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Substrate Recognition and Activation Mechanism of D-Amino Acid Oxidase: A Study Using Substrate Analogs

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We investigated the mechanism of recognition and activation of substrate by D-amino acid oxidase (DAO) by thermodynamical and spectrophotometric methods using zwitterionic ligands [*N*-methylisonicotinate (NMIN), trigonelline, and homarine] and monoanionic ligands as model compounds of the substrate and the product. In terms of the charge within the substrate D-amino acid, monoanionic (*e.g.*, benzoate), zwitterionic (*e.g.*, NMIN), and dianionic (*e.g.*, terephthalate) ligands are thought to be good models for neutral, basic, and acidic amino acids, respectively, because when a substrate binds to DAO, as previously reported, the α-ammonium group ($-NH_3^+$) probably loses a proton to become neutral ($-NH_2$) before the oxidation. Zwitterionic ligands can also be good model compounds of product in the purple complex (the complex of reduced DAO with the product imino acid), because the imino nitrogen of the imino acid is in a protonated cationic form. We also discuss electrostatic interaction, steric effect, and charge-transfer interaction as factors which affect the affinity of substrate/ligand for DAO. Monoanionic ligands have high affinity for neutral forms of oxidized and semiquinoid DAO, while zwitterionic ligands have high affinity for anionic forms of oxidized, semiquinoid, and reduced DAO; this difference was explained by the electrostatic interaction in the active site. The low affinity of homarine (*N*-methylpicolinate) for oxidized DAO, as in the case of *o*-methylbenzoate, is due to steric hindrance: one of the *ortho* carbons of benzoate is near the phenol carbons of Tyr228 and the other *ortho* carbon is near the carbonyl oxygen of Gly313. The correlation of the affinity of *meta*- and *para*-substituted benzoates for oxidized DAO with their Hammet's σ values are explained by the HOMO-LUMO interaction between the phenol group of Tyr224 and the benzene ring of benzoate derivative. The pK_a of neutral flavin [N(3)-H of oxidized flavin, N(5)-H of semiquinoid flavin, and N(1)-H of reduced flavin] decreases by its binding to the apoenzyme. The magnitude of the decrement is oxidized flavin < semiquinoid flavin < reduced flavin. The largest factor in the substantially low pK_a of reduced flavin in DAO is probably the steric hindrance between the hydrogen atom of H-N(1)(flavin) and the hydrogen atom of H-N of Gly315, which becomes significant when a hydrogen is bound to N(1) of flavin.

Key words: D-amino acid oxidase, enzyme-ligand interaction, flavoenzyme, nuclear magnetic resonance, Raman spectra.

Porcine kidney D-amino acid oxidase [D-amino acid:O_2 oxidoreductase (deaminating), EC 1.4.3.3] (DAO), which has FAD as the prosthetic group, is one of the most extensively investigated flavoenzymes. DAO catalyzes the dehydrogenation of the substrate D-amino acid to the corresponding imino acid; the imino acid is spontaneously hydrolyzed to 2-ketoacid and ammonia. Among D-amino acids, neutral amino acids are preferred substrates, basic amino acids are poor substrates, and acidic amino acids are not substrates for DAO (*1*). The reduced enzyme is reoxidized by molecular oxygen, with the production of H_2O_2. The kinetic mechanism of DAO reaction with neutral (upper loop) and basic (lower loop) amino acids as substrates is illustrated in Scheme 1: Eo is oxidized DAO, Er is reduced DAO, S is a substrate D-amino acid, P is the imino acid derived from the substrate, and square brackets show the enzyme species inferred from kinetic studies (*1*). Purple intermediate (Er···P), which exhibits a charge-transfer absorption band, is a complex between reduced DAO and the imino acid derived from a substrate; the intermediate is not observed with basic D-amino acid substrates. However, the detailed molecular mechanism of the substrate specificity and the catalytic reaction is not clear at present.

With the recent development of analytical methods for optically active substances, the presence of D-amino acids in the animal tissues has been demonstrated. The distribution of D-serine in rat brain region is positively correlated with the distribution of *N*-methyl-D-aspartate (NMDA) receptor (*2–4*). The localization of DAO was inversely correlated with the distribution of free D-serine in mammalian

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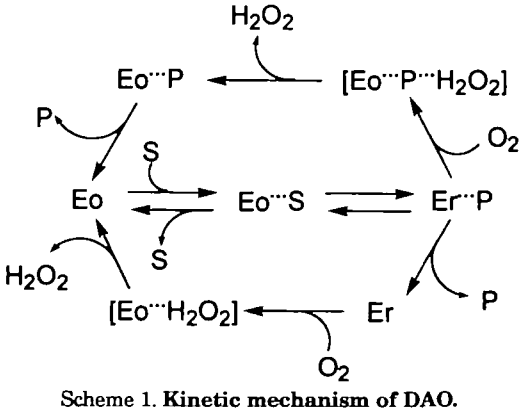
Abbreviations: DAO, D-amino acid oxidase; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; NMIN, *N*-methylisonicotinate; RR spectra, resonance Raman spectra.

brains (*5*). Furthermore, serine racemase, which catalyzes the direct racemization of L-serine to D-serine, was recently purified from rat brain (*6*). Thus, it was established that both biosynthetic and degrading enzymes for D-serine exist in mammalian brain (*6*). In addition to these data suggestive of the physiological role of DAO, the three-dimensional structure of DAO has recently been solved (*7*, *8*). Such progress spurred interest in the physiological role and the catalytic mechanism of DAO in recent years.

On the basis of the three-dimensional structure of DAO-ligand complexes (*7–9*), several reaction mechanisms for the reductive-half reaction of DAO have been proposed (*8*, *9*): (i) the electron-proton-electron transfer mechanism (*9*); (ii) the ionic mechanism (*9*); and (iii) the hydride transfer mechanism of the α-hydrogen (*8*). In all reaction mechanisms, the redox reaction accompanies the transfer of a charge: on the whole a hydride ion equivalent transfers to the oxidized flavin. Electrostatic interaction is generally important in enzyme-substrate interaction, as also in DAO, because the active center is surrounded by hydrophobic residues and a few polar side-chains: electrostatic effects are small in aqueous solution, but are large in hydrophobic media. Therefore, information on the ionic atmosphere and the charged forms of the flavin and the substrate bound to DAO in each step of the reaction pathway is essential for elucidation of the substrate specificity and the reaction mechanism. We previously reported that trigonelline, which has a zwitterionic structure similar to a substrate D-amino acid, is a useful ligand for elucidation of the charged form of a substrate bound to DAO (*10*). Trigonelline binds to oxidized DAO, lowering the pK_a value at N(3)-H of the flavin ring: that is, the insertion of a positive charge into the active site results in thermodynamical destabilization of the active site; the active site is stabilized by a proton release. On the basis of this phenomenon, we discussed proton release in the catalysis of DAO; when a substrate D-amino acid with a dissociable proton, different from trigonelline, binds to DAO, the ammonium group ($-NH_3^+$) probably loses a proton to become neutral ($-NH_2$) before the oxidation (*11*). Furthermore, trigonelline is also a good ligand for semiquinoid and the reduced form of DAO, in addition to the oxidized form of DAO (*12*). The reactivity of the complex between reduced DAO and trigonelline with molecular oxygen is similar to that of the purple intermediate, suggesting that the complex is a good model for elucidation of the nature of the purple intermediate. In addition, the complex of anionic semiquinoid DAO with trigonelline is O_2-insensitive and not reduced by dithionite or photoirradiation (*12*). These phenomena of the DAO-trigonelline complex are not observed with the complex of DAO with a monoanionic ligand such as benzoate. Therefore, comparison of the interaction modes of DAO with zwitterionic and monoanionic ligands seems very valuable for the elucidation of the substrate specificity and the reaction mechanism.

Although many monoanionic ligands such as benzoate derivatives are known, only a few zwitterionic ligands are found. In this paper, we extend our previous study and generalize our conclusion (*10*) using other zwitterionic ligands as well as monoanionic ligands; we found that *N*-methylisonicotinate (NMIN) (I) is another good zwitterionic ligand for DAO in three redox states, hence a useful active site probe. We compared the interaction of monoanionic and zwitterionic ligands with DAO in three redox states by spectrophotometric and thermodynamical analyses, and then scrutinized the electrostatic interaction in the active site and on the charged forms of flavin and substrate bound to DAO. We discuss other factors for substrate binding, *i.e.*, steric effect and charge-transfer interaction. We also discuss the mechanism responsible for the difference in reactivity with DAO among neutral, basic, and acidic D-amino acids.



Scheme 1. **Kinetic mechanism of DAO.**

MATERIALS AND METHODS

Porcine kidney D-amino acid oxidase and the apoenzyme were purified as described elsewhere (*13–15*). Benzoate was removed from the holoenzyme purified in the form of its benzoate complex by the method reported previously (*16*). The enzyme reconstituted with [4,10α-$^{13}C_2$]FAD was prepared by the reported procedure (*17*). The concentration of

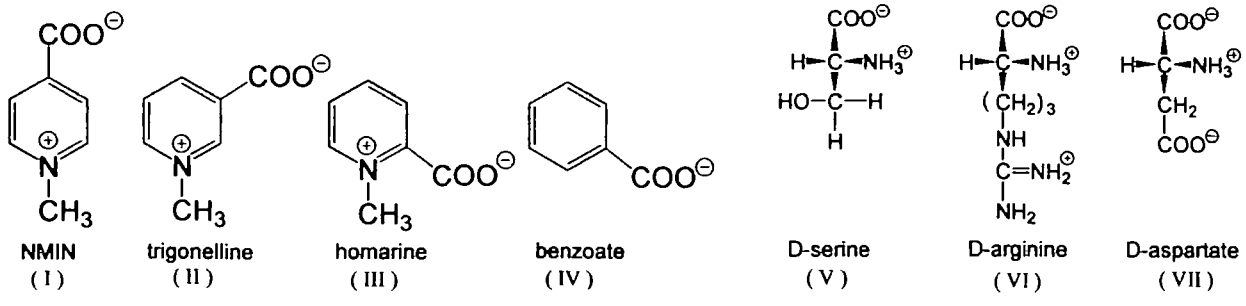


Chart 1.

oxidized DAO was determined spectrophotometrically, using the molar absorption coefficient of 11,300 $M^{-1}cm^{-1}$ at 455 nm.

NMIN and homarine (*N*-methylpicolinic acid) were prepared from isonicotinic acid and picolinic acid, respectively, by *N*-methylation with CH_3I according to the procedure for the synthesis of labeled homarine (*18*). Other chemicals were of the highest grade available from commercial sources.

Visible absorption spectra were measured with Hitachi 220A or U-2000 spectrophotometers thermostated at 25°C. Transient kinetic measurements were performed on a rapid-scanning and stopped-flow spectrometer system, Otsuka Electronics RA-415S and RA-401. Raman spectra were obtained with a JASCO NR-1800 spectrometer (Japan Spectroscopic) with a He-Ne laser (NEC GLG 5900) or an Ar ion laser (INNOVA 70-2) as a light source. The wavenumber axis of the Raman spectra was calibrated for indene. The resonance Raman (RR) spectra were measured in 50 mM sodium pyrophosphate buffer (pH 8.3) at room temperature (*ca.* 25°C).

^{13}C-NMR spectra were measured in Wilmad 5-mm NMR tubes with a Varian UNITY Plus 500 spectrometer operating at 125.7 MHz under proton irradiation. The ^{13}C-chemical shift was scaled in ppm downshift relative to the methyl-carbon signal of external 3-(trimethylsilyl)propionate-d_4. ^{13}C-NMR spectrum of the reduced DAO solution containing 60 mM benzoate was measured at 25°C. The sample was prepared by directly adding a molar excess of solid dithionite to a preparation in an NMR tube. The sample was flushed with nitrogen gas, then the NMR tube was sealed under vacuum. The NMR measurement was carried out in the sealed tube.

Spectrophotometric titration of oxidized DAO, semiquinoid DAO, or reduced DAO with ligands was carried out as described elsewhere (*10, 19, 20*).

RESULTS

Oxidized DAO—Figure 1 shows the absorption spectra of the FAD part of the oxidized DAO in the absence (a) and presence (b) of NMIN (I) at pH 8.5. The spectral changes observed indicate the complex formation of DAO with NMIN. The peak of the second absorption band around 375 nm shifts to a shorter wavelength; these spectral changes are similar to those in the complexation with trigonelline (II) (*10*). Such spectral changes are, therefore, due to the release of the flavin N(3)-imino proton as the result of decreased pK_a value. Contrary to the zwitterionic ligands, benzoate (IV) (monoanionic ligand) suppresses the proton release from flavin N(3)-H (*21, 22*). These results indicate that monoanionic ligands have higher affinity for the neutral form of flavin [N(3)-H], whereas zwitterionic ligands have higher affinity for the anionic form [$N(3)^-$].

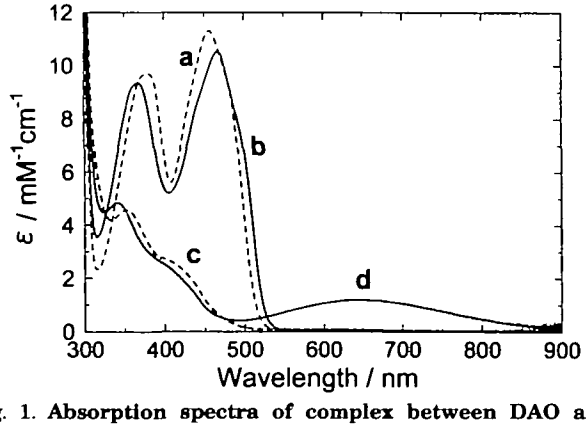


Fig. 1. **Absorption spectra of complex between DAO and NMIN.** Spectra were measured in 100 mM sodium pyrophosphate buffer, pH 8.5. The concentrations were: (a) DAO (43 μM); (b) DAO (39 μM), NMIN (6.7 mM); (c) DAO (42 μM), D-arginine (16 mM); (d) DAO (39 μM), NMIN (6.6 mM), D-arginine (14 mM).

The deprotonation of flavin N(3)-imino group causes a marked increase in the absorption coefficient at 360 nm, which provides a sensitive means for determination of the pK_a value at the flavin N(3)-imino group (*21*). Figure 2 shows the pH-dependence of the molar absorption coefficients of DAO-NMIN complex at 360 nm between pH 5.6 and 10.2; the data for unliganded DAO and DAO-trigonelline complex are also shown for comparison. We determined the pK_a value of the flavin 3-imino group in DAO-NMIN complex to be 8.1 by the same procedure as that for DAO or DAO-trigonelline complex (*10*); the pK_a values in unliganded DAO and DAO-trigonelline complex are *ca.* 9.2 and 8.1, respectively (Table I). The low pK_a value of DAO-NMIN complex, compared with that of DAO, clearly shows that the proton of flavin N(3)-H can be released by the complex formation. The pK_a value increases in complexation with benzoate (*21*) (Table I).

For detailed understanding of the binding reaction between NMIN and DAO, we measured transient absorption

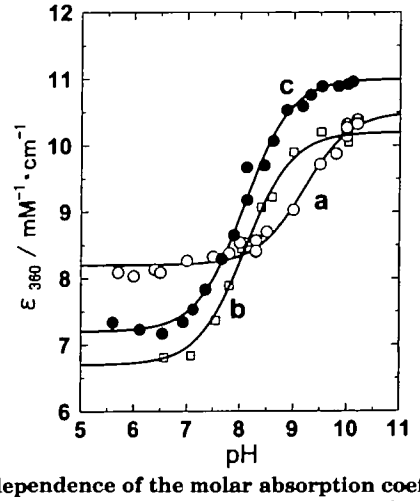


Fig. 2. **pH-dependence of the molar absorption coefficients of DAO (○), DAO-trigonelline complex (●), and DAO-NMIN complex (□) at 360 nm.**

TABLE I. **pK_a values of flavin in three redox states.**

	pK_a (Free)	pK_a (DAO)	pK_a (Free) – pKa (DAO)	pK_a (T)	pK_a (N)	pK_a (B)
Oxidized	10 (N(3))	9.2	0.8	8.1[a]	8.1	>10.9[b]
Semiquinoid	8.3 (N(5))	4.0	4.3			
Reduced	6.7 (N(1))	<1	>5.7			

T, N, and B represent the complexes of DAO with trigonelline, NMIN, and benzoate, respectively. [a]Value from Nishina *et al.* (*10*) and [b]value from Massey and Ganther (*21*).

spectra. Figure 3A shows the rapid-scan spectra for the binding of NMIN with DAO, and Fig. 3B shows the time course of the absorbance changes at 450 and 504 nm at pH 8.3. The "20 ms" spectrum (trace b in Fig. 3A) is distinctly different from that of either DAO or the DAO-NMIN complex in an equilibrium state [320 ms spectrum (trace c in Fig. 3A)], and cannot be reconstituted from the combination of these individual spectra. The "20 ms" spectrum is that of the intermediate species, indicating the presence of two phases, *i.e.*, rapid and slow phases; the slow phase (Fig. 3B) is not detected in the case of benzoate-binding (*22*). The binding process involves an intermediate species which is rapidly formed, in analogy to the complexation with trigonelline (*10*). We presume that the slow relaxation process is due to the slow proton release from the 3-imino group of FAD, triggered by the complexation with NMIN. Both of the apparent rate constants of the absorbance changes at 450 and 504 nm for the slow phase are 19 s^{-1}, which are 5 times larger than those for trigonelline complexation (*10*).

The relationship between the apparent dissociation constant (K_d) for DAO-NMIN complex and pH is shown in Fig. 4; the pH profiles for benzoate- (*22*) and trigonelline-complexes (*10*) are also shown for comparison. The K_d values for NMIN are larger than those for trigonelline over the whole pH region examined, but the pH profiles for these ligands are very similar to each other and very different from that for benzoate; the dissociation constant with NMIN or trigonelline decreases with increasing pH 8 to 9.5, while the constant with benzoate increases. The difference is associated with a proton release from N(3)-H of FAD as described in "DISCUSSION."

As the affinity of homarine (III) to oxidized DAO is too weak for spectrophotometric titration, the dissociation constant (34 mM) of homarine at pH 8.3 was obtained by a competitive binding method (*20*), *i.e.*, analysis of the effect of homarine on the titration curve of *o*-aminobenzoate.

Semiquinoid DAO—Figure 5 shows the absorption spectra of anionic semiquinoid DAO in the absence and presence of NMIN. The anionic semiquinone of DAO was prepared by anaerobic photoreduction catalyzed by 3-methyllumiflavin with EDTA as an electron donor (*23*). The spectral changes after mixing with NMIN indicate the complex formation of anionic semiquinoid DAO with NMIN. The spectrum of the complex is similar to that of the complex of the anionic semiquinone with picolinate (*24*) or trigonelline; the NMIN complex shows hypochromism of the absorption band around 370 nm, the loss of the shoulder at 400 nm, and appearance of a long wavelength absorption band extending beyond 800 nm, in comparison with that of free anionic semiquinoid DAO. The relationships between K_d and pH for NMIN- and trigonelline-semiquinoid DAO are shown in Fig. 6. The K_d values are apparently pH-independent, as are those with D-alanine (*19*). The dissociation constants of anionic semiquinoid DAO with trigonelline and NMIN are 25 and 320 μM, respectively.

Light irradiation of DAO in the presence of EDTA at all pH values (from 6.0 to 8.5) examined failed to show any evidence for the formation of the neutral semiquinone, which is in accord with the previous reports (*23–25*). Though the neutral semiquinone form is not observed in the presence of such zwitterionic ligands, neutral semiquinone can be formed by addition of excess benzoate to the anionic semiquinone form of DAO (*25*). Figure 7 shows the absorption spectrum of semiquinoid DAO and that of semiquinoid DAO in the complex with benzoate. These spectral patterns are similar to those reported by Yagi *et al.* (*25*). We obtained the dissociation constant between semiquinoid DAO and benzoate at various pHs by spectrophotometric titration: the constant was 21 mM at pH 8.4, which is comparable to 20 mM obtained at pH 8.3 (*25*). The relationship between –log K_d and pH is shown in the inset of Fig. 7. The value of –log K_d for benzoate decreases monotonously with the increase of pH value, and the slope is nearly minus one,

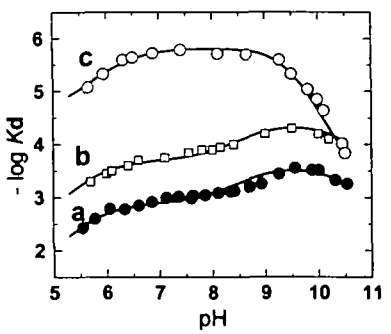
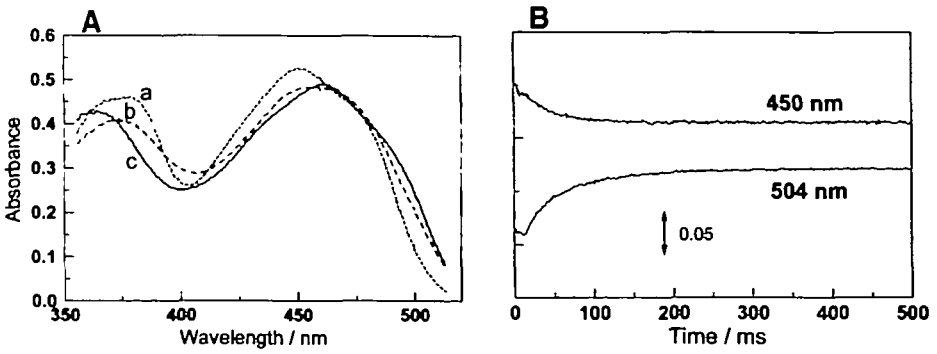


Fig. 4. **pH-dependence of the apparent dissociation constant of DAO with NMIN (a), trigonelline (b), and benzoate (c).** The concentrations of the enzyme used were *ca.* 40 μM. The data for trigonelline and benzoate are cited from Nishina *et al.* (*10*) and Quay and Massey (*22*), respectively. The solid line in (a) is the theoretical curve fitted to Eq. 4 in Ref. *10* with $pK_1 = 9.3$, $pK_2 = 9.0$, $pK_3 = 10.2$, $pK_5 = 8.1$, $pK_8 = 5.8$, and $K_{a1} = 10^{3.0}$ M^{-1}. The parameters are the same for trigonelline (*10*) except for K_{a1}.

Fig. 3. **Rapid-scanning spectra for the binding of NMIN with DAO (A) and absorbance changes at 450 and 504 nm after mixing DAO and NMIN (B).** The experiments were carried out in 100 mM sodium pyrophosphate buffer, pH 8.3. A: The spectrum of DAO (a) was measured by rapid-scanning by mixing DAO and buffer solution without NMIN. The concentrations of the solutions before mixing were: DAO (80.6 μM); NMIN (10.1 mM). (b) after 20 ms, (c) after 320 ms. B: The concentrations of the solutions before mixing were: DAO (80.6 μM); NMIN (10.1 mM).



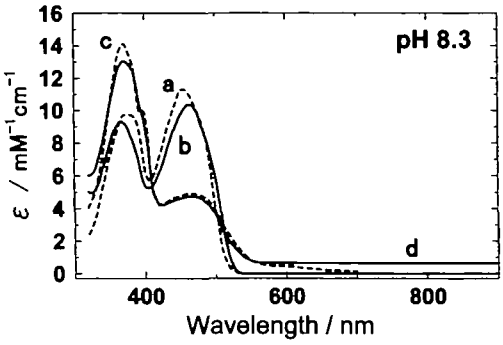


Fig. 5. **Absorption spectra of the complex of NMIN with semiquinoid DAO prepared by photoirradiation.** Spectra were measured in 100 mM sodium pyrophosphate buffer, pH 8.3. (a) DAO (39 μM) without photoirradiation; (b) DAO (36 μM), NMIN (7.6 mM) without photoirradiation; (c) DAO plus EDTA (2 mM) and 3-methyllumiflavin (3 μM) under anaerobic conditions, after 2-min photoirradiation; (d) after the addition of NMIN (1.3 mM) from side arm of anaerobic cell to sample (c).

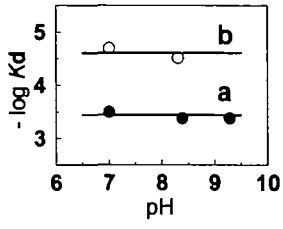


Fig. 6. **pH dependence of the apparent dissociation constant of anionic semiquionoid DAO and NMIN (a) and trigonelline (b).** The concentrations of the enzyme used were *ca.* 40 μM.

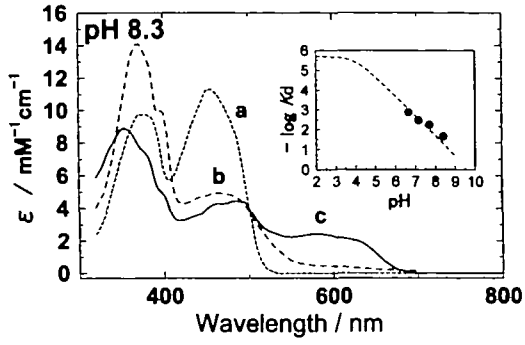


Fig. 7. **Absorption spectra of semiquinoid DAO and its benzoate complex.** Spectra were observed in 100 mM sodium phosphate buffer, pH 7.0. (a) DAO (37 μM) plus EDTA (2 mM) and 3-methyllumiflavin (2.5 μM) under anaerobic conditions; (b) after 2-min photoirradiation; (c) after the addition of benzoate (10 mM) from the side arm of the anaerobic cell. The inset shows the pH-dependence of the apparent dissociation constant between semiquinoid DAO and benzoate. The dashed line is the theoretical curve fitted to Eq. 1 with $K_a = 10^{-4.05}$ and $K_d^0 = 2$ μM (see "DISCUSSION").

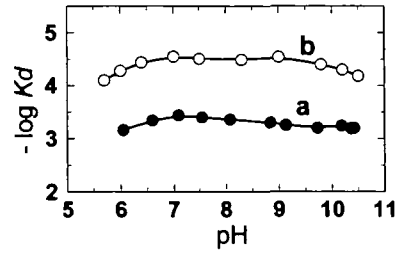


Fig. 8. **pH-dependence of the apparent dissociation constant of reduced DAO with NMIN (a) and trigonelline (b).** The concentrations of the enzyme were *ca.* 40 μM. The data for trigonelline are cited from Nishina *et al.* (*12*).

indicating that one proton is associated with the complex formation; the proton should be the one at N(5)-H of semiquinoid flavin. Therefore, benzoate can bind to neutral semiquinoid DAO but not to the anionic form. These results indicate that the zwitterionic ligands have fairly high affinity for the anionic semiquinone, and that a monoanionic ligand (benzoate) has higher affinity for the neutral semiquinone than for the anionic semiquinone.

Reduced DAO—Reduction of DAO by D-arginine in the presence of NMIN produces an absorption spectrum having an absorption band around 650 nm (Fig. 1d); the spectrum is similar to that of reduced DAO-picolinate complex (*20*). Reduction of DAO with D-arginine in the absence of NMIN produces no such absorbance (Fig. 1c). This indicates that NMIN binds to reduced DAO, accompanying a charge-transfer absorption band. The spectrophotometric titration with NMIN of reduced DAO prepared by D-arginine (data are not shown) indicates that the ligand binds to reduced DAO in a 1:1 molar ratio, as in the case of picolinate- (*20*) or trigonelline-binding (*12*). The relationship between the apparent dissociation constant (K_d) and pH is shown in Fig. 8; the pH profile for trigonelline (*12*) is also shown for comparison. The K_d values for NMIN are larger than those for trigonelline over the whole pH region examined, but the pH profiles are similar to each other. The dissociation constant of anionic reduced DAO [the flavin in reduced DAO is in the anionic reduced form (*26*)] with trigonelline and NMIN around pH 8 are 32 and 400 μM, respectively.

Figure 9 shows a RR spectrum of the complex of reduced DAO with NMIN and a Raman spectrum of free NMIN. The spectrum of the complex has a similar spectral pattern to those of DAO purple complexes (*17*, *27*, *28*) and to that of the complex of reduced DAO with picolinate (*20*). The 1,609-cm^{-1} band is derived from a C(4*a*)=C(10*a*) stretching mode of reduced flavin (*29*). The 1,641 and 1,377 cm^{-1} bands are probably derived from a ring stretching mode and a COO^- symmetric stretching mode of NMIN, respectively. In line with the complex of oxidized DAO with the ligand [*o*-aminobenzoate or aminoethylcysteine-ketimine (*30*)], the band of COO^- stretching of NMIN in the complex shifted to lower frequencies than that free in solution (1,385 cm^{-1}). Such low-frequency shifts unequivocally indicate that COO^- interacts with chemical groups in the active site of DAO. The X-ray crystal structure analysis of DAO-benzoate (*7*, *8*) and DAO-*o*-aminobenzoate (*9*) complexes showed that the carboxylate group of the ligands makes an ion-pair with the guanidino group of Arg283 and a hydrogen bond with the hydroxyl group of Tyr228 (*7–9*). The X-ray analysis of reduced DAO in the complex with the reaction product 2-imino acid showed the existence of the same interactions as those with oxidized DAO (*31*, *32*). Therefore, the low-frequency shifts result from such interactions.

Previously we examined the affinity of benzoate for reduced DAO by a competitive binding method using picoli-

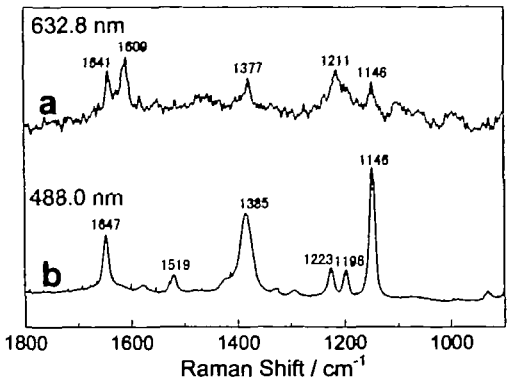


Fig. 9. **Resonance Raman spectrum of the complex between reduced DAO and NMIN excited at 632.8 nm, and Raman spectrum of NMIN excited at 488.0 nm.** Spectra were recorded in 100 mM sodium pyrophosphate buffer, pH 8.3. Concentrations were: (a) DAO (0.52 mM), NMIN (56 mM), D-arginine (20 mM); (b) NMIN (250 mM).

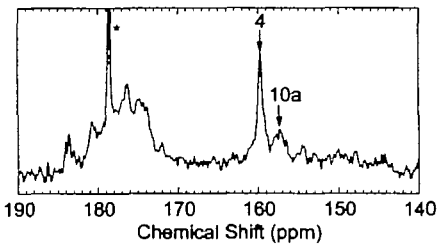
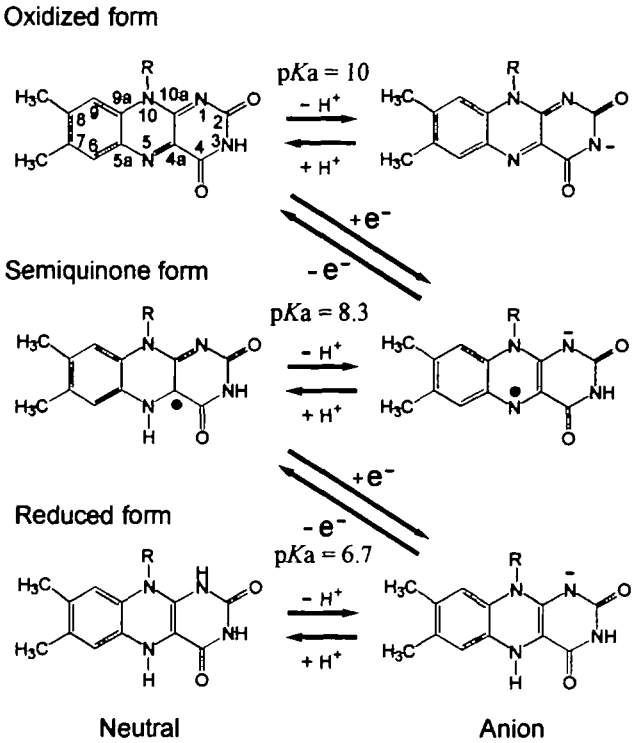


Fig. 10. **^{13}C-NMR spectrum of DAO reconstituted with [4,10α-$^{13}C_2$]FAD in the reduced state.** Concentrations were: DAO (0.8 mM), benzoate (60 mM), and dithionite (100 mM) (157,000 acquisitions). The asterisk indicates the signal for the carboxylate of benzoate.

nate as a ligand at pH 8.3. Even if benzoate should have affinity for reduced DAO, the value of the dissociation constant is greater than 10 mM at pH 7.0 (*20*), *i.e.*, a monoanionic ligand such as benzoate does not bind to anionic reduced DAO. Benzoate can bind to neutral semiquinoid DAO, although a monoanionic ligand such as benzoate has little affinity for anionic semiquionoid or anionic reduced DAO. Thus benzoate can probably also bind to neutral reduced DAO. Therefore, it is expected that anionic reduced DAO is converted into neutral reduced DAO in the presence of benzoate, as observed in the case of semiquinoid DAO. In order to examine the presence of neutral reduced DAO, we measured the ^{13}C-NMR spectrum of reduced DAO reconstituted with [4,10α-$^{13}C_2$]FAD in the presence of a high concentration of benzoate (60 mM) at pH 5.7. The chemical shift values of 10α-^{13}C of neutral and anionic forms of reduced FMN are 145.9 and 157.1 ppm, respectively (*26*, *33*). As shown in Fig. 10, the signal for 10α-^{13}C was observed at 157.3 ppm but not at around 146 ppm. This indicates that neutral reduced flavin scarcely exists under these conditions. As discussed later, the pK_a value at N(1) of reduced flavin in DAO is probably too low for anionic reduced flavin to be converted into the neutral form even in the presence of benzoate.

DISCUSSION

1. Interaction Mode of DAO with Substrate Analogs—The



Scheme 2. **Some structures of neutral and anionic flavin in the three redox states.**

interaction mode of substrate analogs with DAO in three redox states is discussed in this section, and the effect of the net charge of the analogs and the chemical groups in the cavity of the active site will be discussed in detail in the following sections.

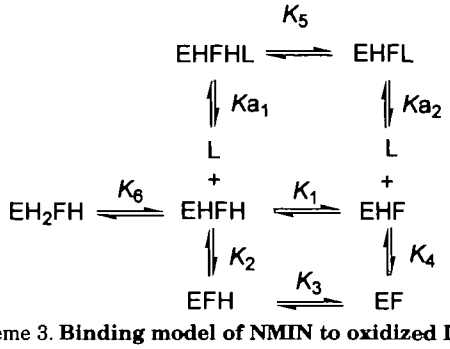
Oxidized DAO—The pH profile (Fig. 4a) for the affinity of NMIN to oxidized DAO was analyzed on the basis of the same model (Scheme 3) as used for trigonelline (*10*).

E, EH, and EH_2 represent the different protonated forms of the apoprotein: FH and F represent the neutral and anionic form of FAD (Scheme 2), respectively; and L represents NMIN. The equilibrium constants, K_1–K_6, and association constants, K_{a1} and K_{a2}, are defined in Scheme 3. The solid line for NMIN in Fig. 4 is drawn by using the same parameters for trigonelline except for K_{a1}; $10^{3.0}$ M^{-1} was used as K_{a1} instead of $10^{3.7}$ M^{-1} for trigonelline. The line fits the experimental values well, indicating that the affinity of NMIN for oxidized DAO is five times weaker than trigonelline and that both pH dependencies on the affinity are similar to each other. The K_{a2} is $10^{4.9}$ M^{-1} (trigonelline) or $10^{4.2}$ M^{-1} (NMIN). This indicates that the zwitterionic ligands have higher affinity for the anionic form [flavin N(3)-] than for the neutral form [flavin N(3)-H]. The effect of net charges will be discussed in detail in the following sections.

The affinity of the ligands (I, II, III) also depends on the position of the *N*-methyl group with respect to the carboxylate. The dependence is similar to that observed with methylbenzoate, a monoanionic ligand; the affinity of *m*-methylbenzoate is six times higher than that of *p*-methylbenzoate, and that of the *ortho*-derivative is very low (*21*). The low affinity of the *ortho*-derivative is attributed to steric hindrance of the substituent group (*34*). The structure of the active site of DAO-benzoate complex is shown in Fig. 11 (*7*).

The distance between one of the *ortho* carbons of benzoate and the phenol carbons of Tyr228 is very short (3.29–4.71 Å for six carbons), while the other *ortho* carbon is near the carbonyl oxygen of Gly313. In the case of DAO-*o*-aminobenzoate complex, the distance between the backbone carbonyl oxygen of Gly313 and the amino nitrogen of *o*-aminobenzoate is 2.6 Å (*9*). Therefore, the methyl group at the *ortho* position results in steric hindrance. *o*-Aminobenzoate has higher affinity than *meta*- or *para*-aminobenzoate, which is probably due to the hydrogen bonding between the amino group and the carbonyl oxygen of Gly313 (*9*). *o*-Hydroxybenzoate also has higher affinity than the *meta* or *para* derivatives (*21*), probably due to the expected hydrogen bonding between the hydroxyl group and Gly313 C=O.

The affinity of *meta*- and *para*-substituted benzoates for oxidized DAO correlates with their Hammet's σ values (*34–36*). This suggests that the active site is large enough to accomodate these derivatives, and that the substituent at *meta*- or *para*-positions, whose size is similar to a methyl group, does not give rise to steric hindrance with chemical groups in the active site; this is explained by the X-ray crystallographic analysis (*7–9*). The carboxylate group of benzoate makes an ion-pair with the guanidino group of Arg283 and a hydrogen bond with the hydroxyl group of Tyr228 (*7*, *8*) (Fig. 11). When the substituent group has a high electron-withdrawing character, the negative charge at the carboxyl group of benzoate derivative should decrease. We can thus expect that the electrostatic interaction in the ion-pair with the guanidino group should weaken, resulting in lower affinity. Contrary to this, however, the affinity definitely increases as the electron-withdrawing character of the substituent group increases (*34–36*). Therefore, another interaction must be involved in addition to the electrostatic attractive force. Fonda and Anderson (*34*) proposed that a non-polar region containing an aromatic residue exists on the enzyme, and that the binding of benzoate would then involve an electrostatic interaction plus π orbital overlapping of aromatic rings of the ligand and a protein moiety. The expected aromatic amino acid residue should be Tyr224, as shown in Fig. 11. The more electron-deficient the aromatic ring of the inhibitor becomes (benzoate derivative with electron-withdrawing substituent), the more effectively the compound interacts with DAO. In this context, the phenol group of Tyr224 should act as an electron donor and the benzene ring of benzoate derivative as an electron acceptor, and the HOMO-LUMO interaction



Scheme 3. **Binding model of NMIN to oxidized DAO.**

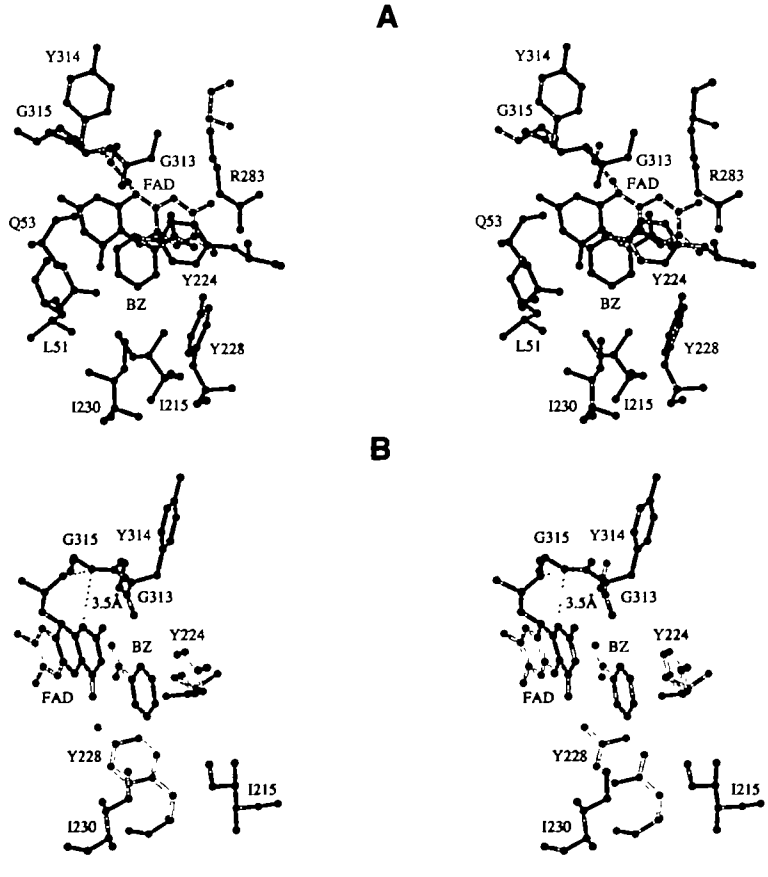


Fig. 11. **Stereo views of the active site in DAO-*o*-aminobenzoate complex.** Structures were constructed by MOLSCRIPT (*44*) using the coordinates of DAO-benzoate complex [refinement of the initial structure (*7*)].

between them should be important for the ligand binding. It is, therefore, deduced that the interaction between oxidized flavin and the benzene ring of a benzoate derivative is smaller than that between the phenol group of Tyr224 and the benzene ring. If the interaction were stronger, the relationship between K_d and the σ value would be the opposite of the observed relationship (*34–36*), because oxidized flavin should act in this case as an electron acceptor and the benzene ring as an electron donor. Indeed, X-ray crystallographic analysis clearly shows that the interaction of the benzene ring of benzoate or *o*-aminobenzoate with the Tyr224 phenol ring is greater than that with flavin ring (*7, 9*).

Semiquinoid DAO and Reduced DAO—Anionic semiquinoid DAO converts into neutral semiquinoid DAO in the presence of benzoate (Fig. 7, b and c), indicating that benzoate can bind to neutral semiquinoid DAO but not to the anionic form. The reaction scheme for the complex formation of semiquinoid DAO with benzoate can thus be assumed to be as follows:

$$EF_{1e}H \overset{K_a}{\rightleftharpoons} EF_{1e}^- + H^+ \quad (1)$$

$$EF_{1e}H{\cdot}L \overset{K_d^0}{\rightleftharpoons} EF_{1e}H + L \quad (2)$$

Here, EF_{1e}^-, $EF_{1e}H$, and L are the anionic semiquinoid DAO, neutral semiquinoid DAO, and benzoate, respectively. The values of K_a and K_d^0 are the acid dissociation constant of semiquinoid flavin and the dissociation constant between the neutral semiquinoid DAO and benzoate, respectively. The apparent dissociation constant, K_d, is given by the following equations:

$$K_d = \frac{([EF_{1e}^-] + [EF_{1e}H])[L]}{[EF_{1e}H{\cdot}L]} \quad (3)$$

$$K_d = K_d^0 (1 + K_a/[H^+]) \quad (4)$$

The value of $-\log K_d$ linearly decreases with increasing pH, and thus we cannot obtain the pK_a value of semiquinoid flavin in DAO directly from the data alone (Fig. 7, inset). If we assume that K_d^0 takes the same value as the dissociation constant (2 μM) between oxidized DAO and benzoate, however, we can estimate $pK_a = 4$ (Table I) by fitting (dashed line in Fig. 7 inset). The assumption can be justified, because the isoalloxazine ring of FAD in both oxidized and neutral semiquinoid DAO is in the neutral form, and thus the electrostatic interaction benzoate receives in the active site should be very similar, if not identical, in the two forms.

The pH profiles of the affinities of NMIN and trigonelline for reduced DAO in the acidic pH region (Fig. 8) are similar to those of the ligands and benzoate for oxidized DAO (Fig. 4). This suggests that the acidic residue associated with binding is common in both redox states. The flavin in reduced DAO is in anionic form (*26*). Thus, the decrease of the affinity at pHs higher than 10, particularly that observed with trigonelline, should be due to a basic group of the apoenzyme.

D-Alanine has a significant affinity (K_d = 2.8 mM) for anionic semiquinoid DAO (*19*), while the binding of D-alanine to reduced DAO cannot be observed (*20*). As D-alanine can exist in the same zwitterionic form as trigonelline or NMIN, an electrostatic repulsion cannot be responsible for its inability to bind to reduced DAO. Anionic semiquinoid flavin has no hydrogen atom at N(5), but anionic reduced flavin (Scheme 2) has a hydrogen atom at this position. According to the molecular modeling of DAO-D-leucine complex, the α-proton is directed toward the lone-pair orbital of N(5) of oxidized flavin (*9*). Thus steric hindrance should arise between the α-proton of D-alanine and the N(5)-H hydrogen of reduced flavin in the binding of D-alanine to reduced DAO. This steric hindrance probably impedes access of D-alanine to reduced DAO.

If we assume, in analogy to the case of the semiquinoid DAO, that benzoate can bind to the neutral reduced form (K_d^0 = 2 μM) but cannot bind to the anionic reduced form, we can also use Eq. 4 to estimate the pK_a at N(1)-H of reduced flavin in DAO. Suppose for the moment that the pK_a of N(1)-H is 1, the apparent dissociation constant (K_d) of benzoate for reduced DAO is calculated to be *ca.* 100 mM at pH 5.7 of the NMR experiment. Thus, the proportion of the benzoate-bound neutral reduced DAO, under the experimental conditions ([DAO] = 0.79 mM and [benzoate] = 60 mM), is calculated to be 37% of the total enzyme. Thus, if the pK_a is larger than 1, we should be able to observe a signal of neutral reduced flavin. However, we can detect only a signal of anionic reduced flavin (Fig. 10). Therefore, we can estimate that pK_a is below 1 (Table I).

The following conclusions can be drawn from the present results. When flavin is in a neutral form in any of the three redox states (Scheme 2), a monoanionic ligand has higher affinity toward the enzyme than a zwitterionic ligand. When flavin is in an anionic form, on the other hand, a zwitterionic ligand has higher affinity. There are only three chemical groups that can have a charge in the active site in the physiological pH region, *i.e.*, Arg283, flavin, and a ligand bound to the active site. Therefore, the active site is more stable with a combination of chemical groups that result in no net charge as a whole in the active site, *i.e.*, (i) Arg283 (positive), flavin (neutral), ligand (anion); (ii) Arg283 (positive), flavin (anion), ligand (zwitterion). Such conclusions are also discussed in detail in the following sections.

2. Electrostatic Interaction in Three Redox States—Figure 12 compares the affinities of trigonelline and NMIN for DAO in the three redox states. The affinities of both ligands

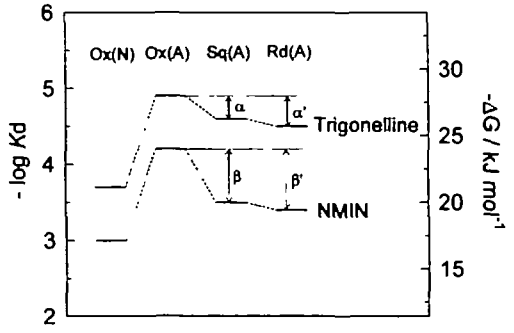


Fig. 12. **The affinities of zwitterinonic ligands (NMIN and trigonelline) for DAO in three redox states.** Ox(N), Ox(A), Sq(A), and Rd(A) show neutral oxidized, anionic oxidized, anionic semiquinoid, and anionic reduced forms of DAO, respectively. The K_d values for oxidized DAO were obtained by analysis of the pH profiles (Fig. 4), and the values for semiquinoid and reduced forms of DAO are those around pH 8. The K_d value of benzoate for oxidized DAO (neutral form) is 2 μM, and benzoate has substantially no affinity for DAO (anionic form) in the three redox states.

for oxidized DAO, whose flavin is in the anionic form, are stronger than those for DAO whose flavin is in the neutral form; and the differences between anionic and neutral forms of flavin are similar in the two ligands. This suggests that the electrostatic interaction between the positive charge of the ligand and the negative charge of flavin contributes equally to the affinity of both ligands. The affinity of both ligands for semiquinoid and reduced enzymes, where the flavin is anionic, is weaker than the affinity for the oxidized enzyme, whose flavin is in the anionic form. This suggests that the electrostatic interaction is weaker in semiquinoid and reduced enzymes than in the oxidized enzyme. When flavin changes from oxidized to semiquinoid or reduced state, the localization of the negative charge of anionic flavin shifts and thus deviates away from the cationic nitrogen atoms of the ligands, as explained below.

We can assume that the positioning of both ligands is essentially the same as that of complexed benzoate, since the active site, as revealed by crystallography, can accommodate these ligands without steric hindrance. Although the negative charge of flavin is delocalized within the flavin ring, a high charge density at particular atoms is maintained. The charge at each atom has been calculated by *ab initio* MO calculation of 7,8-demethyllumiflavin (*37*); the charge at some atoms and groups are as follows: in anionic oxidized flavin: N(1), $0.347e^-$; N(3), $0.479e^-$; C(4)=O, $0.105e^-$; N(5), $0.263e^-$; in anionic semiquinoid flavin: N(1), $0.425e^-$; N(3)-H, $0.224e^-$; C(4)=O, $0.085e^-$-; N(5), $0.266e^-$; in anionic reduced flavin, N(1), $0.440e^-$; N(3)-H, $0.218e^-$; C(4)=O, $0.141e^-$; N(5)-H, $0.158e^-$. The negative charge at N(3) is the largest in anionic oxidized flavin, while the charge at N(1) is the largest in anionic semiquinoid and reduced enzymes. This movement of the negative charge is responsible for the weak electrostatic interaction in semiquinoid or reduced state, for the N(1) position is remote from the aromatic ring of the ligands (Fig. 11).

Between two possible orientations of trigonelline, *i.e.*, those in which the *N*-methyl group is near or distant from flavin N(3), the "near" orientation is expected to avoid steric hindrance. The affinity difference between anionic oxidized DAO and anionic reduced/semiquinoid DAO is smaller with trigonelline (α, α′ in Fig. 12) than that with NMIN (β, β′), indicating that the electrostatic interaction of the positive charge of trigonelline with anionic semiquinoid or reduced flavin is stronger than that in the case of NMIN. Thus, the nitrogen atom of trigonelline should be situated nearer to flavin than that of NMIN. This is supported by the crystallographic structure of DAO-trigonelline complex (Mizutani, H. *et al.* unpublished results).

3. The pK_a Values of Flavin Bound to DAO at Three Redox States—Table I summarizes the pK_a of neutral flavin bound to DAO [N(3)-H of oxidized flavin, N(5)-H of semiquinoid flavin, and N(1)-H of reduced flavin]. In each redox state, the pK_a decreases by binding to the apoenzyme, but the decrements are very different from one redox state to another. This is probably related to the position of the removable proton and the localization of the negative charge of anionic flavin species as described above. Massey and Ganther (*21*) have proposed the existence of a positively charged group in DAO close to the isoalloxazine ring of the flavin ring to explain the lower pK_a found for the ionization of the 3-imino group of oxidized FAD bound to DAO than that for free FAD in H_2O. The guanidino group of Arg283 found within the active site is sufficent for such a positive charge. The positive charge should also be responsible for the pK_a decrease in the cases of semiquinoid and reduced flavins. In addition to the positive charge, the large shift of the pK_a in semiquinoid or reduced flavins is probably also due to the positive end of α-helix dipole located in the vicinity of N(1) (*8*, *9*); the dipole stabilizes the negative charge of the N(1) position, lowering the pK_a of reduced flavin substantially. The largest factor in the substantially low pK_a of reduced flavin in DAO is probably the steric hindrance between the hydrogen atom of H-N(1) (flavin) and the hydrogen atom of H-N of Gly315. This hindrance becomes significant when a hydrogen is bound to N(1) of flavin; the close contact between these hydrogen atoms is predicted from the structure shown in Fig. 11b.

When excess benzoate is present, the pK_a value of oxidized flavin at N(3)-H is expected to increase; the binding of benzoate neutralizes the positive charge of guanidino group of Arg283 and suppresses proton release from the active site. On the other hand, the binding of a zwitterinonic ligand (trigonelline or NMIN) lowers the pK_a value from 9.2 to 8.1 (10 and present study). Although the binding of such a ligand neutralizes the positive charge of the guanidino group, as does benzoate, the positive charge present in the ligand remains and its location is closer to 3-imino group of flavin than that of the guanidino group of Arg283. Thus an electrostatic effect in the complex with a zwitterionic ligand is stronger than that of free DAO, and hence enhances proton release. A zwitterionic species brought within the active site creates an environment for releasing a proton therefrom. Zwitterionic ligands such as trigonelline and NMIN, which lack a labile proton to be released, lower the pK_a value of flavin 3-imino hydrogen. On the other hand, in a zwitterionic substrate D-amino acid which possesses a labile proton on the protonated amino group, the cationic amino group will release a proton, thereby maintaining the electrostatic neutrality when the substrate comes into the active site (*10*, *11*). This is probably important for the substrate activation in the catalysis.

4. Substrate Specificity of DAO—DAO can oxidize many kinds of D-amino acids and is thus considered to have low substrate specificity. However, DAO has a strict substrate specificity from the standpoint of the charge within the substrate D-amino acid (V–VII); neutral D-amino acids are preferred, basic D-amino acids are poor, and acidic D-amino acids are not substrates for DAO. Recently, D-serine (*2*, *38–40*) and D-aspartate (*41*, *42*) were reported in mammalian tissues, especially in the nervous system. Therefore, the specificity of DAO to oxidize D-serine but not D-aspartate is probably important in neurotransmission. When the substrates come into the active site, the cationic α-amino group ($-NH_3^+$) will lose a proton; thus neutral, basic, and acidic amino acids become monoanionic, zwitterionic, and dianionic forms, respectively. Therefore, monoanionic (benzoate), zwitterionic (trigonelline or NMIN), and dianionic (terephthalate) ligands can be good models for each substrate. The substrate specificity is correlated with the affinity of such ligands for oxidized DAO; monoanionic ligands such as benzoate and *m*-methylbenzoate have high affinity; zwitterionic ligands such as trigonelline and NMIN have affinity lower than the monoanionic ligands; and dianionic ligands such as terephthalate (*21*) have no affinity. The affinity difference among the monoanionic, zwitterionic,

and dianionic ligands may be one of the factors in substrate specificity.

The reaction of basic D-amino acids proceeds in the lower loop in Scheme 1, and the purple intermediate cannot be detected, contrary to that of neutral D-amino acids. This is another reason why basic D-amino acids are poor substrates, because the reactivity of the free reduced DAO with O_2 is smaller than that of the intermediate (*43*).

In the case of neutral amino acids, the model ligands of the substrate (S in Eo···S) and the product (P in Er···P) (Scheme 1) are a monoanion (such as benzoate) and a zwitterion (such as trigonelline or NMIN), respectively. Both ligands cancel the net charge in the active site of oxidized DAO (the positive charge of Arg283) and reduced DAO (the positive charge of Arg283 and the negative charge of reduced flavin), respectively; this is responsible for their high affinity. On the other hand, in the cases of basic and acidic amino acids, the binding of S and P brings another charge in addition to the charge(s) cancelling the net charge in the active site; this hinders their binding to DAO in the oxidized and reduced forms, because the active site is surrounded by hydrophobic residues (*7*, *8*). These are several reasons: (i) the disadvantage of the transfer of a charged compound from aqueous solution to hydrophobic region; (ii) van der Waals repulsive force between electron clouds. The size of the van der Waals radius of the chemical group with a negative charge is generally larger than that of the group with a positive charge, and the van der Waals repulsive force received in the active site is larger in an acidic D-amino acid (with a negative charge) than that in a basic D-amino acid (with a positive charge). Therefore, acidic amino acids cannot become substrates, while basic D-amino acids can become substrates albeit with poor affinity. The product of basic D-amino acids has another positive charge in addition to the positive charge of the imino group; this positive charge probably weakens the affinity of the product to DAO, and the product is quickly repelled from the active site, too quickly to form the purple intermediate.

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