Analysis of the Role of the Active Site Residue Arg98 in the Flavoprotein Tryptophan 2-Monooxygenase, a Member of the L-Amino Oxidase Family†

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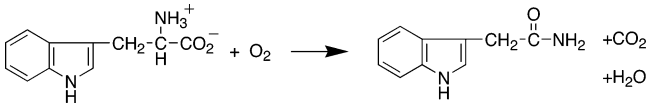
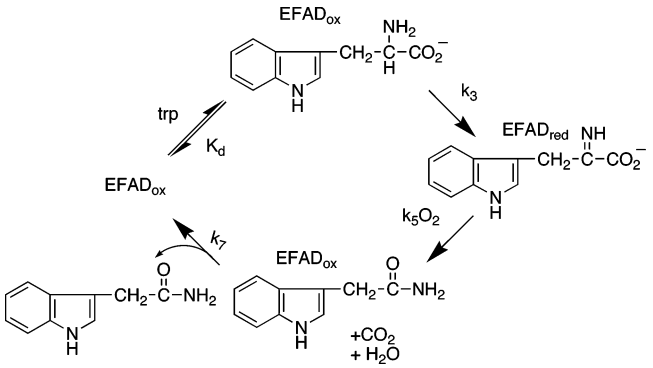
ABSTRACT: The flavoprotein tryptophan 2-monooxygenase catalyzes the oxidative decarboxylation of tryptophan to indoleacetamide. We have previously identified tryptophan 2-monooxygenase as a homologue of L-amino acid oxidase [Sobrado, P., and Fitzpatrick, P. F. (2002) *Arch. Biochem. Biophys*. *402*, 24−30]. On the basis of the sequence comparisons of the different LAAO family members, Arg98 of tryptophan 2-monooxygenase can be identified as an active site residue which interacts with the carboxylate of the amino acid substrate. The catalytic properties of R98K and R98A tryptophan 2-monooxygenase have been characterized to evaluate the role of this residue. Mutation of Arg98 to lysine decreases the first-order rate constant for flavin reduction by 180-fold and the second-order rate constant for flavin oxidation by 26-fold, has no significant effect on the K_d value for tryptophan or the K_i value for the competitive inhibitor indoleacetamide, and increases the K_i value for indolepyruvate less than 2-fold. Mutation of this residue to alanine decreases the rate constants for reduction and oxidation an additional 5- and 2-fold, respectively, and increases the K_d value for tryptophan and the K_i value for indolepyruvate by 31- and 17-fold, respectively, while having an only 2-fold effect on the K_i value for indoleacetamide. Both mutations increase the value of the primary deuterium isotope effect with tryptophan as a substrate, consistent with a later transition state. Both mutant enzymes catalyze a simple oxidase reaction, producing indolepyruvate and hydrogen peroxide. The pH dependences of the V/K_{trp} values for the mutant enzymes show that the anionic form of the substrate is preferred but that the zwitterionic form is a substrate. The results are consistent with the interaction between Arg98 and the carboxylate of the amino acid substrate being critical for correct positioning of the substrate in the active site for efficient catalysis.

Tryptophan 2-monooxygenase (TMO)[1] is an FAD-containing enzyme that catalyzes the oxidative decarboxylation of tryptophan to indoleacetamide (Scheme 1). This reaction is the first step in the biosynthesis of the plant hormone indoleacetic acid by plant pathogens such as *Agrobacterium tumefaciens* and *Pseudomonas savastanoi*; the resulting production of indoleacetic acid induces the formation of galls at the site of infection (*1*, *2*). The kinetic mechanism of TMO has been determined with tryptophan as a substrate and can be divided into two half-reactions (Scheme 2). In the reductive half-reaction, the α-C−H bond of the amino acid is broken and a hydride equivalent is transferred to the FAD, forming the reduced enzyme with the imino acid still bound. In the oxidative half-reaction, the reduced enzyme−imino acid complex reacts with molecular oxygen, forming oxidized FAD and the amide product (*3*). The values of each of the rate constants in Scheme 2 have been determined for recombinant TMO (*3*, *4*).

Scheme 1



Scheme 2



We have recently identified TMO as a member of the L-amino acid oxidase (LAAO) family of flavoproteins (*5*). LAAOs are common components of snake venom and mammalian milk (*6*), and the snake venom enzyme has been shown to induce apoptosis (*7*). The mammalian homologue *Fig1* (*8*, *9*) has been implicated in regulation of B cells by

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[1] Abbreviations: TMO, tryptophan 2-monooxygenase; LAAO, L-amino acid oxidase; LB, Luria Bertani; ACES, *N*-(2-acetamido)-2-aminoethanesulfonic acid.

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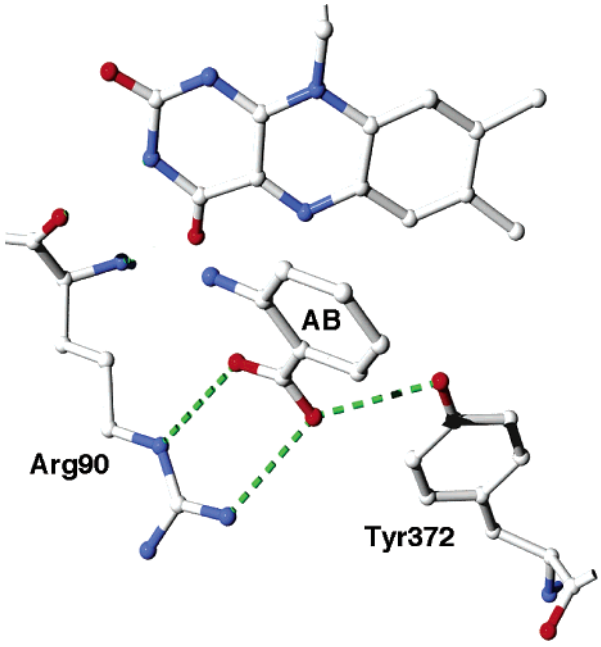

FIGURE 1: Active site of *C. rhodostoma* L-amino acid oxidase with aminobenzoate (AB) bound, showing the interactions with Arg90 and Tyr372. The figure was created using PDB entry 1F8S.

interleukin-4 (*10*) and autoimmune diseases (*11*). The only member of this family for which a crystal structure is available is the LAAO from *Calloselasma rhodostoma* (*12*); to date, there have been no reported analyses of the roles of active site residues in this enzyme or any other member of the LAAO family. In the structure of *C. rhodostoma* LAAO, the carboxylate of the competitive inhibitor *o*-aminobenzoate interacts with conserved residues Arg90 and Tyr372 (Figure 1); binding of the amino acid substrate is assumed to involve a similar interaction (*12*). Arg90 of LAAO corresponds to Arg98 in TMO (Figure 2). This report describes analyses of the contribution of this residue to catalysis in TMO and by extension in the entire LAAO family.

EXPERIMENTAL PROCEDURES

Materials. L-Tryptophan was purchased from USB (Cleveland, OH). Indoleacetamide, indolepyruvic acid, and glucose oxidase were from Sigma (St. Louis, MO). DEAE-Sephacel and phenyl-Sepharose were from Amersham Pharmacia (Uppsala, Sweden). *Escherichia coli* M15 (pREP4) was from Qiagen (Valencia, CA), and catalase was from Roche (Basel, Switzerland). [α-^{2}H]-L-Tryptophan was synthesized via the method of Kiick and Phillips (*13*).

Methods. Enzyme activity assays were performed on a Hansatech (Norfolk, U.K.) oxygen monitoring system with a computer-interfaced graphical mode and on a Yellow Springs Instrument (Yellow Springs, OH) model 5300 oxygen electrode. UV−visible spectra were recorded on a Hewlett-Packard diode array spectrophotometer. Rapid reaction kinetic measurements were performed on an Applied Photophysics SX.18MV stopped-flow spectrophotometer. The stopped-flow spectrophotometer was made anaerobic by filling the system with a solution of 0.03 μM glucose oxidase and 1 mM glucose in 1 mM EDTA and 50 mM Tris (pH 8.5) the night before the experiment was performed. Substrate and enzyme solutions were made anaerobic by cycles of vacuum and flushing with oxygen free argon.

DNA Manipulation. The pQE51-based plasmid previously used for expression of wild-type TMO (*5*) was used as the template for construction of plasmids for expression of the mutant enzymes. Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene). The protein coding regions for the plasmids used for protein expression were sequenced to ensure that no unwanted mutations were incorporated during the polymerase chain reaction.

Enzyme Assays. Standard enzyme assays were carried out in 50 mM Tris, 250 μM oxygen, 1 mM EDTA, 0.5 mM dithiothreitol (pH 8.3) at 25 °C, at different concentrations of [α-H]tryptophan or [α-^{2}H]tryptophan. Different oxygen concentrations were obtained by bubbling the assay mixture with the appropriate O_2/N_2 gas mixtures. Enzyme activity as a function of pH was measured in air-saturated 52 mM Tris, 52 mM ethanolamine, and 100 mM ACES. Because the *V*/*K* value for tryptophan is essentially independent of the concentration of oxygen (*4*), this allowed measurement of the *V*/*K* values but yielded apparent V_{max} values. The extent of formation of hydrogen peroxide was determined by measuring the decrease in the rate of oxygen consumption upon addition of catalase (0.01 mg/mL) at 17 mM tryptophan. The products of tryptophan oxidation by the mutant enzymes were determined by HPLC for reaction mixtures containing 1 mM tryptophan and 50 mM Tris (pH 8.3), as described previously (*4*).

Protein Expression and Purification. For growth of the wild-type and Arg98 mutant enzymes, a single colony of M15 *E. coli* (pREP4) containing the appropriate plasmid was used to inoculate a 60 mL culture of LB (100 μg/mL ampicillin and 25 μg/mL kanamycin). After overnight growth at 37 °C, six flasks containing 1.5 L of LB (100 μg/mL ampicillin and 25 μg/mL kanamycin) were inoculated with 10 mL of the overnight culture. The cultures were incubated at 37 °C until A_{600} reached a value of 0.6−0.8. At this point, 1.5 mL of 0.5 M isopropyl β-D-thioglucanopyranoside was added to each flask, and the temperature was decreased to 28 °C. After incubation for an additional 6−8 h, the cells were harvested by centrifugation at 5000*g* for 30 min at 4 °C. Purification of the wild-type enzyme was performed as described in ref *14*. For purification of the Arg98 mutant enzymes, the cell pellets were resuspended in 250 mL of 50 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol, 50 μM indoleacetamide, 0.5 mM phenylmethanesulfonyl fluoride, and 0.2 mg/mL lysozyme (pH 8.3). After the cell suspension had been stirred for 30 min at 4 °C, the cells were lysed by sonication. Cell debris and insoluble proteins were precipitated by centrifugation at 20000*g* for 30 min at 4 °C. The resulting supernatant was brought to 0.5% (w/v) polyethyleneimine, using a 0.1 g/mL stock solution in 50 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol (pH 8.3). After centrifugation at 20000*g* for 30 min at 4 °C, the supernatant was brought to 60% saturation in ammonium sulfate. The solution was stirred at 4 °C for 20 min, and the protein was precipitated by centrifugation at 20000*g* for 30 min at 4 °C. The resulting pellets were resuspended in 20 mL of 50 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol, 50 μM indoleacetamide, and 10% glycerol (pH 8.3) and dialyzed against two changes of 2 L of the same buffer. After centrifugation at 20000*g* for 20 min at 4 °C, the protein sample was loaded onto a DEAE-Sephacel column (3 cm × 20 cm) previously equilibrated with the same buffer. The mutant enzymes were

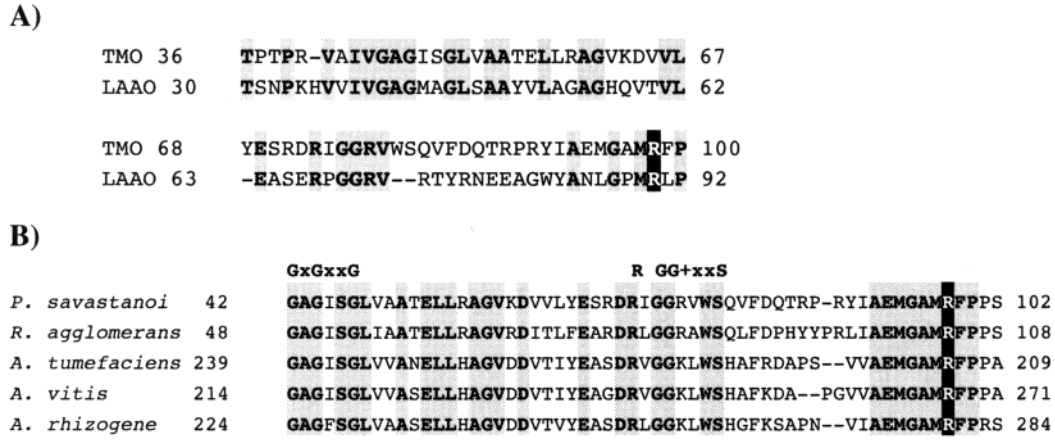

FIGURE 2: (A) Amino acid sequence alignment of the FAD binding domain of *C. rhodostoma* L-amino acid oxidase with the corresponding sequence of *P. savastanoi* TMO. The alignment was generated with the program PSI-Blast (*25*) using the amino acid sequence of TMO as a probe. The numbering for LAAO is that used in ref *12*. (B) Sequences of the putative FAD binding domains of TMOs. The GxGxxG motif of the putative nucleotide binding region and the GG motif of the family of L-amino acid oxidases are indicated (*26*). The conserved amino acids are highlighted. The conserved arginine residue is shown in white letters.

eluted with a linear gradient (total volume of 600 mL) of 0 to 200 mM NaCl. Both enzymes eluted around 150 mM NaCl. Fractions containing TMO were identified by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, pooled, brought to 60% ammonium sulfate saturation, and precipitated by centrifugation at 20000*g* for 20 min. The resulting pellet was resuspended in 50 mM Tris, 1 mM EDTA, 50 μM indoleacetamide, 0.5 mM dithiothreitol, and 10% glycerol (pH 8.3) and dialyzed against the same buffer. Purified enzymes were stored at −80 °C at a concentration of >50 μM. Using this method, 50−90 mg of enzyme was normally obtained. The concentrations of the wild-type and mutant enzymes were calculated from the absorbance at 466 nm using an extinction coefficient of 11.4 mM^{-1} cm^{-1} (*14*).

Removal of Indoleacetamide. To 5 mL of purified enzyme (~30−60 μM) was added an equal volume of a saturated solution of ammonium sulfate in 50 mM Tris and 1 mM EDTA (pH 8.3). After 20 min at 4 °C, the protein was precipitated by centrifugation at 20000*g* for 20 min. The enzyme was resuspended in 5 mL of 100 mM phenylalanine, 50 mM Tris, and 1 mM EDTA (pH 8.3) and incubated at 4 °C for 20 min, during which the protein solution went from bright yellow to light yellow. The enzyme was then precipitated by centrifugation for 20 min at 20000*g*. This procedure was repeated twice. The final pellet was suspended in 5 mL of 100 mM phenylalanine, 50 mM Tris, and 1 mM EDTA (pH 8.3) and dialyzed against two buffer changes of 1 L of 50 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol, and 10% glycerol (pH 8.3). The presence of indoleacetamide was monitored by HPLC with fluorescence detection; no traces of the compound were detected in the final enzyme samples.

Data Analysis. The kinetic data were analyzed using the programs Kaleidagraph (Synergy Software, Reading, PA) and Igor (Wavemetrics, Lake Oswego, OR). Initial rate data were fit to the Michaelis−Menten equation. Isotope effect values were obtained from fits of the data to eq 1. This equation describes separate isotope effects on V_{max} and V/K_{trp}

$$v = \frac{VA}{K_m[1 + F_i(E_{VK}k)] + A[1 + F_i(E_V)]} \quad (1)$$

where F_i is the fraction of the heavy atom, E_{VK} is the isotope effect on $V/K - 1$, and E_V is the isotope effect on $V_{max} - 1$. The inhibition constants for the wild-type and R98K enzymes were obtained using eq 2, which describes the behavior of a competitive inhibitor where K_i is the inhibition constant.

$$v = \frac{VA}{K_m\left(1 + \frac{I}{K_i}\right) + A} \quad (2)$$

The K_i values for the R98A enzyme were obtained by analyzing the effect of different inhibitor concentrations at 2.0 mM tryptophan ($K_m = 3.3$ mM) by the method of Dixon (*15*). The V/K_{trp} values at different pH values for the wild-type enzyme were fit to eq 3

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

where K_1 and K_2 are the dissociation constants for the ionizable groups and C is the pH-independent V/K_{trp} value. The V/K_{trp} values for the Arg98 mutant enzymes as a function of pH were fit to eq 4

$$\log Y = \log \frac{Y_L + \frac{Y_H K_1}{H}}{1 + \frac{K_1}{H}} \quad (4)$$

where K_1 is the dissociation constant for the ionizable group, Y_L is the activity at low pH, and Y_H is the activity at high pH. Stopped-flow traces were fit to eq 5 which describes a biphasic exponential decay

$$A_{total} = A_\infty + A_1 e^{-\lambda_1 t} + A_2 e^{-\lambda_2 t} \quad (5)$$

where λ_1 and λ_2 are the first-order rate constants for each phase, A_1 and A_2 are the absorbances of each species at time t, and A_∞ is the final absorbance. The pseudo-first-order rate

Table 1: Steady State Kinetic Parameters for Wild-Type and Mutant Tryptophan 2-Monooxygenases[a]

	wild-type	R98K	R98A
V_{max} (s^{-1})[b]	13.2 ± 0.7[c]	1.05 ± 0.05	0.21 ± 0.003
V/K_{trp} (mM^{-1} s^{-1})[d]	360 ± 37[c]	1.5 ± 0.15	0.042 ± 0.006
K_{trp} (mM)[d]	0.04 ± 0.005[c]	0.30 ± 0.04	3.3 ± 0.7
V/K_{O_2} (mM^{-1} s^{-1})[b]	140 ± 18[c]	7.5 ± 2	3.2 ± 0.2
K_{O_2} (mM)[b]	0.09 ± 0.01[c]	0.14 ± 0.05	0.067 ± 0.005
$^{D}V_{max}$[e]	1.22 ± 0.07	3.1 ± 0.2	4.3 ± 0.2
$^{D}(V/K_{trp})$[d]	1.16 ± 0.25	1.8 ± 0.3	5.7 ± 1.5
$K_{indoleacetamide}$ (μM)[d]	16 ± 0.8[f]	20 ± 2	45 ± 15
$K_{indolepyruvate}$ (μM)[d]	40 ± 9	71 ± 11	670 ± 150

[a] Conditions: 50 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol at pH 8.3 and 25 °C. [b] Measured by varying the concentration of oxygen at 20 mM tryptophan. [c] From ref *4*. [d] Measured by varying the concentration of tryptophan at 250 μM oxygen. [e] Determined by comparing the rates with 10 mM [α-H]tryptophan and 10 mM [α-^{2}H]tryptophan at 1.2 mM oxygen. [f] From ref *14*.

constants for the rapid phase at different concentrations of tryptophan were analyzed using eq 6

$$k_{obs} = \frac{k_3 A}{K_d + A} \quad (6)$$

here k_3 is the rate constant for flavin reduction at saturating substrate concentrations and K_d is the apparent dissociation constant for the substrate.

RESULTS

Steady State Kinetic Parameters. To analyze the contribution to catalysis of Arg98 in TMO, this residue was changed to lysine and alanine. The steady state kinetic parameters of the mutant enzymes with tryptophan as a substrate are given in Table 1. With the R98K enzyme, the V_{max} and V/K_{trp} values are 1 and 2 orders of magnitude lower, respectively, than the wild-type values. In the case of the R98A enzyme, the decreases are even more substantial. The reactivity of the reduced enzyme with molecular oxygen is also affected in the mutant enzymes. The V/K_{O_2} values decrease 18- and 44-fold in the R98K and R98A enzymes, respectively. These results strongly suggest that the presence of an arginine at position 98 is essential for efficient catalysis in TMO.

The binding of the competitive inhibitors indoleacetamide and indolepyruvate (*14*) to the oxidized enzyme is differentially altered by the two mutations. The $K_{indoleacetamide}$ value for the R98K enzyme is unchanged from the wild-type value, and that for the R98A enzyme is 2-fold higher. In contrast, the $K_{indolepyruvate}$ value is altered only slightly in the R98K enzyme but increases by 1 order of magnitude in the R98A enzyme (Table 1). These results are consistent with a positive charge at position 98 being important for binding of carboxylate-containing ligands in the active site of TMO.

The effect of the mutation of Arg98 on the rate constant for C−H bond cleavage was probed by measuring deuterium kinetic isotope effects with tryptophan as the substrate. For the wild-type enzyme, C−H bond cleavage is not rate-limiting, as indicated by the small deuterium isotope effects on both the V_{max} and V/K_{trp} values (Table 1) (*3*). The conservative substitution of Arg98 with lysine increases both the $^{D}V_{max}$ and $^{D}(V/K_{trp})$ values significantly, while the R98A mutation increases them still further (Table 1).

Products from Tryptophan Oxidation. The final product formed from tryptophan by the mutant proteins was determined by HPLC of reaction mixtures after turnover of tryptophan by each. In the case of both mutant enzymes, the only detectable product was indolepyruvate rather than indoleacetamide (results not shown). The formation of indolepyruvate suggested that the mutant enzymes carry out a simple oxidase reaction rather than an oxidative decarboxylation. In that case, hydrogen peroxide should also be a product. Whether hydrogen peroxide was formed during turnover of the mutant enzymes was determined by measuring the effect of catalase on the rate of oxygen consumption. With tryptophan as a substrate for the wild-type enzyme, the presence of catalase has no effect on the rate of oxygen consumption, indicating that no hydrogen peroxide is released into solution (*14*). In contrast, the rate of oxygen consumption by both Arg98 mutant enzymes decreases in the presence of catalase. The decrease in the rate of oxygen consumption was used to calculate the amount of hydrogen peroxide produced during catalysis for these enzymes. For the R98K enzyme, 0.90 ± 0.06 mol of hydrogen peroxide was formed per mole of tryptophan oxidized, while for the R98A enzyme, 0.97 ± 0.03 mol of hydrogen peroxide was produced.

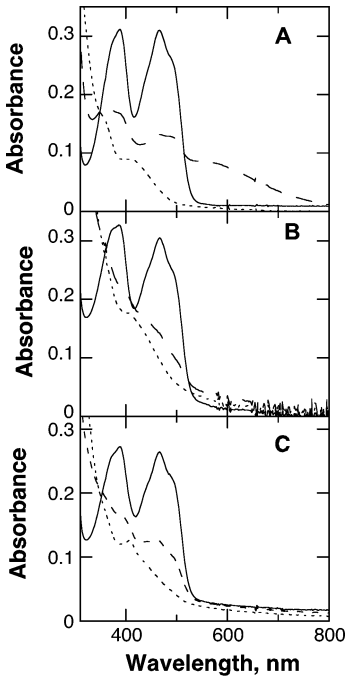

FIGURE 3: Spectral changes during reduction of tryptophan 2-monooxygenase by tryptophan in the absence of oxygen. (A) Spectra of the wild-type enzyme before (—) and 20 s (— — —) and 30 min (- - -) after addition of 1 mM tryptophan. (B) Spectra of the R98A enzyme before (—) and 30 s (— — —) and 180 s (- - -) after addition of 10 mM tryptophan. (C) Spectra of the R98K enzyme before (—) and 40 s (— — —) and 280 s (- - -) after addition of 1.25 mM tryptophan. Conditions: 50 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol at pH 8.3 and 25 °C.

Spectral Changes during Anaerobic Reduction. In the case of the wild-type enzyme, addition of substrate under anaerobic conditions results in a rapid decrease in the absorbance of the flavin between 350 and 520 nm and a simultaneous increase in absorbance beyond 520 nm. This is followed by a slow decrease at all wavelengths until the spectrum of the reduced enzyme is obtained (Figure 3) (*4*). The transient long wavelength absorbance is due to a charge transfer interaction between the imino acid intermediate and the reduced flavin in the active site which is lost when the imino acid dissociates

Scheme 3

$$EFl_{ox} + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EFl_{ox}S \xrightarrow{k_3} EFl_{red}P \xrightarrow{k_9} EFl_{red} + P$$

Table 2: Rapid Reaction Kinetic Parameters for Wild-Type and Mutant 2-Tryptophan Monooxygenases[a]

enzyme	k_3 (s^{-1})	K_d (mM)
wild-type[b]	139 ± 4	0.11 ± 0.015
R98K	0.77 ± 0.02	0.085 ± 0.011
R98A	0.16 ± 0.01	3.4 ± 0.8

[a] Conditions: 50 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol at pH 8.3 and 25 °C. [b] Values from ref *4*.

from the reduced enzyme. For the mutant enzymes, the rapid decrease in absorbance between 350 and 520 nm is also observed, but there is no increase in absorbance beyond 520 nm. The spectra of the fully reduced mutant enzymes are obtained more quickly than with the wild-type enzyme (Figure 3).

Rapid Reaction Kinetics. The rate constants for flavin reduction in the absence of oxygen (k_3 in Scheme 2) were determined by following the absorbance at 466 nm of the enzyme-bound flavin in the stopped-flow spectrophotometer. For both mutant enzymes, the decrease in absorbance was biphasic with only the observed first-order rate constant for the fast phase being substrate-dependent, similar to the behavior of the wild-type enzyme (*4*) and consistent with the kinetic mechanism of Scheme 3, where P is the indolepyruvate imine. The value of k_3 decreases close to 200-fold with the R98K enzyme and almost 1000-fold for the R98A enzyme compared to the value for the wild-type enzyme (Table 2). The rate constants for the slow phase (k_9 in Scheme 3) increase to 0.016 ± 0.005 and 0.026 ± 0.007 s^{-1} for the R98K and R98A enzymes, respectively, compared to the value for the wild-type enzyme [0.0078 ± 0.0004 s^{-1} (*4*)]. In the case of the R98A enzyme, the K_d value determined from the rapid reaction analysis is identical to the K_{trp} value for this enzyme, consistent with the isotope effects on the V_{max} and V/K values being equal to the intrinsic isotope effect. For the R98K enzyme, the K_d value is significantly less than the K_{trp} value. For this protein, the ^DV and $^D(V/K)$ values differ significantly, so the method of Klinman and Matthews (*16*) can be used to calculate the K_d value from the steady state kinetic measurements. This yields a value of 0.11 ± 0.05 mM, in agreement with the rapid reaction value.

pH Effects. The V/K_{trp}−pH profiles for the mutant enzymes exhibit low and high activity plateaus with a single pK_a value of 9.5−10 for a group that must be unprotonated for high activity (Figure 4 and Table 3). In contrast, the V/K_{trp}−pH profile for the wild-type enzyme is bell-shaped, consistent with the presence of one group with a pK_a value of 5.3 which must be unprotonated for catalysis and another group with a pK_a value of 9.9 which must be protonated for activity (*4*).

DISCUSSION

The catalytic properties of R98K and R98A TMO are fully consistent with Arg98 interacting with the carboxylate of the amino acid substrate in the active site of the enzyme. They also support the identification of TMO as a member of the LAAO family and of the larger flavin amine oxidase superfamily (*17*). The combination of steady state and rapid

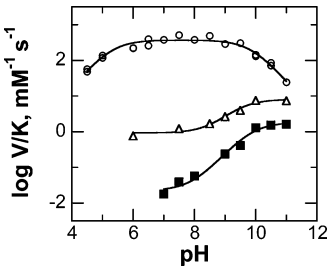

FIGURE 4: V/K_{trp}−pH profiles of wild-type (○), R98K (△), and R98A (■) tryptophan 2-monooxygenase. The lines are from fits of the data to eq 3 for the wild-type enzyme and to eq 4 for the mutant proteins. The data for the wild-type enzyme are from ref *3*.

Table 3: pK_a Values for Wild-Type and Mutant Tryptophan 2-Monooxygenases

enzyme	parameter	pK_1	pK_2	equation
wild-type[a]	V/K_{trp}	5.34 ± 0.05	9.89 ± 0.05	3
R98K	V/K_{trp}	9.50 ± 0.19		4
R98A	V/K_{trp}	9.86 ± 0.17		4

[a] Values from ref *3*.

Table 4: Intrinsic Rate Constants for Wild-Type and Mutant Tryptophan 2-Monooxygenases

	wild-type[a]	R98K	R98A
K_d (mM)	0.11 ± 0.05	0.08 ± 0.01	3.4 ± 0.8
k_3 (s^{-1})	139 ± 4	0.77 ± 0.02	0.16 ± 0.01
k_5 (mM^{-1} s^{-1})	196 ± 7	7.5 ± 2	3.2 ± 0.2
k_7 (s^{-1})	14	>8	>2

[a] From ref *3*.

reaction kinetic data for the mutant enzymes allows determination of the effects of the mutations on each of the kinetic constants in the mechanism of Scheme 2 (Table 4). The K_d value and the first-order rate constant for flavin reduction (k_3) can be directly measured in the stopped-flow spectrophotometer, the value for k_5 corresponds to the V/K_{O_2} value, and the value of k_7 can be calculated.

The changes in the absorbance spectra of the wild-type and mutant enzymes when they are mixed with tryptophan in the absence of oxygen are consistent with the kinetic mechanism of Scheme 3 (*4*). Binding of the amino acid to the enzyme is followed by reduction of the flavin and oxidation of the amino acid in a single first-order step; this step is readily monitored in the wild-type enzyme due to the reduction in flavin absorbance at 450 nm and the simultaneous appearance of a charge transfer absorbance band at longer wavelengths due to the formation of the reduced enzyme−imino acid complex (*4*). The imino acid then slowly dissociates from the reduced enzyme with a rate constant much slower than turnover. This slow step is not along the normal catalytic pathway which involves product release from the oxidized enzyme. The K_d value for binding of tryptophan to the oxidized enzyme can be determined from the concentration dependence of the pseudo-first-order rate for reduction (*18*). The K_d value is not altered by mutation of Arg98 to lysine, while it increases close to 30-fold in the R98A enzyme. This indicates that the conservation of a positive charge at position 98 is essential for effective binding of tryptophan in TMO. The changes in the inhibition constants for indoleacetamide and indolepyruvate support this conclusion and demonstrate the need for a carboxylate in the ligand if the loss of a positively charged amino acid

residue is to have a significant effect on ligand binding. The results with both the substrate and inhibitors are most consistent with an interaction of Arg98 with the carboxylate group of the substrate. The effect of removing this positive charge produces comparable changes in the free energy of binding of tryptophan and indolepyruvate (1.6−1.8 kcal/mol).

The greatest quantitative effect of both mutations on enzyme activity is on k_3, the rate constant for the chemical step in which a hydride equivalent is transferred from the substrate tryptophan to FAD (Table 2).[2] With R98K TMO, the value of k_3 has decreased 180-fold, even though the K_d value for tryptophan is not altered, while the mutation to alanine slows this chemical step by an additional 1 order of magnitude. This is consistent with altered binding of substrates and products in the active sites of both mutant enzymes. Disruption of the electrostatic interaction between the amino acid carboxylate and Arg98 would be expected to result in less than optimal positioning of the substrate in the active site. In a number of flavoproteins which catalyze transfer of a hydride equivalent to the flavin, the carbon from which the hydrogen is lost binds an average of 3.5 Å from flavin N5 (*20*), consistent with a requirement for a precise alignment of the amino acid and flavin in the active site. Loss of the arginine residue responsible for anchoring the carboxylate of the amino acid would disrupt this positioning, with a consequent decrease in the rate of catalysis.

In the case of wild-type TMO, the reaction of oxygen with the reduced enzyme−imino acid complex occurs in a single pseudo-first-order process with no detectable intermediates (*4*). Oxidative decarboxylation of the imino acid to form indoleacetamide occurs simultaneously with flavin oxidation. Kinetically, the reaction behaves as a simple second-order reaction, with a rate constant equal to the V/K value for oxygen determined from steady state analyses. Thus, the V/K_{O_2} values for the mutant enzymes yield k_5, the second-order rate constant for the reaction of the reduced enzyme−imino acid complex with molecular oxygen. The k_5 values for both enzymes decrease, but by substantially less than the k_3 values. Again, the effect is larger for the R98A enzyme. In addition, there is a qualitative change in the oxidative half-reaction, in that decarboxylation of the imino acid bound to indoleacetamide no longer occurs. Instead, hydrogen peroxide and indolepyruvate are produced. This is not due to dissociation of the bound imino acid prior to the reaction with oxygen. This step can be monitored as the slow phase in the reductive half-reaction seen in the stopped-flow experiments. While the rate constant for this slow phase is faster in the mutant enzyme, in both cases it is substantially slower than turnover, establishing that the product not does not dissociate from the mutant enzymes until after the FAD has been oxidized. Both the decreased rate constants for oxidation and the altered product profiles can be rationalized by the mutation of Arg98 affecting the relative orientation of the imino acid and the flavin, due to either an increased distance or an altered angle between the new C=N bond of the oxidized amino acid and the plane of the reduced flavin. In both mutants, this altered interaction is reflected in the fact that the reduced enzyme−imino acid complex no longer exhibits the long wavelength absorbance band which arises from a charge transfer interaction between the reduced flavin and oxidized amino acid. Such charge transfer transitions are highly dependent on the orientation of the partners (*21*).

The rate constant for product release, k_7, was not measured directly for either mutant enzyme. However, it is possible to place a lower limit on this parameter for each. For the mechanism of Scheme 2, $1/V_{max} = 1/k_3 + 1/k_7$. Since the measured values of k_3 are clearly not greater than the measured values of V_{max}, k_7 must be at least 10 times greater than k_3. This provides lower limits for k_7 of 8 and 2 s^{-1} for the R98K and R98A enzymes, respectively, compared to the value for the wild-type enzyme of 14 s^{-1}. Thus, it is a reasonably safe assumption that neither mutation results in a significant decrease in the value of k_7, although an increase in this parameter cannot be ruled out.

For wild-type TMO, the kinetic isotope effect on k_3 is 2.4 with tryptophan as the substrate; this is probably the intrinsic isotope effect in that case (*3*). Both mutant enzymes exhibit values of $^{D}V_{max}$ significantly greater than 2.4. These results suggest that the transition state for C−H bond cleavage in the wild-type enzyme is early, becoming later in the mutant enzymes. With the R98A enzyme, the $^{D}V_{max}$ and $^{D}(V/K)_{trp}$ values are not significantly different from one another and are probably identical. In contrast, the $^{D}(V/K)_{trp}$ value for the R98K enzyme is significantly less than the $^{D}V_{max}$ value. The $^{D}V_{max}$ value is likely to be the intrinsic isotope effect on k_3, given the identity of V_{max} and k_3. The decreased $^{D}(V/K)_{trp}$ value with this mutant enzyme suggests that there is still a small external commitment with tryptophan as the substrate for this enzyme form; i.e., $k_3/k_2 \sim 1.5$. This reflects a significant decrease from the commitment of approximately 10 in the wild-type enzyme (*3*).

The shapes of the V/K_{trp}−pH profiles are different from the shape of the wild-type profile for both mutant enzymes, showing a single pK_a value instead of a bell-shaped profile. The mutant enzymes were not sufficiently stable to determine if the pK_a value of ~6 in the wild-type enzyme is still present. However, the pK_a of 9.9 for a group in the wild-type enzyme which must be protonated for catalysis is clearly absent in the pH profiles of both mutant enzymes. A straightforward explanation for this result would be that this pK_a is that of Arg98. While it is theoretically possible for an arginine residue to have a pK_a value as low as 10, it is more likely that Tyr413 is the group responsible for this pK_a. As shown in the following paper (*22*), this pK_a value is also missing in the pH profiles of the Y413F and Y413A enzymes. Tyr413 of TMO corresponds to Tyr372 of LAAO; the role of the latter residue is to bind the carboxylate of the amino acid substrate (Figure 1). A reasonable explanation for the loss of the pK_a when either Arg98 or Tyr413 is mutated is that the dominant interaction with the substrate carboxylate is that with Arg98, consistent with the greater effect on kinetic parameters when Arg98 is mutated. The altered position of the substrate in the active site, reflected in the altered reduction and oxidation kinetics, results in the loss of the interaction between the carboxylate and Tyr413.

Unlike the wild-type enzyme, both mutant enzymes exhibit a pK_a value of ~9.7 for a group whose deprotonation results in increased activity. This is probably due to the amino group of the substrate tryptophan, with a pK_a of 9.4. With wild-

[2] The mechanism of C−H bond cleavage and flavin reduction has not been established for any LAAO, but a reasonable assumption is that the reaction occurs by hydride transfer. The active site is a mirror image of that of D-amino acid oxidase (*12*), which does catalyze hydride transfer from the amino acid substrate to the flavin cofactor (*19*).

type TMO and tryptophan as the substrate, the pK_a of the substrate amino group is not evident in the V/K−pH profile (Figure 4). However, with the slower substrate methionine, the V/K profile shows a third pK_a of 9.4 for a group whose deprotonation increases reactivity by severalfold in addition to the two pK_a values seen in the V/K_{trp} profile (*3*). Thus, the protonation state of the substrate becomes more important as the external commitment to catalysis of the substrate decreases, because of either altered substrate structure, as with methionine, or mutagenesis of Arg98. These data suggest that the enzyme preferentially binds the anionic form of the amino acid substrate, but can also form a productive complex with the zwitterion.[3] A study of the pH dependence of the reductive half-reaction of *Crotalus adamanteus* LAAO over the pH range of 5−9 found evidence for a single pK_a of greater than 9 for a group which must be deprotonated for activity (*23*). The authors assigned this pK_a to the amino group of the substrate phenylalanine. This is consistent with the model presented here for TMO, but suggests that the zwitterion does not bind to LAAO. The identity of the base in TMO responsible for removing the amino group proton from the bound zwitterion is not clear. In the structure of *C. rhodostoma* LAAO with anthranilate bound, His223 can be positioned with imidazole N3 within hydrogen bonding distance of the anthranilate nitrogen, leading to the proposal that this residue deprotonates the substrate amino moiety (*12*). However, this histidine is not conserved in several LAAOs or any TMO, while it is present in *C. adamanteus* LAAO. The lack of conservation of His223 and the pH dependence of the *C. adamanteus* enzyme suggest that His223 is not responsible for deprotonating the substrate amino group. Given the preference of the enzyme for the unprotonated form of the substrate, it is possible that the base which deprotonates the zwitterion is in the tunnel which leads to the active site rather than an actual active site residue.

The reductive half-reaction of LAAO and TMO, the two-electron oxidation of an amino acid, is also catalyzed by D-amino acid oxidase. In the yeast *Rhodotorula gracilis* D-amino acid oxidase, mutation of Arg285, which is proposed to bind the carboxylate of the amino acid substrate, has effects similar to those seen here for TMO. The V_{max} and k_3 values decrease ~400-fold with alanine as substrate for the R285K enzyme and 1 order of magnitude more for the R285A enzyme. In addition, the V/K value for oxygen decreases 12- and 250-fold in the R285K and R285A enzymes, respectively, and the long wavelength species is not observed in the Arg285 mutant enzymes (*24*). The pH dependence of the mutant enzymes was not described. The similarities of the effects of this mutation in the two enzymes illustrate the degree to which convergent evolution has produced similar active sites for amino acid oxidation by flavoproteins and are consistent with similar chemical mechanisms for both D-amino acid oxidase and the members of the LAAO family.

[3] The assays which determined the pH dependence of the V/K_{trp} value also yielded the pH dependence for the apparent V_{max} value. These pH−rate profiles for both mutant enzymes show high and low activity plateaus with a single pK_a for a group that must be unprotonated for maximal activity, as does that for the wild-type enzyme (*3*). For the R98K and R98A enzymes, the pK_a value is ~9.2, significantly higher than the wild-type enzyme pK_a value of 6.2. However, because they were not determined at saturating concentrations of oxygen, the pK_a values must also be considered apparent.

In conclusion, the results presented here are consistent with the proposed role of Arg98 of TMO in binding the substrate carboxylate and support the assignment of Arg98 as the residue homologous to Arg90 of the LAAOs. Together with the results in the following paper (*22*), they illustrate the dominant role of the interaction between the amino acid substrate and the active site arginine/tyrosine pair in proper positioning of the substrate for C−H bond cleavage.

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