O₂ Reactivity of Flavoproteins

DYNAMIC ACCESS OF DIOXYGEN TO THE ACTIVE SITE AND ROLE OF A H⁺ RELAY SYSTEM IN D-AMINO ACID OXIDASE*†‡§¶*

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Molecular dynamics simulations and implicit ligand sampling methods have identified trajectories and sites of high affinity for O₂ in the protein framework of the flavoprotein D-amino-acid oxidase (DAAO). A specific dynamic channel for the diffusion of O₂ leads from solvent to the flavin Si-side (amino acid substrate and product bind on the Re-side). Based on this, amino acids that flank the putative O₂ high affinity sites have been exchanged with bulky residues to introduce steric constraints. In G52V DAAO, the valine side chain occupies the site that in wild-type DAAO has the highest O₂ affinity. In this variant, the reactivity of the reduced enzyme with O₂ is decreased ≥100-fold and the turnover number = 1000-fold thus verifying the concept. In addition, the simulations have identified a chain of three water molecules that might serve in relaying a H⁺ from the product imino acid =NH⁺ group bound on the flavin Re-side to the developing peroxide on the Si-side. This function would be comparable with that of a similarly located histidine in the flavoprotein glucose oxidase.

The early proposal by Lakowicz and Weber (1) that O₂ diffuses just about freely through proteins has forged the thinking of biochemists for decades. However, evidence is accumulating that O₂ access is guided and controlled. It thus appears that our understanding of the mechanisms of O₂ migration to catalytic centers is currently experiencing a shift of paradigms. Many of the early works on O₂ migration between affinity sites concern myoglobin (2–5) and enzymes from the respiratory chain (6, 7). Studies on lipooxygenase-1 dealt with the effect of steric perturbations on the reaction with O₂ and suggested a distinct O₂ pathway (8, 9). Later, the role of O₂ channels in the stereocchemistry of the 15-lipoxygenase reaction was addressed using a combination of computational and experimental methods (10). A similar approach led to the identification of O₂ access pathways in copper amine oxidase (11).

In monooxygenases and oxidases from the flavoprotein family, O₂ (re)oxidizes the reduced flavin cofactor. Although there is agreement on the physical mechanisms of the initial step(s) of electron transfer from the flavin to O₂ (12–14), key questions regarding the control of O₂ reactivity with the reduced flavin cofactor are still open. There are examples for channels in flavoproteins that guide O₂ to the reaction site (15–17). In this context, several questions emerge. To what extent are these channels controlling O₂ reactivity? Do they have a relevant O₂ affinity? What role do H₂O molecules play? This study addresses these issues using D-amino-acid oxidase (EC 1.4.3.3, DAAO), a prominent member of the flavoprotein oxidase family that has played a central role in mechanistic studies of this enzyme class (18, 19). DAAO catalyzes the oxidative deamination of D-amino acids to yield α-keto acids, ammonia, and hydrogen peroxide.

In DAAO, the catalytic center is located at the end of a funnel serving as entrance for the substrate D-amino acid. In this channel, the cofactor flavin is shielded by the bound D-amino acid substrate or by the product imino acid from access of small molecules originating from the solvent region (18, 19). Because catalysis by DAAO with the best substrates follows a ternary complex mechanism (20), it is unlikely that O₂ accesses the reduced flavin via the substrate channel. Inspecting DAAO three-dimensional structures (18, 19), putative channels can be envisaged that would connect solvent and the flavin pocket (see supplemental Fig. 1). What makes a good O₂ access channel? Ideally, it is a continuous region between solvent and the active site where the average probability of finding O₂ is high. This probability depends on many factors like the dynamics of protein side chains and H₂O molecules or the hydrophobicity of the environment.

Implicit ligand sampling (ILS) is an efficient method, based on molecular dynamics (MD) simulations, to compute the probability density for small, weakly interacting particles such as O₂ at all positions in a molecular system (4). From this probability, useful quantities can be calculated as follows: (i) occupancy, the probability to find a particle in a certain volume region; (ii) Gibbs free energy costs ΔG(O₂) of relocating O₂.

* The abbreviations used are: DAAO, D-amino-acid oxidase; ILS, implicit ligand sampling; IP, imino pyruvate; MD, molecular dynamics; WT, wild type.

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‡ The online version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–7 and Tables 1 and 2.

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from a reference state (e.g. solvent) into a certain region; and (iii) affinity for O\textsubscript{2} for a given region expressed as an apparent dissociation constant K_{d,app}.

Our approach combines the strengths of computer simulations and biochemical experiments. Using ILS, we predict high affinity regions for O\textsubscript{2} inside the protein and in the vicinity of the flavin and extract the most likely diffusion pathways. To verify these predictions, the protein side chains expected to interfere sterically with the high affinity sites were subjected to mutagenesis, and their kinetic parameters are compared with those of the parent DAAO. In addition, the simulations have uncovered a H\textsubscript{2}O chain that is proposed to play a key role in the chemical reaction of O\textsubscript{2}.

EXPERIMENTAL PROCEDURES

Computational Procedures—The sites of high O\textsubscript{2} affinity were determined using the ILS method (4). The underlying MD trajectories were based on the model of DAAO from Rhodotorula gracilis built from the three-dimensional structure 1c0p in which the ligand d-Ala was replaced by IP (19). Flavin is in the reduced anionic state (FAD\textsubscript{red}H\textsuperscript{–}), i.e. the species that reacts with O\textsubscript{2} (20). In the MD simulations, the protein is solvated in physiological NaCl solution, and periodic boundary conditions were applied; no explicit O\textsubscript{2} is present in the system. The ILS calculations were based on 26-ns MD runs after a 4-ns equilibration period. Before applying the ILS procedure, the protein in the 26,000 frames of each trajectory had to be aligned. To improve the alignment, the ILS free energy maps were generated for each monomer P1 and P2 of dimeric yeast DAAO independently. The two maps compare well and were combined into one, thereby doubling the number of samples. To compute the free energy maps, an oxygen probe molecule was placed in 20 different orientations on each position on a fine grid with grid points 0.33 Å apart. The results of the ILS calculation of each frame were averaged, and the resulting free energy grid was down-sampled to 1 Å resolution. All MD simulations were performed using the program NAMD (21). The ILS runs and all molecular graphics were carried out with VMD (22).

Biochemical Procedures—Site-saturation mutagenesis at position 52 or 201 was carried out using the DAAO cDNA subcloned into the pT7-HisDAAO plasmid as template; ∼280 clones were screened using d-Ala as substrate and at low (2.5% = 30 μM) O\textsubscript{2} saturation (23). The G52C/G52T/G52V DAAO variants were identified based on their limited activity in the screening procedure compared with WT DAAO; the clones encoding for T201L/T201V variants were identified based on their enhanced activity compared with WT DAAO. Production and purification of the variants were according to Ref. 23. Purification yields were ≥65%; purity was >90% by SDS-PAGE analysis. Activity was determined using an oxygen consumption assay at pH 8.5, 25 °C, with 28 mM d-Ala and 0.253 mM dioxygen.

Spectral experiments were carried out at 15 °C in 50 mM potassium phosphate, pH 7.5, containing 2 mM EDTA, 10% glycerol, and 5 mM 2-mercaptoethanol. Semiquinone forms of ∼10 μM DAAO were produced by light irradiation under anaerobic conditions containing 5 mM EDTA and 0.5 μM 5-deazaflavin. The thermodynamic stability of the semiquinone forms and reduction potentials of DAAO variants were evaluated according to Ref. 24.

Steady State and Pre-steady State Stopped-flow Experiments—Such experiments were performed in 50 mM sodium pyrophosphate, pH 8.5, containing 1% (v/v) glycerol and 0.5 mM 2-mercaptoethanol at 15 °C and as detailed in Ref. 20, 23; indicated concentrations are final, i.e. after mixing. The enzyme-mono-turnover technique (25) was used to assess steady state kinetic parameters by mixing equal volumes of ∼10 μM air-saturated enzyme with a solution of 30 mM d-Ala equilibrated with different [O\textsubscript{2}]. Evaluation of 455 nm traces was according to Gibson et al. (25) and as detailed in Ref. 23. For anaerobic experiments, solutions contained 100 mM glucose, 0.1 μM glucose oxidase, and 30 mM catalase. For reductive half-reaction experiments, oxidized DAAO was reacted with increasing [d-Ala] in the absence of dioxygen. For the study of the (re)oxidation, the free reduced DAAO (E-Fl\textsubscript{red}) was generated by reacting oxidized DAAO anaerobically with an ∼3-fold excess d-Ala. Obtained E-Fl\textsubscript{red} was then reacted with buffer solutions saturated with different O\textsubscript{2} concentrations. Reaction rates were estimated from fits of traces at 455 and 530 nm using the application Biokine32 (BioLogic) or with SPECFIT/32® (Spectrum Software Associates, Chapel Hill, NC) for global analysis (23).

RESULTS

DAAO Has Two High Affinity Sites for O\textsubscript{2} Close to the Flavin—The ILS calculations take the three-dimensional structure of the DAAO-imino pyruvate (IP) complex (19) as a starting point. They render the three-dimensional distribution of the free energy potential ΔG(O\textsubscript{2}) of O\textsubscript{2} relative to its potential in the bulk solvent. The lower the free energy for a given point in space, the higher is the affinity for O\textsubscript{2} and therefore the average O\textsubscript{2} occupancy. Fig. 1A shows ΔG(O\textsubscript{2}) in the vicinity of the active site in the form of an isoenergy surface. On the Si-side of flavin, there are two high affinity regions: site A is adjacent to the N(5)–C(4a) locus, whereas site B is close to the xylene edge of the flavin (Fig. 1A). Both sites correspond to small voids in the crystal structure with enough volume to hold a molecule such as O\textsubscript{2} or H\textsubscript{2}O. Remarkably, there is no high affinity site on the substrate side (Re) in direct contact with flavin, the closest one being found behind the IP product at ∼9 Å (site D, Fig. 1A); it has less affinity than the ones on the Si-side. It is conceivable that the IP product with its hydration shell plugs the entrance and effectively shields the isoxazoline Re-side.

Several Paths Lead O\textsubscript{2} to the Active Center—The three-dimensional structures of DAAO from yeast (19), human (26), and pig (27) have in common a narrow water-filled channel connecting the solvent and the Si-side of the flavin (see Figs. 1 and 2 and supplemental Fig. 1). In yeast DAAO, the entrance is flanked by Val-41, Ser-42, Pro-140, Leu-203, and Arg-227 and the flavin (see Figs. 1A and 2). It is conceivable that the IP product with its hydration shell plugs the entrance and effectively shields the isoxazoline Re-side.
The colors and the location of the flavin plane correspond to the ones in right and product IP in the binding pocket. The substrate entrance is on the A, section through the protein (shown in the form of the isosurface at red, blue, gray surface representation) with flavin cofactor and product IP in the binding pocket. The substrate entrance is on the right; the putative O2 access channels are on the left. Areas that are considered to be part of the solvent have a light blue tint and dashed outline. Regions of low free energy potential \( \Delta G(O_2) \) denote high affinity sites for \( O_2 \) and are shown in the form of the isosurface at \(-3 \) kJ/mol (transparent blobs in green, red, blue, and yellow). There are two high affinity sites for \( O_2 \) close to the flavin on the Si-side; site A is \( \sim3.5 \) Å from the N(5)-C(4a) FAD locus; site B is \( \sim5 \) Å from the flavin benzene ring. Site D is further away from flavin (∼9 Å) on the Re-side and is part of the solvent phase at the substrate entrance. Minimum energy paths between the different sites and the solvent are displayed as tubular structures whose thickness scales with \( \Delta G(O_2) \). The pathway connecting site A with the solvent via site C is shown in green, and the one connecting sites B and C in blue. At the protein surface, the path branches out into several catchment areas for \( O_2 \). There is an alternative path (red) to site A on the Si-side with three different entrances. The clipping plane through the protein is rendered transparent in an oval region to show the red path where it dips behind the surface. B, free energy profiles along the different \( O_2 \) channels. The colors and the location of the flavin plane correspond to the ones in A. The 

\[ \text{Oxygen Diffusion in \( \delta \)-Amino Acid Oxidase} \]

\[ \text{Catalytic center of DAAO seen from the flavin Re-side.} \]

The flavin in its reduced state is complexed with the product imino pyruvate. High affinity sites for \( O_2 \) are shown in form of nested isoenergy surfaces (∼3, ∼7, and ∼11 kJ/mol beginning from the outermost surface) using the same color codes as in Fig. 1, A and B. Four \( H_2O \) molecules around the complex may serve as a \( H^- \) relay chain between the Re- and Si-sides as described in the text and in Fig. 5. The H-bond network is shown in pink. W1 corresponds to a \( H_2O \) seen in the crystal structure (19).

\[ \text{Overview of \( O_2 \) access pathways to the active site of DAAO.} \]

A, section through the protein (gray surface representation) with flavin cofactor and product IP in the binding pocket. The substrate entrance is on the right; the putative \( O_2 \) access channels are on the left. Areas that are considered to be part of the solvent have a light blue tint and dashed outline. Regions of low free energy potential \( \Delta G(O_2) \) denote high affinity sites for \( O_2 \) and are shown in the form of the isosurface at \(-3 \) kJ/mol (transparent blobs in green, red, blue, and yellow). There are two high affinity sites for \( O_2 \) close to the flavin on the Si-side; site A is \( \sim3.5 \) Å from the N(5)-C(4a) FAD locus; site B is \( \sim5 \) Å from the flavin benzene ring. Site D is further away from flavin (∼9 Å) on the Re-side and is part of the solvent phase at the substrate entrance. Minimum energy paths between the different sites and the solvent are displayed as tubular structures whose thickness scales with \( \Delta G(O_2) \). The pathway connecting site A with the solvent via site C is shown in green, and the one connecting sites B and C in blue. At the protein surface, the path branches out into several catchment areas for \( O_2 \). There is an alternative path (red) to site A on the Si-side with three different entrances. The clipping plane through the protein is rendered transparent in an oval region to show the red path where it dips behind the surface. B, free energy profiles along the different \( O_2 \) channels. The colors and the location of the flavin plane correspond to the ones in A. The 

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to the increase of the O₂ “effective concentration” at the reaction center.

**Protein Dynamics Reveals a Proton Relay System**—Comparing the x-ray structure (100 K) with the MD trajectories (298 K) yields interesting clues. The putative O₂ channel of the flavin Si-side is flexible, and the H₂O molecules inside are not fixed but in constant exchange with the bulk water phase. No H₂O is observed at site A in any of the four different DAAO x-ray structures (18, 19, 26, 27), and only one structure hosts H₂O in site B. In the simulations, the presence of H₂O at these sites is transient, and throughout the MD trajectories, H₂O molecules diffuse in and out of sites A and B. A lone H₂O in the hydrophobic cavity A or B is not favorable, but an H-bond chain connecting it to hydrophilic regions can stabilize H₂O in these positions significantly. Notably, part of the time there is a state with a contiguous chain of four H-bonded H₂O molecules crossing the flavin plane between the IP and site A that might be used as H⁺ relay system (Figs. 2 and 5 and supplemental Fig. 1B).

**Biochemical Properties of G52X DAAO Variants**—The general properties of the G52X variants are very similar to those of WT DAAO (see supplemental material). From this, we conclude that there are negligible structural differences in the immediate flavin environment. A difference between WT and G52X DAAO variants is in the midpoint redox potential (Eₘ) that is lowered from −109 mV (WT) to −185 mV in G52V DAAO. Note that a decrease of Eₘ by ≈80 mV is expected to increase the rate of reaction with O₂ by at least one order of magnitude (28), an effect that would thus be opposite to those resulting from the steric factors.

The substitution of Gly-52 significantly affects the steady state kinetic parameters in that, compared with WT DAAO, kcat, Kₘ, D-Ala, and Kₘ,O₂ are 1000-, 70-, and 20-fold lower (supplemental Table 1). The catalytic equations of DAAO consist of two half-reactions as shown in Scheme 1 (20, 29), where a) is the reductive half-reaction (substrate dehydrogenation + flavin reduction) and b) is the oxidative half-reaction (O₂ reaction with the reduced enzyme flavin). To verify that in the G52X DAAOs the observed consequences of the exchange are due to

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**Scheme 1.** Minimal kinetic scheme for the catalytic cycle of DAAO with d-Ala as substrate (sequential mechanism). S, amino acid substrate; P, imino acid product that dissociates in solvent; ox/red, oxidation state of the flavin (20, 29). In parentheses are reported the kinetic steps related to the alternative ping-pong mechanism, as observed only for basic d-amino acids (20, 29).

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**FIGURE 3.** Effect of the G52V substitution on O₂ affinity of DAAO. A, free energy maps for WT DAAO from Fig. 1A (green, blue) are superimposed on the G52V DAAO structure model. The free energy distribution for G52V DAAO is in magenta, and A* is its high affinity site. B, comparison of O₂ free energy profiles along the channel for WT and G52V DAAO. The O₂ energy at the position of site A* in G52V variant is 10 kJ/mol higher than in WT DAAO.

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**FIGURE 3a.** The O₂ access hypothesis was tested computationally as well as experimentally. Based on the three-dimensional structure (19), it can be predicted that mutation of the residue Gly-52 would interfere with the high affinity site A (Fig. 3) by partially filling the space expected to be occupied by O₂ in a complex prior to electron transfer. G52C/G52V/G52T DAAO variants were prepared by site-saturation mutagenesis, and their biochemical properties were investigated with particular focus on O₂ reactivity (see supplemental material). The effect of the G52V substitution was assessed computationally. Fig. 3A shows how the position of the valine side chain in the G52V variant (obtained by rotamer optimization and local energy minimization) overlaps with the volume of site A in WT DAAO. The pathways obtained from ILS calculations with G52V and WT DAAO are compared in Fig. 3B. In the variant, the high affinity site A* has substantially less affinity for O₂, and the energy minimum is shifted 3 Å away from flavin and the original site A (WT DAAO). At the same time, site B remains unaffected by the mutation. Remarkably, tracing the H₂O molecules along the channel reveals that they are more mobile in G52V DAAO, which might explain the lower energy barriers observed for the variant. The valine side chain in the G52V variant not only displaces O₂ but also H₂O from site A. Thus, it interferes with the formation of the transient H₂O chain, which in WT DAAO provides a stabilizing anchor for the other H₂O molecules in the mostly hydrophobic O₂ access channel.
the reactivity of reduced flavin with O$_2$, we have compared the rates of the half-reactions with those of WT DAAO. Thus, the rate of flavin reduction by $\alpha$-Ala, the best substrate (20), is 2-fold faster for G52V DAAO compared with WT DAAO (Fig. 4A and supplemental material). $K_d$ and the rate of product dissociation (Scheme 1a, $k_d$) are identical (supplemental Table 1). Because the chemical steps underlying substrate dehydrogenation are likely the chemically “difficult” ones, we assume that the corresponding catalytic machinery is essentially unaltered in DAAO following substitution of Gly-52.

Reactivity of Free, Reduced G52V DAAO with O$_2$ Is Lowered ≈100-Fold—In contrast to the reductive half-reaction, the reactivity of the DAAO variants in the oxidative half-reaction is drastically lowered (Fig. 4B). The profile that represents the reaction of the free reduced variant with O$_2$ ($k_r$) reflects a ≈100-fold lower rate (Table 1, Fig. 4B, and supplemental Fig. 6). The rate of the reaction of the reduced enzyme-IP complex with O$_2$ for G52V DAAO could not be assessed directly because of the unfavorable equilibria for the formation of the complex itself (see supplemental material). However, a lower limit for $k_r$ corresponding to an ≈100-fold decrease compared with WT DAAO was obtained by simulation of steady state measurements (see supplemental material). Because the parameters for the reductive half-reaction are essentially the same for WT and G52V DAAO, the ≈1000-fold decrease in $k_{cat}$ should result from effects on parameters connected with the oxidative half-reaction (Table 1). Because similar catalytic parameters have been determined for the three G52X DAAO variants (supplemental Table 1), we assume that the decrease of O$_2$ reactivity is not due to a specific effect of the valine isopropyl side chain. The drastic difference in catalytic properties of G52V versus WT DAAO is perhaps best illustrated by the data in Fig. 4C. In this, the absorbance at 455 nm (ordinate) reflects the relative concentrations of oxidized and reduced enzyme species present during turnover. The absorbance value at a given point during turnover also corresponds to the net ratio of the rates of steps involved in enzyme reduction and enzyme (re)oxidation under the specific conditions at this point in time. In these experiments, the enzyme is present in the oxidized form at the very beginning of the reaction, and its absorbance is indicated by Start in Fig. 4C. Upon mixing WT DAAO with $\alpha$-Ala in the presence of O$_2$, there is a very rapid absorbance decrease by some 10% that occurs in the first ≈10 ms of the reaction (data not shown, difference between “Start” absorbance and initial absorbance data points). Then the enzyme enters a stationary phase (turnover) up to ≈5 s and subsequently gradually returns to the original absorbance value within 20 s. Of importance in the present context is the observation that during turnover WT DAAO is present mainly (≈90%) in its oxidized form. Consequently, for WT DAAO, the net velocity of the reductive half-reaction is ≈1/10 that of the oxidative half-reaction. In sharp contrast to this, for G52V DAAO essentially total conversion to the reduced form occurs within 1–2 s (Fig. 4C). Then the enzyme remains reduced until consumption of the reductive substrate $\alpha$-Ala and subsequently returns to the oxidized state within 100 s (Fig. 4C). For G52V DAAO, the ratio of the net velocities for the reductive and oxidative half-reactions is ≈100 (see also supplemental material). The kinetic constants that were extracted from these experiments (Table 1 and supplemental Fig. 7) are consistent with a catalytic mechanism for the G52V variant in which the decrease of $k_{cat}$ compared with WT DAAO

![Figure 4. Comparison of rapid reaction kinetics for WT and G52V DAAO using $\alpha$-Ala as substrate.](image)

**TABLE 1**

| Steady state | Reductive half-reaction | Oxidative half-reaction |
|--------------|-------------------------|-------------------------|
| $k_{cat}$ ($s^{-1}$) | $K_{m,app,\alpha$-Ala} ($mM$) | $K_{m,O_2}$ ($mM$) |
| Wild-type | 330 ± 30 | 2.6 ± 0.4 | 3.0 ± 0.2 |
| G52V | 0.33 ± 0.03 | 0.036 ± 0.005 | 0.15 ± 0.03 |

*Data are from Ref. 20.
*Data are estimated from simulations of steady state kinetics (see supplemental Fig. 7).
results largely from the decrease of the rate of reoxidation of the reduced enzyme product complex with \( O_2 \) \( (k_3, \text{Scheme 1}) \). In a similar approach used to test the role of site B, residue Thr-201 was exchanged into Leu and Val. In contrast to the G52X variants, however, the overall catalytic properties of the T201L/T201V DAAOs are not significantly different from those of WT enzyme \( (\text{supplemental Table 2}) \).

**Overall Kinetic Mechanism of G52V DDAO**—From the time courses reported in Fig. 4C \( (\text{see also supplemental Figs. 4 and 7}) \), it is apparent that during turnover the DAAO variants are largely present in the reduced form, whereas WT DAAO is mainly in the oxidized form during the course of the same experiment. These observations and the evidence from the rapid reaction studies indicate that the major effect resulting from the substitution of Gly-52 in yeast DAAO leads to an alteration of the rate(s) of reaction of the reduced enzyme flavin with \( O_2 \). The validity of the estimated rate constants and of the overall kinetic mechanism was tested by simulation of the time courses of flavin absorbance changes during enzyme-monitored turnover experiments. For this, the sequences of kinetic steps of Scheme 1 and the experimentally employed \( \alpha \)-Ala and oxygen concentrations were used. An example of the quality of the simulation procedure is given in supplemental Fig. 7 for G52V DDAO. From this analysis, values for \( k_3 = 2.4 \pm 0.8 \times 10^3 \text{ M}^{-1} \text{ s}^{-1} \) and \( k_6 \approx 300 \text{ M}^{-1} \text{ s}^{-1} \) were estimated. This confirms that the main kinetic change following substitution of Gly-52 in DAAO is related to a \( \approx 100\)-fold decrease in rate constants involved in the reoxidation of reduced flavin by \( O_2 \), \( k_3 \), and \( k_6 \) in Fig. 4.

**DISCUSSION**

**Implications for the Reaction Mechanism**—Sites A and B (Fig. 1) lie close to the flavin on the \( \text{Si} \)-side. Site A is in contact with the isoalloxazine system, with its center being \( \approx 3.5 \text{ Å} \) from the flavin C(4a) position. Because the highest density of the reduced flavin negative charge is at positions C(4a)-N(5) \( (30) \), site A would constitute an ideal location for efficient \( O_2 \) reactivity. However, the negative charge is delocalized over the whole isoalloxazine system, with a substantial fraction being found in the C(7)-C(8) area \( (31) \). Site B at \( \approx 5 \text{ Å} \) from the flavin xylene edge would thus also be suitable for electron transfer. The relative importance of sites A and B can be inferred from the mutagenesis results. Obstruction of site A as in G52X DAAOs reduces the \( O_2 \) reactivity up to 100-fold, which sets an upper limit for a chemical reactivity via site B in the low percent range compared with site A. This is supported by the absence of major kinetic effects upon the insertion of a sterically demanding substituent in site B \( (\text{T201L DAAO}) \). On the other hand, sites A and B are close \( (\approx 8 \text{ Å}) \); they are connected via site C and intertransfer of \( O_2 \) proceeds through a low activation energy barrier \( (\text{Fig. 1B}) \). Thus, site B increases the effective \( [O_2] \) in the vicinity of site A. The change in dielectric constant within site B resulting from the \( \text{CH(OH)} \cdot \text{CH}_2 \) to \( \text{CH} \cdot \text{(CH}_3)_2 \) exchange following mutagenesis at position 201 might increase its affinity for \( O_2 \) in a \"storage role,\" and this results in the observed and somewhat higher reactivity of the T201L DAAO with \( O_2 \).

The additional sites of \( O_2 \) affinity \( (\text{Fig. 1A}) \) could also play relevant roles. Site C is located just under the protein surface at the entrance of the channel leading to the high affinity sites A and B. Together with the affinity sites at the protein surface, it constitutes a region where \( O_2 \) is \"collected/concentrated\" for further transport and utilization (catchment area). The energetic profiles of the various paths of \( O_2 \) between solvent and the sites of high affinity in the vicinity of the flavin \( (\text{Fig. 1B}) \) emphasize that once \( O_2 \) has entered the protein frame, it can move about freely and get to the point of chemical reactivity with a high frequency; the \( O_2 \) storage functionality can be ascribed to the entire channel system. Furthermore, storage should not be understood in the sense that a single \( O_2 \) molecule finds its way into the channel and is kept there until the reaction occurs. Instead, the \( O_2 \) equilibration between channel and solvent is much faster than the reaction time. Hence, many \( O_2 \) molecules enter and leave the channel before eventually one of them participates in the reaction. The affinity of the channel system increases the probability for each \( O_2 \) to stay close to flavin thus enhancing its bimolecular reaction term.

An unexpected finding is the uncovering of a chain of ordered \( \text{H}_2\text{O} \) molecules that could represent a \( \text{H}^+ \) relay system. Its importance becomes evident in view of a key conclusion from the work of Klinman and co-workers \( (32) \) on the reactivity of \( \text{(reduced)} \) glucose oxidase with \( O_2 \). This is best described in their statement: \"...a protonated active site histidine at low pH accelerates the second-order rate constant for one electron transfer to dioxygen through electrostatic stabilization of the superoxide anion intermediate.\" One difference between the active sites of DAAO and glucose oxidase is that in the former there is no amino acid residue at the active site or its vicinity that might have a role analogous to that of the His in glucose oxidase \( (32) \). This role is proposed to be exerted by the \( \text{H}^+ \) relay chain and to consist
in the transfer of a H\(^+\) from the \(=\text{NH}_2\) function of bound IP to the developing, negatively charged O\(_2\) species. This H\(^+\) relay is shown in Figs. 2 and 5.

This interpretation also provides an answer to the long standing puzzle that the reactivity of the reduced DAAO-IP complex with O\(_2\) is substantially higher than that of the free form (Scheme 1). The opposite would be expected because in the free enzyme there is no apparent hindrance in O\(_2\) access. An important feature of the mechanism shown in Fig. 5 is its rearrangement and balance of charges. In the E-Fl

\(^{\text{red}}\)-P complex (Fig. 5, left), the opposed charges on reduced flavin and IP are an important thermodynamic factor for complex stability, whereas the negative charge on the reduced flavin is required to enhance reactivity with O\(_2\) (32, 33). Upon transfer of electrons and H\(^+\) to O\(_2\), the resulting complex (Fig. 5, right) is now neutral. Consequently, the rate of product dissociation is enhanced providing a molecular rationale for the sequential kinetic mechanism of DAAO (and related enzymes) in turnover with neutral \(\alpha\)-amino acids (19, 29).

Because H\(_2\)O\(_2\) is only somewhat larger than O\(_2\) or H\(_2\)O, the O\(_2\) channel and the H\(^+\) relay channel might in principle also serve for its release in a way similar to the “in-out” diffusion of H\(_2\)O. Furthermore, if a path via a S\(_i\) \(\rightarrow\) Re migration and via the H\(^+\) channel would be feasible, once H\(_2\)O\(_2\) is on the Re-side of the flavin, it would likely leave through the substrate channel following IP release. In the three-dimensional structure of DAAO, a small molecule on the flavin Re-side near the N(5)-C(4a) position was detected at low occupancy (19). This observation could be reconciled with the presence of H\(_2\)O\(_2\) that might have become trapped at that position before leaving the active center via the substrate entrance.

Comparison within the Flavoprotein Oxidase Family—For an efficient activation of O\(_2\), the reduced flavin ought to be in its anionic form (14, 34, 35), a condition found in most oxidases. A second prerequisite is the presence of a system that provides a H\(^+\) that neutralizes the developing O\(_2\)\(^{=\text{O}}\)HOO\(^-\). This role has been assigned to a His in glucose oxidase (12, 32) and to a Lys in DAAO (supplemental Fig. 2), and it is placed such as to suggest a H\(^+\) donor role in O\(_2\) activation also on the flavin Si-side. Thus, D- and L-\(\alpha\)-amino-acid oxidases would share the flavin Si-side for O\(_2\) activation; however, they would use two variants of the same theme for H\(^+\) transfer in the chemical activation of O\(_2\).

Conclusions—This work demonstrates the great potential of joint computational/experimental research. MD simulations allowed individuating specific paths and positions suitable for the diffusion and reactivity of O\(_2\) and thus the prediction of effects resulting from steric constrictions. Meanwhile, biochemical experiments provided the required verification. The uncovering of steric and chemical factors that affect O\(_2\) reactivity brings up the question of their relative importance. Although a clear-cut differentiation is not possible, we conclude the following.

(i) In free, reduced WT DAAO, O\(_2\) has unrestricted access via both the O\(_2\) and the substrate channels. There is no H\(^+\) relay chain. The observed O\(_2\) reaction rate should thus be the intrinsic one (step \(k_3\) in Scheme 1).

(ii) In G52V DAAO, there is a steric hindrance to O\(_2\) access and no H\(^+\) relay chain. The \(\approx 100\)-fold difference of the rates in i and ii should thus be due to steric factors. Note that this decrease might be up to 10-fold higher because the (75 mV) more negative \(E_{\text{m}}\) in G52V variant compared with WT DAAO partially counters the steric effects.

(iii) A lower limit estimate of the effect of the H\(^+\) relay chain can be obtained by comparing the rates of reoxidation of reduced WT DAAO in the free (no H\(^+\) relay) and IP complexed state (with H\(^+\) relay), corresponding to \(k_3\) and \(k_4\) rate constants (Scheme 1). This difference is \(\approx 10\)-fold for yeast DAAO (20).

REFERENCES

1. Lakowicz, J. R., and Weber, G. (1973) Biochemistry 12, 4161–4170
2. Scott, E. E., Gibson, Q. H., and Olson, J. S. (2001) J. Biol. Chem. 276, 5177–5188
3. Srajer, V., Ren, Z., Teng, T. Y., Schmidt, M., Ursby, T., Bourgeois, D., Pradervand, C., Schildkamp, W., Wulff, M., and Moffat, K. (2001) Biochemistry 40, 13802–13815
4. Cohen, J., Arkhipov, A., Braun, R., and Schulten, K. (2006) Biophys. J. 91, 1844–1857
5. Olson, J. S., Soman, J., and Phillips, G. N., Jr. (2007) IUBMB Life 59, 552–562
6. Hofacker, I., and Schulten, K. (1998) Proteins 30, 100–107
7. Soulimane, T., Buse, G., Bourenkov, G. P., Bartunik, H. D., Huber, R., and Than, M. E. (2000) EMBO J. 19, 1766–1776
8. Knapp, M. J., Seebeck, F. P., and Klinman, J. P. (2001) J. Am. Chem. Soc. 123, 2931–2932
9. Knapp, M. J., and Klinman, J. P. (2003) Biochemistry 42, 11466–11475
10. Saam, J., Ivanov, I., Walther, M., Holzhüttner, H. G., and Kuhn, H. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 13319–13324
11. Johnson, B. J., Cohen, J., Welford, R. W., Pearson, A. R., Schulten, K., Klinman, J. P., and Wilmut, C. M. (2007) J. Biol. Chem. 282, 17767–17776
12. Kemal, C., Chan, T. W., and Bruce, R. C. (1977) J. Am. Chem. Soc. 99, 7272–7286
13. Roth, J. P., Wincek, R., Nodet, G., Edmondson, D. E., McIntire, W. S., and Klinman, J. P. (2004) J. Am. Chem. Soc. 126, 15120–15131
14. Klinman, J. P. (2007) Acc. Chem. Res. 40, 325–333
15. Couломbe, R., Yue, K. Q., Ghisla, S., and Vrieling, A. (2001) J. Biol. Chem. 276, 30435–30441
16. Vrieling, A., and Ghisla, S. (2009) FEBS J. 276, 6826–6843
17. Baron, R., Riley, C., Chenprakhon, P., Thotsaporn, K., Winter, R. T., Aliferi, A., Forneris, F., van Berkel, W. J., Chaiyen, P., Fraaije, M. W., Mattevi, A., and McCammon, J. A. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 10603–10608
18. Bollegioni, L., Piubelli, L., Sacchi, S., Pilone, M. S., and Molla, G. (2007) Cell. Mol. Life Sci. 64, 1373–1394
19. Umhau, S., Bollegioni, L., Molla, G., Diederichs, K., Welte, W., Pilone, M. S., and Ghisla, S. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 12463–12468
20. Pollegioni, L., Langkau, B., Fischer, W., Ghisla, S., and Pilone, M. S. (1993) J. Biol. Chem. 268, 13850–13857
21. Kalé, L., Skoel, R., Bhandarkar, M., Brunner, R., Gursoy, A., Krawetz, N., Phillips, J., Shinozaki, A., Varadarajan, K., and Schulten, K. (1999) J. Comp. Phys. 151, 283–312
22. Humphrey, W., Dalle, A., and Schulten, K. (1996) J. Mol. Graph. 14, 33–38
23. Rosini, E., Bollegioni, L., Ghisla, S., Orru, R., and Molla, G. (2009) FEBS J. 276, 4921–4932
24. Pollegioni, L., Porrini, D., Molla, G., and Pilone, M. S. (2000) Eur. J. Biochem. 267, 6624–6632
25. Gibson, Q. H., Swoboda, B. E., and Massey, V. (1964) J. Biol. Chem. 239, 3927–3934
26. Kawazoe, T., Tsuge, H., Pilone, M. S., and Fukui, K. (2006) Protein Sci. 15, 2708–2717

J. Biol. Chem.
Oxygen Diffusion in \textit{d}-Amino Acid Oxidase

27. Todone, F., Vanoni, M. A., Mozzarelli, A., Bolognesi, M., Coda, A., Curti, B., and Mattevi, A. (1997) \textit{Biochemistry} \textbf{36}, 5853–5860

28. Yorita, K., Misaki, H., Palfey, B. A., and Massey, V. (2000) \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{97}, 2480–2485

29. Porter, D. J., Voet, J. G., and Bright, H. J. (1977) \textit{J. Biol. Chem.} \textbf{252}, 4464–4473

30. Tilocca, A., Gamba, A., Vanoni, M. A., and Fois, E. (2002) \textit{Biochemistry} \textbf{41}, 14111–14121

31. Hall, L., Orchard, B., and Tripathy, S. (1987) \textit{Int. J. Quantum Chem.} \textbf{31}, 217–242

32. Su, Q., and Klinman, J. P. (1999) \textit{Biochemistry} \textbf{38}, 8572–8581

33. Massey, V. (1994) \textit{J. Biol. Chem.} \textbf{269}, 22459–22462

34. Favaudon, V. (1977) \textit{Eur. J. Biochem.} \textbf{78}, 293–307

35. Massey, V. (2002) \textit{Int. Congr. Ser.} \textbf{1233}, 3–11

36. Zhao, G., Bruckner, R. C., and Jorns, M. S. (2008) \textit{Biochemistry} \textbf{47}, 9124–9135

37. Binda, C., Li, M., Hubalek, F., Restelli, N., Edmondson, D. E., and Mattevi, A. (2003) \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{100}, 9750–9755

38. Son, S. Y., Ma, J., Kondou, Y., Yoshimura, M., Yamashita, E., and Tsukihara, T. (2008) \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{105}, 5739–5744