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Three-Dimensional Structure of the Purple Intermediate of Porcine Kidney D-Amino Acid Oxidase. Optimization of the Oxidative Half-Reaction through Alignment of the Product with Reduced Flavin[1]

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The three-dimensional structure of the purple intermediate of porcine kidney D-amino acid oxidase (DAO) was solved by cryo-X-ray crystallography; the purple intermediate is known to comprise a complex between the dehydrogenated product, an imino acid, and the reduced form of DAO. The crystalline purple intermediate was obtained by anaerobically soaking crystals of oxidized DAO in a buffer containing excess D-proline as the substrate. The dehydrogenated product, Δ^1-pyrrolidine-2-carboxylate (DPC), is found sandwiched between the phenol ring of Tyr 224 and the planar reduced flavin ring. The cationic protonated imino nitrogen is within hydrogen-bonding distance of the backbone carbonyl oxygen of Gly 313. The carboxyl group of DPC is recognized by the Arg 283 guanidino and Tyr 228 hydroxyl groups through ion-pairing and hydrogen-bonding, respectively. The $^+$HN=C double bond of DPC overlaps the N(5)–C(4a) bond of reduced flavin. The electrostatic effect of the cationic nitrogen of DPC is suggested to shift the resonance hybridization of anionic reduced flavin toward a canonical form with a negative charge at C(4a), thereby augmenting the electron density at C(4a), from which electrons are transferred to molecular oxygen during reoxidation of reduced flavin. The reactivity of reduced flavin in the purple intermediate, therefore, is enhanced through the alignment of DPC with respect to reduced flavin.

Key words: D-amino acid oxidase, flavoenzyme, oxidative half-reaction, purple intermediate, X-ray crystallography.

D-Amino acid oxidase [EC 1.4.3.3] (DAO) catalyzes the dehydrogenation of a substrate D-amino acid into the corresponding imino acid, forming an intermediate comprising the reduced form of DAO and the dehydrogenated product, 2-imino acid. Reduced flavin in reduced DAO is reoxidized by molecular oxygen, thereby closing the catalytic cycle, while the product, 2-imino acid, is released from the active site and nonenzymatically hydrolyzed to 2-ketoacid and ammonia when 2-imino acid is acyclic. Scheme 1 illustrates the reaction of DAO with D-proline, in which the product Δ^1-pyrrolidine-2-carboxylate (DPC) does not undergo nonenzymatic hydrolysis. Reactions of flavoenzymes generally consist of two half-reactions, *i.e.*, reductive and oxidative half-reactions. The former is the process by which a substrate is oxidized at the expense of flavin reduction, while, in the latter, reduced flavin is reoxidized by yet another substrate or an electron acceptor, which is oxygen in the case of oxidases. Accordingly, the formation of the purple intermediate and reoxidation of reduced flavin by oxygen are the reductive and oxidative half-reactions of DAO, respectively. This enzyme was discovered in animal tissues by H.A. Krebs in the 1930s and later recognized as the first FAD enzyme to be discovered (*1*, *2*). Among DAO species found widely distributed in the biosphere, porcine kidney DAO has been the most extensively investigated and thus has been regarded as a prototype not only of the DAO family but also of the flavooxidase group. The three-dimensional structure of DAO was recently solved by us (*3*) and independently by Mattevi *et al.* (*4*), and we have subsequently introduced a new reaction mechanism for the reductive half-reaction based on the explicit active-site geometry (*5*). In the present study, we solved the crystal structure of the purple intermediate of DAO with D-proline as the substrate and deduced the mechanism underlying the oxidative half-reaction of DAO.

The purple intermediate of DAO has been recognized as

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Abbreviations: DAO, D-amino acid oxidase; DPC, Δ^1-pyrrolidine-2-carboxylate, HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital.

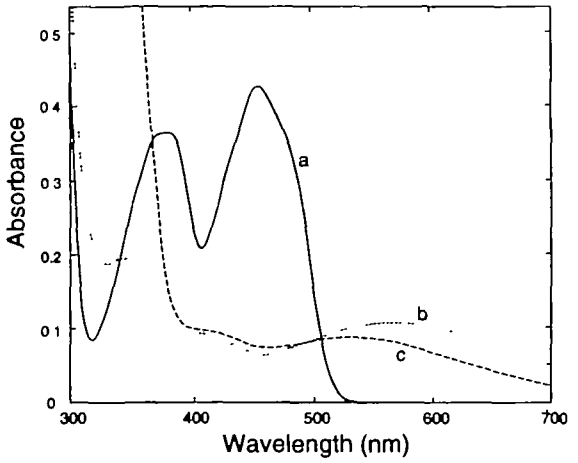


Fig. 1 **Absorption spectra of DAO and the purple intermediate with D-proline or D-alanine as the substrate.** The concentrations were (a) DAO 55 μM, (b) DAO 55 μM, D-proline 91 mM; (c) DAO 55 μM, D-alanine 91 mM, ammonium sulfate 0.16 mM, sodium pyruvate 55 mM.

an intermediate observed during catalysis and is characterized by a broad absorption band in the long wavelength region extending beyond 700 nm (*6–9*) (Fig. 1), the broad long-wavelength band being referred to as a charge-transfer band. Although the purple intermediate had generally been regarded as a charge-transfer complex between reduced flavin and imino acid (*10*, *11*), it was our resonance Raman and ^{13}C-NMR studies that unequivocally demonstrated the purple intermediate comprises a charge-transfer complex between anionic reduced flavin and the zwitterionic form of imino acid (*12–15*). The importance of the purple intermediate with respect to the kinetic mechanism lies in the fact that reoxidation of reduced flavin by oxygen proceeds by way of this particular intermediate prior to release of the product imino acid (Scheme 1). This notion was substantiated by the considerably greater reaction rate constant of the purple intermediate with oxygen than that of free reduced DAO (*16*). This experimental evidence indicates that the reactivity of reduced flavin to oxygen is markedly enhanced in the purple intermediate. We have ascribed this enhanced reactivity to increased electron density at C(4a) of reduced flavin in the purple intermediate on the basis of ^{13}C-NMR spectroscopic observations with ^{13}C-labeled FAD reconstituted into DAO (*15*). However, we did not know the molecular basis underlying the electron-density augmentation at C(4a) of reduced flavin in the purple intermediate, because detailed structural information on the active-site was not available then. The aim of the present study was to reveal this molecular basis and we indeed made it clear by crystallographic analysis of this particular intermediate with D-proline as the substrate.

MATERIALS AND METHODS

DAO was obtained as previously described from *Escherichia coli* into which an expression vector carrying DAO cDNA had been introduced (*17*). Chemicals used were of the highest grade commercially available and were used as supplied.

Visible absorption spectra were obtained with a Hitachi U-2000 spectrophotometer thermostated at 25°C. Molecular orbital calculation of Δ^1-pyrrolidine-2-carboxylate (DPC) was carried out on Cerius^2 provided by Molecular Simulations, San Diego, using MOPAC6 (*18*). The three-dimensional structures in Figs. 3 and 5A were constructed with the program MOLSCRIPT (*19*).

The crystalline form of the purple intermediate was prepared by soaking crystals of oxidized DAO in a solution containing excess D-proline under anaerobic conditions as follows. Yellow crystals of the DAO-benzoate complex, which had been obtained as previously described (*17*), were freed of benzoate soaking in 100 mM sodium citrate buffer, pH 6.3, containing 200 mM sodium acetate, 30% (w/v) polyethylene glycol 4000, and 5 mM D-alanine. The yellow crystals then became colorless, indicating that DAO was in the free reduced state. The colorless crystals were then immersed in the same buffer from which D-alanine was omitted, allowing the reduced DAO to be reoxidized by dissolved oxygen. Shortly afterwards the colorless crystals regained the yellow color indicating that DAO had returned to the oxidized state. Following the procedure for preparing the purple intermediate with D-proline as the substrate (*12*), we soaked the yellow crystals of reoxidized DAO in a buffer, 0.1 M sodium citrate, pH 6.5, containing 40% (w/v) polyethylene glycol 4000 and 50 mM D-proline, under anaerobic conditions. The crystals gradually assumed a purple color (Fig. 2), indicating formation of the purple intermediate. That DAO in the crystals was in the purple intermediate was confirmed by microscopic spectroscopy of the crystals (H. Mizutani *et al.*, unpublished results). The purple crystals were quickly scooped and immersed in liquid nitrogen, and then kept until use for diffraction analysis. The crystalline state of the crystals did not deteriorate, since polyethylene glycol 4000 present in the buffer served as an antifreeze.

X-ray diffraction data were collected at 100 K by flash cooling with synchrotron radiation at SPring 8 (Harima) on a beam line 41XU (Proposal No. 199A0075-NL-np). The data were processed with software provided by Rigaku.

The crystals of the purple intermediate of DAO were isomorphous as to those of the DAO-benzoate complex with two subunits correlated by a non-crystallographic twofold axis in an asymmetric unit. Structure refinement of the DAO purple intermediate was carried out with the coordinates of the DAO-benzoate complex at 2.5 Å resolution (refined from the initial structure (*3*)) from which benzoate and water molecules were removed for the initial model. Simulated annealing and subsequent several rounds of least-squares refinement using XPLOR (*20*) further refined the structure. After each round of refinement, the model obtained was refitted to the electron density against $2Fo - Fc$ omit difference map using program O (*21*). Electron density corresponding to Δ^1-pyrrolidine-2-carboxylate (DPC), the dehydrogenated product from D-proline, was clearly identified within the active site. At this stage, the restraint on the twofold non-crystallographic symmetry was removed and water molecules were picked up on the basis of the peak height and distance criteria from the difference Fourier map, and further inspected against $2Fo - Fc$ difference Fourier maps. Water molecules with thermal factors of greater than 80.0 Å^2 after refinement were excluded from the list. The C-terminal seven amino acid residues were invisible in the electron density map as in the case of the

DAO-benzoate (*3*) or DAO-*o*-aminobenzoate (*5*) complex. The quality of the final model was assessed by means of a Ramachandran plot and by analysis of the model geometry with program PROCHECK (*23*) from the CCP4 program package (*24*). The Ramachandran plot showed that Gln 28 and Ser 300 of each subunit fell outside generously allowed regions, but these residues are unequivocally discernible in the electron density map. The final R and R_{free} factors for all reflections between 10.0 and 2.5 Å resolution for the purple intermediate were 0.232 and 0.298, respectively. The mean positional error of the atoms from a Luzzati plot (*22*) was 0.33 Å. The detailed crystallographic data set for the purple intermediate is given in Table I. The coordinates of the structure have been deposited with the Brookhaven Protein Data Bank under PDB ID code 1EVI.

Fig. 2. **Photograph of DAO crystals of the purple intermediate (see text for the method of preparation).** Approximate dimensions of the crystals: 1 × 0.4 × 0.2 mm.

TABLE I. **Crystallographic data set for the purple intermediate of DAO.**

Space group	$P2_12_12_1$, No. 19
Cell constant	
a (Å)	107.73
b (Å)	91.37
c (Å)	71.24
Temperature	100 K
Wavelength (Å)	0.708
Z (mol/unit cell)	8
Resolution range (Å)	10–2.5
No. of observed reflections	86,285
No. of unique reflections (>2σ(F))	23,434
Completeness (%)	95.4
R_{merge}[a] (%)	6.7
No. of protein atoms	5,458
No. of water atoms	307
No. of heterogen atoms	122
Residues not well defined in electron density	341–347 741–747
R[b] (%)	23.2
R_{Free}[c]	29.8
Rms deviations from ideal geometry:	
Bond lengths (Å)	0.006
Bond angles (deg.)	1.39
Dihedral angles (deg.)	24.21
Improper torsion angles (deg.)	1.08

[a] $R_{merge} = \Sigma |I_{obs} - \langle I_{obs}\rangle| / \Sigma \langle I_{obs}\rangle$. [b] $R = \Sigma ||F_{obs}| - |F_{calc}|| / \Sigma |F_{obs}|$ and $|F_{obs}|$ and $|F_{calc}|$ are the observed and calculated structure factor amplitudes, respectively. [c] Free R was calculated using 10% randomly selected reflections.

RESULTS

The Crystal Structure of the Purple Intermediate of DAO—The overall structural feature of the purple intermediate is similar to that of the DAO-benzoate complex (*3*) with respect to the dimeric structure and the folding pattern of each subunit. The RMS difference in Cα between the two structures is 0.454 Å; the deviation seems to be greater toward the periphery than in the core of the proteins. This indicates that the overall structure is unaltered when the enzyme is in both the resting oxidized state and the intermediary stage during the reaction sequence. The subunit three-dimensional structure of the purple intermediate is shown in Fig. 3. The active site resides in the boundary region between the two domains, *i.e.*, the α/β and pseudo-barrel domains (*3*). The dehydrogenated product of D-proline, DPC, is found within the active site, and is sandwiched between the phenol ring of Tyr 224 and the reduced flavin ring (Figs. 4 and 5). Although N and Cβ of DPC can not be assigned unequivocally to the electron density corre-

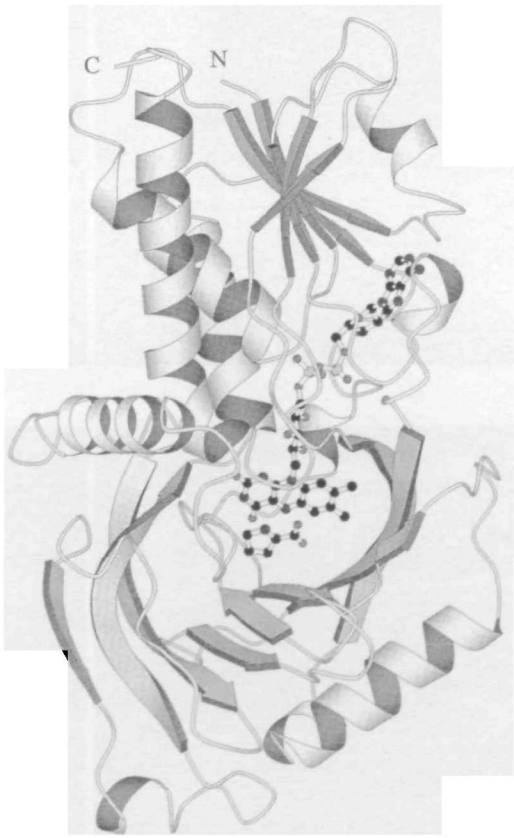


Fig. 3. **The subunit three-dimensional structure of the purple intermediate of DAO with D-proline as the substrate.** FAD and DPC are presented as ball-and-stick models.

sponding to DPC solely from the present crystallographic data obtained at 2.5 Å resolution, we assigned them as shown in Fig. 6 on the following grounds. The hydrogen-bonding distance (3.0 Å) between N/Cβ of DPC and the carbonyl oxygen of Gly 313 nicely fits with the assignment represented. We have shown previously, by means of molecular mechanics simulation based on the DAO-substrate analog complex structure, that the substrate amino group forms a hydrogen bond with Gly 313 C=O in the Michaelis complex prior to the electron transfer from the substrate to flavin with the substrate α-proton pointing toward N(5) of flavin (*5*). Therefore, D-proline binds to the active site, forming a hydrogen bond between NH of D-proline and Gly 313 C=O. Electron transfer as well as proton transfer from D-proline to flavin then results in the orientation of DPC, as shown in Fig. 6, without cleavage of this hydrogen bond. Taking the alternative orientation of DPC with Cβ close to Gly 313 C=O would require surmounting a great energy barrier for 180° rotation along the Cα-COO^- bond, thus making the alternative orientation essentially impossible. It is noteworthy that the reduced flavin ring system is in a planar conformation, rather than in the butterfly conformation found for the free reduced form of flavin due to the sp^3 hybridization at N(5) and N(10). The carboxylate group of DPC is recognized by the Arg 283 guanidino and Tyr 228 hydroxyl groups through ion-pairing and hydrogen-bonding, respectively. The protonated cationic nitrogen of DPC is within hydrogen-bonding distance (3.0 Å) of the Gly 313 backbone carbonyl oxygen, which is also hydrogen-bonded to a neighboring water molecule. Gly 313 is within the loop located on the N-terminal side of the C-terminal α-helix, which has a kink towards the N-terminal end (Fig. 3). The N-terminal end of this α-helix points toward the O=C(2)–N(1) moiety of reduced flavin in such a way that the α-helix dipole stabilizes the negative charge of the reduced flavin, although the dipole moment may be somewhat weakened by the kink. Figure 5 shows the mutual geometrical alignment between reduced flavin and DPC. An important characteristic of this alignment with respect to DAO catalysis is the overlapping between the $^+$HN=C double bond of DPC and C(4a)–N(5) of reduced flavin, which positions the cationic nitrogen of DPC close (3.3 Å) to C(4a) of reduced flavin.

Charge-Transfer Interaction between DPC and Reduced Flavin—We previously demonstrated that the purple intermediate of DAO with D-proline as the substrate is associated with a charge-transfer interaction between the anionic reduced form of flavin and zwitterionic DPC by means of

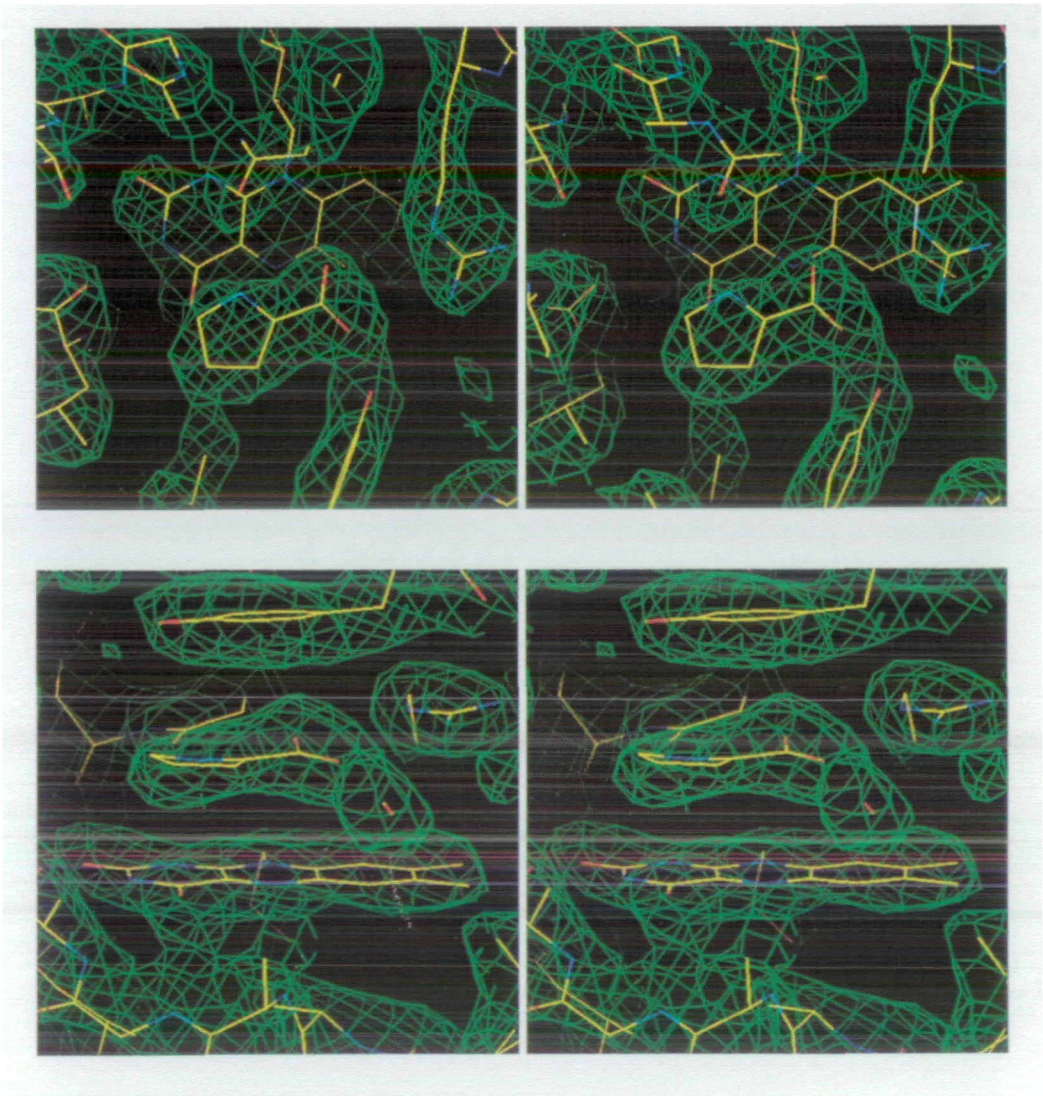


Fig. 4. **Active-site stereoview of the (2|*Fo*| − |*Fc*|) omit map.** The map was contoured at 1σ above the *re*-face of flavin (top) and above N(5) of flavin (bottom) using program O (*21*)

resonance Raman and NMR spectroscopy (*12–15*). Since reduced flavin acts as an electron donor in redox reactions, it should also act as an electron donor in the charge-transfer interaction involving reduced flavin. In the charge-transfer interaction in the purple intermediate, therefore, anionic reduced flavin and zwitterionic DPC act as the electron donor and acceptor, respectively. As a result of this, the charge-transfer interaction in the purple intermediate should be framed through the overlapping between the highest occupied molecular orbital (HOMO) of anionic reduced flavin and the lowest unoccupied molecular orbital (LUMO) of zwitterionic DPC. The mutual alignment between DPC and reduced flavin, as revealed by the crystal structure (Fig. 5), indeed ensures the HOMO-LUMO interaction, as illustrated in Fig. 6; the HOMO-LUMO interaction is optimum in terms of the orbital symmetry as well as

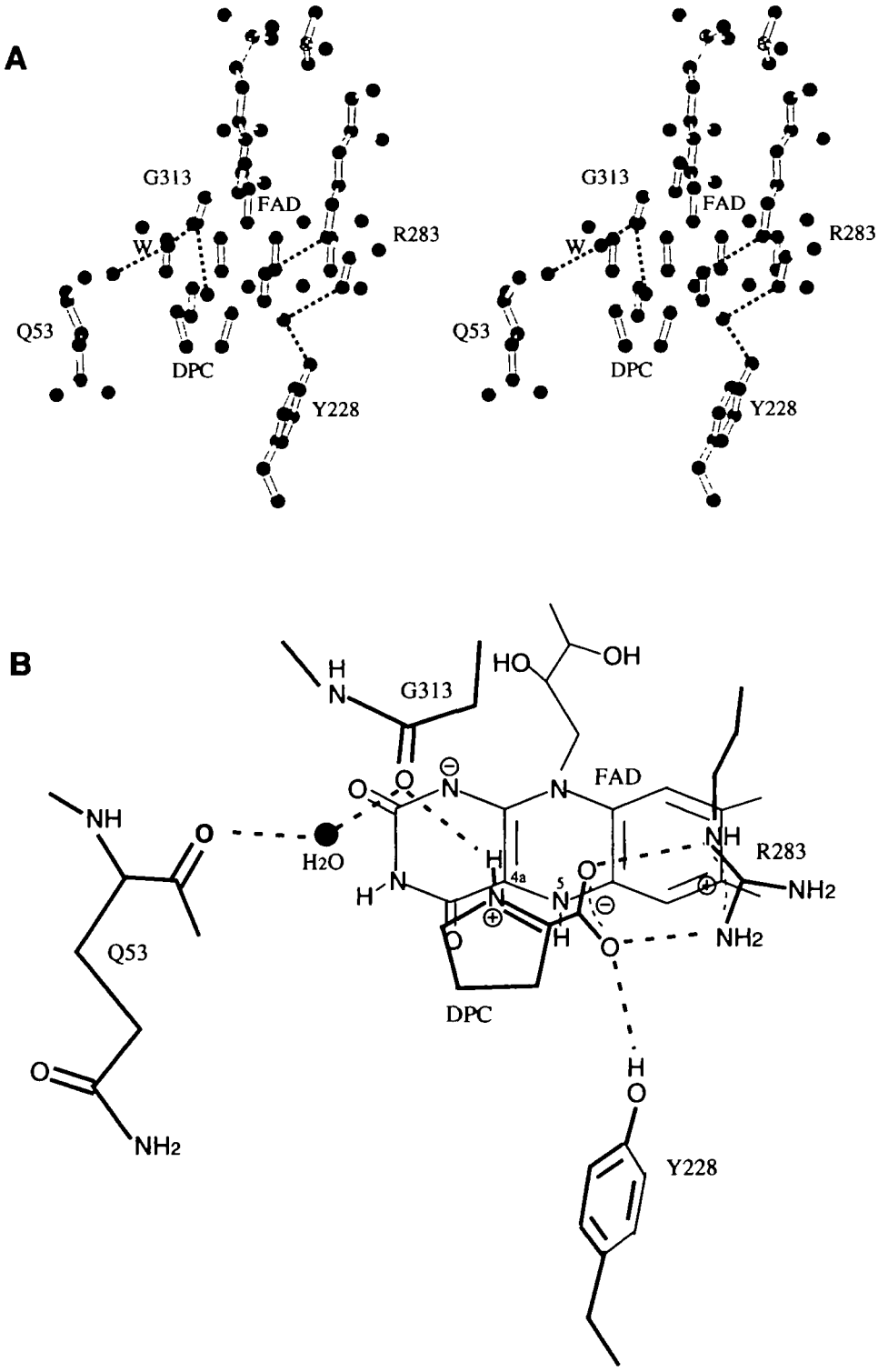


Fig. 5. **The alignment between reduced flavin and DPC.** A: Stereoview of the DPC binding-site structure. B Illustration of the DPC-binding mode.

the magnitude of the molecular orbital coefficients at the overlapping positions. Note that the overlapping between the N=C double bond of DPC with flavin C(4a)–N(5) is slightly deviated, so that the overlapping of the DPC nitrogen extends toward C(4) of reduced flavin.

DISCUSSION

We have successfully applied the cryocrystallographic method to elucidation of the detailed three-dimensional structure of the purple intermediate of DAO. In the present study, polyethylene glycol 4000 used as a precipitant happened to act as an antifreeze and also protected the crystals from cracking in liquid nitrogen or at 100 K during diffraction-data collection. Since the purple intermediate is a reaction intermediate with high reactivity toward oxygen, this cryogenic method is appropriate for protecting the intermediate from further reaction with oxygen. The crystals of the purple intermediate maintained the same purple color during the diffraction-data collection, indicating that the crystals stayed in this intermediary state throughout the crystallographic procedure.

Imino acids produced on dehydrogenation of acyclic D-amino acids, *e.g.*, D-alanine, D-leucine, *etc.*, undergo nonenzymatic hydrolysis after they are released from the active site, leading to 2-ketoacids. The dehydrogenated product from a cyclic D-amino acid such as D-proline, however, is stable against hydrolysis even after it is released from the active site into the bulk aqueous environment (Scheme 1). The final product from D-proline, therefore, is DPC, while those from acyclic D-amino acids are 2-ketoacids. (Since D-proline itself is an imino acid, the product DPC cannot sim-

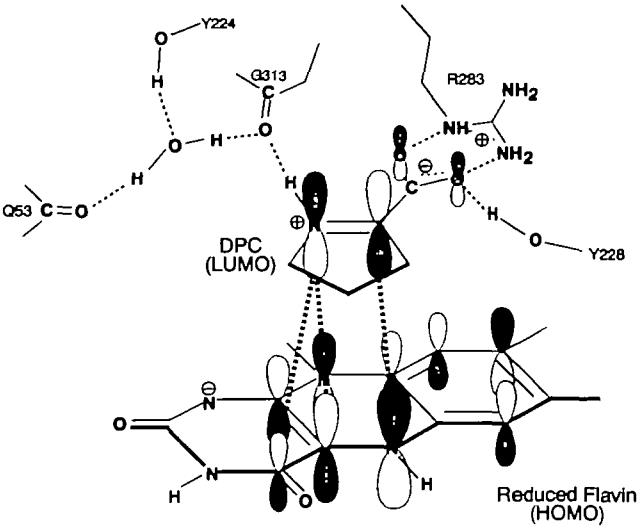
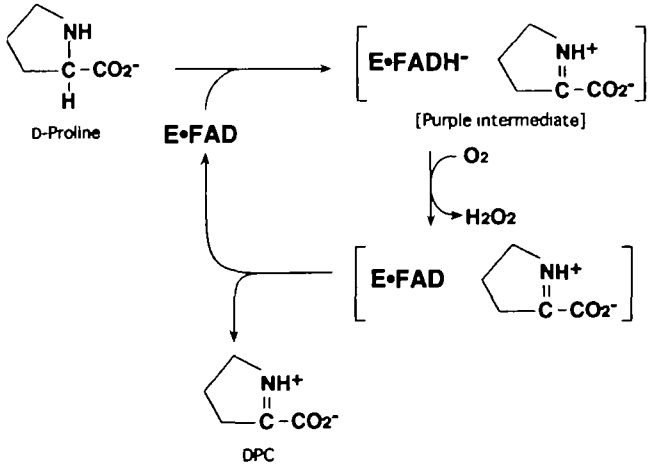
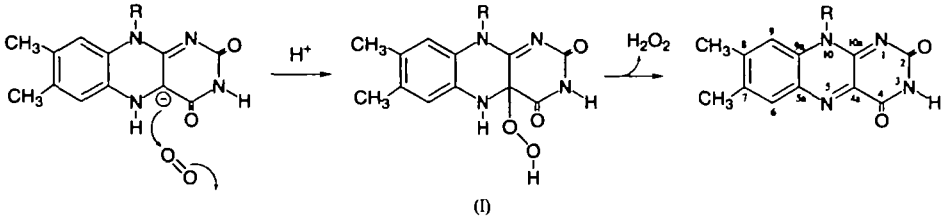


Fig. 6. **The charge-transfer interaction between LUMO of DPC and HOMO of reduced flavin in the purple intermediate of DAO.** HOMO of anionic reduced flavin is cited from Hall *et al.* (*25*).



Scheme 1 **Reaction catalyzed by DAO.**



Scheme 2. **Reaction of anionic reduced flavin with molecular oxygen.**

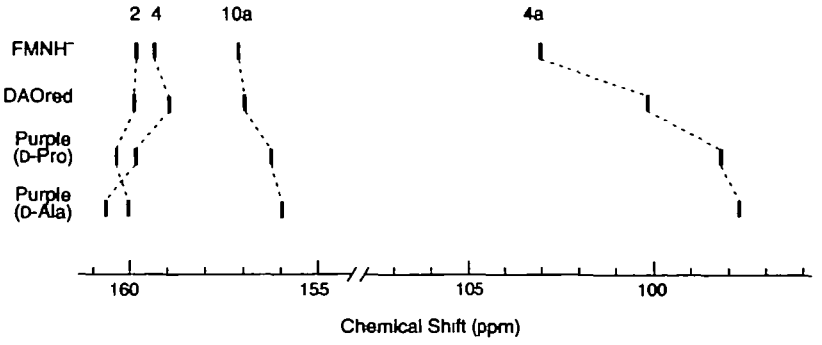
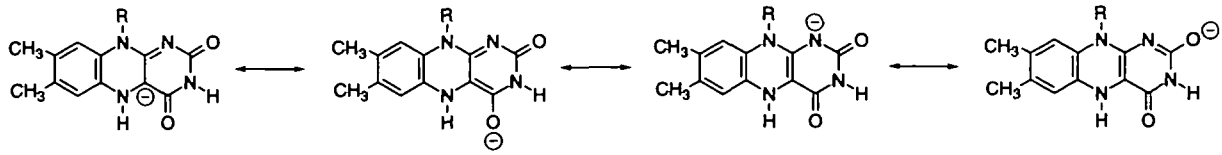


Fig 7 **^{13}C-Chemical shift correlation diagram for the selectively ^{13}C-enriched flavin with various states of DAO.** FMNH⁻, DAOred, Purple (D-Ala), and Purple (D-Pro) represent anionic reduced FMN, the free reduced form of DAO, purple intermediate with D-alanine as the substrate, and purple intermediate with D-proline as the substrate, respectively Data are cited from Miura and Miyake (*15*).



(II) (III) (IV)

Scheme 3 **Resonance hybridization of anionic reduced flavin.**

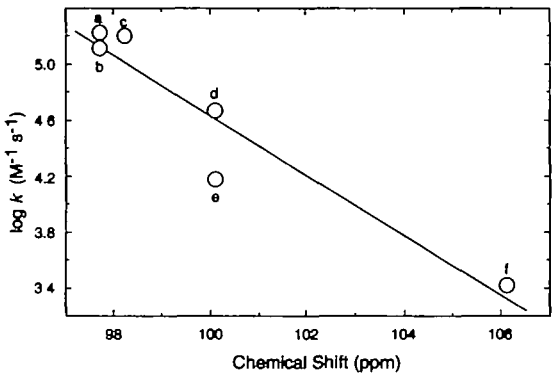


Fig. 8 **Correlation between the ^{13}C(4a)-chemical shift of reduced flavin and the reaction rate constant of reduced flavin to oxygen.** a and b, purple intermediate of DAO with D-alanine as the substrate, c, purple intermediate of DAO with D-proline as the substrate, d and e, free reduced form of DAO; f, free reduced form of medium-chain acyl-CoA dehydrogenase. The chemical shift data for a–f are cited from Miura and Miyake (*15*), and those for f from Miura *et al* (*27*). The rate constants for a and d are cited from Porter *et al* (*16*), those for b, c, and e from Nishina *et al.* (*28*), and those for f from Johnson *et al* (*29*) All the data were measured at 25°C.

ply be classified as an imino acid but is, strictly speaking, an unsaturated cyclic imino acid.) Due to this non-hydrolyzable property of DPC, formation of the purple intermediate is easier with D-proline than with acyclic D-amino acids; the intermediate can be prepared by incubating DAO with excess D-proline under anaerobiosis (Fig. 2). The purple intermediate obtained with D-proline is stable for at least a few days even at room temperature only if oxygen is successfully excluded.

The purple intermediate of DAO is well quoted not only by the peculiar purple color arising from the charge-transfer interaction between reduced flavin and a dehydrogenated product but also particularly by its catalytic importance, *i.e.* the oxidative half-reaction of DAO proceeds by way of this particular intermediate (*16*). This catalytic role of the purple intermediate is achieved through the enhanced reactivity of reduced flavin toward molecular oxygen. We previously ascribed this enhanced reactivity to the increased electron density at C(4a) of reduced flavin in the purple intermediate in comparison with that of free reduced DAO by means of ^{13}C-NMR spectroscopy applied to selectively ^{13}C-labeled FAD reconstituted into DAO (*15*). It is generally accepted that in the reaction of anionic reduced flavin with oxygen, electrons are transferred from C(4a) of the reduced flavin to oxygen (Scheme 2). The formation of oxygenated flavin, 4a-hydroperoxy-1,5-dihydroflavin (I), cannot proceed through a single step, since this step is spin forbidden; the ground state of molecular oxygen is in the triplet state whereas that of reduced flavin is the singlet state. Bruice *et al.* have proposed that the reaction proceeds *via* two single-electron transfer steps from C(4a) to oxygen intervened by triplet-singlet spin conversion (Eq. 1) to circumvent the spin-forbidden reaction process (*26*).

$$\mathrm{FlH^-} + \mathrm{O_2} \longrightarrow \mathrm{FlH{\cdot}\uparrow\uparrow O_2^{-}{\cdot}} \rightarrow \mathrm{FlH{\cdot}\uparrow\downarrow O_2^{-}{\cdot}}$$
$$\xrightarrow{\mathrm{H^+}} \mathrm{FlH\text{-}OOH} \longrightarrow \mathrm{Flox} + \mathrm{H_2O_2} \qquad (1)$$

$\mathrm{FlH^-}$, $\mathrm{FlH{\cdot}}$, FlOOH, and Flox are anionic reduced flavin, the neutral semiquinone form of flavin, 4a-hydroperoxy-1,5-dihydroflavin (I), and oxidized flavin, respectively. The initial electron transfer in Eq. 1 is from C4a of reduced flavin to oxygen. Accordingly, the higher the electron density at C4a of reduced flavin, the faster the reaction with oxygen. Figure 7 shows the ^{13}C-chemical shift correlation for selectively labeled ^{13}C atoms in the isoalloxazine nucleus with various states of DAO. The chemical shifts of C(4a) of reduced flavin are remarkably upfield-shifted relative to those of free reduced DAO or anionic reduced FMN in an aqueous solution suggesting strongly that C(4a) in the purple intermediate has greater electron density than that in free reduced DAO or anionic reduced FMN. This indication is corroborated by the correlation between the ^{13}C(4a)-chemical shift of the reduced flavin and the reaction rate constant with molecular oxygen (Fig. 8). The reaction of reduced flavin is faster when the chemical shift of C(4a) is at a higher field, and hence there is higher electron density at C(4a). However, we did not know the molecular basis for the augmentation of the electron density at C(4a) because detailed structural information on the active site in the purple intermediate was unavailable at that time. We can understand the molecular basis, as explained below, of the electron density increase now that we have obtained the detailed three-dimensional structure of the active site in the purple intermediate.

The anionic reduced form of flavin exists as resonance hybridization among the canonical forms, as shown in Scheme 3. Of these, the contributor (II) with a negative charge at C(4a) is responsible for the reaction with oxygen, as depicted in Scheme 2. The crystal structure of the purple intermediate clearly shows that the cationic nitrogen of DPC is located close to C(4a) of reduced flavin (Figs. 5 and 6). Due to the electrostatic effect exerted by the positive charge at N(1) of DPC, the canonical form (II) with a negative charge at C(4a) significantly contributes in the resonance state of the anionic reduced flavin in the purple intermediate, hence the increased electron density at C(4a). This notion is supported by the results of our resonance Raman study (*30*) on the purple intermediates (complexes of reduced enzymes with dehydrogenated products) of flavooxidases, in which the reaction of reduced flavin with oxygen is enhanced, and flavodehydrogenases, in which the reactivity of reduced flavin to oxygen is suppressed. It was

found that the Raman bands for the C(4a)=C(10a) stretching of reduced flavin were observed at lower frequencies with oxidases including DAO, whereas the corresponding bands with dehydrogenases were observed at higher frequencies. In oxidases, the canonical form (II) greatly contributes to the resonance hybridization (Scheme 3), leading to a lower bond order for C(4a)–C(10a) as well as a higher electron density at C(4a), thus resulting in the enhanced reactivity to molecular oxygen. In dehydrogenases, on the other hand, the canonical form (III) or (IV) contributes considerably to the resonance state (Scheme 3), resulting in a higher C(4a)–C(10a) bond order and a lower electron density at C(4a), the reaction of reduced flavin with oxygen consequently being suppressed. The large contribution of (III) to the resonance in the case of medium-chain acyl-CoA dehydrogenase is attributable to a very strong hydrogen bond (2.55 Å) between N(1) of reduced flavin and the hydroxyl group of Thr 136 (*31*). In contrast, no hydrogen bond was found at N(1) of reduced flavin in the purple intermediate of DAO.

We have revealed in the present study that the oxidative half-reaction of DAO is optimized through the alignment of the product, DPC, with respect to reduced flavin. This mechanism is elegant and hitherto unknown for other flavoenzymes, and may well be one of the general mechanisms underlying the oxidative half-reactions of flavooxidases.

A problem still remains, however, as to how molecular oxygen can gain access to reduced flavin in the purple intermediate. It has been revealed from the crystal structure that the *si*-face of flavin is shielded by a hydrophobic stretch, V47-A48-A49-G50 (*3*), which makes oxygen approach from the *si*-face unlikely. Since the *re*-face of the reduced flavin in the purple intermediate is shielded only partly by DPC (Fig. 5), the open space on the *re*-face above the C(10a)–N(10) region can allow access of oxygen. In the reoxidation of reduced flavin with oxygen, LUMO of oxygen is required to overlap with HOMO of anionic reduced flavin. Although we lack convincing structural evidence at this stage, access of oxygen to the *re*-face above the C(10a)–N(10) region does not seem to interfere with this HOMO-LUMO interaction. 4a-Hydroperoxy-1,5-dihydroflavin (I) has not been detected in the reoxidation of DAO, let alone its crystal structure in DAO, so there is still ambiguity as to whether or not the reoxidation by oxygen proceeds *via* 4a-hydroperoxy-1,5-dihydroflavin (I), and the stereochemistry of 4a-hydroperoxy-1,5-dihydroflavin (I) needs to be settled if the reoxidation should involve this oxygenated form. It would be worth the effort to attempt detection of 4a-hydroperoxy-1,5-dihydroflavin (I) or the complex of the purple intermediate with oxygen by means of cryocrystallography, a beneficial method for unstable states. This would ultimately answer the remaining question as to how oxygen approaches reduced flavin in the purple intermediate during the oxidative half-reaction.

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