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Mechanistic Studies of an Amine Oxidase Derived from D-Amino Acid Oxidase

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Abstract

The flavoprotein D-amino acid oxidase has long served as a paradigm for understanding the mechanism of oxidation of amino acids by flavoproteins. Recently, a mutant D-amino acid oxidase (Y228L/R283G) that catalyzed the oxidation of amines rather than amino acids was described [Yasukawa, K., et al. (2014) *Angew. Chem., Int. Ed.* 53, 4428–4431]. We describe here the use of pH and kinetic isotope effects with (*R*)- α -methylbenzylamine as a substrate to determine whether the mutant enzyme utilizes the same catalytic mechanism as the wild-type enzyme. The effects of pH on the steady-state and rapid-reaction kinetics establish that the neutral amine is the substrate, while an active-site residue, likely Tyr224, must be uncharged for productive binding. There is no solvent isotope effect on the $k_{\text{cat}}/K_{\text{m}}$ value for the amine, consistent with the neutral amine being the substrate. The deuterium isotope effect on the $k_{\text{cat}}/K_{\text{m}}$ value is pH-independent, with an average value of 5.3, similar to values found with amino acids as substrates for the wild-type enzyme and establishing that there is no commitment to catalysis with this substrate. The $k_{\text{cat}}/K_{\text{O}_2}$ value is similar to that seen with amino acids as the substrate, consistent with the oxidative half-reaction being unperturbed by the mutation and with flavin oxidation preceding product release. All of the data are consistent with the mutant enzyme utilizing the same mechanism as the wild-type enzyme, transfer of hydride from the neutral amine to the flavin.

Graphical abstract

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Table S1 and Figures S1–S3 showing steady-state kinetics at a fixed ratio of substrate concentrations, alternate analyses of the $k_{\text{cat}}/K_{\text{m}}$ and k_{red} –pH profiles, and the pL profiles (PDF)

Notes: The authors declare no competing financial interest.

As one of the first flavoproteins to be described,¹ pig kidney D-amino acid oxidase (DAAO) has long served as the paradigm for understanding the mechanism by which flavoproteins oxidize amino acids. While the mechanism of DAAO and related flavoproteins has been controversial,² a combination of structural,³ solution,⁴ and computational⁵ studies support a concerted mechanism involving direct hydride transfer from the neutral carbon–nitrogen moiety of the amino acid to the flavin. Other flavoenzymes with the same fold as DAAO will catalyze the oxidative N-demethylation of *N*-methylamino acids; mechanistic studies of these enzymes support a similar mechanism.^{6,7}

Members of a separate family of flavoproteins with the same fold as monoamine oxidase (MAO) will catalyze the oxidation of aliphatic amines and L-amino acids. Mechanistic studies of the oxidation of amino acids by members of the MAO family are consistent with the same hydride transfer mechanism accepted for DAAO,^{8,9} but there is more controversy regarding the mechanism of oxidation of amines by this enzyme family. In some cases, the mechanism has been proposed to change depending upon the identity of the substrate or specific amine oxidase.^{10–12} These results suggest that the mechanism of oxidation of amines by flavoproteins might differ from the mechanism of oxidation of amino acids. An alternative hypothesis is that both families of enzymes have evolved to catalyze the oxidation of carbon–nitrogen bonds by a common mechanism, with individual enzymes differing only in how the substrate must be bound to properly position the reacting carbon–nitrogen bond for oxidation. There is no need for an active-site base or acid in such a mechanism because of the pK_a value of aliphatic amines.¹³ The structures of members of the MAO family are consistent with such a model, in that these enzymes contain a highly conserved flavin binding domain. The divergent substrate binding domain is made of two insertions into the sequence of the flavin domain.¹⁴

Although members of the DAAO structural family have been reported to utilize only amino acids as substrates, Yasukawa et al.¹⁵ recently described a mutant DAAO that is active on the *R* enantiomers of primary and secondary amines but not amino acids. The change in specificity required mutation of two residues that interact with the amino acid carboxylate, Arg283 and Tyr228, to glycine and leucine, respectively. The crystal structure of the mutant protein in the presence of (*R*)- α -methylbenzylamine (Figure 1) showed that the amine binds with the carbon containing the hydride that is transferred to a position adjacent to N5 of the FAD, similar to the position of an amino acid in the wild-type enzyme, but with the substrate orientation flipped so that the amino group is above the xylene ring of the cofactor rather than the pyrimidine ring. Y228L/R283G DAAO catalyzes the same reaction as MAO, so that this mutant enzyme affords the possibility of testing the hypothesis that flavin-dependent

amine oxidases utilize a conserved mechanism and diverge only in substrate binding. We describe here mechanistic studies of the oxidation of (*R*)- α -methylbenzylamine by Y228L/R283G DAAO.

Experimental Procedures

Materials

2-Amino-2-methyl-1,3-propanediol was from Acros. (*R*)- α -Methylbenzylamine, sodium borodeuteride, and titanium isopropoxide were from Sigma-Aldrich. Racemic [2-²H]- α -methylbenzylamine was synthesized as previously described.¹⁶ The pJ201 vector encoding pig kidney D-amino acid oxidase with the Y228L and R283G mutations with codons optimized for expression in *Escherichia coli* was obtained from DNA 2.0 (Menlo Park, CA). The plasmid pGro7 for expression of GroES and GroEL was from TaKaRa Bio Inc. DNA ligase and the restriction enzymes DpnI, EcoRI, and NdeI were purchased from New England BioLabs. Glucose oxidase from *Aspergillus niger* was purchased from MP Biomedicals. XL10-Gold ultracompetent cells were obtained from Agilent Technologies. The Q-Sepharose Fast Flow Hi-Trap column was obtained from GE Healthcare. DNA sequencing was performed by GenScript.

Expression and Purification of Y228L/R283G DAAO

The DNA encoding Y228L/R283G DAAO was subcloned from pJ201 into the pET21b vector using the NdeI and HindIII sites at the 5' and 3' ends, respectively, to generate a 6430 bp plasmid designated pSyntheticY228L R283G DAAO-pET21b. The sequence of the plasmid was verified by DNA sequencing. This plasmid was used to transform BL21(DE3) *E. coli* containing the pGro7 plasmid. A single colony from the transformed cells was used to inoculate two 250 mL culture flasks each containing 70 mL of LB broth with 100 μ g/mL ampicillin, 50 μ g/mL chloramphenicol, and 500 μ g/mL L-arabinose. These cultures were grown for 16 h at 37 °C while being shaken (250 rpm). Aliquots (12 mL) of these cultures were used to inoculate 12 Fernbach flasks each containing 1.5 L of LB broth with 100 μ g/mL ampicillin, 50 μ g/mL chloramphenicol, and 500 μ g/mL L-arabinose. The cultures were allowed to grow while being shaken (250 rpm) for 1 h at 37 °C; the temperature was then lowered to 18 °C. When the cultures reached an A_{600} of 0.6, protein expression was induced by adding isopropyl β -D-1-thiogalactopyranoside to a final concentration of 0.2 mM. After further growth at 18 °C for 14–15 h, the cells were harvested by centrifugation at 6200g for 15 min at 4 °C. The cell paste (~60 g) was stored at –80 °C until it was used for protein purification.

Except for the chromatography, which was performed at room temperature, all purification steps were performed at 4 °C. The thawed cell paste (~60 g) was resuspended in 400 mL of 24 mM 2-amino-2-methyl-1,3-propanediol buffer (pH 9.2) containing 0.1 mg/mL PMSF, 1 μ M leupeptin, 1 μ M pepstatin A, 300 μ M FAD, and 0.1 mg/mL lysozyme. The cell suspension was stirred for 30 min and then passed through a 16 gauge needle twice. The cells were then lysed with a Branson model 450 sonifier, using three cycles of 6 min at a duty cycle of 30% at level 3. The resulting suspensions were centrifuged at 27000g for 30 min, and streptomycin sulfate was slowly added to the resulting supernatant to a final

concentration of 2.5% (w/v). The mixture was stirred for 1 h and then centrifuged at 27000g for 30 min. Solid ammonium sulfate (81.3 g) was slowly added to the resulting supernatant (450 mL). After 1 h, the precipitated protein was collected by centrifugation at 27000g for 30 min. The protein pellet was resuspended in 25 mL of buffer A [10 mM Tris and 10% glycerol (pH 8.0)] containing 300 μ M FAD, 0.25 μ M leupeptin, and 0.25 μ M pepstatin A and dialyzed against three 1 L changes of buffer A containing 0.25 μ M leupeptin and 0.25 μ M pepstatin A. The sample was then loaded at a flow rate of 1.0 mL/min onto a 5 mL Q-Sepharose Fast Flow Hi-Trap column that had been equilibrated with buffer A. The column was washed at a rate of 2.5 mL/min with buffer A until the absorbances at 280 and 450 nm were near zero. The protein was eluted at a rate of 2.5 mL/min with a 50 mL gradient from 0 to 250 mM KCl in buffer A. Fractions (2.0 mL) eluting at 50–250 mM KCl were yellow in color. Fractions that were more than ~95% pure as judged by sodium dodecyl sulfate–polyacrylamide electrophoresis were combined and dialyzed against three 1 L changes of 20 mM sodium pyrophosphate and 10% glycerol (pH 8.5). The purified protein (~22 mL) was filtered through a 0.2 μ m filter and stored in aliquots at –80 °C. The purified protein showed maximal absorbance at 452 nm with an A_{274}/A_{452} ratio of 8.34. The enzyme concentration was determined using the molar extinction coefficient for the wild-type enzyme (11300 M⁻¹ cm⁻¹). Approximately 110 mg of purified protein was obtained from 18 L of bacterial cell culture.

Steady-State Kinetic Assays

Initial rates of amine oxidation were determined by following oxygen consumption with a Yellow Springs Instrument model 5300 oxygen monitor. All assays were conducted at 25 °C. For assays not performed in air-saturated buffer, the appropriate mixture of oxygen and nitrogen was bubbled into the assay mixture for 10 min prior to starting the reaction. Measurement of $k_{\text{cat}}/K_{\text{amine}}$ values and k_{cat} values as a function of pH was performed in air-saturated buffer, where the concentration of oxygen (250 μ M) is above the K_{m} for oxygen at pH 8.6 of 170 μ M. Assays (2 mL) contained ~50 nM enzyme and 10 μ M FAD in 0.2 M HEPES (pH 8.0), 0.02 M sodium pyrophosphate (pH 8.5), 0.2 M CHES (pH 8.6–9.2), or 0.2 M EDTA (pH 9.4–11). The enzyme lost activity too rapidly above pH 11 for assays to be reliable. Stock solutions of (*R*)- α -methylbenzylamine were prepared at a concentration of 0.2 or 0.3 M in the appropriate buffer, and the pH was adjusted to the desired value. Assays were performed in duplicate or triplicate.

Rapid-Reaction Kinetic Measurements

All stopped-flow kinetic measurements were performed on an Applied Photo physics SX-20 stopped-flow spectrophotometer in single-mixing mode with single-wavelength detection at 25 °C under anaerobic conditions. Prior to use, the instrument was equilibrated for 4–8 h with buffer containing 5 mM glucose and 70 nM glucose oxidase. The assay buffers were 0.2 M HEPES (pH 7.0–8.0), 0.2 M CHES (pH 8.6), 0.2 M EDTA (pH 9.0–10.0), and 0.2 M CHAPS (pH 10.5). All buffers except 0.2 M CHES also contained 10% glycerol, which was found to be necessary for enzyme stability. The enzyme precipitated upon prolonged incubation above pH 10.2, preventing data from being obtained at high pH.

For each experiment, a solution of 30 μM enzyme and 5 mM glucose was placed in a glass tonometer, with glucose oxidase (to yield a final concentration of 70 nM) in a side arm. The contents of the tonometer were made anaerobic by 10 cycles of vacuum followed by equilibration with oxygen-free nitrogen; glucose oxidase was then introduced from the side arm, and five additional cycles were performed. Substrate solutions containing various concentrations of (*R*)- α -methylbenzylamine and 5 mM glucose were prepared in the appropriate buffer and bubbled with oxygen-free nitrogen for 10 min. Glucose oxidase (final concentration of 70 nM) was then added, and the solution was bubbled for an additional 3–5 min.

Data Analysis

All fitting of data was performed using KaleidaGraph (Synergy Software). Initial rates of oxygen consumption (v/E) were fit to the Michaelis–Menten equation to obtain the steady-state kinetic parameters. Deuterium isotope effects on the $k_{\text{cat}}/K_{\text{m}}$ value were determined directly from the individual $k_{\text{cat}}/K_{\text{m}}$ values for deuterated and nondeuterated substrate because the high K_{m} value made it impossible to determine k_{cat} values with sufficient accuracy. Apparent rate constants for flavin reduction by the amine substrate were calculated from fits of the absorbance at 450 nm as a function of time to eq 1, which describes a biphasic reaction

$$A_t = \Delta A_1 e^{-k_{1\text{obs}} t} + \Delta A_2 e^{-k_{2\text{obs}} t} + A_{\infty} \quad (1)$$

where A_1 and A_2 are the magnitudes of the two phases, $k_{1\text{obs}}$ and $k_{2\text{obs}}$ are the rate constants for the two phases, and A_{∞} is the absorbance at the end of the reaction. The observed rate constants as a function of amine concentration were fit to eq 2

$$k_{\text{obs}} = \frac{k_{\text{red}} [\text{S}]}{K_{\text{d}} + [\text{S}]} \quad (2)$$

where k_{obs} is the observed rate constant, k_{red} is the maximal rate constant at a saturating substrate concentration, $[\text{S}]$ is the substrate concentration, and K_{d} is the apparent dissociation constant for the enzyme–substrate complex. pH profiles were fit to eq 3, which describes a bell-shaped pH profile that decreases at both high and low pH values, or eq 4, which describes a pH profile that decreases below a single $\text{p}K_{\text{a}}$.

$$\log Y = \log \frac{c}{1 + H/K_1 + K_2/H} \quad (3)$$

$$\log Y = \log \frac{c}{1 + H/K} \quad (4)$$

Results

Steady-State Kinetics of Y228L/R283G DAAO

Steady-state kinetic data for most substrates for wild-type pig DAAO are described well by the standard equation for ping-pong kinetics (eq 5)

$$e/v = \frac{k_{\text{cat}} [\text{amine}] [\text{O}_2]}{[\text{amine}] K_{\text{O}_2} + [\text{O}_2] K_{\text{amine}} + [\text{amine}] [\text{O}_2]} \quad (5)$$

where the $k_{\text{cat}}/K_{\text{m}}$ value for each substrate is unaffected by the concentration of the other.¹⁷ To determine if this was the case for the mutant protein, the concentrations of (*R*)- α -methylbenzylamine and oxygen were varied in a fixed ratio; the concentration of the amine was set at 100-fold that of oxygen based on preliminary measurements of the K_{m} values for the two substrates. The data from such an experiment for an enzyme exhibiting ping-pong kinetics are fit well by the Michaelis–Menten equation, while the data for an enzyme exhibiting sequential kinetics are not.^{18,19} The data for Y228L/R283G DAAO fit well to the Michaelis–Menten equation [$R^2 = 0.997$ (Figure S1)], establishing that the kinetic mechanism was not altered by the mutation. This experiment also yielded a k_{cat} value for (*R*)- α -methylbenzylamine of $88 \pm 4 \text{ s}^{-1}$ at pH 8.6. Because the individual $k_{\text{cat}}/K_{\text{m}}$ values are independent of the concentration of the other substrate, the $k_{\text{cat}}/K_{\text{amine}}$ value was then determined by varying the concentration of the amine at 250 μM oxygen in a separate analysis, yielding a value of $2.9 \pm 0.4 \text{ mM}^{-1} \text{ s}^{-1}$; similarly, varying the concentration of oxygen at 100 mM amine gave a $k_{\text{cat}}/K_{\text{O}_2}$ value of $520 \pm 80 \text{ mM}^{-1} \text{ s}^{-1}$. Figure 2A shows the $k_{\text{cat}}/K_{\text{m}}$ -pH profile for Y228L/R283G DAAO. The data exhibit a maximum at pH 10, decreasing below and above this pH, and fit well to eq 3 (Table S1). The two $\text{p}K_{\text{a}}$ values obtained from fitting the data to eq 3 differ by only 0.3, too little to allow reliable values for both $\text{p}K_{\text{a}}$ s to be obtained,²⁰ so the data were fit to eq 3 with the two $\text{p}K_{\text{a}}$ values set to be identical. This yielded an average $\text{p}K_{\text{a}}$ value for the two groups of 10.04 ± 0.03 . The $\text{p}K_{\text{a}}$ of the substrate is 9.6; fitting the data to eq 3 with the acidic $\text{p}K_{\text{a}}$ value fixed at 9.6 gave a comparable fit (Figure S2) and a basic $\text{p}K_{\text{a}}$ value of 10.4 ± 0.1 for a group in the free enzyme whose protonation state is critical for binding and/or catalysis. In contrast, the k_{cat} value was essentially independent of pH above pH 8.5, with a small decrease at pH 8 (Figure 2B). The k_{cat} -pH profile could be fit to eq 4 to obtain an estimate of the $\text{p}K_{\text{a}}$ responsible for the decrease as 7.8 ± 0.4 .

Rapid-Reaction Kinetics

The kinetics of reduction of Y228L/R283G DAAO by (*R*)- α -methylbenzylamine were analyzed by anaerobic stopped-flow spectroscopy. Representative changes in the absorbance of the enzyme-bound flavin during reduction by 25 mM α -methylbenzylamine are shown in

nm, Figure 3. There was a biphasic decrease in the absorbance at 450 nm (Figure 3A). When the reaction was followed at 550 there was a transient increase in absorbance with a magnitude that was ~3% of the change at the lower wavelength (Figure 3B). The rate constants for the two phases were comparable at the two wavelengths. There were no spectral changes indicative of formation of a transient flavin radical (results not shown). The rate constant for the rapid phase varied with the concentration of the amine (Figure 4A); this concentration dependence could be fit to eq 2 to obtain values for the apparent K_d for binding of 37 ± 10 mM, and the limiting rate constant for reduction, k_{red} , of 220 ± 20 s⁻¹. The slow phase had an average value of 11.3 ± 3.8 s⁻¹ from 5 to 100 mM amine; this is significantly slower than k_{cat} , suggesting it is not catalytically relevant. The pH dependence of the k_{red} value for the fast phase was determined as a function of pH. The results are shown in Figure 4B. The data fit well to eq 3, but the fit yielded an acidic pK_a value that was higher than the basic pK_a value (Table S1). In such a case, only the average of the two pK_a values can be determined reliably, so the data were fit to eq 3 with $pK_1 = pK_2$ to yield an average pK_a value of 9.3 ± 0.1 . The two groups responsible for the pH dependence of the k_{red} value are likely the same two groups affecting the k_{cat}/K_m -pH profile, with their pK_a values slightly perturbed in the enzyme-substrate complex.

Isotope Effects

Deuterium isotope effects on k_{cat}/K_{amine} values provide insight into the magnitude of the rate constant for carbon-hydrogen bond cleavage relative to other steps in the reductive half-reaction and into the transition state for carbon-hydrogen bond cleavage. Accordingly, the $^D(k_{cat}/K_{amine})$ value was determined at pH 10, the pH optimum, as well as above and below the pH optimum. The $^D(k_{cat}/K_{amine})$ value was independent of pH (Table 1), with an average value of 5.3. The deuterium isotope effect on the k_{red} value was determined at pH 9.5, yielding a value of 6.3 ± 0.6 . To determine the solvent isotope effects, k_{cat} and k_{cat}/K_{amine} values were determined in D₂O over the pD range of 9-11.3 (Figure S3). The resulting k_{cat}/K_{amine} profile was fit to eq 3 for which $pK_1 = pK_2$, while the k_{cat} profile was fit to eq 4. In both cases, the pK_a values from the fits were shifted up by ~0.5 unit, but there was no change in the shapes of the profiles. The pH-independent values (c in eqs 3 and 4) in H₂O and D₂O were then used to calculate the solvent isotope effects on the k_{cat}/K_{amine} and k_{cat} values as 1.02 ± 0.08 and 1.42 ± 0.14 , respectively.

Discussion

The steady-state kinetic mechanism for wild-type DAAO (Scheme 1) can be described as a modified ping-pong mechanism, in that the amino acid substrate binds to the enzyme and is oxidized before oxygen reacts with the reduced enzyme-product complex, with product release from the oxidized enzyme as the final step.²¹ Because amine oxidation is effectively irreversible, the k_{cat}/K_m value for each substrate is independent of the concentration of the other and the steady-state kinetic equation is identical to that for an enzyme utilizing a typical ping-pong mechanism.^{17,22} The effect of varying the concentrations of both (*R*)- α -methylbenzylamine and oxygen on the kinetics (Figure S1) establishes that Y228L/R283G DAAO utilizes the same kinetic mechanism.

For the mechanism in Scheme 1, the k_{cat} value equals $k_3k_7/(k_3 + k_7)$, where k_3 is k_{red} and k_7 is the rate constant for product release. For the mutant enzyme, the k_{red} value of 220 s^{-1} at pH 8.6 is significantly larger than the k_{cat} value of 88 s^{-1} at the same pH; this is consistent with product release being partially rate-limiting for the mutant enzyme. The k_{red} - and k_{cat} -pH profiles have different shapes, in that the k_{cat} value is pH-insensitive at pH 9–11 whereas the k_{red} value is highly sensitive to pH over this pH range; this provides further evidence that amine oxidation is not rate-limiting for turnover. The $k_{\text{cat}}/K_{\text{O}_2}$ value for Y228L/R283G DAAO of $520 \text{ mM}^{-1} \text{ s}^{-1}$ is ~20-fold greater than the second-order rate constant for the oxidation of reduced wild-type DAAO with no ligands and within the range of values reported with amino acid substrates for wild-type DAAO.^{21–24} suggesting that the Y228L and R283G mutations did not significantly perturb the reaction with oxygen.

The pH-independent $^D(k_{\text{cat}}/K_m)$ value for the mutant protein, with an average value of 5.3 ± 0.3 , is not meaningfully different from the $^Dk_{\text{red}}$ value at the pH optimum of 6.3 ± 0.6 , consistent with this being the intrinsic isotope effect on amine oxidation. The observation that the value is pH-independent establishes that carbon–hydrogen bond cleavage is much slower than binding, with no significant forward or reverse commitments²⁵. In contrast, with the best-studied substrate for wild-type DAAO, D-alanine, there is a significant commitment to catalysis because of the rate constant for hydride transfer being faster than that for dissociation of the amino acid substrate from the active site of the oxidized enzyme ($k_3 \gg K_2$).^{21,22} As a result, the $^D K_{\text{cat}}/K_m$ value with D-alanine is strongly pH-dependent. The k_{cat}/K_m for oxidation of (*R*)- α -methylbenzylamine by Y228L/R283G DAAO is 1–3 orders of magnitude lower than the values for the wild-type enzyme with several amino acid substrates.^{21–23} This is due to a combination of the weak binding of the substrate, with a K_d value of ~40 mM, and a relatively low value for k_{red} , the rate constant for amine oxidation.

Primary deuterium isotope effects on carbon–hydrogen cleavage reactions are highly sensitive to the contribution of quantum-mechanical tunneling to the reaction,^{27–29} so that the magnitude of a deuterium isotope effect provides only a qualitative measurement of the extent of bond dissociation in the transition state. Indeed, previous measurements of the intrinsic deuterium isotope effect for wild-type pig DAAO show that the value varies with the substrate. Still for the two of the best-characterized substrates for the wild-type enzyme, D-alanine and D-serine, the intrinsic deuterium isotope effects of 5.7 and 4.5, respectively, are quite close to the value of 5.3 for the Y228L/R283G enzyme, while the value of 3.6 for glycine is slightly smaller.³⁰ The value for oxidation of (*R*)- α -methylbenzylamine by Y228L/R283G DAAO includes a secondary isotope effect, unlike the values for oxidation of amino acids by the wild-type enzyme; the value of the secondary isotope effect is likely to be between 1.0 and 1.2.³¹ Deuterium isotope effects have also been measured for amine oxidation by a number of members of the MAO family of flavoprotein amine oxidases. For MAO from different sources with a variety of amine substrates, the values vary from ~6 to ~14,^{32–35} slightly larger than the value reported here. For mouse *N*-acetylpolyamine oxidase, the intrinsic deuterium isotope effect with *N,N*-dibenzyl-1,4-diaminobutane is 6.7, close to the value reported here.³⁶ For the L-amino acid oxidase tryptophan 2-mono-oxygenase, the isotope effects vary from 2.4 for the physiological substrate tryptophan to 5.3–5.7 for the slow substrates alanine and methionine.^{37,38} Overall, the $^D(k_{\text{cat}}/K_m)$ value for Y228L/R283G

DAAO does not provide evidence of any change in mechanism from that for the wild-type enzyme.

The pH-independent value of $k_{\text{cat}}/K_{\text{amine}}$ for Y228L/R283G DAAO is unchanged in D₂O. The same result has been found for the wild-type enzyme with D-serine as the substrate.³⁰ The lack of a solvent isotope effect on $k_{\text{cat}}/K_{\text{amine}}$ establishes that a proton on the substrate nitrogen is not in flight in the transition state for carbon–hydrogen bond cleavage. As was the case with the wild-type enzyme,⁴ the lack of a solvent isotope effect on the $k_{\text{cat}}/K_{\text{amine}}$ value for the mutant enzyme can be explained by the neutral form of the amine being required for productive binding. There is a significant solvent isotope effect on the k_{cat} value for Y228L/R283G DAAO. The wild-type enzyme also exhibits a solvent isotope effect on k_{cat} when D-alanine is the substrate.²⁴ In that case, the solvent isotope effect was attributed to proton transfer during product release. A similar explanation is reasonable for the mutant protein. Qualitatively, the observation of solvent isotope effects on the k_{cat} values for both the wild-type and the mutant enzyme suggests that product release is unaffected by the mutations.

Both $k_{\text{cat}}/K_{\text{amine}}$ and k_{red} -pH profiles reflect contributions of one moiety that must be protonated for catalysis and another that must be unprotonated. The $\text{p}K_{\text{a}}$ values determined from the $k_{\text{cat}}/K_{\text{amine}}$ profile are due to either the free enzyme or the free substrate. Because there is no significant forward commitment to catalysis for the mutant enzyme, the $\text{p}K_{\text{a}}$ values seen in the $k_{\text{cat}}/K_{\text{amine}}$ profile equal the intrinsic values. The $\text{p}K_{\text{a}}$ of 9.6 can be assigned to the substrate, which must be unprotonated for productive binding, while the $\text{p}K_{\text{a}}$ of 10.4 must be due to a group on the enzyme that must be protonated. Examination of the active site (Figure 1) allows assignment of this $\text{p}K_{\text{a}}$ to Tyr224 or the flavin N3. The k_{red} -pH profile (Figure 4B) reflects the protonation state of moieties in the enzyme–substrate complex required for amine oxidation. The two $\text{p}K_{\text{a}}$ values can again be assigned to the substrate and a group on the enzyme. The average $\text{p}K_{\text{a}}$ in this profile is lower than in the $k_{\text{cat}}/K_{\text{amine}}$ profile; this can be attributed to perturbation of the $\text{p}K_{\text{a}}$ values in the active-site environment.

Our results are consistent with the catalytic mechanism of DAAO being unchanged in the mutant enzyme. Productive binding of the substrate involves the neutral amine, and product release limits k_{cat} . Binding of the amine substrate and its oxidation is slower than is the case for many substrates for the wild-type enzyme, but the k_{red} value of 220 s⁻¹ still reflects robust activity. The activity of the mutant enzyme is quite impressive given that the amine group of the substrate does not form hydrogen bonds directly with active-site residues (Figure 1). While members of the DAAO structural family have been reported to use only amino acids as substrates, the MAO structural family of flavoproteins contains both amine oxidases and L-amino acid oxidases. It has been suggested that the mechanism of members of the MAO family can utilize different catalytic mechanisms for different substrates.¹⁰ These results with Y228L/R283G DAAO do not support such a hypothesis for the DAAO family.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DAAO D-amino acid oxidase

MAO monoamine oxidase

Figure 1.
Active site of Y228L/R283G DAAO, based on Protein Data Bank entry 3WGT. MBA denotes (*R*)- α -methylbenzylamine.

Figure 2. Effect of pH on the $k_{\text{cat}}/K_{\text{amine}}$ and k_{cat} values for Y228L/R283G DAAO with (R)- α -methylbenzylamine as a substrate at 25 °C. The line in panel A is from a fit of the data to eq 3 for which $pK_1 = pK_2$. The line in panel B is from a fit to eq 4.

Figure 3. Changes in the absorbance of the flavin during reduction of Y228L/R283G DAAO by 25 mM (*R*)- α -methylbenzylamine at pH 8.6 and 25 °C. The lines are from fits to eq 1.

Figure 4. Kinetics of reduction of Y228L/R283G DAAO by (R)- α -methylbenzylamine. (A) Dependence of the rate constant for the fast phase of flavin reduction on the amine concentration at pH 8.6. (B) Effect of pH on the k_{red} value. The lines are from fits of the data to eq 2 (A) or eq 3 for which $pK_1 = pK_2$ (B).

Scheme 1. Steady-State Kinetic Mechanism for D-Amino Acid Oxidase

Table 1
Deuterium Kinetic Isotope Effects for Y228L/R283G DAAO

pH	$D(k_{\text{cat}}/K_m)$
8.6	5.42 ± 0.43
9.0	5.35 ± 0.48
10.0	5.42 ± 0.29
11.0	4.88 ± 0.75
average	5.3 ± 0.3