation of the amino and carboxyl groups. These features altogether can be taken as the evidence that the charge donor in the charge–transfer complex between *o*-aminobenzoate and DAO is the amino group. The charge–transfer in this case, therefore, is from the lone pair of the amino nitrogen to the oxidized flavin and bears the $n \rightarrow \pi$ nature. This would not require a staggered sandwich-type arrangement between the aromatic ring of *o*-aminobenzoate and the plane of the flavin nucleus. Another charge–transfer ligand with both an amino and a carboxyl group is α-amino-β-cyanoacrylate (**XII**), which was assigned as the



(XII)

oxidation product in the reaction of DAO with β-cyano-D-alanine (*29*). This compound (**XII**) forms a strong charge–transfer complex with oxidized DAO accompanying a broad absorption band (Fig. 4) and a negative CD band in the long wavelength region (*29*); these are the characteristics shared by *o*-aminobenzoate. This ligand (**XII**) resembles the substrate D-amino acid even more closely than *o*-aminobenzoate in terms of the geometrical configuration of the amino and carboxyl groups. We interpret these unique properties of *o*-aminobenzoate and α-

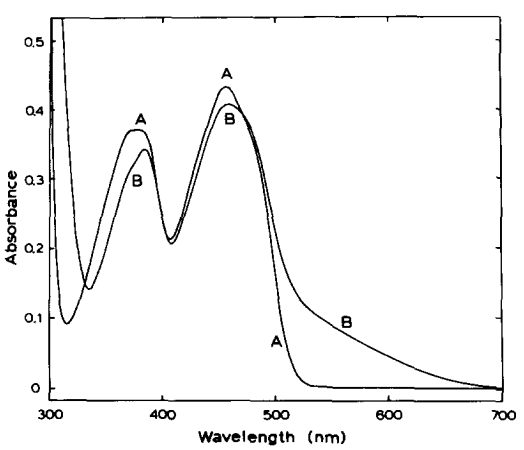
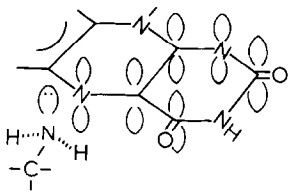


FIG. 4. The aerobic reaction of DAO with β-cyano-D-alanine (*29*). Absorption spectra of DAO (39 μM) before (A) and after (B) the aerobic incubation with β-cyano-D-alanine (120 μM).

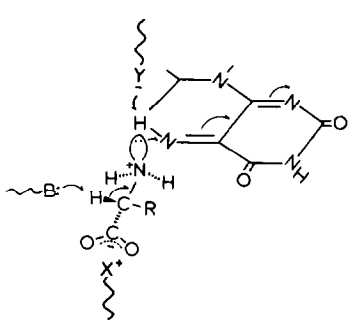


SCHEME 5

amino-β-cyanoacrylate as described below. The carboxylate and amino groups of these ligands are bound to the carboxylate and amino binding sites, respectively, for the substrate D-amino acid, thereby orienting the amino *n*-orbital to overlap with the flavin π-orbital (Scheme 5). This orbital overlap enables the charge-transfer of $n \rightarrow \pi$ type from the ligand nitrogen to flavin. The $n \rightarrow \pi$ type charge-transfer from the amino group of amino benzoate has been implicated by circular dichroism studies (*27*). We postulate, accordingly, in the reaction with DAO that the substrate D-amino acid forms, prior to electron transfer, a transient $n \rightarrow \pi$ charge-transfer complex with the oxidized flavin, the amino and carboxyl groups being fixed at the corresponding binding sites (Scheme 6). This charge-transfer from the substrate to flavin may lower the pK_a of the α-proton somewhat but cannot be expected to be strong enough to allow the carbanion as a discrete entity. However, in this reaction scheme, the formation of the carbanion is not prerequisite and thus the activation energy in the whole process would be significantly lower than that which the carbanion theory would require. The reductive half reaction should proceed quite smoothly by way of the concerted electron transfer without going over the huge potential barrier.

As to the $n \rightarrow \pi$ nature of the charge-transfer between aminobenzoate or α-amino-β-cyanoacrylate with DAO-bound flavin, there has been no direct supporting evidence, despite extensive study on the charge-transfer interaction with these ligands (*24, 27, 28, 30*). In connection with this, we have recently reported the $n \rightarrow \pi$ character in the charge-transfer interaction between phenolic compounds and the oxidized flavin in the old yellow enzyme by ^{13}C NMR spectroscopy; phenolate oxygen is the site of charge-donation to flavin (*31*).

It should be emphasized that the concerted mechanism described here does not



SCHEME 6

contradict the experimental results obtained with the β-halo-substituted D-amino acids (*20–23*) or the suicide substrate, D-propargylglycine (*32–34*). The relevance of the concerted theory to the former case has already been discussed. In the latter case, the active species for protein modification is not the carbanion but the primary product of the reductive half reaction in question (*34*). The isotope effects observed for the α-proton (*20, 21, 35–37*) have likely been interpreted as the experimental supports for the carbanion theory. However, these can equally support the concerted sequence of the reductive half reaction; the α-C–H bond is undoubtedly cleaved in either mechanism.

The experimental results which were interpreted to provide the direct evidence for the carbanion mechanism came from the reaction of DAO with nitroethane (*38*). As far as the pK_a is concerned, however, this substrate analog is quite different from D-alanine; the pK_a of the C(1)–H is as low as 8.6 (*10*), while that of the D-alanine α-proton is well over 20. It is undoubtedly the carbanion that forms the covalent adduct with flavin in this system. Indeed the carbanion of nitroethane was used as the substrate and the reaction rate was lower by more than two orders of magnitude when the neutral form of nitroethane was used as the substrate. Moreover, the enzyme turns over only with the release of nitrite anion, whereas the reaction with D-amino acid does not accompany decarboxylation. On the basis of these facts, nitroethane is not considered a true substrate analog. In this respect, it should be recalled that sulfite anion forms a covalent adduct with the oxidized flavin in DAO (*39, 40*); sulfite anion cannot be considered to be a substrate analog as far as its structure is concerned. One simple explanation may be that electron-deficient flavin in DAO is capable of reacting with an electron-rich anion species which may not structurally resemble the substrate.

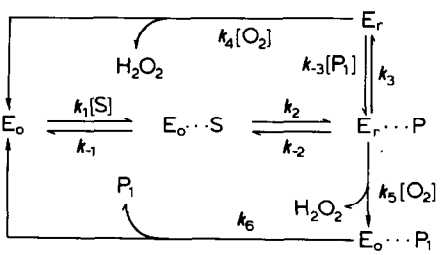
V. LIGAND–FLAVIN CHARGE–TRANSFER INTERACTION

Although the catalytic significance that various charge–transfer interactions may exhibit has been discussed in the preceding chapters, it seems still worthwhile to examine them in more detail. If the $n \rightarrow \pi$ type charge–transfer preexists between the substrate D-amino acid and the oxidized flavin prior to the electron transfer, the amino group of the D-amino acid should be inevitably in its neutral form. This would seem contradictory to pH profiles of K_m and V_{max}/K_m, which are in favor of the protonated cationic form of the amino group in the binding of the substrate to the enzyme (*41, 42*). However, this apparent discrepancy seems to afford further details of the reaction as given below in association with the mechanism depicted in Schemes 4–6. Fitzpatrick and Massey reported, in their study on the stoichiometry of proton uptake and release, that the reductive half reaction accompanies release of a proton with formation of the charge–transfer complex between reduced flavin and imino acid (**X**) (*43*). We interpret that the proton released in the reductive half reaction originates in the protonated cationic amino group of the substrate. The amino group of the substrate D-amino acid is originally in the cationic protonated state. It is this species that is bound to the enzyme at a specific binding site, probably with a negative charge (group Y in Scheme 6),

which traps a proton from the amino group, rendering the amino group neutral and, therefore, suitable for *n*-donation to the oxidized flavin. The proton trapped at the amino-binding site is then released upon formation of the charge–transfer complex between imino acid and reduced flavin as the result of electron transfer from the substrate to flavin. That the amino nitrogen in the transient charge–transfer complex prior to electron transfer is in the neutral state is in accordance with the finding that the amino group of *o*-aminobenzoate is neutral in the charge–transfer complex with the enzyme (*43*). If one proton is released from the amino group upon formation of the purple intermediate as the result of the reductive half reaction, the imino acid in the purple intermediate should be cationic at the imino nitrogen as in **X** (Scheme 4). In fact, the resonance Raman studies on the purple intermediate provided evidence that the imino acid cationic at the imino nitrogen is complexed to the reduced flavin (*44–46*). The model study of the purple intermediates also supports this conclusion (*47, 48*). We have found in our recent ^{13}C NMR studies on the purple intermediates (*49, 50*) that flavin in this intermediate is in the anionic reduced state. That the imino nitrogen is cationic and that the reduced flavin is anionic argue in favor of the imino portion as the charge acceptor and the anionic reduced flavin as the charge donor. Furthermore, ^{13}C NMR studies also indicated that the electron density at C(4a) is substantially higher in the purple intermediate than in the uncomplexed reduced form (*49, 50*); the nucleophilicity of the 4a-position is accordingly raised in the purple intermediate relative to the free reduced form. This gives relevance to the 4a-position in the purple intermediate as the site of the reaction with molecular oxygen in the reoxidation of the reduced flavin, i.e., the oxidative half reaction. This justifies electronically that the rate of oxidation by molecular oxygen is higher with the purple intermediate than with uncomplexed reduced DAO ($k_5 > k_4$, Scheme 7) (*37*) and that the oxidative half reaction does proceed via oxidation of the purple intermediate by molecular oxygen (lower loop in Scheme 7) prior to product release (*37, 51*).

The reoxidation of the purple intermediate (**X** in Scheme 4) by molecular oxygen produces, as the immediate product, the charge–transfer complex between imino acid and the oxidized flavin (**XI** in Scheme 4). The imino group of the imino acid in this intermediate was proven to be in the neutral form as in **XI** (Scheme 4) by resonance Raman spectroscopy (*24*). This neutrality rationalizes the imino portion as the charge donor and the oxidized flavin as the charge acceptor.

As the last epitome in this chapter, flavin–protein interaction in these reaction



SCHEME 7

intermediates deserves brief discussion. In our recent ^{13}C NMR studies on DAO reconstituted with ^{13}C-enriched FADs, we observed that the electronic state of flavin in terms of the electron density at specific sites of flavin is modulated in those reaction intermediates discussed herein as well as in the charge–transfer complexes with competitive inhibitors (*30, 49, 50*). Particularly significant with regard to the flavin–protein interaction are C(2) and C(4) of the isoalloxazine nucleus. The ^{13}C chemical shifts for these carbon resonances underwent changes which were dependent on the ligands. These positions are known to be the sites of hydrogen bonds between the carbonyl oxygens attached and the amino acid residues of the protein moiety. The hydrogen bondings at these positions are among the most critical factors that influence the flavin reactivity in either the oxidized or the reduced form as demonstrated by molecular orbital calculations (*52–54*). Taking these considerations into account, we concluded that the binding of the substrate (intermediates) modulates these flavin–protein interactions, thereby tuning the flavin reactivity during the catalytic reaction sequence. With regard to this conclusion, the postulation by Stankovich and her co-workers should be referred to. They presented evidence that binding of benzoate, a potent competitive inhibitor, to DAO does critically regulate the redox potential of flavin and extended this to a hypothesis that flavin reactivity is modulated by substrate binding (*55, 56*).

VI. EPILOGUE

We have introduced in this review our hypothesis that the reductive half reaction of DAO proceeds in a concerted manner such that the α-proton abstraction and the electron transfer from the substrate to flavin are concertedly coupled with no intervention of the discrete carbanion intermediate. This hypothesis lacks an unequivocal experimental basis that would establish it with no alternatives, just as the carbanion theory has not been proved by explicit experimental evidence. However, the energetical consideration with regard to the pK_a of the α-proton of D-alanine (Section II) has led to the concerted reaction hypothesis which rationalizes those lines of evidence obtained thus far more favorably than does the carbanion theory. The necessity of increasing the kinetic acidity of the α-proton of a D-amino acid in DAO reaction was also pointed out by Brown and Hamilton (*57*).

Although we have limited our mechanistic consideration solely to DAO, the reverse reaction of flavocytochrome b_2 with 3-halopyruvates by Urban and Lederer (*58*) deserves a few remarks here. Their experimental system is strongly related to that of the reverse reaction of DAO with chloropyruvate and ammonia (*15, 16*) discussed in Section III, although with different results and an explanation different from that of DAO (*15, 16*). Based on the fact that the reverse reaction with 3-halopyruvate does undergo reductive elimination of hydrogen halide, they propose the complex of the carbanion (α-deprotonated β-halolactate) and oxidized enzyme as the common intermediate ("T") for oxidation and elimination in the forward reaction with β-halolactate. Although their results reconcile the carbanion mechanism, it is tempting to suppose the $n \rightarrow \pi$ type charge–transfer complex between the hydroxyl oxygen of (halo)lactate and oxidized flavin

for the intermediate T. This charge–transfer complex would correspond to that of Scheme 5 and would partition to oxidation and elimination, both of which proceed in concerted fashions as in Scheme 3, where $-NH_2$ can be replaced by –OH. The carbanion mechanism of Urban and Lederer leaves the pK_a problem of the α-proton still open; the α-proton of lactate can be regarded equally as high as that of D-alanine.

In connection with the pK_a energetics, we may be allowed to refer to the mechanism put forward recently for acyl-CoA dehydrogenases (*59–61*), a group of flavoenzymes which catalyze α,β-unsaturation of fatty acyl-CoA. The pK_a value of the α-proton, which is abstracted by a protein nucleophile, in fatty acyl-CoA can be regarded considerably lower than the α-proton of D-alanine; the generation of carbanion at the α-position of fatty acyl-CoA would seem favorable, at least in comparison with D-alanine. Nevertheless, the α-proton abstraction from fatty acyl-CoA is coupled in a concerted fashion with the transfer of the β-hydride to oxidized flavin, precluding the discrete carbanion formation (*59–61*).

For the still better understanding of the mechanistic details, one of the most critical experiments that await completion is the X-ray crystallographic analysis of DAO itself and ideally of the complexes with substrate analogs and reaction intermediates; the preliminary results of the crystal structure have been reported (*62*). This should, no doubt, offer not only such static information as the interaction mode of flavin with the protein moiety or the orientation of the specific amino acid residues participating in the reaction but also dynamic information on the transient substrate (intermediate)–flavin complex or the elegant movement of the catalytic domain along the reaction sequence. These may give an ultimate clue to the unanswered question, which is the very motif of the present review.

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