Identification of Tyr413 as an Active Site Residue in the Flavoprotein Tryptophan 2-Monooxygenase and Analysis of Its Contribution to Catalysis†

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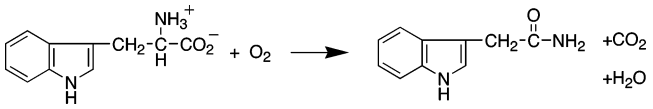
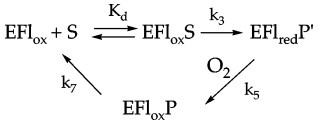
ABSTRACT: The flavoenzyme tryptophan 2-monooxygenase catalyzes the oxidation of tryptophan to indoleacetamide, carbon dioxide, and water. The enzyme is a homologue of L-amino acid oxidase. In the structure of L-amino acid oxidase complexed with aminobenzoate, Tyr372 hydrogen bonds with the carboxylate of the inhibitor in the active site. All 10 conserved tyrosine residues in tryptophan 2-monooxygenase were mutated to phenylalanine; steady state kinetic characterization of the purified proteins identified Tyr413 as the residue homologous to Tyr372 of L-amino acid oxidase. Y413F and Y413A tryptophan 2-monooxygenase were characterized more completely with tryptophan as the substrate to probe the contribution of this residue to catalysis. Mutation of Tyr413 to phenylalanine results in a decrease in the value of the first-order rate constant for reduction of 35-fold and a decrease in the rate constant for oxidation of 11-fold. Mutation to alanine decreases the rate constant for reduction by 200-fold and that for oxidation by 33-fold. Both mutations increase the K_d value for tryptophan and the K_i values for the competitive inhibitors indoleacetamide and indole pyruvate by 5−10-fold. Both mutations convert the enzyme to an oxidase, in that the products of the catalytic reactions of both are indolepyruvate and hydrogen peroxide. The V/K_{trp}−pH profiles for the Tyr413 mutant enzymes no longer show the pK_a value of 9.9 seen in that for the wild-type enzyme, allowing identification of Tyr413 as the active site residue in the wild-type enzyme which must be protonated for catalysis. Substitution of Tyr413 abolishes the formation of the long wavelength charge transfer species observed in the wild-type enzyme. The data are consistent with the main role of Tyr413 being to maintain the correct orientation of tryptophan for effective hydride transfer and imino acid decarboxylation.

The FAD-containing enzyme tryptophan 2-monooxygenase (TMO,[1] EC 1.13.12) catalyzes the oxidation of tryptophan to indoleacetamide, carbon dioxide, and water (Scheme 1) (*1*). The enzyme belongs to a group of flavoenzymes that catalyze the oxidative decarboxylation of amino acids. Other members of this group are L-phenylalanine oxidase (*2*), L-arginine 2-monooxygenase (*3*), and L-lysine 2-monooxygenase (*4*, *5*). TMO is the only enzyme in this group for which both the kinetic and chemical mechanisms have been studied in detail. Scheme 2 shows the kinetic mechanism for TMO determined with tryptophan as the substrate (*6*). Although the kinetic mechanism of TMO is well-understood, structural information about this and related flavoenzymes is lacking. We have identified the flavoenzyme

Scheme 1



Scheme 2



L-amino acid oxidase (LAAO) as a homologue of TMO (*7*). Although the amino acid sequences of TMO and LAAO are only 24% identical, both enzymes oxidize L-amino acids to form imino acid intermediates in their respective reductive half-reactions (*6*, *8*); TMO differs from LAAO in catalyzing the decarboxylation of this intermediate in the oxidative half-reaction. The structure of *Calloselasma rhodostoma* LAAO with the inhibitor *o*-aminobenzoate bound has been determined at 2.0 Å resolution (*8*). The carboxylate of the inhibitor and by implication the amino acid substrate are bound to Arg90 and Tyr372 (Figure 1). Arg98 of TMO corresponds to Arg90 of LAAO (*9*). We describe here the identification of Tyr413 as the residue corresponding to Tyr372 of LAAO and the use of site-directed mutagenesis to analyze the role of this residue in catalysis.

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

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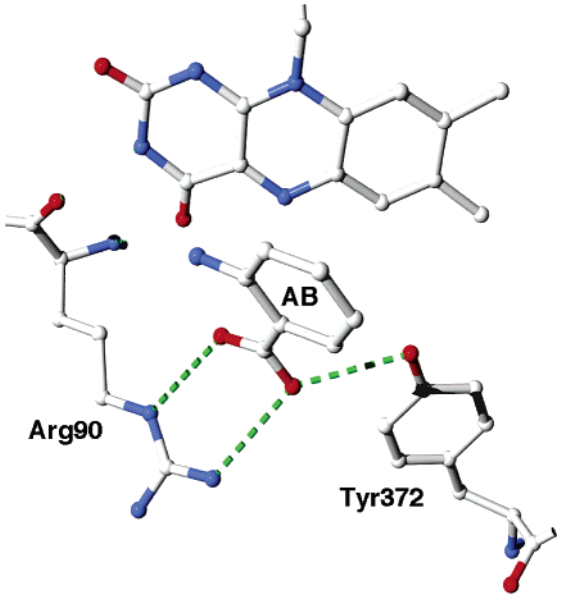

FIGURE 1: Active site of L-amino acid oxidase with aminobenzoate (AB) bound showing the interactions with Arg90 and Tyr372. The figure was created using PDB entry 1F8S.

EXPERIMENTAL PROCEDURES

Materials. The sources of substrates and proteins are as described in the preceding paper (*9*).

DNA Manipulation. The plasmid pQE51 (Qiagen, Valencia, CA) was used for expression of all proteins described here (*7*). Site-directed mutagenesis was performed using the QuikChange protocol (Stratagene). Plasmids were first transformed into XL1Blue cells for selection and plasmid preparations. Plasmid purification was performed using standard kits from Qiagen.

Protein Expression and Purification. Wild-type and mutant enzymes were expressed and purified as described in refs *9* and *10*. The wild-type enzyme in the absence of indoleacetamide is stable if stored at −80 °C for several months. This is not true for the Y413A and Y413F enzymes. The Y413A enzyme loses activity after several days at −80 °C and thus was used immediately after removal of indoleacetamide. The Y413F enzyme lost all activity in the absence of indoleacetamide.

Enzyme Assays. TMO activity was routinely measured in 2.5 mM tryptophan, 250 μM oxygen, 1 mM EDTA, 0.5 mM dithiothreitol, and 50 mM Tris (pH 8.3) at 25 °C by monitoring the rate of oxygen consumption as previously described (*10*). All other assays were as described previously (*9*).

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 effects were calculated using eq 1. This equation describes separate isotope effects on V_{max} and V/K_{trp}

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

where F_i is the fraction of heavy atom substitution in the substrate, E_V is the isotope effect on $V_{max} - 1$, and E_{VK} is the isotope effect on $V/K_{trp} - 1$. Inhibition constants were obtained by fitting the data to eq 2 which describes the behavior of a competitive inhibitor where K_i is the inhibition constant.

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

The pK_a values were calculated using eqs 3 and 4. Equation 3 describes a pH profile that is bell-shaped and has two pK_a values

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

where K_1 and K_2 are the dissociation constants for the ionizable groups and C is the pH-independent value of the kinetic parameter. Equation 4 is similar to eq 3 but with only one ionizable group which must be unprotonated.

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

Stopped-flow traces were fit to eq 5 which describes a biphasic exponential decay

$$A_{total} = (A_a - A_c)e^{\lambda_1 t} + \frac{\lambda_1(A_b - A_c)}{\lambda_1 - \lambda_2}(e^{-\lambda_2 t} - e^{-\lambda_1 t}) + A_c \quad (5)$$

where A is the measured absorbance, λ_1 and λ_2 are the first-order rate constants for the two phases, A_a and A_b are the absorbances of species A and B at time t, respectively, and A_c is the final absorbance. The pseudo-first-order rate constants for flavin reduction as a function of amino acid concentration were fit to eq 6

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

where k_3 is the rate constant for flavin reduction at a saturating substrate concentration and K_d is the apparent dissociation constant for the substrate.

RESULTS

Phenylalanine Scanning. In the crystal structure of LAAO with aminobenzoate bound, Tyr372 hydrogen bonds to the carboxylate of the inhibitor in the active site (Figure 1). The homology of TMO and LAAO suggests that a similar tyrosine residue is present in the active site of TMO. Residues 36−100 in TMO are 44% identical to the homologous residues in LAAO, which contain the conserved FAD-binding site. This level of identity aided in the identification of Arg98 as an active site residue in TMO (*9*). However, the C-terminal three-quarters of the enzymes are only 24% identical (Figure 2). This low level of identity makes it difficult to confidently identify a tyrosine residue in the C-terminal half of TMO as corresponding to Tyr372 in LAAO. Ten tyrosines are conserved in the TMO sequences available in the database. To identify the tyrosine that is important for catalysis in TMO, each conserved tyrosine was

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TMO    1                                    MYDHFNSPSIDILYDYGPFLKKCEMTGGIGSYSAG          35
LAAO -18                                    ADDRNPLAECFQENDYEEFLEIARNGLKA                29

TMO   36  TPTPR-VAIVGAGISGLVAATELLRAGVKDVVLYESRDRIGGRVWSQVFDQTRPRYIAEM                   94
LAAO  30  TSNPKHVVIVGAGMAGLSAAYVLAGAGHQVTVL-EASERPGGRV--RTYRNEEAGWYANL                   86

TMO   95  GAMRFPPSATGLFHYLKKFGISTSTTFPDPGVVDTELHY-RGKRYHWPAGKKPPELFRRV                 153
LAAO  87  GPMRLPEKHRIVREYIRKFDLRLNEFSQEN---DNAWYFIKNIRKKVGEVKKDPGLLKYP                 143

TMO  154  YEGWQSLLSEGYLLEG--GSLVAPLDITAMLKSGRLEEAAIAWQGWLNVFRDCSFYNAIV                 211
LAAO 144  VKPSEAGKSAGQLYEESLGKVVEELKRT----------------------NCSYILNKY                 180

TMO  212  CIFTGRHPPGGDRWARPEDFELFGSLGIGSGGFLPVFQAGFTEILRM-VINGYQSDQRLI                 270
LAAO 181  DTYSTKEYLIKEGDLSPGAVDMIGDLLNEDSGYY----VSFIESLKHDDIFAYEKRFDEI                 236

TMO  271  PDGISSLAARLADQSFDGKALRDRVCF-SRVGRISREAEKIIIQTEAGEQR----VFDRV                 325
LAAO 237  VDGMDKLPTAMY------RDIQDKVHFNAQVIKIQQNDQKVTVVYETLSKETPSVTADYV                 290

TMO  326  IVTSSNRAMQMIHCLTDSESFLSRDVARAVRETHLTGSSKLFILTRTKFWIKNKLPTTIQ                 385
LAAO 291  IVCTTSRAVRLIKF---NPPLLPKK-AHALRSVHYRSGTKIFLTCTTKFW---------E                 337

TMO  386  SDGLVRGVYCLD------YQPDEPEGHGV-VLLSYTWEDDAQKMLAMPDKKTRCQVLVDD                 438
LAAO 338  DDGIHGGKSTTDLPSRFIYYPNHNFTNGVGVIIAYGIGDDANFFQAL-DFKDCADIVFND                 396

TMO  439  LAAIH----PTFASYLLPVDGDYERYVLHHDWLTDPHSAGAFKLNYPGEDVYSQRLFFQP                 494
LAAO 397  LSLIHQLPKKDIQSFCYP--------SVIQKWSLDKYAMGGITTFTP----YQFQHFSDP                 444

TMO  495  MTANSPNKDTGLYLAGCSCSFAGGWIEGAVQTALNSA----CAVLRSTGGQLSKGNPLDC                 550
LAAO 445  LTASQGR----IYFAGEYTAQAHGWIDSTIKSGLRAARDVNLASENPSGIHLSNDNEL--                 498

TMO  491  INASYRY                                                                     557
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FIGURE 2: Amino acid sequence alignment of *Pseudomonas savastanoi* tryptophan 2-monooxygenase and *C. rhodostoma* L-amino acid oxidase. The alignment was generated with the program PSI-Blast (*15*) using the amino acid sequence of TMO as a probe. The numbering for LAAO is that used in ref *8*. The residues shown in italics were not used in the alignment. The conserved amino acids are in bold. Tyr372 of LAAO and Tyr413 of TMO are in larger letters.

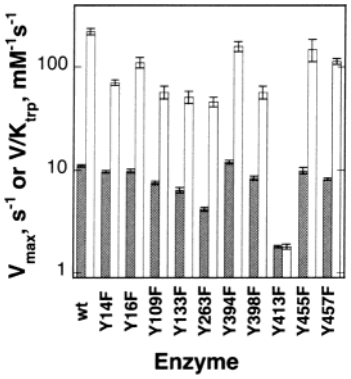

FIGURE 3: Results of phenylalanine scanning mutagenesis of TMO. Apparent steady state V_{max} (gray bars) and V/K_{trp} (white bars) values for wild-type and mutant TMO were determined as described in Experimental Procedures.

mutated to phenylalanine; the mutant enzymes were expressed and purified, and the steady state kinetic parameters were determined with tryptophan as the substrate. Only the mutation of Tyr413 decreases the V_{max} and V/K_{trp} values more than 4-fold (Figure 3). This result identifies Tyr413 as the most critical tyrosine residue in TMO and supports the alignment of this residue with Tyr372 of LAAO.

Steady State Kinetics of Y413F and Y413A TMO. Since scanning mutagenesis suggested that Tyr413 is important for catalysis, the steady state kinetics of the Y413F enzyme were further characterized. In addition, the Y413A enzyme was expressed and characterized. The results of the kinetic analyses are given in Table 1. The most pronounced effect is on the V/K_{trp} values, which decrease at least 2 orders of magnitude for both enzymes. Both mutations cause a decrease in the V_{max} values, with a greater effect in the case of the Y413A enzyme. Smaller changes were seen in the V/K_{O_2} values. The inhibition constants for the competitive inhibitors indoleacetamide and indolepyruvic acid were also measured. The K_i values for both inhibitors increase for both mutant proteins, with larger effects seen for the Y413F enzyme. The K_i value for indolepyruvate shows a larger increase than the K_i value for indoleacetamide with both enzymes.

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

	wild-type[e]	Y413F	Y413A
V_{max} (s^{-1})[b]	13.2 ± 0.7	4.0 ± 0.3	0.69 ± 0.03
V/K_{trp} (mM^{-1} s^{-1})[c]	360 ± 37	1.8 ± 0.1	0.35 ± 0.02
K_{trp} (mM)[c]	0.04 ± 0.005	1.0 ± 0.1	1.48 ± 0.14
V/K_{O_2} (mM^{-1} s^{-1})[b]	140 ± 18	18 ± 3	6 ± 1
K_{O_2} (mM)[b]	0.09 ± 0.01	0.22 ± 0.05	0.12 ± 0.02
$^{D}V_{max}$[d]	1.22 ± 0.07[f]	1.9 ± 0.1	4.4 ± 0.3
$^{D}(V/K_{trp})$[c]	1.16 ± 0.25[f]	2.1 ± 0.1	4.0 ± 0.4
$K_{indoleacetamide}$ (μM)[c]	16 ± 0.08	76 ± 6	47 ± 5
$K_{indolepyruvate}$ (μM)[c]	40 ± 9[f]	388 ± 50	216 ± 35

[a] Standard 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 2.5 or 20 mM tryptophan for the wild-type and mutant enzymes. [c] Measured by varying the tryptophan concentration at 230 μM oxygen. [d] Determined by comparing the rates with 10 mM [α-H]tryptophan and 10 mM [α-^{2}H]tryptophan at 1.2 mM oxygen. [e] From refs *6*, *10*, and *11*. [f] This work.

To obtain information about the effects of mutation of Tyr413 on the rate of C−H bond cleavage, we measured the primary deuterium kinetic isotope effects with tryptophan as the substrate. In the wild-type enzyme, the $^{D}V_{max}$ and $^{D}(V/K_{trp})$ values are very close to unity, indicating that C−H bond cleavage is not rate-limiting (Table 1) (*11*). For the Y413F enzyme, the $^{D}V_{max}$ and $^{D}(V/K_{trp})$ values both increase

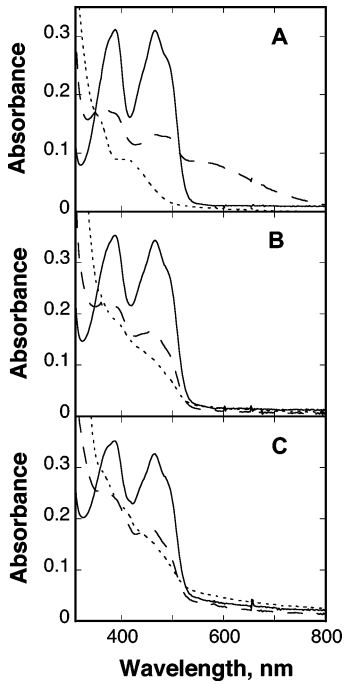

FIGURE 4: Spectral changes during reduction of TMO by tryptophan. (A) The reduction of the wild-type enzyme in the absence of oxygen before (—), 20 s after (– – –), and 30 min after (- - -) addition of 1 mM tryptophan. For the Y413A (B) and Y413F (C) enzymes, the spectra were taken before (—), 15 s after (– – –), and 10 min after (- - -) addition of 3 mM tryptophan. Conditions: 50 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol at pH 8.3 and 25 °C.

to ~2. With the Y413A enzyme, both isotope effects increase further to an average value of 4 (Table 1).

Spectral Changes during Flavin Reduction. When wild-type TMO is mixed with tryptophan in the absence of oxygen, the absorbance between 350 and 520 nm decreases and that above 520 nm increases (Figure 4) (*6*). These changes in absorbance are very rapid and are followed by a slow decrease in absorbance at all wavelengths until the fully reduced enzyme spectrum is formed. The long wavelength absorbance is due to the reduced enzyme−imino acid intermediate; with the wild-type enzyme in the absence of oxygen, this intermediate dissociates at a rate of 0.0078 ± 0.004 s^{-1} to produce the free reduced enzyme (*6*). In the reduction of the Y413A and Y413F enzymes, the rapid decrease in absorbance between 320 and 520 nm is also observed, but the spectrum of the reduced flavin is obtained at a rate of 0.015 ± 0.004 s^{-1}. Neither mutant enzyme shows any absorbance at long wavelengths (Figure 4). The reduction of the Y413A enzyme was also monitored in the stopped-flow spectrophotometer with a diode array detector, and no long wavelength absorbance was detected (data not shown).

Rapid Reaction Kinetics. The rate constant for flavin reduction by tryptophan was measured directly by monitoring the decrease in absorbance at 466 nm in the stopped-flow apparatus under anaerobic conditions. This was only possible for the Y413A enzyme. The mutant enzymes are purified with indoleacetamide bound, as is the wild-type enzyme (*10*). The indoleacetamide must be removed for rapid reaction kinetic analyses. When this was done by the method developed for the wild-type enzyme, which involves dialysis against methanol (*10*), both mutant proteins lost all activity. With an alternative method in which the indoleacetamide-complexed enzyme was reduced several times with excess phenylalanine to displace the indoleacetamide (*9*), it was possible to obtain the fully active Y413A enzyme without indoleacetamide. However, this enzyme was unstable in the absence of indoleacetamide and had to be used immediately for stopped-flow experiments. Even with the modified protocol, the Y413F enzyme had no activity once the indoleacetamide was removed, so rapid reaction analyses could not be carried out with this mutant protein.

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 Y413A Tryptophan 2-Monooxygenases[a]

enzyme	k_3 (s^{-1})	K_d (mM)	$^Dk_3$
wild-type[b]	139 ± 4	0.11 ± 0.015	2.4 ± 0.06
Y413A	0.71 ± 0.02	1.2 ± 0.1	4.2 ± 1.2[c]

[a] Conditions: 50 mM Tris, 1 mM EDTA, and 0.5 mM dithiothreitol at pH 8.3 and 25 °C. [b] From ref *6*. [c] Determined by comparing the rates of flavin reduction at 10 mM [α-H]tryptophan and 10 mM [α-^{2}H]tryptophan.

With Y413A TMO, as with the wild-type enzyme, flavin reduction is biphasic; only the rate constant for the fast phase varies with the concentration of tryptophan (*6*). These results are consistent with the applicability to this mutant enzyme of the mechanism of Scheme 3, which also applies to wild-type TMO (*6*). The effect of the tryptophan concentration on the observed first-order rate constant for the fast phase could be fit to eq 6 to yield the first-order rate constant for reduction, k_3, and the K_d value for tryptophan. The k_3 value for Y413A TMO is 195-fold lower than the wild-type value, and the K_d value is 13-fold higher than the wild-type value (Table 2). The $^Dk_3$ value is greater for the mutant enzyme than for the wild-type enzyme and equal to the isotope effects on the V_{max} and V/K_{trp} values.

Characterization of Products. The products formed by oxidation of tryptophan by the mutant enzymes were determined by HPLC analysis of reaction mixtures. For both enzymes, no significant indoleacetamide formation could be detected. Instead, a peak with the same retention time as indolepyruvate was formed. Thus, the mutant enzymes catalyze the same two-electron oxidation of tryptophan in the reductive half-reaction as the wild-type enzyme, but differ in that their oxidative half-reactions produce oxidized flavin without the attendant decarboxylation of the imino acid. In that case, hydrogen peroxide should also be produced in the oxidative half-reaction. The amounts of hydrogen peroxide formed in the reactions catalyzed by Y413F and Y413A TMO were determined by measuring the decrease in the rate of oxygen consumption in the presence of catalase. For the wild-type enzyme with tryptophan as a substrate, catalase has no effect on the rate of oxygen consumption, indicating that no hydrogen peroxide is produced (*10*). With the Y413F and Y413A enzymes, the rates decrease in the presence of catalase. The magnitude of the change in the rate of oxygen consumption can be used to calculate the amount of hydrogen peroxide released per oxidized tryptophan during catalysis. The Y413F enzyme produces 0.75 ± 0.01 mol of hydrogen peroxide per tryptophan consumed, while 0.95 ± 0.02 mol

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

enzyme	parameter	pK_a	pK_b	equation
wild-type[b]	V/K_{trp}	5.34 ± 0.05	9.89 ± 0.05	3
Y413F	V/K_{trp}	6.20 ± 0.06		4
Y413A	V/K_{trp}	6.12 ± 0.06		4

[a] Conditions: 250 μM O_2, 100 mM ACES, 52 mM Tris, and 52 mM ethanolamine at 25 °C. [b] From ref *11*.

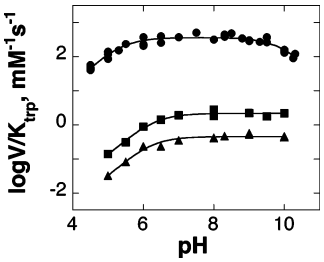

FIGURE 5: pH dependence of V/K_{trp} for wild-type (●), Y413F (■), and Y413A (▲) tryptophan 2-monooxygenase. The lines are fits of the data to eq 3 for the wild-type enzyme and to eq 4 for the mutant enzymes.

of H_2O_2 is produced by the Y413A enzyme, consistent with both mutations converting the enzyme to an oxidase.

pH Studies. The effect of pH on the V/K_{trp} value was determined for both mutant enzymes. The wild-type V/K_{trp}−pH profile is bell-shaped, consistent with the involvement of two ionizable groups, one with an apparent pK_a value of 5.3 and the other with an apparent pK_a value of 9.9 (*11*). The V/K_{trp}−pH profiles of both the Y413F and Y413A enzymes exhibit a single pK_a with a value close to 6 for a group that must be deprotonated for activity (Table 3 and Figure 5).

DISCUSSION

The results of mutagenesis of the conserved tyrosine residues in TMO shown in Figure 3 identify Tyr413 as the residue homologous to Tyr372 of LAAO. This result validates the sequence alignment shown in Figure 2 and further supports the homology between LAAO and TMO. The detailed characterization of the effects of mutagenesis of Tyr413 confirms its importance for catalysis and provides insight into the role of this residue and of Tyr372 in LAAO.

The effect of the mutation on the inhibition constants for competitive inhibitors and on the K_d value for tryptophan can be used as a measure of the contribution of Tyr413 to binding. The $K_{indolepyruvate}$ values increase 5−10-fold for both mutant enzymes, while the $K_{indoleacetamide}$ value increases by half as much. The K_d value for tryptophan for the Y431A enzyme also exhibits a 10-fold increase. The greater increase in the $K_{indolepyruvate}$ values and the K_d value for the amino acid substrate is consistent with Tyr413 interacting with the carboxylate of the substrate. The increase in $K_{indoleacetamide}$ suggests that Tyr413 has a weak interaction with the carbonyl oxygen of indoleacetamide or that the binding of the indole ring has been affected by the mutations. The decreases in affinity for the substrate and the inhibitors seen when Tyr413 is mutated are smaller in magnitude than that found for the R98A enzyme (*9*). This suggests that the interaction between the substrate carboxylate and Arg98 plays the dominant role in substrate binding. The changes in binding energies for indolepyruvate are −1.3 and −1.0 kcal/mol for the Y413F and Y413A enzymes, respectively, while a change in the binding energy for the substrate of −1.4 kcal/mol can be calculated from the K_d value. These values are close to the values expected for the elimination of a hydrogen bond (*12*). The smaller increase in the K_i value for indoleacetamide is consistent with the decreased strength of a hydrogen bond involving a neutral carbonyl rather than a charged carboxylate. The distance of 2.96 Å from the Tyr372 phenolic oxygen in LAAO to one of the carboxylate oxygens of aminobenzoate in the active site confirms the existence of this hydrogen bond for that enzyme.

If this tyrosine is to form a hydrogen bond with a carboxylate oxygen, the phenolic oxygen must be protonated. The pH dependences of the V/K_{trp} values for the Y413F and Y413A enzyme show only one pK_a value of 6.0 for a residue that must be deprotonated for activity. This is different from the wild-type pH profile where two pK_a values are observed, one for a group that must be deprotonated for activity with an apparent pK_a value of 5.3 and another for a group that must be protonated for activity with an apparent pK_a value of 9.9.[2] The lack of the pK_a in the alkaline region of the pH profiles of the Y413A and Y413F enzymes suggests that Tyr413 is the residue responsible for that pK_a. The change in the pH profile when Tyr413 is mutated is clearly different from that seen when Arg98 is mutated, even though both residues are involved in binding the substrate carboxylate. In the case of the R98K and R98A enzymes, the only pK_a that is observed is that of the amino acid substrate (*9*). This suggests that, in the absence of the interaction with Arg98 to properly orient the carboxylate, the hydrogen bond with Tyr413 does not make a meaningful contribution to binding, either because of the increased distance between the carboxylate and the phenolic oxygen or because of the increased ability of the carboxylate to rotate in the active site.

The deletion of the phenol moiety at position 413 of TMO also decreases the rate of C−H bond cleavage. In the case of the Y413A enzyme, this is demonstrated directly by the 195-fold decrease in the value of k_3 determined in rapid reaction analyses. No rapid reaction data are available for the Y413F enzyme due to the loss of activity in the absence of indoleacetamide. Still, the value of k_3 for this enzyme can be calculated from the steady state kinetic parameters using the isotope effects. The relationships between the kinetic constants of Scheme 2 and steady state kinetic parameters V_{max} and ^DV are given by eqs 7 and 8 (*13*).

$$V_{max} = \frac{k_3 k_7}{k_3 + k_7} \tag{7}$$

$$^DV = \frac{^Dk_3 + k_3/k_7}{1 + k_3/k_7} \tag{8}$$

The deuterium kinetic isotope effect on k_3 for the wild-type enzyme is 2.36 ± 0.06 (*6*). If one assumes that the intrinsic isotope effect on k_3 is not altered in the Y413F enzyme,[3] it is possible to calculate the values of k_3 and k_7 (6 ± 2 and 12 ± 3, respectively). Thus, mutation of Tyr413 to phenyl-

[2] In the case of wild-type TMO, the large commitment to catalysis for tryptophan alters the pK_a values seen in the V/K_{trp} profile by ∼1 pH unit so that the intrinsic pK_a values are ∼6 and ∼9 (*11*).

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

	wild-type[a]	Y413F	Y413A
K_d (mM)	0.11 ± 0.05	1.0 ± 0.1	1.2 ± 0.1
k_3 (s^{-1})	139 ± 4	6 ± 2	0.71 ± 0.02
k_5 (mM^{-1} s^{-1})	196 ± 7	18 ± 3	6 ± 1
k_7 (s^{-1})	14	12 ± 3	≥ 14

[a] From ref *6*.

alanine decreases the rate of the C−H bond cleavage step by ~23-fold. This mutation has no effect on the rate constant for product release, k_7 (Table 4). It is not possible to calculate a value for k_7 for the Y413A enzyme, since reduction has become fully rate limiting in that enzyme. However, the value of k_7 must be at least 20-fold greater than k_3 so that a lower limit of 14 can be placed on the value of k_7. Thus, there is no decrease in the rate of product release upon replacement of Tyr413 with alanine.

For Y413A TMO, $^{D}V_{max}$ and $^{D}(V/K_{trp})$ have an average value of 4.2. This is larger than the intrinsic isotope effect for the wild-type and Y413F enzymes but is comparable to the value for the R98A enzyme (*9*). These results are consistent with an early transition state for the wild-type and Y413F enzymes with tryptophan as a substrate and a later and more symmetric transition state for the Y413A enzyme. The increase in the value of the intrinsic isotope effect as Tyr413 is increasingly modified parallels the changes seen when Arg98 is altered (*9*). In both cases, the transition state for C−H bond cleavage becomes later as the reaction becomes slower.

The reaction of the reduced enzyme with oxygen is also affected by mutagenesis of Tyr413 to either phenylalanine or alanine, as seen by the reduced V/K_{O_2} values, although the effects are smaller in magnitude than those on the reductive half-reaction (Table 1). The V/K_{O_2} value for TMO is equal to k_5, the second-order rate constant for reaction for the reduced enzyme−imino acid complex with oxygen which produces the oxidized enzyme and simultaneously decarboxylates the acid (*6*). The effects of mutagenesis of Tyr413 on the oxidative half-reaction are similar to those seen upon mutagenesis of Arg98: decreased reactivity with oxygen and conversion of the enzyme from a decarboxylase to a simple oxidase (*9*). The structure of the reduced enzyme−imino acid complex is altered by mutagenesis of Tyr413, since the long wavelength charge transfer absorbance band is not observed and the imino acid dissociates more rapidly from the mutant enzymes than from the wild-type enzyme. This is consistent with Tyr413 contributing to binding of the imino acid intermediate, thereby contributing to the proper orientation of the indole ring with respect to the isoalloxazine ring of the FAD in the reduced enzyme. The similar effects of mutating Arg98 and Tyr413 on the oxidative half-reaction support the conclusion drawn from studies of the R98K and R98A enzymes that the interaction between the substrate carboxylate and the enzyme is critical for proper positioning of the oxidized substrate for oxidative decarboxylation.

Together with the results of the preceding paper (*9*), the characterization of the effects of mutagenesis of Tyr413 described herein establishes the importance of the Arg98/Tyr413 pair in TMO for binding the carboxylate of the amino acid substrate and properly positioning the amino acid for oxidation. They also confirm the identification from sequence comparison of TMO as a member of the L-amino acid oxidase family. Consequently, these results should be applicable to the other members of the L-amino acid oxidase family, which has seen no significant structure−function analysis to date.

[3] The effect of the Y413F mutation on the V/K_{trp}−pH profile provides an independent estimate of the value of the intrinsic isotope effect on k_3. In the case of the mechanism of Scheme 2, the relationship between the true pK_a value and that observed in the V/K_{trp} profile is given by eq 9 (*14*). For the wild-type TMO, the intrinsic pK_a for a group in the free enzyme which must be deprotonated for catalysis is 6.0 ± 0.2 (*11*). The pK_a value of 6.1−6.2 in the V/K_{trp}−pH profiles for the Tyr413 mutant enzymes is consistent with a decrease in the value of k_3/k_2 of <0.2 and probably to zero. Use of a k_3/k_2 value of 0−0.2 and the observed $^{D}(V/K)_{trp}$ value of 2.1 ± 0.1 in eq 10 yields an intrinsic isotope effect on k_3 of 2.1−2.3, not significantly different from the value of $^{D}k_3$ for the wild-type enzyme.

$$\Delta pK_a = \log(1 + k_3/k_2) \quad (9)$$

$$^{D}(V/K_{trp}) = \frac{^{D}k_3 + k_3/k_2}{1 + k_3/k_2} \quad (10)$$

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