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Insights on the Mechanism of Amine Oxidation Catalyzed by D-Arginine Dehydrogenase Through pH and Kinetic Isotope Effects

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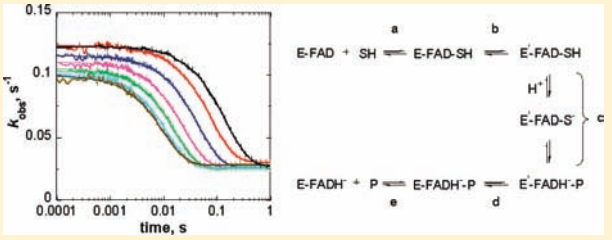
ABSTRACT: The mechanism of amine oxidation catalyzed by D-arginine dehydrogenase (DADH) has been investigated using steady-state and rapid reaction kinetics, with pH, substrate and solvent deuterium kinetic isotope effects (KIE) as mechanistic probes, and computational studies. Previous results showed that 85−90% of the flavin reduction reaction occurs in the mixing time of the stopped-flow spectrophotometer when arginine is the substrate, precluding a mechanistic investigation. Consequently, leucine, with slower kinetics, has been used here as the flavin-reducing substrate. Free energy calculations and the pH profile of the K_d are consistent with the enzyme preferentially binding the zwitterionic form of the substrate. Isomerization of the Michaelis complex, yielding an enzyme−substrate complex competent for flavin reduction, is established due to an inverse hyperbolic dependence of k_{cat}/K_m on solvent viscosity. Amine deprotonation triggers the oxidation reaction, with cleavage of the substrate NH and CH bonds occurring in an asynchronous fashion, as suggested by the multiple deuterium KIE on the rate constant for flavin reduction (k_{red}). A pK_a of 9.6 signifies the ionization of a group that facilitates flavin reduction in the unprotonated form. The previously reported high-resolution crystal structures of the iminoarginine and iminohistidine complexes of DADH allow us to propose that Tyr_{53}, on a mobile loop covering the active site, may participate in substrate binding and facilitate flavin reduction.



INTRODUCTION

The oxidation of primary amines plays important roles in biology and is crucial to several biochemical processes, such as neurotransmission,[1] cell growth and differentiation[2] and neoplastic cell proliferation.[3,4] The enzymes that oxidize C−N bonds have been shown to utilize $NADP^+$, copper and 2,4,5-trihydroxyphenylalanine quinone (TPQ), or flavins.[5−8] Glutamate dehydrogenase serves as the prototype for the $NADP^+$-dependent class of primary amine oxidizing enzymes,[5] copper-amine oxidase for the Cu^{2+}-TPQ enzymes,[6] and D-amino acid oxidase and monoamine oxidase for the flavin-dependent family.[7,9−11]

Amine oxidation in glutamate dehydrogenase has been proposed to proceed through a direct hydride transfer from the C_α atom of glutamate to the $NADP^+$.[12] The enzyme-catalyzed deprotonation of the substrate α-NH_3^+ group in the active site of the enzyme is proposed to trigger the reaction.[12] Mechanistic studies on copper amine oxidase have elucidated the involvement of a Schiff base of the TPQ with the amino group of the substrate, which remains bound to the reduced cofactor after substrate oxidation.[13] The oxidation of the reduced TPQ by molecular oxygen through two one-electron steps and a TPQ semiquinone intermediate complete the turnover of the enzyme.[14] With D-amino acid oxidase (DAAO), the results of mechanistic studies with physiological substrates and of structural studies with the enzyme in complex with inhibitors are most consistent with a hydride transfer mechanism of catalysis.[7,15,16] Lack of solvent kinetic isotope effect (KIE) and an inverse ^{15}N isotope effect of 0.996 on the k_{cat}/K_m value with D-serine as substrate are consistent with a hydride transfer mechanism from the C_α atom of the anionic amine to the flavin N(5) atom.[17,18] Similar inverse ^{15}N isotope effects have been reported for tryptophan monooxygenase (TMO) and *N*-methyltryptophan oxidase (MTOX).[19,20] What promotes the formation of the anionic amine in the Michaelis complexes of these enzymes is not clear. Computational studies on DAAO have also supported a hydride transfer mechanism from the anionic amino acid to the flavin.[21] With monoamine oxidase (MAO) the inactivation of the enzyme by *trans*-2-phenylcyclopropylamine was initially used to propose that oxidation of primary amines involves radical intermediates.[22−25] A nucleophilic mechanism for amine oxidation in which a flavin C(4a) adduct is formed prior to proton transfer from the substrate C_α atom to the flavin N(5) atom has also been proposed.[26−28] Nitrogen KIE for the oxidation of benzylamine and perdeuterated benzylamine by MAO B are consistent with rehybridization of the nitrogen atom and CH bond cleavage not being concerted, in keeping with radical and nucleophilic mechanisms.[29] The proposals for amine oxidation in MAO implicate the anionic amine as the competent form of the substrate that is oxidized. However, the mechanism of amine deprotonation has not been investigated.

We recently subcloned, expressed and purified to high levels a novel member of the class of flavin-dependent, amine-oxidizing enzymes: D-arginine dehydrogenase (DADH).[30,31] The enzyme from *Pseudomonas aeruginosa* catalyzes the flavin-linked oxidation

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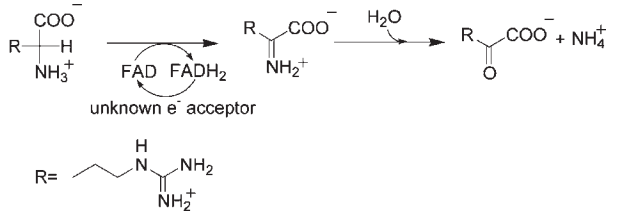
Scheme 1. Oxidation of D-Arginine Dehydrogenase by DADH



of D-arginine to iminoarginine, which is non-enzymatically hydrolyzed to 5-guanidino-2-oxopentanoic acid and ammonia (Scheme 1). DADH is strictly a dehydrogenase, in that during turnover its reduced FAD reacts with an electron acceptor other than molecular oxygen,[31] presumably ubiquinone. The enzyme displays broad substrate specificity, being able to oxidize all D-amino acids except for D-glutamate, D-aspartate, and glycine.[30,32] In the crystal structure of the enzyme in complex with iminoarginine resolved to 1.3 Å the side chain of Glu_{87} forms an ionic interaction with the guanidinium group of the ligand (Figure 1),[30] providing a firm rationale for D-arginine being the best substrate with a k_{cat}/K_m value of 10^6 M^{-1} s^{-1}.[31] Several ionic and hydrogen-bond interactions are established between the carboxylate of iminoarginine and the side chains of Tyr_{53}, Arg_{222}, Arg_{305}, and Tyr_{249}, suggesting that these residues may also play a role in substrate binding (Figure 1). Similar interactions are seen in the structure of the enzyme in complex with iminohistidine.[30] Flavin reduction (k_{red}) with arginine occurs with a rate constant ≥ 700 s^{-1}, precluding mechanistic investigations of the reductive half-reaction catalyzed by the enzyme with this substrate by using a stopped-flow spectrophotometer.[31]

In the present study, the mechanism for amine oxidation catalyzed by DADH has been investigated with leucine as substrate using steady-state and rapid kinetics and mechanistic probes, such as pH effects, substrate and solvent deuterium KIEs, and solvent viscosity effects. leucine was chosen as the flavin-reducing substrate because it is the slowest substrate for DADH for which the kinetic parameters, k_{cat} and K_m, had been previously determined using the steady-state kinetic approach.[30] The mechanistic investigation, complemented with free energy calculations and molecular dynamics simulations, establishes that the pK_a of the amino moiety of leucine in the Michaelis complex is consistent with the zwitterionic form of leucine being the preferred substrate. The substrate amine is then deprotonated, triggering the transfer of a hydride equivalent to the enzyme-bound flavin. Substrate deprotonation and flavin reduction occur asynchronously. The mechanistic conclusions are interpreted in view of the recently reported high-resolution structures of DADH.[30]

EXPERIMENTAL SECTION

Materials. Leucine was from TCI America (Portland, OR). Leucine-d_{10} was purchased from CDN Isotopes (Canada). Sodium deuterium oxide (99%) was from Isotec Inc. (Miamisburg, OH). Deuterium oxide (99.9%), glucose and glucose oxidase were obtained from Sigma-Aldrich (St. Louis, MO). DADH was prepared as described previously.[31] All of the other reagents were of the highest purity commercially available.

Rapid Reaction Kinetics. Reductive half-reactions were carried out using an SF-61DX2 Hi-Tech KinetAsyst high performance stopped-flow spectrophotometer, thermostatted at 25 °C. Unless otherwise stated, the observed rate constants for flavin reduction (k_{obs}) were determined at varying concentrations of leucine in 20 mM sodium pyrophosphate, pH 10.3, under pseudofirst order conditions where the final concentration after mixing of the enzyme was 10 μM and that of the substrate between 0.5 and 50 mM. The stopped-flow traces obtained anerobically were identical to those obtained aerobically. Leucine and leucine-d_{10} were used as substrates to determine the substrate deuterium KIE. For the determination of the solvent deuterium KIE and the proton inventories in solvents containing varying mole fractions of D_2O, the pD values were adjusted using DCl and NaOD based on the empirical relationship (eq 1) that exists between the pH-meter reading and the pD value at varying mole fractions of D_2O (n).[33] Solvent viscosity effects were measured in the presence of 9% glycerol as viscosigen, in both the tonometer containing the enzyme and the syringes containing the organic substrates. The resulting relative viscosity at 25 °C was 1.26, which is slightly above the value of 1.23 representing a 100% solution of D_2O.[34] The pL dependence of the reductive half-reaction of DADH was studied in the stopped-flow spectrophotometer as described above in the pL range from 7.0 to 11.0. Sodium phosphate (20 mM final) was used in the pL range from 7.0 to 8.0; sodium pyrophosphate was used in the pL range from 8.5 to 10.3 and EDTA in the pL range from 10.5 to 11.0. Instability of the enzyme precluded extending the pL studies at pL values lower than 7.0.

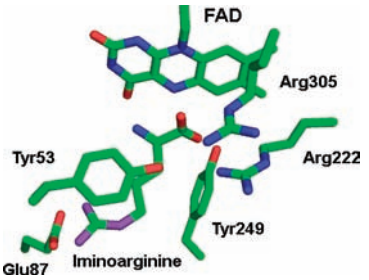


Figure 1. Active site of DADH in complex with iminoarginine, with selected amino acid residues shown. The structure is from Protein Data Bank entry 3NYE.

$$(\Delta pH)_n = 0.067n^2 + 0.3314n \quad (1)$$

Steady-State Kinetics. The steady-state kinetic parameters with leucine were determined by measuring initial rates of reaction with an oxygen electrode. The determinations were carried out in 20 mM sodium pyrophosphate, pH 10.3 and 25 °C, at varying concentrations of leucine and a fixed, saturating concentration of 1 mM for PMS ($K_m \approx$ 10 μM) as the electron acceptor, as previously described.[31] Solvent viscosity effects were determined at pH 10.3 using glycerol as viscosigen. The values for the relative viscosities of glycerol-containing solutions were taken from Weast[35] and adjusted for 25 °C. Substrate deuterium KIEs were determined with leucine and leucine-d_{10}. For the determination of the pH profiles, the same buffers used in the rapid reaction kinetics were employed. All reaction rates are expressed per molar concentration of enzyme-bound flavin.

Data Analysis. Data were fit with the KaleidaGraph software (Synergy Software, Reading, PA) and the Hi-Kinetic Studio Software Suite (Hi-Tech Scientific, Bradford-on-Avon, U.K.).

Time-resolved flavin reductions were fit to eq 2, which describes a single exponential process for flavin reduction. k_{obs} represents the observed first-order rate constant associated with the absorbance changes at 449 nm at a given concentration of substrate, t is time, A is the absorbance at 449 nm at any given time, B is the amplitude of the absorbance change, and C is the absorbance at infinite time.

$$A = B\,e^{-k_{obs}t} + C \quad (2)$$

Reductive half-reaction parameters were determined by using eq 3. k_{obs} is the observed first-order rate constant for the reduction of the enzyme-bound flavin at any given concentration of substrate (S), k_{red} is the limiting first-order rate constant for flavin reduction at saturating concentrations of substrate, and K_d is the apparent dissociation constant for the dissociation of the enzyme−substrate complex into free enzyme and substrate.

$$k_{obs} = \frac{k_{red}S}{K_d + S} \quad (3)$$

The substrate deuterium KIEs were calculated using eq 4, which applies for a KIE on the k_{red} value. Solvent deuterium and multiple KIEs were calculated using eq 5, which describes a KIE on the k_{red} value and a solvent effect on the K_d value. F_i is the fraction of heavy atom, $^{D}k_{red}$ is the KIE on k_{red}, and $^{SE}(K_d)$ is the solvent effect due to pH on the apparent K_d value elicited by substituting water with D_2O at those pL values where K_d is not pH-independent.

$$k_{obs} = \frac{k_{red}S}{(K_d + S)[1 + F_i(^{D}k_{red} - 1)]} \quad (4)$$

$$k_{obs} = \frac{k_{red}S}{K_d\left[1 + F_i\left(\frac{^{D}k_{red}}{^{SE}(K_d)}\right) - 1\right] + S[1 + F_i(^{D}k_{red} - 1)]} \quad (5)$$

The pL dependence of the k_{red} value was determined by fitting the data with eq 6, where $k_{red}(lim)$ is the limiting, pL-independent rate constant for flavin reduction at high pL, and pK_a is the apparent value for the ionization of a group that must be unprotonated for flavin reduction.

$$\log k_{red} = \log \frac{k_{red}(lim)}{1 + \frac{10^{-pH}}{10^{-pK_a}}} \quad (6)$$

The pH dependence of the K_d value was determined by fitting the data with eq 7, which describes a curve with a slope of +1 at high pH and a plateau region that defines a limiting, pH-independent K_d value at low pH, i.e., $K_d(lim)$.

$$\log(K_d) = \log[K_d(lim) \times (1 + 10^{pH - pK_a})] \quad (7)$$

The proton inventory on the k_{red} value was fit with eq 8.[33] Here, $(k_{red})_n$ is the limiting first-order rate constant for flavin reduction in n mole fraction D_2O, $(k_{red})_{H2O}$ is the limiting first-order rate constant for flavin reduction in H_2O, and Φ_T is the isotopic fractionation factor of the transition state proton.

$$\frac{(k_{red})_n}{(k_{red})_{H_2O}} = 1 - n + n\phi_T \quad (8)$$

Estimating the ΔpK_a of the amino group of leucine by molecular dynamics simulations. The change in the microscopic pK_a of the amine moiety of leucine as it moves from water to the binding site of DADH was estimated using eq 9. Here $\Delta\Delta G$ is the relative binding free energy of protonated and unprotonated leucine to DADH. $\Delta\Delta G$ was calculated by perturbing leucine using thermodynamic integration[36,37] combined with molecular dynamics (MD) simulations from a protonated to a deprotonated amino group in explicit TIP3P[38] water and in the active site of DADH, using the 1.06 Å resolution structure with PDB ID 3NYC. The carboxylic group was modeled in the anionic form. The protonation state of the ionizable amino acids in DADH were modeled as unprotanated for glutamate and aspartate, protonated for arginine and lysine, and neutral for histidine, since the experiments were carried out at relatively high pH of around 10. The system was then neutralized with 16 Na^+ ions, after solvating with 13914 TIP3P water molecules in a periodic octahedron box. The water molecules were placed such that the edges of the box were at least 10 Å away from the surface of the protein. The AMBER 9 suite of programs[39] and the modified version of the Cornell et al. force field[40,41] were used to carry out all molecular dynamics and free energy simulations, as previously described.[42] All simulations were carried out at a constant temperature of 300 K and constant pressure of 1 bar. The temperature was controlled using the Langevin thermostat with a collision frequency of 1 ps^{-1}. A time step of 2 fs was used to numerically solve Newton's equation of motion. Electrostatic interactions were treated using particle mesh Ewald (PME) method,[43] and all bonds involving H-atoms were constrained using the SHAKE algorithm.[44] The cutoff for all long-range, nonbonded interaction was set to 9 Å.

$$\Delta pK_a = \frac{1}{2.303kT}(\Delta\Delta G) \quad (9)$$

The potential energy function, $V(\lambda)$, defining the transformation from the protonated state to the unprotonated state of leucine is controlled by λ, where $\lambda = 0$ for the protonated state and $\lambda = 1$ for the unprotonated state. The integral over $\langle\partial V(\lambda)/\partial\lambda\rangle\lambda$, from $\lambda = 0$ to 1, gives the free energy change. The integral was calculated using a 7-points Gaussian quadrature at seven discrete λ values: 0.02544, 0.12923, 0.29707, 0.50000, 0.70292, 0.87076, and 0.97455. The partial atomic charges for the protonated and unprotonated form of leucine and the flavin were derived using the standard two-step RESP method[45] from the electrostatic potential calculated at the HF/6-31G* level of theory using quantum mechanics. The flavin was modeled in the fully oxidized form (FAD) because this form represents the state of the flavin before the hydride transfer reaction. The general Amber force field (GAFF) parameters were used to describe FAD.[46] Molecular dynamics simulations (10^6 steps (2 ns)) were performed for each λ value, and data from the second half were used. The first half was considered to be an equilibration phase. Each simulation was repeated a minimum of three times with a different initial random seed. The error of the computed free energy change was estimated by calculating the standard deviation of the results from the different runs.

RESULTS

Time-Resolved Flavin Reduction. The time-resolved reduction of DADH was investigated in a stopped-flow spectrophotometer at pH 10.3 and 25 °C by monitoring the loss of absorbance of the oxidized flavin at 449 nm upon mixing the enzyme with the reducing substrate leucine. Pseudofirst order conditions with ~10 μM enzyme and 0.5−50 mM reducing substrate were maintained, and the resulting stopped-flow traces were fit to single exponentials to obtain observed rate constants (k_{obs}) for flavin reduction at any given substrate concentration (Figure 2A). The k_{obs} value was hyperbolically dependent on the concentration of the reducing substrate (Figure 2B), allowing for the determination of the limiting rate constant for flavin reduction (k_{red}) and the apparent equilibrium constant for the dissociation of the substrate from the Michaelis complex (K_d). The best fit of the data was obtained with the curve extrapolating to the plot origin (Figure 2B), consistent with flavin reduction being irreversible and in agreement with previous results with histidine as the reducing substrate.[31] The UV−visible absorbance spectrum of the hydroquinone obtained at the end of the reduction process showed a well-resolved peak at 360 nm (Figure 2C), consistent with the presence of the anionic form of the flavin hydroquinone.

pH Effects on k_{red} and K_d. The effects of pH on the k_{red} and K_d values for DADH with leucine as the reducing substrate were determined in the pH range 7.0−11.0. This was carried out with

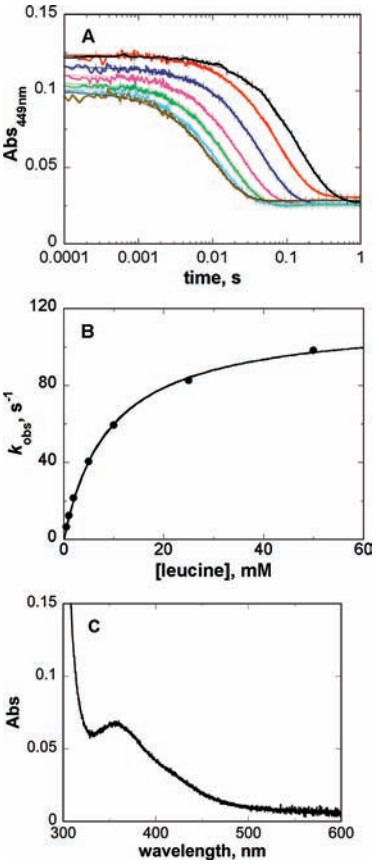


Figure 2. Time-resolved flavin reduction of DADH with leucine as the reducing substrate at pH 10.3 and 25 °C. Panel A shows the reduction traces of the enzyme-bound flavin (10 μM) with 0.5 mM (black), 1 mM (red), 2 mM (blue), 5 mM (purple), 10 mM (green), 25 mM (cyan), and 50 mM (brown) leucine. All traces were fit with eq 2. Time indicated is after the end of the flow, i.e. 2.2 ms. In the interest of clarity, one experimental point every 5 ms is shown (vertical lines). Panel B shows the observed rate constant for flavin reduction as a function of leucine concentration. Data were fit to eq 3, yielding $k_{red} = 115 \pm 2\ s^{-1}$ and $^{app}K_d = 9.2 \pm 0.4$ mM ($R^2 = 0.999$). Panel C shows the reduced DADH at pH 10.3.

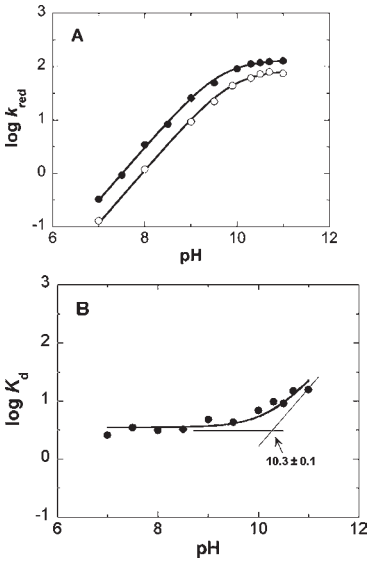


Figure 3. Effects of pH on the reductive half-reaction of DADH with leucine as the reducing substrate. Panel A shows the pL profiles of k_{red} in aqueous and deuterated buffers. Data were fit to eq 5, yielding $k_{red}(lim) = 133 \pm 5\ s^{-1}$, $pK_a = 9.6 \pm 0.1$ ($R^2 = 0.999$) in H_2O and $k_{red}(lim) = 85 \pm 4\ s^{-1}$, $pK_a = 9.9 \pm 0.1$ ($R^2 = 0.999$) in D_2O. Panel B shows the pH profile of K_d. Data were fit to eq 6, yielding $K_d(lim) = 3.5 \pm 0.3$ mM and $pK_a = 10.3 \pm 0.1$ ($R^2 = 0.89$).

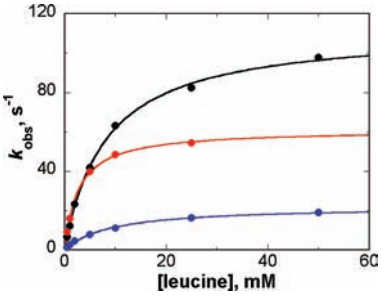


Figure 4. Observed rate constants as a function of substrate concentration. Black curve represents substrate reduction of DADH with leucine as a substrate in H_2O. Red curve represents substrate reduction of DADH with leucine as a substrate in D_2O. Blue curve represents substrate reduction of DADH with leucine-d_{10} as a substrate in H_2O. Data were acquired at pL 10.3 and fit to eqs 4 and 5.

the goal of determining the ionization states and the apparent pK_a values of groups that participate in the reaction of amine oxidation. At all pH values tested, and ensuring that pseudo-first-order conditions were maintained, the kinetics of the reductive half-reaction followed the general trends of Figure 2, allowing us to establish k_{red} and K_d values as a function of pH. At pH ≥7.0, the UV−visible absorbance spectrum of the reduced flavin was that of the anionic hydroquinone. The k_{red} increased with increasing pH and reached a plateau at high pH with a limiting value of $133 \pm 5\ s^{-1}$, defining an apparent pK_a value of 9.6 ± 0.1 (Figure 3A). The K_d was pH-independent below pH 9.5 and increased with increasing pH values, defining an apparent pK_a of ∼10.3 in H_2O (Figure 3B).

Substrate Deuterium KIE on k_{red}. To report on the status of the substrate CH bond in the transition state(s) for the amine oxidation catalyzed by the enzyme, the substrate deuterium KIE on the k_{red} value was determined at pH 10.3. In aqueous solution the $^{D}(k_{red})_{H2O}$ had a value of 5.1 ± 0.1 ($R^2 = 0.9996$) (Figure 4), consistent with cleavage of the substrate CH bond being manifested in the transition state for the reaction of flavin reduction. There was no KIE associated with the K_d value, and the best fit of the data was obtained by using eq 4. Upon substitution of water with D_2O there was a small, but significant, decrease in the magnitude of the KIE with a $^{D}(k_{red})_{D2O}$ value of 4.7 ± 0.1 ($R^2 = 0.9996$).

Solvent Deuterium KIE on k_{red}. Substitution of H_2O with D_2O yielded a pD profile for k_{red} similar to that seen in H_2O, with a limiting k_{red} value of $85 \pm 4\ s^{-1}$ and a pK_a value of 9.9 ± 0.1 (Figure 3A). The solvent KIE on k_{red} was determined at pL 10.3, where the enzyme was stable over prolonged time and artifactual contributions originating from pH effects are minimal. This was carried out in order to probe the status of bonds involving solvent exchangeable protons in the transition state(s) for the amine oxidation catalyzed by the enzyme. With leucine as substrate there was a normal $^{D2O}(k_{red})_{H}$ of 1.77 ± 0.01 ($R^2 = 0.9995$) (Figure 4), in good agreement with the pL-independent KIE of 1.6 ± 0.1 determined from the pL profile. The best fit of the data

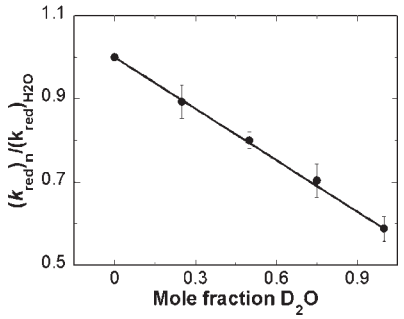


Figure 5. Proton inventory for the reduction rate, k_{red}, using leucine at pL 10.3.

was obtained by using eq 5, which allowed also for a solvent effect on the K_d value of 2.8 ± 0.1 to be calculated. Since at pL 10.3 the K_d value is not pL-independent (Figure 3), the solvent effect is likely due to a pH effect elicited by the substitution of water with D_2O. Consequently, the solvent effect on K_d was not considered further in this study. Upon replacing leucine with leucine-d_{10} there was a small, but significant, decrease in the $^{D2O}(k_{red})_D$ value to 1.60 ± 0.04 (R^2 = 0.9989).

As a control that the solvent effect on k_{red} is not due to the increased viscosity of D_2O as compared to H_2O, the k_{red} value was determined in aqueous solutions in the absence and presence of 9% glycerol, which is isoviscous with D_2O.[34,47] At pH 10.3, the k_{red} was similar when determined in the presence and absence of glycerol, with a $(k_{red})_{H2O}/(k_{red})_{glycerol}$ ratio of 1.00 ± 0.01 (R^2 = 0.9992 by using eq 4). These results established that the solvent deuterium KIE on k_{red} reports on solvent-exchangeable proton(s) that are in flight in the transition state for the reaction of flavin reduction.

Proton Inventory on k_{red}. The proton inventory technique was used to gain insights on the number of solvent-exchangeable protons responsible for the solvent deuterium KIE observed in the time-resolved reduction of DADH. The k_{red} value was determined at pL 10.3 in solutions with varying mole fractions of deuterium oxide, and the data were analyzed in a plot of the reciprocal of the KIE as a function of the mole fraction D_2O (Figure 5).[48] The resulting k_{red} proton inventory was linear, suggesting that a single solvent-exchangeable proton is in flight in the transition state for the reaction of flavin reduction (Figure 5).

Multiple Deuterium KIEs on k_{red}. Multiple deuterium KIEs were determined at pL 10.3 to elucidate whether the substrate and solvent deuterium KIE determined independently on the k_{red} value occurred in the same or in multiple kinetic steps. The $^{D,D2O}k_{red}$ value, representing the effect of substituting leucine in H_2O with leucine-d_{10} in D_2O, was 7.8 ± 0.1 (calculated by using eq 5). For comparison the product of the individual substrate and solvent deuterium KIEs, i.e., $^{D}(k_{red})_{D2O} \times {}^{D2O}(k_{red})_H$, is 9.0 ± 0.2.

Steady-State Kinetics. Previous results demonstrated a Ping Pong Bi−Bi steady-state kinetic mechanism for DADH with either arginine or histidine and that the K_m value for PMS is ∼10 μM.[31] Consequently, when the steady-state kinetic parameters are determined at fixed 1 mM PMS as in this study, they approximate well the true k_{cat}, K_m, and k_{cat}/K_m values for the amino acid substrate.

The effects of pH on the steady-state kinetic parameters with leucine as the varying substrate were determined with the method of the initial rates in the pH range from 7.0 to 10.3,[49] by coupling the consumption of PMS to that of oxygen using an oxygen electrode. The k_{cat} at any given pH was on average only 0.7 times lower than the k_{red} (Table 1). Similarly, the K_m was not different from the K_d determined by using a stopped-flow spectrophotometer (Table 1).

Table 1. Effects of pH on the Steady-State and Rapid Reaction Kinetic Parameters for DADH with Leucine as Substrate[a]

pH	k_{cat}, s^{-1}	$K_{m\ D\text{-}leu}$, mM	k_{red}, s^{-1}	$K_{d,\ leu}$, mM	k_{cat}/k_{red}	K_m/K_d
7.0	0.26 ± 0.01	3.1 ± 0.2	0.33 ± 0.01	2.6 ± 0.1	0.8	1.2
7.5	0.64 ± 0.01	3.6 ± 0.3	0.93 ± 0.01	3.5 ± 0.1	0.7	1.0
8.0	2.3 ± 0.1	2.6 ± 0.1	3.5 ± 0.1	3.0 ± 0.3	0.7	0.9
8.5	7.0 ± 0.2	3.4 ± 0.2	8.4 ± 0.3	3.2 ± 0.4	0.8	1.1
9.0	17 ± 1	4.7 ± 0.6	26 ± 1	4.8 ± 0.4	0.7	1.0
9.5	31 ± 1	3.8 ± 0.3	50 ± 2	4.3 ± 0.5	0.6	0.9
10.0	73 ± 3	6.6 ± 0.5	91 ± 1	6.8 ± 0.2	0.8	1.0
10.3	92 ± 1	10.9 ± 0.1	113 ± 1	9.7 ± 0.3	0.8	1.1
10.5			119 ± 2	9.1 ± 0.3		
10.7			125 ± 5	15 ± 1		
11.0			129 ± 4	16 ± 1		

[a] Conditions: 20 mM buffer, pH from 7 to 11 and 25 °C. Rapid reaction kinetic parameters were calculated with eq 2; steady-state kinetic parameters were obtained by using the Michaelis−Menten equation; in all cases, the R^2 of the fits were ≥0.991. By assuming pH-independence, the k_{cat}/k_{red} ratio has an average value of 0.74 ± 0.03, the K_m/K_d of 1.0 ± 0.1.

Solvent Viscosity Effects on k_{cat}/K_m and k_{cat}. The effect of solvent viscosity on the k_{cat}/K_m and k_{cat} values with leucine was determined at pH 10.3 using glycerol as viscosigen. Both the normalized k_{cat}/K_m and k_{cat} values increased with inverse hyperbolic patterns with increasing relative viscosity of the solvent (Figure 6). These results are consistent with solvent viscosity reporting on equilibria of complexes of the enzyme with the substrate or the product.

Substrate Deuterium KIE on k_{cat}/K_m and k_{cat}. The substrate deuterium KIEs on the k_{cat}/K_m and k_{cat} values were determined with leucine and leucine-d_{10} at pH 10.3. The $^{D}(k_{cat}/K_m)$ was 5.9 ± 0.2, and the $^{D}k_{cat}$ was 3.8 ± 0.1, suggesting that the cleavage of the substrate CH bond contributes to different extents to the reductive half-reaction and the overall turnover of the enzyme.

Relative Binding Free Energies of the Protonated and Unprotonated Forms of Leucine in the Enzyme−Substrate Complex. The relative free energy ($\Delta\Delta G$) for binding of the protonated and unprotonated forms of leucine to DADH was calculated using molecular dynamics simulations and thermodynamics integration to be +7.7 ± 2.1 kcal/mol. His_{48}, which in the active site of the enzyme is ∼6 Å away from the leucine substrate, was initially modeled in the neutral state. The free energy simulations were repeated with His_{48} in the protonated form, yielding a $\Delta\Delta G$ value of +6.8 ± 1.0 kcal/mol. By using eq 9 and the estimated $\Delta\Delta G$, an increase of ∼5.0 units could be calculated for the pK_a of the amino moiety of leucine when bound in the active site of the enzyme as compared to the value of 9.6 in bulk solution.

DISCUSSION

In the present study, the reductive half-reaction of DADH was investigated using leucine, a substrate with slow reaction kinetics, substrate and solvent deuterium KIEs, solvent viscosity and pH effects, and molecular dynamics simulations. The results

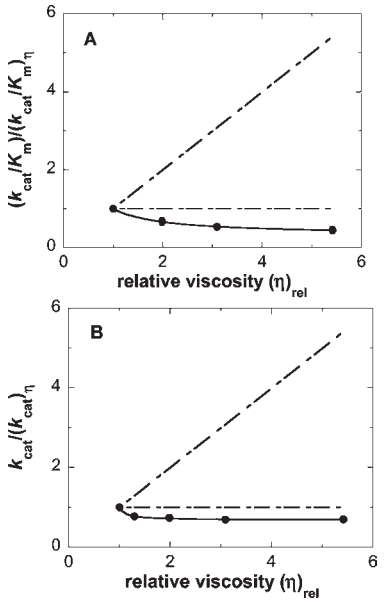
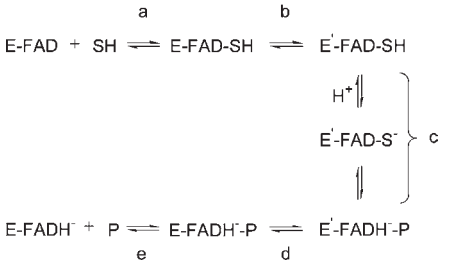


Figure 6. Effects of solvent viscosity on the steady-state kinetic parameters for the D-arginine dehydrogenase with leucine as a substrate. Panel A shows the normalized k_{cat}/K_m values with leucine as a function of the relative solvent viscosity. Panel B shows the normalized k_{cat} values as a function of the relative solvent viscosity. The dashed lines with a slope of 1 indicate the expected results for a fully diffusion-limited reaction. The values for the relative viscosities of the solvent were taken from Weast[35] and adjusted for 25 °C. Reaction rates were measured at varying concentrations of leucine and fixed 1 mM PMS in 20 mM sodium pyrophosphate, pH 10.3, at 25 °C.

are consistent with the minimal kinetic mechanism in Scheme 2. The enzyme forms a Michaelis complex (step a in Scheme 2) by preferentially binding the zwitterionic form of the amino acid substrate rather than the anionic amino acid. An isomerization of the Michaelis complex yields the enzyme−substrate complex that will undergo amine oxidation (b). Amine oxidation is triggered by the deprotonation of the substrate amine, resulting in the asynchronous transfer of a hydride equivalent to the enzyme-bound flavin (c). Finally, the reduced enzyme−iminoleucine complex isomerizes (d) before the fast release of the product from the reduced enzyme (e). Evidence in support of the mechanism in Scheme 2 is presented below.

In the reductive half-reaction, DADH binds preferentially the zwitterionic amino acid substrate rather than its anionic counterpart. Evidence supporting this conclusion comes from calculations of the relative binding free energy using molecular dynamics simulations, showing that the pK_a of the amino moiety of leucine is increased from its solution value of 9.6 when the amino acid is bound to the active site of the enzyme. The magnitude of the pK_a perturbation may have been overestimated due to systematic errors in the molecular mechanics force field, the lack of an explicit representation for the electronic polarizability, or other approximations that are used to define the system in silico.[37] Nonetheless, these results unequivocally show that the pK_a of the substrate amine increases when the substrate is in the active site of the enzyme, consistent with preferred binding of the zwitterionic form of the substrate. Independent evidence supporting the preferred binding of the zwitterionic substrate comes from the pH profile of the K_d value determined in a stopped-flow spectrophotometer, which shows small K_d values when an

Scheme 2. Reductive Half-reaction of DADH with Leucine [a]



[a] E-FAD and E′-FAD, oxidized enzymes; E-FADH$^-$ and E′-FADH$^-$, reduced enzyme with anionic hydroquinone; SH, zwitterionic substrate; S$^-$, anionic substrate; P, imino product.

ionizable group with apparent pK_a of ~10.3 is protonated. This is the expected result for binding of the zwitterionic substrate to yield the initial enzyme−substrate complex, irrespective of whether the observed pK_a is assigned to the substrate amino moiety or to an ionizable side chain in the active site of the enzyme. Preferred binding of the zwitterionic substrate to the enzyme likely exploits an electrostatic interaction of the positive charge on the substrate amine and a negative charge on the enzyme. A reasonable candidate for such an interaction is Tyr_{53}, which is <4.2 Å from the imino moiety of iminoarginine in the crystal structure of the enzyme in complex with the product of the reaction.[30] Interestingly, in the ligand-free conformation of the enzyme Tyr_{53} points outward to the solvent, being part of a conformationally flexible loop that has been proposed to control substrate accessibility to the active site of the enzyme.[30] Thus, it is plausible that the electrostatic interaction of Tyr_{53} with the substrate amine is used to facilitate the access of the amino acid substrate to the active site of the enzyme. Preferred binding of the zwitterionic substrate to a flavin-dependent amine oxidase was previously shown for an active-site mutant of MSOX where Tyr_{317} is replaced with phenylalanine based on the pH dependence of K_d.[50]

Reduction of DADH occurs through the asynchronous cleavage of the substrate NH and CH bonds, resulting in the transfer of a hydride equivalent[51] from the substrate to the enzyme-bound flavin. Evidence for the transfer of a hydride equivalent, as opposed to two consecutive one-electron transfers, comes from the lack of observed transients in the stopped-flow traces of substrate reductions, showing that the oxidized enzyme is directly reduced to the hydroquinone without stabilization of a flavin semiquinone. To accommodate these observations with a radical mechanism for amine oxidation one would have to postulate that the second electron transfer occurred at least 20-times faster than the first electron transfer. Support for a stepwise mechanism comes from substrate, solvent, and multiple deuterium KIEs on the rate constant for flavin reduction. The $^{D,D2O}k_{red}$ value obtained with leucine in water and leucine-d_{10} in D_2O is significantly lower, with a value of 7.8, than the product of the individual substrate and solvent deuterium KIEs (i.e., 9.0). This is consistent with the kinetic step involving a solvent exchangeable proton, which is probed by the solvent deuterium KIE, not being concerted with the cleavage of the substrate CH bond, which is probed by the substrate deuterium KIE.[52] The decreases in $^{D}k_{red}$ upon substituting water with D_2O and $^{D2O}k_{red}$ upon replacing leucine with leucine-d_{10}, although small, reinforce the conclusion that these KIEs probe kinetic steps not occurring in the same transition state.[53,54]

The $^{D}k_{red}$ of ~5.0 immediately establishes that flavin reduction is associated with the cleavage of the substrate CH bond, irrespective of the mechanism of transfer of the hydride equivalent. In principle, the $^{D2O}k_{red}$ of ~1.8 may originate from the cleavage of the substrate NH bond or the formation of the flavin NH bond on the N(1) atom (or both) or an enzyme conformational change associated with flavin reduction. The latter can be ruled out due to the lack of solvent viscosity effects on k_{red} in the presence of glycerol, which would be sensitive to a protein conformational change. The UV−visible absorbance spectrum of the flavin after reduction, with a well-defined peak in the near-UV region,[55] is typical of a flavin with the N(1) atom being unprotonated.[56] This rules out protonation of the reduced flavin as a step probed by $^{D2O}k_{red}$ at pH 10.3. Consequently, the single proton observed in the k_{red} proton inventory establishes that the $^{D2O}k_{red}$ reports on the deprotonation of the substrate NH group. Thus, amine deprotonation in the active site of DADH triggers the amine oxidation reaction. In other flavin-dependent amine dehydrogenases, such as DAAO, MSOX, MTOX and MAO B, it was previously shown that amine oxidation occurs from the deprotonated substrate, although the timing of amine deprotonation was not addressed.[8,19,21,50,57−59]

The Michaelis complex isomerizes, yielding an enzyme−substrate complex that is competent for the subsequent reaction of flavin reduction. Evidence in support of an internal equilibrium of the enzyme in the reductive half-reaction comes from the effect of increasing relative solvent viscosity on the normalized k_{cat}/K_m for leucine. The hyperbolic increase to a limiting value of the normalized k_{cat}/K_m with increasing glycerol concentrations demonstrates that the solvent effect does not originate from events associated with substrate binding, for which linear dependencies with slopes comprising between 0 and +1 are predicted, depending upon the extent of diffusion control of the reaction.[60] In contrast, a viscosity-sensitive isomerization of the enzyme−substrate complex is consistent with the observed solvent viscosity pattern.[61] With DADH, an internal equilibrium of the enzyme−substrate complex was previously reported with D-histidine as substrate.[31] In that case, the crystallographic structure of the enzyme in complex with the reaction product, iminohistidine, demonstrated that the ligand binds in two quasi-orthogonal conformations, of which one is likely competent for amine oxidation based on the relative orientation of the groups participating in catalysis.[30]

Two ionizations of groups that participate in the reductive half-reaction of DADH are seen in the pH profiles of k_{red} and K_d. Flavin reduction requires an unprotonated group with pK_a of 9.6 in the enzyme−substrate complex, as indicated by the pH profile of k_{red} that increases to a limiting value at high pH. A reasonable role for this group is to act as a catalytic base that abstracts the amino proton from the zwitterionic substrate that is preferentially bound to the enzyme. Tyr_{53}, which lies on a flexible loop at <4.2 Å from the imino moiety of the reaction product, is a reasonable candidate for such a role.[30] The alternate possibility of the pK_a being that of the substrate amine undergoing oxidation appears less likely because the solvent deuterium KIE >1 is consistent with amine deprotonation occurring in the kinetic step probed by k_{red}. A protonated group with an apparent pK_a value of ~10.3 favors the binding of the substrate to the enzyme. Evidence for this conclusion comes from the pH profile of the K_d value determined in a stopped-flow spectrophotometer, which decreases to a limiting value with decreasing pH. In the crystal structures of DADH in complex with iminoarginine or iminohistidine, the interaction of the substrate carboxylate with the enzyme is mediated by the side chains of Arg_{222}, Arg_{305}, and Tyr_{249}.[30] A similar pattern for binding of the carboxylate moiety of the substrate is present in glycolate oxidase,[62,63] for which mutagenesis studies on the spinach enzyme showed a 20-fold increase in the K_d value when the Tyr_{24} was replaced with phenylalanine.[64] Thus, it is conceivable that in DADH Tyr_{249} may be the protonated group that facilitates substrate binding. Alternatively, due to the multitude of side chains that can ionize in the active site of the enzyme, including His_{48}, Glu_{87}, Tyr_{53}, Arg_{222}, Arg_{305}, and Tyr_{249}, and the amino moiety of the substrate, the pK_a of ~10.3 may represent a macroscopic value that is contributed by multiple sites.

The overall turnover of DADH with leucine as substrate is limited primarily by the transfer of the hydride equivalent from the amino acid substrate to the flavin, with a minor contribution form an isomerization of the reduced enzyme−product complex occurring before product release. Evidence supporting this conclusion comes from the comparison of the KIEs determined by using rapid kinetics and the steady-state kinetic approach, and of the values for k_{cat} and k_{red}. The $^{D}k_{cat}$ is significantly smaller than $^{D}(k_{cat}/K_m)$, with a value of 3.8 as compared to 5.8. This is consistent with a first-order kinetic step other than that in which the substrate CH bond is cleaved being partially rate limiting for the overall turnover of the enzyme. The substrate deuterium KIE of 5.8 in $^{D}(k_{cat}/K_m)$ compares very well with the intrinsic values between 5.7 and 6.0 previously reported for DAAO with D-alanine and D-serine[17] and TMO with L-alanine as substrate.[19] Thus, the $^{D}(k_{cat}/K_m)$ value approximates well the intrinsic deuterium KIE for the oxidation of leucine by DADH. This allows for the use of eqs 10 and 11 to estimate the rate constant for the kinetic step that contributes along with CH bond cleavage to the overall turnover of the enzyme. This approach yields a C_{Vf} value of 0.7, which translates into a k_x value of ~160 s^{-1} at pH 10.3. Equation 12 shows that k_{cat} is equal to 0.6 × k_{red}, in good agreement with the value of 0.7 experimentally determined from the ratio k_{cat}/k_{red}. Evidence for the isomerization of the reduced enzyme−product complex contributing to the overall turnover of the enzyme comes from the solvent viscosity effect on the normalized k_{cat} and previous steady-state kinetic results showing that the reductive and oxidative half-reactions are independent of each other.[31] The presence of such an isomerization is suggested by the hyperbolic increase to a limiting value of the normalized k_{cat} value with increasing glycerol concentrations. These data also immediately rule out product release as contributing to the overall turnover of the enzyme because, if this were the case, a linear dependence with the slope comprising between 0 and +1 of the normalized k_{cat} would be observed at increasing relative solvent viscosity.[60] The k_{cat}/K_{PMS} values of 10^6 M^{-1} s^{-1} previously determined with D-arginine and D-histidine are consistent with the oxidative half-reaction with 1 mM PMS not contributing to the overall turnover of the enzyme, since it has an overall rate constant of 1000 s^{-1}.

$$^{D}k_{cat} = \frac{^{D}\left(\frac{k_{cat}}{K_m}\right) + C_{Vf}}{1 + C_{Vf}} \quad (10)$$

$$k_x = \frac{k_{red}}{C_{Vf}} \quad (11)$$

$$k_{cat} = \frac{k_{red}}{k_{red} + C_{Vf}} \quad (12)$$

CONCLUSION

In summary, mechanistic and computational approaches have been used to elucidate the reductive half-reaction of DADH with the slow substrate leucine. Binding of the zwitterionic substrate is preferred over the anionic substrate. A kinetically relevant isomerization of the enzyme−substrate complex is demonstrated. The transfer of a hydride equivalent from the neutral amino acid substrate to the enzyme-bound flavin occurs in an asynchronous fashion. Previous studies on other flavin-dependent amine oxidases/dehydrogenases have all concluded that flavin reduction occurs from the anionic amine of the substrate.[8,19,21,50,57−59] However, only in the case of an active-site mutant of MSOX it was demonstrated that the zwitterionic substrate was the preferred form of the substrate that binds to the enzyme.[50] This raised the question of how and when the substrate amine is deprotonated in the active site of these enzymes. With DADH we have shown that amine deprotonation is linked to amine oxidation, although the two bond cleavages occur in an asynchronous fashion.

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