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A Lysine Conserved in the Monoamine Oxidase Family Is Involved in Oxidation of the Reduced Flavin in Mouse Polyamine Oxidase

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Abstract

Lysine 315 of mouse polyamine amine oxidase corresponds to a lysine residue that is conserved in the flavoprotein amine oxidases of the monoamine oxidase structural family. In several structures, this lysine residue forms a hydrogen bond to a water molecule that is hydrogen-bonded to the flavin N(5). Mutation of Lys315 in polyamine oxidase to methionine was previously shown to have no effect on the kinetics of the reductive half-reaction of the enzyme (Henderson Pozzi, M., Gawandi, V., and Fitzpatrick, Paul F. (2009) Biochemistry 48, 1508–1516). In contrast, the mutation does affect steps in the oxidative half-reaction. The k_{cat} value is unaffected by the mutation; this kinetic parameter likely reflects product release. At pH 10, the k_{cat}/K_m value for oxygen is 25-fold lower in the mutant enzyme. The k_{cat}/K_{O2} value is pH-dependent for the wild-type enzyme, decreasing below a pK_a of 7.0, while this kinetic parameter for the mutant enzyme is pH-independent. This is consistent with the neutral form of Lys315 being required for more rapid flavin oxidation. The solvent isotope effect on the k_{cat}/K_{O2} value increases from 1.4 in the wild-type enzyme to 1.9 in the mutant protein, and the solvent inventory changes from linear to bowed. The effects of the mutation can be explained by the lysine orienting the bridging water so that it can accept the proton from the flavin N(5) during flavin oxidation. In the mutant enzyme the lysine amine would be replaced by a water chain.

A large number of flavoproteins catalyze the oxidation of amines, with molecular oxygen as the final electron acceptor [1]. Based on their sequences and the three-dimensional structures of a growing number of flavoprotein amine oxidases, most of these enzymes can be grouped into two distinct structural families. D-Amino acid oxidase is the paradigm member of the structural family that also contains sarcosine oxidase, glycine oxidase, and dimethylglycine oxidase [2–5]. Monoamine oxidase is the most-studied member of the family that also contains polyamine oxidase, the histone lysine specific demethylase LSD1, and the L-amino acid oxidases [6–10]. The general reaction of flavoprotein amine oxidases can be divided into two half-reactions. The reductive half-reaction consists of the transfer of a hydride equivalent from the substrate to the flavin, producing reduced flavin and oxidized amine. This step is typically irreversible; therefore, these enzymes exhibit ping pong patterns in steady-state kinetic analyses [11]. While the mechanism of amine oxidation has been controversial [12–14],

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mechanistic and structural results are most consistent with direct transfer of a hydride from the amine substrate to the flavin [2,15–20]. In the less-studied oxidative half-reaction, two electrons are transferred from the reduced flavin to molecular oxygen, forming H_2O_2 [21,22].

Polyamine and spermine oxidases are involved in the catabolism of polyamines in a variety of eukaryotic cells. The mammalian polyamine oxidases (PAOs)[1] prefer N1-acetylated spermine and spermidine as substrates, oxidizing the substrate on the *exo* side of the secondary nitrogen (Scheme 1) [23]. Spermine oxidases catalyze a similar reaction, but are more active with non-acetylated spermine and spermidine [24,25]. In contrast, plant polyamine oxidases catalyze the oxidation on the *endo* side of the secondary nitrogen [26], a reaction also catalyzed by putrescine oxidases [27]. To date, no structure of a mammalian polyamine or spermine oxidase has been described. While there are structures of polyamine oxidases from maize and yeast, the former is more accurately a spermine oxidase based on its substrate specificity [28] and the latter is reported to catalyze the oxidation of N1-acetylspermine and spermine equally efficiently [29]. All of the published structures of proteins in the monoamine oxidase family show a conserved lysine residue in the active site [6–8,30,31]. In several of these structures there is a water molecule in an appropriate location to form hydrogen bonds to the lysine amino group and the FAD N(5). This is illustrated in Figure 1 for maize polyamine oxidase. When this lysine in that enzyme is mutated to methionine, the rate constant for reduction of the flavin by spermidine is reported to be 1400-fold slower than the value for the wild-type enzyme, suggesting that this residue plays a critical role in amine oxidation [32]. Mutation of the corresponding lysine in LSD1, Lys661, to alanine yielded a mutant protein with no detectable activity in a gel-based assay [33], consistent with a critical role in that enzyme also. While the lack of kinetic data for the LSD1 K661A enzyme precludes determination of which steps in the reaction are affected by the mutation, amine oxidation is rate-limiting for that enzyme, at least with peptide substrates [19]. Alignment of sequences of mouse polyamine oxidase with other members of the monoamine oxidase family, including maize PAO and LSD1 (Figure 2), shows that Lys315 in mouse PAO corresponds to the conserved lysine residue. Surprisingly, mutagenesis of this lysine in mouse PAO to methionine has no effect on the rate constant for flavin reduction at the pH optimum or on the pH dependence of the reductive half-reaction [34], in contrast to the results with maize PAO and LSD1. We report here that mutagenesis of Lys315 in mouse PAO to methionine instead affects the oxidative half-reaction of the enzyme.

EXPERIMENTAL PROCEDURES

Materials

Spermine was purchased from Acros Organics (Geel, Belgium) and N1-acetylspermine was purchased from Fluka (Switzerland). Deuterium oxide was purchased from Cambridge Isotope Laboratories, Inc (Andover, MA). All other materials were of the highest purity commercially available. Wild-type and K315M PAO were expressed and purified as previously described [34].

Assays

Steady-state kinetic assays were performed using a computer-interfaced Hansatech (Hansatech Instruments) oxygen electrode [35]. All assays were initiated by the addition of enzyme. All buffers contained 10% glycerol; 50 mM PIPES, 50 mM Tris-HCl, 50 mM CHES and 50 mM CAPS were used for the pH ranges of 6.6, 7.1–8.6, 9.1–9.6, and 10, respectively. Solvent isotope effects were performed in buffers containing 50 mM CHES (pH 9 or pD 9.4) or 50 mM CAPS (pH 10, or pD 10.4), 10% glycerol. Glycerol buffers with a relative viscosity of 1.3 were prepared as described by Segur and Oberstar [36]. A concentration of 1 mM N1-acetylspermine

[1]Abbreviations used: PAO, polyamine oxidase.

was used in all assays. Due to the hygroscopic nature of N1-acetylspermine, its concentration was determined enzymatically [20].

Data Analysis

Steady-state kinetic parameters were determined based on fits to the Michaelis-Menten equation using the program KaleidaGraph (Adelbeck Software, Reading, PA). Data for the k_{cat}/K_{O2}-pH profile of wild type PAO were fit to eq 1, which applies for a kinetic parameter that decreases below pK due to the protonation of a single moiety; y is the k_{cat}/K_{O2} value at the specific pH being measured, c is the pH-independent value of the k_{cat}/K_{O2}, and K is the ionization constant for a residue which must be unprotonated. Eq 2 was used to fit the k_{cat} pH profile for the wild-type enzyme. This describes a kinetic parameter that decreases to a limiting value at low pH; Y_L and Y_H are the values of the kinetic parameter at the pH extremes and K is the ionization constant for the transtition [37]. Eqs 3–5 were used to fit the proton inventories for wild type and K315M PAO. Eq 3 describes a proton inventory arising from a single proton. Eq 4 describes a proton inventory when two protons with identical fractionation factors contribute to the isotope effect [38]. Eq 5 describes a proton inventory when a large (≥3) number of protons contribute to the isotope effect [38]. In all three equations, n is the mole fraction of D_2O in the buffer, $(k_{cat}/K_{O2})_0$ and $(k_{cat}/K_{O2})_n$ are the k_{cat}/K_{O2} values in water and the indicated mole fraction of D_2O, respectively, and KIE is the solvent isotope effect.

$$\log y = \log\left(\frac{c}{1+H/K}\right)$$

$$\log y = \log\left(\frac{Y_L + Y_H(K/H)}{1+K/H}\right)$$

$$(k_{cat}/K_{O2})_n = (k_{cat}/K_{O2})_0 * (1 - n + (n/KIE))$$

$$(k_{cat}/K_{O2})_n = (k_{cat}/K_{O2})_0 * (1 - n + (n/KIE))^2$$

$$(k_{cat}/K_{O2})_n = (k_{cat}/K_{O2})_0 * (1 - n + (n/KIE))^m$$

RESULTS

Steady-state kinetic parameters

Since our previous characterization of K315M PAO showed that the kinetics of amine oxidation were not significantly affected by the mutation, steady-state kinetic parameters were determined at saturating concentrations of N1-acetylspermine (≥50 K_m), allowing the k_{cat} and k_{cat}/K_{O2} values to be determined.[2] The analyses were carried out at pH 10, the pH optimum

[2]For the wild-type enzyme, the K_m value for N1-acetylspermine is 12 ± 2 μM at pH 8 and 27 ± 6 μM at pH 10, while the values for the mutant enzyme are 8 ± 2 μM at pH 8 and 2 ± 2 μM at pH 10.

[34], and pH 8, the pH of the peroxisome [39]. The values for the wild-type enzyme and the K315M mutant are shown in Table 1. At pH 8 the k_{cat}/K_{O2} value for the mutant protein is one-tenth that of the wild-type enzyme, while the effect at pH 10 is slightly larger. The effect on the k_{cat} value is smaller; the high K_m for oxygen at pH 10 and the limited solubility of oxygen precluded measuring a reliable k_{cat} value for the mutant protein above pH 8.

pH profiles

The values in Table 1 show that the k_{cat}/K_{O2} and k_{cat} values are affected to some extent by pH. Accordingly the effect of pH on these kinetic parameters was determined for the wild-type and mutant enzyme over the pH range of 6.6–10. The k_{cat}/K_{O2}-pH profiles are shown in Figure 3. The k_{cat}/K_{O2} value for the wild-type enzyme is clearly pH-dependent, with a limiting value of 20 ± 1 $mM^{-1}s^{-1}$ at high pH and decreasing below a pK_a of 7.0 ± 0.1. In contrast, the k_{cat}/K_{O2} value for the mutant protein is independent of the pH between pH 6.6 and 10, with an average value of 0.8 ± 0.1 $mM^{-1}s^{-1}$ over that pH range. This is consistent with the mutation resulting in a decrease of 25-fold in the reactivity of the reduced enzyme with oxygen at the pH optimum and with Lys315 being responsible for the pH dependence of the k_{cat}/K_{O2} value.

The k_{cat}-pH profile for the wild-type enzyme is shown in Figure 4. There is a decrease in the value of this kinetic parameter at low pH from a limiting value at high pH. The data were fit to eq 2, which assumes the k_{cat} value reaches a limiting value at low pH, with a pK_a value of 8.8 ± 0.2 for the transition. Eq 2 gave a better fit than a model which assumes that k_{cat} value goes to zero at low pH. It was not possible to construct a k_{cat}-pH profile for the mutant enzyme due to the high K_{O2} value above pH 8. The k_{cat} values for the mutant at pH 8 and below are shown in Figure 4 and establish that the k_{cat} value for the mutant protein is close to the wild-type value over that limited pH range.

Solvent Isotope Effects and Proton Inventory

To gain further insight into the role of Lys315 in the reaction of reduced PAO with oxygen, the solvent isotope effects on the k_{cat}/K_{O2} value for wild-type and K315M PAO were determined. The solvent isotope effect for wild-type PAO at pH 10/pD 10.4, where the k_{cat}/K_{O2} value is pH-independent, is 1.43 ± 0.05. This value is not very different from the relative viscosity of a D_2O solution, 1.3 versus water. To test whether the small isotope effect is simply due to the viscosity of the D_2O buffer, the effect of 10% (w/w) glycerol, which similarly increases the viscosity of the solvent by 30%, was determined. This concentration of glycerol resulted in a slight increase in the k_{cat}/K_{O2} value, for an inverse viscosity effect of 0.93 ± 0.06. Thus, the solvent isotope effect is not due to the viscosity of the D_2O solution. The solvent isotope effect on the k_{cat}/K_{O2} value for the mutant protein was also determined. At pH 10/pD 10.4 the isotope effect is 1.84 ± 0.08. To ensure that data were collected in a pH-insensitive region, the solvent isotope effect was also determined at pH 9/pD 9.4, where the value is 1.98 ± 0.05. These values are within error of each other and give an average solvent isotope effect of 1.91 ± 0.06. These data establish that the mutation has increased the sensitivity of the k_{cat}/K_{O2} value to D_2O.

Solvent isotope effects can be difficult to interpret, in that a large number of protons in the enzyme will exchange with D_2O. A proton inventory experiment, in which the solvent isotope effect is determined at various ratios of H_2O and D_2O, can establish the number of protons in flight in the isotope-sensitive transition state. Accordingly, proton inventories were determined for the wild-type and mutant proteins. The results are shown in Figure 5. For the wild-type enzyme, the data are readily fit by a straight line, consistent with this solvent isotope arising from a single exchangeable proton. Fitting the data to eq 3, which describes a proton inventory in which the isotope effect arises from a single proton in the transition state, gives a solvent isotope effect of 1.39 ± 0.02. In contrast to the result for the wild-type enzyme, the proton

inventory for K315M PAO is bowed, consistent with more than one exchangeable proton contributing to the isotope effect. Use of eq 4, which applies for two exchangeable protons, gave only a slightly better fit. Consequently, the data were fit to eq 5, which describes a solvent isotope effect arising from a large number of protons.

DISCUSSION

Previous analyses of the effect of the K315M mutation on the kinetics of PAO focused on the reductive half-reaction, since the data with the maize enzyme and LSD1 implicated steps in amine oxidation as being substantially slower. However, the mutation does not affect the k_{cat}/K_m value for spermine at any pH and does not affect the rate constant for flavin reduction by N1-acetylspermine at the pH optimum, although there is a small change in the pH dependence of that kinetic parameter [34]. The present results establish that the mutation instead affects the reaction of the reduced flavin in PAO with oxygen.

While the increase in the K_{O2} with pH precludes reliable measurement of the k_{cat} value above pH 8, there is clearly no significant effect of the mutation on this kinetic parameter below that pH. It is thus reasonable to conclude that the K315M mutation does not affect the k_{cat} value of the enzyme. For the wild-type enzyme, the k_{cat} value is more than 20-fold less than the value of k_{red}, the rate constant for flavin reduction at saturating concentrations of the amine substrate, over this pH range [34]. Consequently, the k_{cat} value likely reflects the rate constant for product release rather than a chemical step. The rate constant for this step is clearly pH-dependent, increasing ~100-fold when a group with a pK_a of about 8.8 is deprotonated. Similar pH-dependent rate constants for product release have been seen for other flavin amine oxidases, where they were attributed to pH-dependent conformational changes that alter the rate constant for product release [40,41]. An alternative explanation for the pK_a seen in the k_{cat} pH profile for PAO is that it reflects deprotonation of a substrate nitrogen in the enzyme active site. The active form of the amine substrate for PAO has one charged nitrogen, with the others neutral, although the enzyme will bind the substrate with the secondary nitrogen that is oxidized in a charged form [34]. Since the protonation of a nonreacting nitrogen in the substrate increases the affinity of the enzyme for the substrate, it is reasonable that deprotonation of that same nitrogen in the product would decrease the affinity of the enzyme for the product, increasing the rate constant for product release and thus the k_{cat} value.

The K315M mutation results in a decrease of the k_{cat}/K_{O2} value of 25-fold at the pH optimum. For flavin oxidases, including PAO [42], single turnover kinetic analyses show that the oxidation of the reduced enzyme by oxygen occurs as a second order reaction, with no evidence for an intermediate[3] [21].

As a result the steady-state k_{cat}/K_{O2} value equals the second order rate constant for the reaction of the reduced enzyme with oxygen [44]. The pH dependence of the k_{cat}/K_{O2} value for wild-type and mutant PAO establishes that the protonation state of Lys315 is important for flavin oxidation and specifically that the nitrogen of Lys315 must be uncharged for more rapid flavin oxidation. This is consistent with the nitrogen being a hydrogen bond acceptor, with the water molecule that forms the bridge to the flavin N(5) as the hydrogen bond donor.

The solvent isotope effect on the wild-type enzyme is consistent with the lysine-water-flavin motif (Figure 1) seen in the structures of maize PAO and other members of the monoamine oxidase structural family. Oxidation of the reduced flavin necessarily results in the loss of a proton from the flavin N(5), while the formation of hydrogen peroxide requires a source of protons. The data are consistent with a model in which the role of the lysine is to properly

[3]The detection of a hydroperoxyflavin intermediate in the oxidative half-reaction of pyranose 2-oxidase is a notable exception [43].

orient the water molecule hydrogen-bonded to the flavin N(5), so that a free lone pair of electrons is available to facilitate the transfer of the proton from the flavin N(5) to oxygen. The linear proton inventory would then result from the movement of the N(5) proton. An alternative explanation for the solvent isotope effect and the role of Lys315 is that the proton is transferred from the flavin to the bridging water as a second proton is transferred from the water to the neutral lysine. This would require another amino acid residue to act as the source of the proton for oxygen. Either model yields the hydrogen bond interactions shown in Scheme 2. The latter model could result in a curved proton inventory if both protons were in flight in the transition state for flavin oxidation. The linear proton inventory seen with the wild-type enzyme would be due to asynchronicity in the extent of transfer of the two protons. While the data are fit better by a model for a single proton, the precision of the data cannot rule out the involvement of two protons with different isotope effects, if the isotope effect arising from one is 1.1 or less.

In contrast to the result with the wild-type enzyme, the solvent inventory for the mutant protein is clearly bowed, indicating that multiple protons are in flight in the transition state for flavin oxidation in this case. Our data do not allow us to determine the number of protons involved with any accuracy beyond stating that the best fits occur with three or more protons. This is a general problem with the proton inventory method [38]. The elimination of the amino moiety of Lys315 by the mutation is likely to alter the interaction of the conserved water molecule with the flavin N(5) and result in an additional water molecule in the active to fill the cavity left by the amino group. If the additional water serves as the proton donor to oxygen as the N (5) proton is lost, a curved proton inventory would result. Moreover, transfer of an additional proton between the two water molecules would be required to avoid formation of adjacent hydroxide and hydronium ions. This model thus predicts that there would be at least 3 protons in flight in the key transition state for the mutant protein, consistent with the proton inventory. The alternative model in which the lysine accepts a proton from the bridging water as a second proton is transferred from the flavin is also consistent with the curved proton inventory in the mutant protein. The role of proton acceptor from the bridging water would be be taken by the additional water molecule in the active site. The need to avoid formation of hydroxide suggests that a chain of water molecules would be needed, providing an explanation for the curvature of the proton inventory.[4]

For several flavoprotein oxidases, rapid flavin oxidation requires the presence of a positively charged active site residue, presumably to neutralize the negative charge developing on oxygen as it accepts electrons from the flavin [22,40,45–47]. The role of Lys315 in the PAO reaction is clearly different, since it must be unprotonated for optimal oxygen reactivity. The effect of the K315M mutation on the k_{cat}/K_m value of oxygen is much less than has been observed upon mutating the positively charged residue in other flavin oxidases, suggesting that another residue in PAO must play that role.

While the present results provide insight into the role of Lys315 in the reaction of a mammalian PAO and the effects of mutating this residue, they do not provide an obvious rationale for the results with maize PAO and LSD1. The characterization of the K661A mutant of LSD1 was quite qualitative [33], so the explanation may be that a decrease of ~25-fold in the reaction would have resulted in no detectable activity in the assay. However, the characterization of the K300M mutation in the maize enzyme was done by single-turnover kinetic analyses that measured the rate constant for flavin reduction directly [32]. It may be that this lysine has an additional role in the reductive half-reaction of that enzyme. The sequence identity between

[4]The only structure of a reduced member of the monoamine oxidase family is that of maize PAO. This shows the water bridging the flavin and Lys300, as shown in Figure 1. However, no product is bound in this structure. If the reaction with oxygen involves the reduced enzyme-product complex, as suggested by the low value of k_{cat} compared to k_{red}, the possibility must be considered that the product displaces the bound water. In that case the nitrogen in the newly oxidized carbon-nitrogen bond could fulfill the proposed role of the oxygen in the water molecule.

maize and mouse PAO is only 21%, and they oxidize different sides of the secondary nitrogen in the substrate, so that some differences in the roles of active site residues would not be remarkable. An alternative explanation is that the mutation has a structural effect in the maize enzyme, such that the resting enzyme is in an inactive conformation and the rate constant for reduction reflects the rate constant for a conformational change to an active enzyme. Such a model was recently proposed to explain the effects of mutating a conserved active site histidine in the lactate dehydrogenase flavocytochrome b_2 [48].

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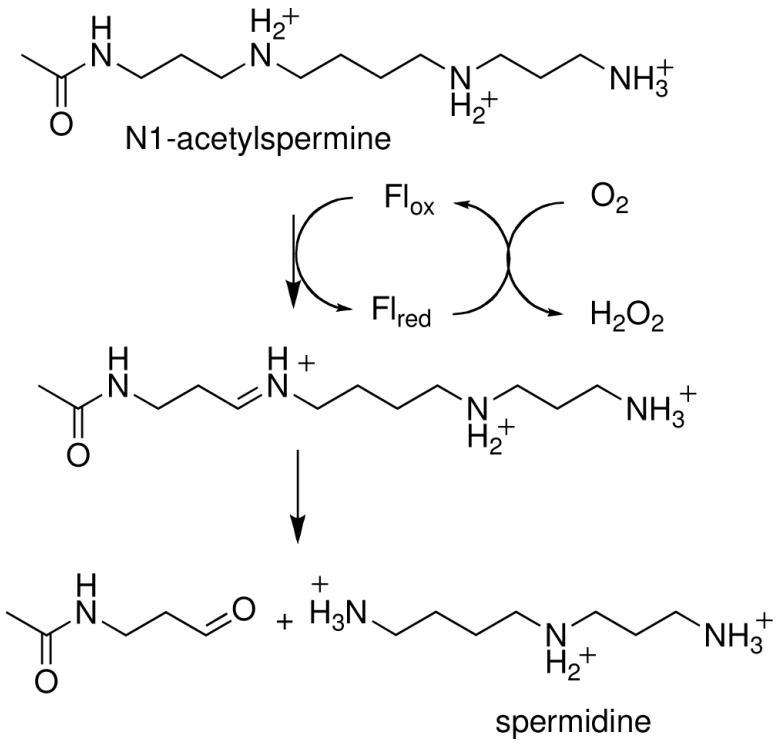
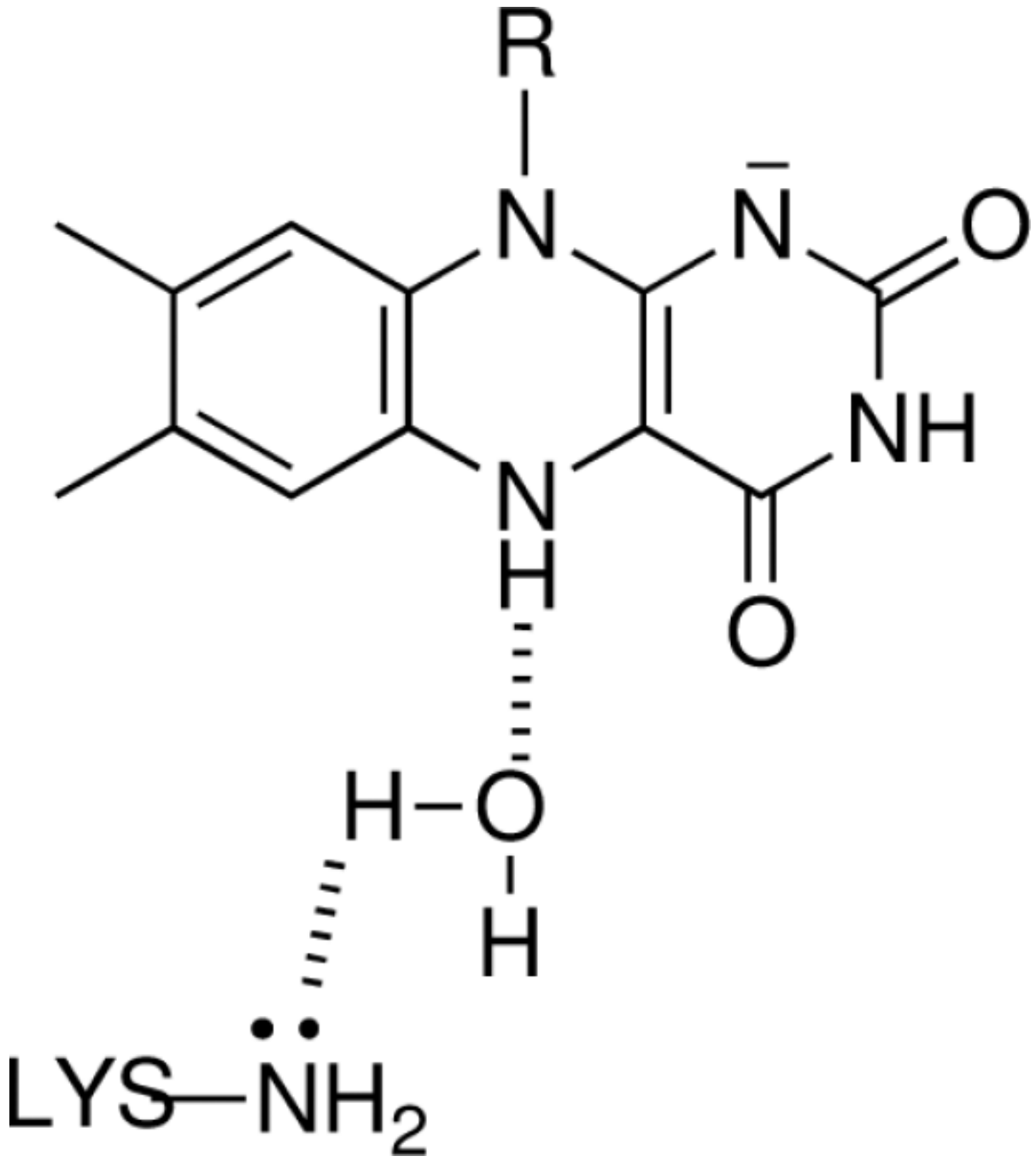


Scheme 1.



Scheme 2.

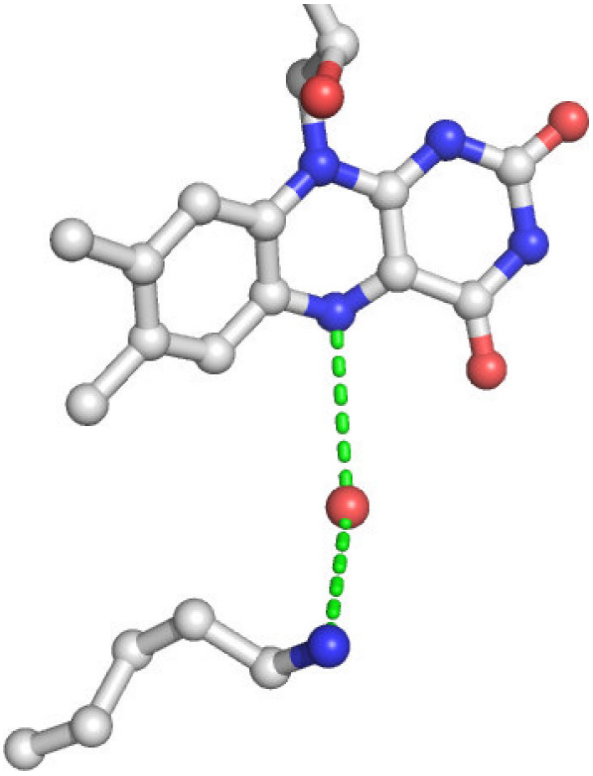


Figure 1.
The water-mediated interaction between the FAD and Lys300 in maize polyamine oxidase. The structure was drawn using pdb file 1h81 [49].

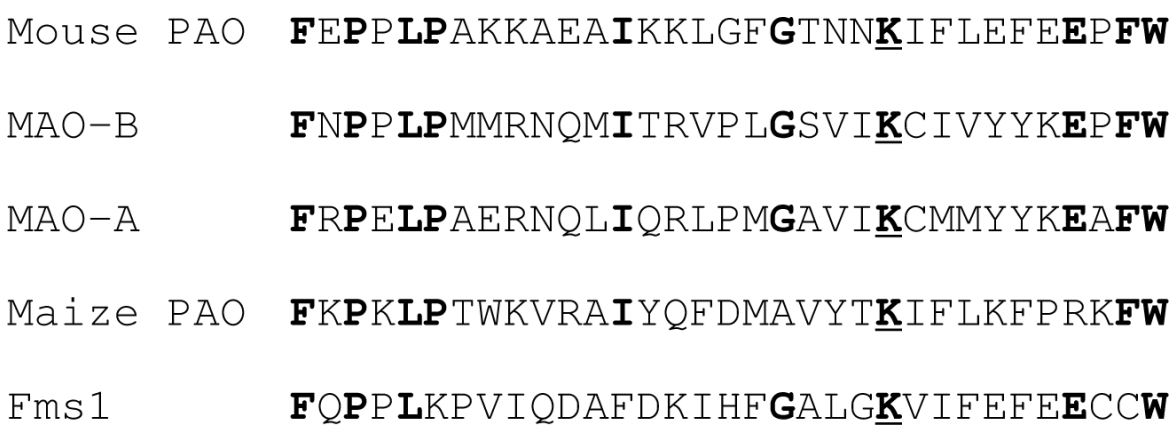


Figure 2.
Multiple sequence alignment for mouse PAO, human MAO-B, human MAO-A, maize PAO, and yeast Fms1. Conserved residues are in bold. The lysine corresponding to Lys315 in mouse PAO is underlined.

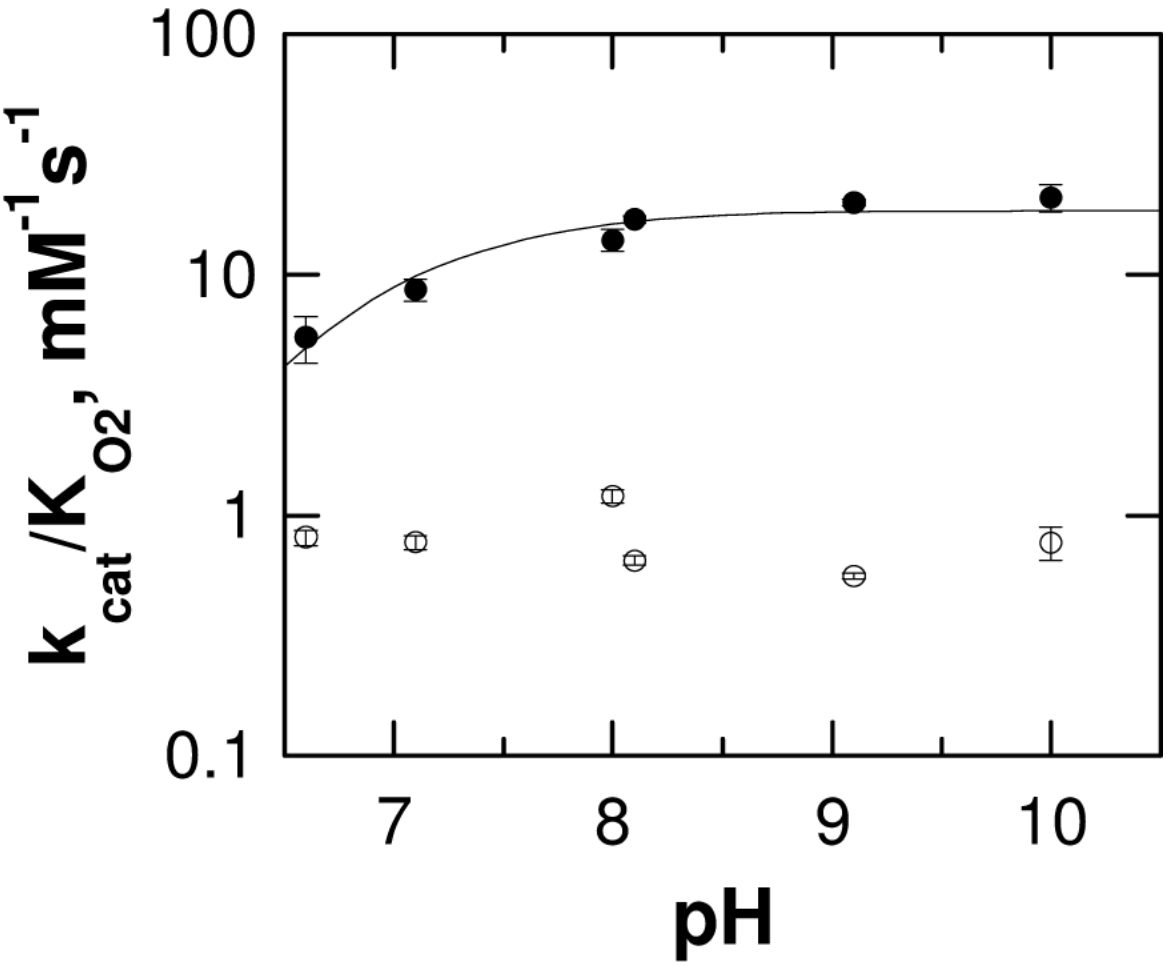


Figure 3.
k_{cat}/K_{O2}-pH profile for wild-type (filled circles) and K315M (open circles) PAO with N1-acetylspermine. The line is from a fit of the data to eq 1.

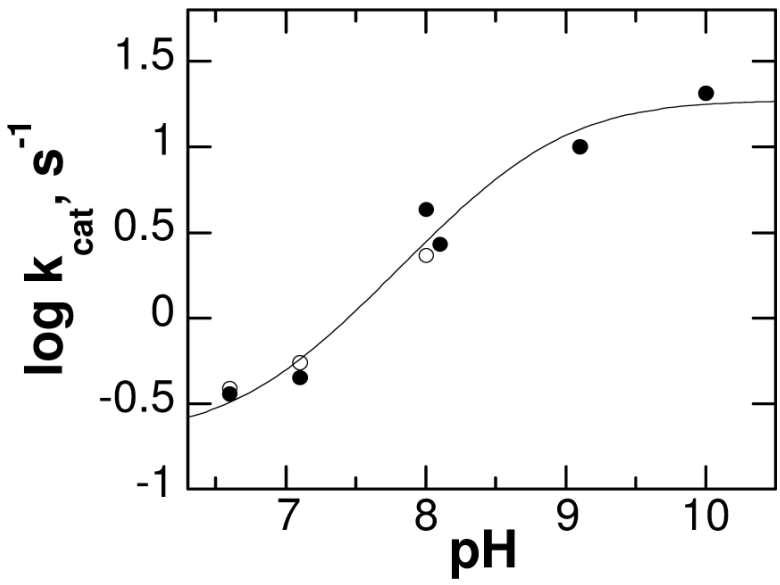


Figure 4.
k_{cat}-pH profile for wild-type (filled circles) and K315M (open circles) PAO with N1-acetylspermine. The line is from a fit to eq 2 of the wild-type enzyme data.

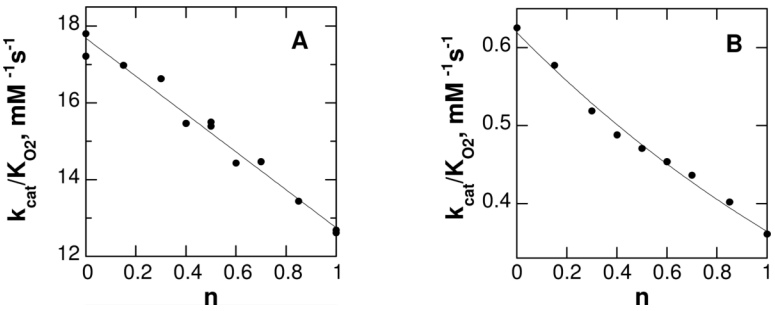


Figure 5.
The effect of the mole fraction of D_2O, n, on the k_{cat}/K_{O2} value at pH 10/pD 10.4 for (A) wild-type and (B) K315M PAO. The lines are from fits to eq 3 in A and to eq 5 in B.

Table 1

Steady-state kinetic parameters for wild type and K315M PAO

	pH 8			pH 10		
enzyme	**k_{cat}/K_{O2} (mM^{-1} s^{-1})**	**K_{O2} (mM)**	**k_{cat} (s^{-1})**	**k_{cat}/K_{O2} (mM^{-1} s^{-1})**	**K_{O2} (mM)**	**k_{cat} (s^{-1})**
Wild-type	14 ± 1	0.32 ± 0.05	4.3 ± 0.2	21 ± 3	0.99 ± 0.25	20.5 ± 2.6
K315M	1.2 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	0.7 ± 0.1	6 ± 6	4 ± 4

*Conditions: 1 mM N1-acetylspermine, 0.05 mM Tris-HCl (pH 8) or CAPS (pH 10), 20° C

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