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Analysis of the roles of amino acid residues in the flavoprotein tryptophan 2-monooxygenase modified by 2-oxo-3-pentynoate: characterization of His338, Cys339, and Cys511 mutant enzymes☆

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Abstract

The flavoprotein tryptophan 2-monooxygenase catalyzes the oxidative decarboxylation of tryptophan to indoleacetamide. His338, Cys339, and Cys511 of the *Pseudomonas savastanoi* enzyme were previously identified as possible active-site residues by modification with 2-oxo-3-pentynoate ([G. Gadda, L.J. Dangott, W.H. Johnson Jr., C.P. Whitman, P.F. Fitzpatrick, Biochemistry 38 (1999) 5822–5828]). The H338N, C339A, and C511S enzymes have been characterized to determine the roles of these residues in catalysis. The steady-state kinetic parameters with both tryptophan and methionine decrease only slightly in the case of the H338N and C339A enzymes; the decrease in activity is greater for the C511S enzyme. Only in the case of the C511S enzyme do deuterium kinetic isotope effects on kinetic parameters indicate a significant change in catalytic rates. The structural bases for the effects of the mutations can be interpreted by identification of L-amino acid oxidase and tryptophan monooxygenase as homologous proteins.

Keywords: Tryptophan monooxygenase; Flavoprotein; Oxidase; Mutagenesis; Isotope effects; L-amino acid oxidase

Tryptophan 2-monooxygenase (TMO)[1] (EC 1.13.12.3) from *Pseudomonas savastanoi* catalyzes the oxidative decarboxylation of tryptophan to indoleacetamide, carbon dioxide, and water (Scheme 1). This reaction is the first of two steps in the bacterial pathway for synthesis of the plant hormone indoleacetic acid [1]. Localized high levels of indoleacetic acid at sites of infection result in the formations of knots or galls on the infected plants [2].

TMO is a member of a family of generally poorly characterized flavoproteins which catalyze the oxidative decarboxylation of amino acids [3,4]. The kinetic mechanism of TMO has been determined with tryptophan as substrate (Scheme 2). In the reductive half-reaction the substrate binds to the enzyme forming the imino acid bound to the reduced flavin. In the oxidative half-reaction, oxygen reacts with the reduced enzyme–imino acid complex to produce indoleacetamide and the oxidized FAD [5]. Kinetic isotope effects have shown that CH bond cleavage is only partially rate limiting with tryptophan as substrate, with product release limiting turnover. Studies of the effects of pH on activity have shown that an amino acid residue in the free enzyme with a pK_a value of 6 must be deprotonated and a second group with a pK_a value of about 10 must be protonated for activity. The temperature dependence of the pK_a value for indoleacetamide binding is consistent with a histidine residue being the ionizable group with the pK_a value of 6 [6].

Chemical modification studies of TMO using several reagents have indicated the presence of histidine and cysteine residues at or near the active site [7]. Recently, Gadda et al. [8] characterized 2-oxo-3-pentynoate as an

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[1] *Abbreviations used:* TMO, tryptophan 2-monooxygenase; LAAO, L-amino acid oxidase; LB, Luria–Bertani.

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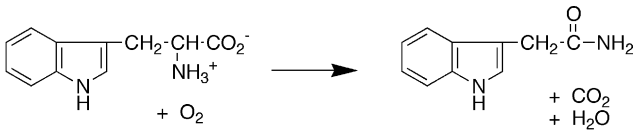
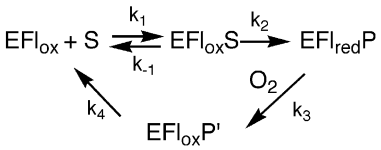


Scheme 1.



Scheme 2.

active-site-directed inactivator of TMO; the sites of modification by 2-oxo-3-pentynoate were identified as the conserved residues Cys339 and Cys511. In these studies, it was also noted that His338 was conserved in all the TMOs described to date. Based on this conservation and the results from kinetic analyses, it was hypothesized that H338 was also in the active site [8]. We have now utilized site-directed mutageneses to investigate the roles of His338, Cys339, and Cys511 in catalysis by TMO. The results of these studies are described in this report.

Experimental procedures

Materials. L-Tryptophan and L-methionine were purchased from USB (Cleveland, OH); indoleacetamide was from Sigma (St. Louis, MO); DEAE–Sephacel and phenyl–Sepharose were from Amersham Pharmacia (Uppsala, Sweden); and *Escherichia coli* M15 (pREP4) was from Qiagen (Valencia, CA).

DNA manipulation. Plasmids containing the genes for the mutant proteins H338N, C339A, and C511A enzymes in the expression plasmid pQE51 (Qiagen) were generous gifts from Dr. A. Spena of the University of Verona. Mutagenesis of Cys511 to serine was performed using the QuikChange protocol (Stratagene). The protein-coding regions for all the plasmids were sequenced to ensure that no unwanted mutations were incorporated during the polymerase chain reaction. Plasmids were transformed into *E. coli* M15.

Protein expression and purification. Wild-type and mutant enzymes were expressed by inoculating six 1.5-L flasks of LB broth containing 100 μg/ml ampicillin and 25 μg/ml kanamycin with 10 ml of an overnight culture. These were incubated at 37 °C until the A_{600} value reached 0.2–0.4. At this point isopropyl β-D-thiogalactopyranoside was added to a final concentration of 500 μM. After an additional 8 h at 30 °C, cells were collected by centrifugation. The cell paste was resuspended in 250 ml 100 mM Tris–HCl, 1 mM EDTA, 50 μM indoleacetamide, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml lysozyme, pH 8.3. The resuspended cells were lysed by sonication for eight cycles of 30 s each. The lysate was centrifuged for 20 min at 20,000*g*, the pellet was discarded, and the supernatant was brought to 0.55% (w/v) with polyethyleneimine, using a 0.1 g/ml stock solution in 100 mM Tris–HCl, pH 8.3. After centrifugation for 20 min at 20,000*g*, solid ammonium sulfate was added to the supernatant to yield 60% saturation. The sample was stirred for 20 min; the precipitate collected by centrifugation for 20 min at 20,000*g* was dissolved in the appropriate buffer for the subsequent column.

The chromatographic steps for purification of the wild-type and mutant C511S enzymes were as described previously [7]. For purification of the C339A and H338N enzymes, the 60% ammonium sulfate pellets were suspended in 20 ml of 50 mM Tris–HCl, 1 mM EDTA, 0.5 mM dithiothreitol, 50 μM indoleacetamide, 10% glycerol, pH 8.3, and dialyzed for 8 h against 2 L of the same buffer with two buffer changes. The sample was centrifuged for 20 min at 20,000*g* and then loaded onto a DEAE–Sephacel column (3 × 15 cm) equilibrated with the same buffer. The enzyme was eluted with a 600-ml gradient from 0 to 300 mM NaCl in the same buffer; the enzyme eluted in the middle of the gradient at ~150 mM NaCl. Fractions containing TMO were pooled, brought to 60% ammonium sulfate saturation, and centrifuged for 20 min at 20,000*g*. The pellet was resuspended in 25 ml of 350 mM ammonium sulfate, 1 mM EDTA, 0.5 mM dithiothreitol, 50 μM indoleacetamide, pH 8.3; clarified by centrifugation; and loaded onto a phenyl–Sepharose column (3 × 15 cm) equilibrated with the same buffer. The enzyme was eluted with a 400-ml gradient of 350–0 mM ammonium sulfate; the enzyme eluted one-third through the gradient. Protein concentrations for the wild-type and mutant enzymes were calculated from the absorbances at 466 nm using an extinction coefficient of 11.4 $mM^{-1}\,cm^{-1}$ [7].

Enzyme assays. Enzyme activity was routinely measured in air-saturated 50 mM Tris–HCl, 1 mM EDTA, 0.5 mM dithiothreitol, pH 8.3, at 25 °C, by monitoring the rate of oxygen consumption in a computer-interfaced Hansatech oxygen monitoring system. Different oxygen concentrations were obtained by bubbling appropriate mixtures of O_2/N_2 into the assay mixture until saturation was reached.

Data analysis. Kinetic data were fit using the programs Kaleidagraph (Adelbeck Software, Reading, PA) and Igor (Wavemetrics, Lake Oswego, OR). Initial rate measurements were fit to the Michaelis–Menten equation. Isotope effects were calculated using Eq. (1), which describes separate isotope effects on the V_{max} and V/K_{aa} values; F_i is the fraction of heavy atom substitution in the substrate, E_v is the isotope effect on V_{max} minus 1, and $E_{V/K}$ is the isotope effect on the V/K_{aa} value minus 1

[9]. Inhibition constants for indoleacetamide were calculated by fitting the data to Eq. (2). This equation depicts the behavior of a competitive inhibitor where K_i is the inhibition constant for that inhibitor

$$v = \frac{VA}{K_M(1 + F_iE_{tk}) + A(1 + F_iE_v)}, \quad (1)$$

$$v = \frac{VA}{K_M(1 + \frac{I}{K_i}) + A}. \quad (2)$$

Results

Protein expression. To probe the roles of His338, Cys339, and Cys511 in catalysis, the H338N, C339A, and C511A enzymes were constructed and expressed in *E. coli*. While both the H338N and the C339A enzymes were readily expressed at levels comparable to the wild-type enzyme, their behavior during purification was different, suggesting changes in the conformation of these enzymes. Specifically, these enzymes did not bind to phenyl–Sepharose in 350 mM potassium phosphate buffer. The purification protocol was modified to account for this difference. Both enzymes would bind to phenyl–Sepharose in the presence of 350 mM ammonium sulfate after prior purification by DEAE–Sephacel chromatography, thereby allowing their purification.

In contrast, it was not possible to obtain pure C511A enzyme in the active form. The enzyme was expressed well and could be purified to homogeneity following the protocol for the C339A and H338N enzymes. However, the resulting protein contained no FAD and was inactive. All attempts to reincorporate the FAD into the mutant were unsuccessful. As an alternative approach, the C511S enzyme was constructed. This enzyme proved to be better behaved and could be expressed and purified following the protocol for the wild-type enzyme. The visible absorbance spectra of the three active mutant proteins were not distinguishable from that of the wild-type enzyme.

Steady-state kinetics. Steady-state kinetic parameters were determined for the mutant enzymes with tryptophan and methionine as the amino acid substrates (Table 1). Neither the H338N nor the C339A mutation has a drastic effect on the kinetic parameters with tryptophan as substrate. The V/K_{trp} value decreases about 7-fold with the H338N enzyme and about 3.5-fold for the C339A enzyme, compared to the wild-type values. The V_{trp} value shows smaller effects. The V/K_{O_2} value decreases 4- and 2-fold for the H338N and the C339A enzymes, respectively. The K_{O_2} values for the H338N and C339A enzymes are similar to the wild-type value. The V_{max} and V/K values for methionine are also very close to the wild-type values.

The steady-state kinetic parameters with both amino acid substrates are altered much more by the C511S substitution (Table 1). The V_{max} value with tryptophan as substrate only decreases about 2.6-fold, but the V/K_{trp} value decreases close to 20-fold. With methionine as substrate, the V_{max} value decreases about 10-fold while the V/K_{met} value remains close to the wild-type value. The effects of this mutation on the oxidative half-reaction are similar to the effects of the C339A and H338N mutations. The V/K_{O_2} value decreases approximately 5-fold and the K_{O_2} value remains close to the wild-type value.

Inhibition by indoleacetamide. In order to examine the effect of the mutations on binding, the inhibition constant for the competitive inhibitor indoleacetamide was determined for all three mutant enzymes (Table 1). The K_i values for the C339A and H338N enzymes are not significantly different from the K_i value for the wild-type enzyme. In the case of the C511S enzyme the K_i value for indoleacetamide increases 4-fold. These results are consistent with the steady-state results with tryptophan as substrate, where only the C511S enzyme exhibits a significant change in the kinetic parameters.

Kinetic isotope effects. Primary deuterium kinetic isotope effects with [2-^{2}H]tryptophan and [2-^{2}H]methionine were measured for all three mutant enzymes

Table 1
Steady-state kinetic parameters of tryptophan monooxygenase mutant proteins

Kinetic parameter	Enzyme			
	Wild-type	H338N	C339A	C511S
V_{trp}, s^{-1}	13.2 ± 0.67	4.0 ± 0.2	10 ± 0.5	5 ± 0.2
K_{trp}, mM	0.036 ± 0.004	0.083 ± 0.016	0.097 ± 0.015	0.25 ± 0.05
V/K_{trp}, $mM^{-1}\,s^{-1}$	360 ± 37	50 ± 8	105 ± 13	19 ± 2
$K_{O_2(trp)}$, mM	0.09 ± 0.01	0.11 ± 0.03	0.15 ± 0.08	0.28 ± 0.17
$V/K_{O_2(trp)}$, $mM^{-1}\,s^{-1}$	140 ± 18	33 ± 6	65 ± 28	30 ± 13
V_{met}, s^{-1}	5.6 ± 0.23	4.8 ± 0.2	$6.1 \pm .42$	0.70 ± 0.01
K_{met}, mM	22.4 ± 1.4	11 ± 1	14 ± 3	2.0 ± 0.16
V/K_{met}, $mM^{-1}\,s^{-1}$	0.25 ± 0.012	0.44 ± 0.06	0.42 ± 0.06	0.33 ± 0.02
$K_{indoleacetamide}$, μM	16 ± 0.8	13 ± 3	28 ± 6	68 ± 8

Note. Conditions: 50 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol, pH 8.3, 25 °C.

Table 2
Primary deuterium isotope effects for tryptophan monooxygenase mutant proteins

Kinetic parameter	Wild-type	H338N	C339A	C511S
$^{D}V_{trp}$	1.22 ± 0.07	1.11 ± 0.07	1.05 ± 0.05	1.23 ± 0.06
$^{D}(V/K_{trp})$	1.16 ± 0.25	1.26 ± 0.15	1.20 ± 0.08	1.92 ± 0.22
$^{D}V_{met}$	2.43 ± 0.15	2.50 ± 0.25	2.62 ± 0.05	2.90 ± 0.06
$^{D}(V/K_{met})$	1.8 ± 0.3	2.20 ± 0.4	2.33 ± 0.08	2.70 ± 0.30

Note. Conditions: 50 mM Tris, 1 mM EDTA, 0.5 mM dithiothreitol, pH 8.3, 25 °C.

(Table 2). For the wild-type enzyme, both the $^{D}V_{trp}$ value and the $^{D}(V/K_{trp})$ value are small, consistent with C–H bond cleavage being only partially rate limiting with this substrate due to its high commitment to catalysis and rate-limiting product release [6]. With the H338N and C339A enzymes, the $^{D}V_{trp}$ and $^{D}(V/K_{trp})$ values are very close to those for the wild-type enzyme. These results indicate that the relatives rates of chemical steps are not greatly affected by these mutations. With the C511S enzyme the $^{D}V_{trp}$ value is not significantly different from the wild-type value, while the $^{D}(V/K_{trp})$ value increases. In the case of the C511S mutant, the results suggest that substitution of this residue by serine makes chemical steps more rate limiting.

With methionine as substrate for the wild-type enzyme, C–H bond cleavage is more rate limiting [6]. For the H338N and C339A enzymes, the values of the isotope effects with methionine are very close to the values for the wild-type enzyme. In contrast, both the ^{D}V and the $^{D}(V/K_{met})$ values are higher for the C511S enzyme (Table 2).

Discussion

Tryptophan monooxygenase is one of several flavoenzymes which catalyze the oxidative deamination of α-amino acids to form amides. Others are lysine monooxygenase [3,10] and phenylalanine oxidase [4]. Unlike the case with these latter two enzymes, the catalytic mechanism of TMO has been analyzed by a range of approaches, so that a detailed kinetic mechanism of the enzyme is available [5,6]. Several active site-directed reagents have been used to identify catalytically important residues. Both thiol- and imidazole-directed reagents inactivate the enzyme in a manner consistent with modification of active site residues, suggesting that cysteinyl and histidinyl residues play critical roles [7]. Cys338 and Cys511 were recently identified as residues modified when TMO is inactivated by 2-oxo-3-pentynoate [8]. Both modification and inactivation are blocked by indoleacetamide, a reversible inhibitor [8], suggesting that these residues are in the active site. Consistent with an important role of these two residues, they are conserved in the several TMO sequences which are available. The adjacent residue His338 is also conserved; its proximity to a residue modified by 2-oxo-3-pentynoate and the inactivation of TMO seen upon treatment with diethylpyrocarbonate [7] suggested that this residue may also be in the active site [8].

The results described here rule out a critical catalytic role for either Cys339 or His338, but do suggest that Cys511 may play an important role. In the case of the C339A enzyme, there are only small changes in the kinetic parameters. The V_{max} value and the V/K values for both substrates decrease 2- to 3-fold. With methionine as substrate there is no significant change in any kinetic parameter for the C339A enzyme. There is also no change in any of the isotope effects with either substrate, consistent with chemical and other steps being decreased by comparable amounts. These results are most consistent with a subtle change in the overall structure of the protein which results in a slightly less active conformation. The lack of an effect on the parameters with methionine as substrate suggests that the conformational change results in a restricted active site which binds indolic compounds less well, but still can bind smaller substrates effectively.

Mutagenesis of His338 to asparagine has a somewhat larger effect, but qualitatively the effect of the mutation is the same as that of the C339A mutation. The decreases in the V/K and V_{max} values with tryptophan are about twice those seen upon mutation of the adjacent residue, but those with methionine are unchanged. In addition, the isotope effects with this enzyme are very close to those seen with both the wild-type and the C339A enzymes. Thus, His338 is likely to be involved in maintaining an active conformation but is not directly involved in catalysis or binding. These results resemble the effects of mutating the conserved histidine in D-amino acid oxidase. This residue, His307 in the pig enzyme, forms an ion pair with an aspartate residue on the surface of the protein near a loop which closes over the active site when substrate is bound [11]. Mutagenesis of this residue to serine decreases the rates of all steady-state kinetic parameters by about 5-fold [12].

Only in the case of C511S TMO do the kinetic parameters with both methionine and tryptophan decrease significantly. Cys511 is clearly important for maintaining the protein structure, since the C511A enzyme proved too unstable to survive purification. Still, the very conservative replacement of the cysteine with serine provides a stable enzyme, so that a thiol at this position is not essential. The kinetic parameters given in Tables 1 and 2 can be used to determine the effect of the C511S mutation on the intrinsic rate constants of Scheme 4. The relationships between the intrinsic rate constants and the steady-state kinetic parameters are given by Eqs. (3)–(7) [13]. Here, $^{D}k_2$ is the intrinsic deuterium kinetic isotope effect on the rate constant for the chemical step, k_2. This has previously been determined with tryptophan

and methionine as substrates for wild-type tryptophan monooxygenase to be 2.4 and 5.3, respectively [5,6]. If it is assumed that the intrinsic isotope effects for the C511S enzyme are unchanged from these values, Eqs. (3)–(7) describe the five intrinsic rate constants in terms of five measurable steady-state kinetic parameters. Table 3 gives the values of $k_1 - k_4$ calculated from the data of Tables 1 and 2 using the following equations:

$$\left(\frac{V}{K}\right)_{aa} = \frac{k_1 k_2}{k_{-1} + k_2}, \tag{3}$$

$$\left(\frac{V}{K}\right)_{O_2} = k_3, \tag{4}$$

$$V_{max} = \frac{k_2 k_4}{k_2 + k_4}, \tag{5}$$

$${}^{D}\left(\frac{V}{K}\right)_{aa} = \frac{{}^{D}k_2 + \frac{k_2}{k_{-1}}}{1 + \frac{k_2}{k_{-1}}}, \tag{6}$$

$${}^{D}V_{max} = \frac{{}^{D}k_2 + \frac{k_2}{k_4}}{1 + \frac{k_2}{k_4}}. \tag{7}$$

With tryptophan as substrate, the values of k_1 and k_{-1} for the wild-type enzyme cannot be determined with any reasonable degree of precision. This is due to the large value of k_2/k_{-1}, which is between 7 and 15 [6]. As a result the isotope effect on the V/K_{trp} value is too close to one to use Eq. (6) in the analysis of the data. Even with that caveat the data of Table 3 indicate that binding of tryptophan is affected, with the rate of association, k_1, decreasing 5-fold or more. The rate of dissociation, k_{-1}, may in fact be increased by the mutation, but the precision of the data and the uncertainty in the value of k_{-1} for the wild-type enzyme make this less than certain. The severalfold increase in the K_d value for indoleacetamide is consistent with the association rate constant decreasing more than the dissociation rate constant for that compound. This decrease in affinity for the amino acid is not seen with methionine as substrate, suggesting that it is the binding of the indole ring of tryptophan which is altered. Indeed, there is a decrease in the K_d value for methionine in the C511S enzyme. The effects on the rate of the chemical step are not significantly different with the two amino acid substrates, with an average decrease of about 6-fold in k_2. The rate constants for steps in the oxidative half reaction, k_3 and k_4, also decrease 5-fold on average with both substrates in the C511S enzyme. Thus, the changes in the steady-state kinetic parameters seen in the C511S enzyme are due to changes in a number of kinetic parameters. In no case does a single rate constant decrease by more than an order of magnitude, establishing that the thiol of Cys511 is not involved in catalytic steps as either an acid/base catalyst or a nucleophile. A reasonable conclusion from these data is that Cys511 is required to maintain the proper structure of the active site, but is not involved in the actual chemistry.

Interpretation of the effects of these mutations and identification of active-site residues of TMO is clearly made more difficult by the absence of direct information on the three-dimensional structure of the protein. To identify possible homologous proteins with known structure to aid in this analysis, a PSI-BLAST [14] search was made using the *P. savastanoi* TMO sequence. This identified the flavoenzyme L-amino acid oxidase as the most closely related enzyme, with *e* values of 10^{-8} to 10^{-12}. The LAAO with the most significant score is that from *Calloselasma rhodostoma*. This LAAO and *P. savastanoi* TMO are 24% identical and 40% similar, well above the levels expected solely from random chance. The similarity of the reactions catalyzed by TMO and LAAO supports the identification of these enzymes as homologous. The reductive half-reactions of LAAO and TMO are the same, the oxidation of an L-amino acid with transfer of a hydride equivalent to the FAD [15]. The enzymes differ in the oxidative half-reactions, in that LAAO does not catalyze a subsequent oxidative decarboxylation to form an amide. A sequence alignment of LAAO and TMO is shown in Fig. 1. Cys339, His338, and Cys511 are not conserved between the two enzymes, suggesting that they are not required for the common catalytic reactions of the two enzymes.

Table 3
Calculated intrinsic rate constants for C511S tryptophan monooxygenase

Kinetic parameter	Tryptophan			Methionine		
	Wild-type enzyme	C511S	$\Delta\Delta G^{\ddagger}$	Wild-type enzyme	C511S	$\Delta\Delta G^{\ddagger}$
c_f	(~8)[a]	0.52 ± 0.25		64.4 ± 1.7	1.53 ± 0.32	
c_{vf}	5.4 ± 1.7	5.1 ± 1.4		62.0 ± 0.24	1.26 ± 0.05	
k_1, $mM^{-1}\,s^{-1}$	(~400)[a]	55 ± 26	1.2 ± 0.3	0.31 ± 0.15	0.54 ± 0.14	-0.33 ± 0.37
k_{-1}, s^{-1}	(~17)[a]	58 ± 38	-0.7 ± 0.5	63.8 ± 1.6	1.04 ± 0.36	0.77 ± 0.36
k_2, s^{-1}	84 ± 44	30 ± 13	0.61 ± 0.49	17 ± 3.2	1.59 ± 0.43	1.4 ± 0.2
	(139 ± 4)[a]		0.91 ± 0.28			
k_3, $mM^{-1}\,s^{-1}$	140 ± 18	30 ± 13	0.92 ± 0.29			
k_4, s^{-1}	16 ± 6.6	6.0 ± 2.1	0.58 ± 0.36	8.4 ± 1.2	1.3 ± 0.34	1.1 ± 0.2

[a] Values from Refs. [5] and [6].

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TMO     1                                    MYDHFNSPSIDILYDYGPFLKKCEMTGGIGSYSAG      35
LAAO  -18                          MNVFFMFSLLFLAALGSCADDRNPLAECFQENDYEEFLEIARNGLKA    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----CAVLRSTGGQLSKGNPL      548
LAAO  445  LTASQGR----IYFAGEYTAQAHGWIDSTIKSGLRAARDVNLASENPSGIHLSNDNEL      498
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Fig. 1. Alignment of *P. savastanoi* tryptophan monooxygenase and *C. rhodostoma* L-amino acid oxidase. The alignment was generated with the program PSI-BLAST, using the TMO sequence as the probe. Residues conserved in both TMO and LAAO are in bold. The asterisks indicate His338, Cys339, and Cys511 of TMO.

The crystal structure of *C. rhodostoma* LAAO has been solved at 2.0 Å resolution [16]. Surprisingly, LAAO has the same fold as monoamine oxidase B [17]. When the program 3D-PSSM [18] is used to thread the sequence of TMO onto proteins of known structure, a highly significant match is obtained with the structure of monoamine oxidase B and an only slightly less significant match with LAAO. [2] The differences in the structural models of TMO built using MAO or LAAO are mostly in the position of surface loops. The FAD binding domain of LAAO consists of three discontinuous regions which correspond to residues 41–69, 276–346, and 496–536 in TMO and thus includes the three residues analyzed here. Fig. 2A shows the locations of His338, Cys339, and Cys511 of TMO in a structure modeled on LAAO. His338 and Cys339 are on a loop on the surface of the protein, adjacent to the adenosine portion of the FAD. Binding of indoleacetamide to the wild-type enzyme results in changes in the FAD spectrum [7], suggesting that there is a conformational change in the region of the protein surrounding the flavin when the inhibitor binds. The decreased reactivity of Cys338 with 2-oxo-3-pentynoate when indoleacetamide is bound [8] can be attributed to propagation of the conformational change upon inhibitor binding to the loop containing this residue, located as it is near the FAD. Conversely, mutagenesis of either His338 or Cys339 could have subtle effects on the interactions of the protein with the FAD, leading to the small and generalized changes in the kinetic parameters described here. The differences in behavior during chromatography of these proteins are also consistent with subtle changes in the protein surface.

TMO Cys511 corresponds to Glu457 in LAAO (Fig. 1). A carboxylate oxygen of this residue forms a hydrogen bond with a ribitol hydroxyl of the FAD in the latter enzyme [16]. Modeling shows that the thiol of a cysteine in the same position would be within hydrogen bonding distance (2.7 Å) of the same flavin hydroxyl[2] (Fig. 2B). LAAO Glu457 is on one end of a loop which ends with Gly464, Trp465, and Ile466. The first two of these residues form hydrogen bonds with the FAD, at N(1) and the C(2) oxygen of the isoalloxazine ring, respectively. Gly464 and Trp465 also form part of the binding site for the amino acid substrate. These three residues correspond to Gly500, Trp501, and Ile502 in TMO and are also conserved in that enzyme (Fig. 1). The conservation of these residues in both TMO and LAAO suggests that they play similar roles in both enzymes. We attribute the properties of the C511S protein to a distortion of the interactions of the protein with the FAD and a loss of flexibility of the active site due to the propinquity of Cys/Glu457 to residues critical for amino acid binding. Replacement of this residue with alanine would be expected to weaken flavin binding, consistent with the formation of inactive apoenzyme observed in attempts to purify C511A TMO. The more conservative C511S mutation would be able to form the hydrogen bond to the flavin, but the much stronger interaction or

[2] P. Sobrado and P. F. Fitzpatrick, unpublished observations.

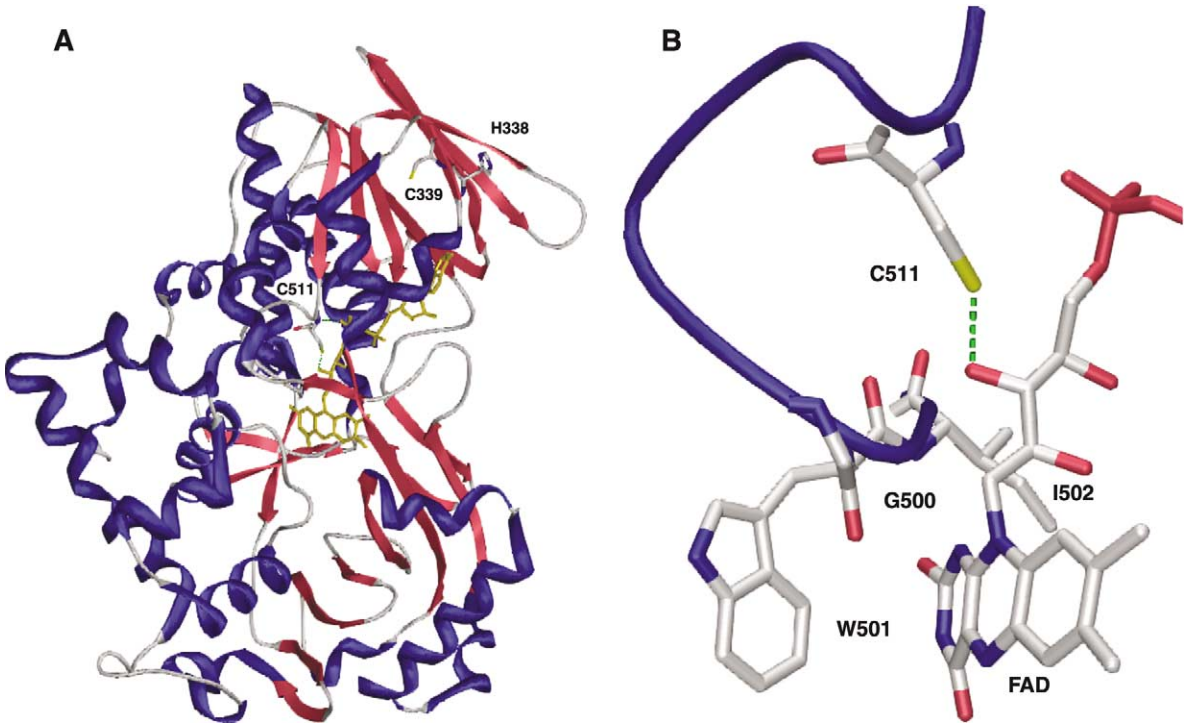

Fig. 2. Modeling of tryptophan monooxygenase using the L-amino acid oxidase structure. (A) Location of His338, Cys339, and Cys511. (B) Proposed interaction between Cys511 and the FAD in tryptophan monooxygenase. The structures were drawn using the program SwisPDBViewer, the PDB file 1F8S, and the alignment of Fig. 1.

oxygen relative to sulfur could alter the dynamics of this region of the protein. Given the propinquity of Cys511/Glu457 to these three conserved active-site residues, a change in dynamics or structure of the peptide backbone around Cys511 could be reflected in the binding site for substrates. The effects of the mutation on the individual catalytic steps are consistent with such a model.

In conclusion, characterization of the effects of mutagenesis of His338, Cys339, and Cys511 rules out critical catalytic roles such as acid/base catalysis for these residues. All three mutations result in changes in the rates of multiple steps in catalysis and binding, with the C511S enzyme showing the greatest change. The structural effects of the mutations can be interpreted upon identification of tryptophan monooxygenase and L-amino acid oxidase as homologous proteins.

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