The environments of Trp-248 and Trp-330 in tryptophan indole-lyase from *Escherichia coli*

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The two tryptophan residues, Trp-248 and Trp-330, in tryptophan indole-lyase (tryptophanase) from *E. coli* have been separately mutated to phenylalanine using site-directed mutagenesis. Both single tryptophan mutant enzymes have full catalytic activity, but exhibit different fluorescence and near-UV circular dichroism spectra. These results indicate that Trp-330 is more deeply buried than is Trp-248, and is in a more asymmetric environment. Neither residue reacts with *N*-bromosuccinimide (NBS), although tryptophan indole-lyase is inactivated by NBS. These results demonstrate that the tryptophan residues in tryptophan indole-lyase are not catalytically essential.

Tryptophan indole-lyase, *E. coli*; Tryptophanase; Site-directed mutagenesis; Tryptophan; Fluorescence; Circular dichroism

1. INTRODUCTION

Tryptophan indole-lyase (tryptophanase) (EC 4.1.99.1) is one of the best-studied pyridoxal-5′-phosphate (PLP) dependent enzymes. The amino acid sequence of the enzyme from *E. coli* has been elucidated both by protein chemistry techniques [1] and by nucleotide sequencing of the cloned *tna*A gene [2,3]. Chemical modification experiments have provided evidence for the participation of a wide range of amino acid residues in PLP binding or in catalysis. These residues include cysteine [4–7], histidine [8], arginine [9], tyrosine [10], methionine [11], and tryptophan [12,13]. We have recently used site-directed mutagenesis of Cys-294 and Cys-298 to confirm that Cys-298 is the reactive sulfhydryl of tryptophan indole-lyase, and is probably located near or in the active site [14]. We have now employed site-directed mutagensis to study the role of the tryptophan residues, Trp-248 and Trp-330, in the structure and catalytic activity of the enzyme. Our results demonstrate that the two residues are not catalytically essential and that they reside in quite distinct environments.

2. EXPERIMENTAL

2.1. *Chemicals*

L-Tryptophan was obtained from US Biochemical Corp., and was recrystallized from water before use. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) was prepared as previously described [15]. Pyridoxal-5′-phosphate (PLP), NADH, and lactate dehydrogenase from rabbit muscle were also obtained from US Biochemical Corp.

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2.2. *Preparation of mutant enzymes*

Site-directed mutagenesis was performed as previously described [14], using the appropriate mutagenic oligonucleotides to replace the desired tryptophan with phenylalanine. The resultant clones were subjected to dideoxy sequencing [16] to establish the presence of the desired mutation. The resultant plasmids were transformed into *E. coli* SVS 370, which contains Tn5 in *tna*A, to ensure that no wild-type enzyme is produced. The cells were grown and the enzymes purified as previously described [14], except that the Sepharose CL-4B step was operated in a column rather than in batch mode. Apoenzymes were prepared by incubation with Tris and cysteine as previously described [14]. Concentrations of wild-type and the mutant enzymes were determined using the Bradford reagent [17] (Pierce Chemical Co.), with wild-type apotryptophan indole-lyase as standard ($A_{278}^{1\%}$ = 7.95 [18]). Tryptophan indole-lyase activity was assayed and kinetic parameters were determined as previously described [14].

2.3. *Inactivation by* N-*bromosuccinimide*

Solutions of the apoenzymes (0.4 mg/ml) were incubated for 5 min with and without 1 mM NBS (recrystallized from water) in 100 μl of 0.1 M potassium phosphate, pH 7.0. 50 μl each of 0.1 M 2-mercaptoethanol and 2 mM PLP were then added, and the solutions were incubated for 1 h at 37°C. After dilution to 1 ml with 0.1 M potassium phosphate, pH 8, the remaining activity was measured with SOPC.

2.4. *Instrumentation*

Enzyme assays were performed using a Gilford Response II equipped with a Peltier-type thermoelectric cell block. UV spectra were obtained on the Gilford Response II, and visible spectra were obtained on either the Gilford Response II or a Cary 14 connected to a Zenith Z-158 computer (On-Line Instruments). Fluorescence spectra were obtained on a Perkin-Elmer MPF-44 spectrofluorimeter. Excitation was at 285 nm, with 10 nm slits for excitation and 5 nm emission slits. Circular dichroism was measured with a Jasco J500 equipped with a DP-501 data processor.

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3. RESULTS AND DISCUSSION

3.1. *Activity of mutant enzymes*

Tryptophan residues in proteins have often been found to have important roles in the maintenance of structure and/or catalysis. In the X-ray crystallographic structure of aspartate aminotransferase, Trp-140 forms an essential element of the PLP binding site [19,20]. It was previously reported that ozonolysis of one or both of the tryptophan residues of tryptophan indole-lyase results in loss of catalytic activity [12,13]. Replacement of either of the tryptophan residues in tryptophan indole-lyase with phenylalanine results in fully active enzymes (Table I), with k_{cat} and k_{cat}/K_m values comparable to those of the wild-type enzyme. In addition, pH-rate profiles for the mutant and wild-type enzymes are identical (data not shown). These results suggest that these mutations result in minimal structural perturbations of the enzyme. The results of this study clearly demonstrate that neither Trp-330 nor Trp-248 is catalytically essential, in contrast to conclusions drawn from the ozonolysis experiments [12,13].

3.2. *UV and visible spectra*

The intensity of the 278 nm peak is significantly reduced for both the W330F and W248F mutant enzymes, as expected for the removal of a tryptophan residue (Table II). The visible spectra of the three enzymes, due to the PLP chromophore, are all very similar, with λ_{max}s of 420 nm and 335 nm (Table II). However, the spectrum of the W330F enzyme exhibits a shift to about 400 nm upon storage at 4°C, which is reversible by incubation at 37°C. Wild-type tryptophan indole-lyase binds a quasi-substrate, L-alanine, and forms an intense absorption peak at 502 nm, assigned to the quinonoid α-carbanion [21]. This peak forms rapidly, and the absorbance change is complete within several seconds [14,22]. However, when solutions of the W330F enzyme that have been stored at 4°C are added to solutions of L-alanine, the formation of the 502 nm peak is extremely slow, requiring longer than 20 min to reach the maximum intensity. In this respect, the W330F mutant enzyme resembles the C298S mutant form of tryptophan indole-lyase [14]. After preincubation at 37°C for 30 min, the W330F mutant enzyme reacts rapidly with L-alanine, forming a strong peak at 502 nm. The cold-adapted W330F enzyme also exhibits a lag and slightly lower initial activity (~70%) when assayed with SOPC. In contrast, the reactions of the W248F enzyme and the wild-type enzyme are not significantly affected by storage at 4°C.

Table I

Kinetic parameters for wild-type and Trp mutants of tryptophan indole-lyase

Enzyme	SOPC		L-Trp		PLP K_d (μM)
	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}\cdot s^{-1}$)	k_{cat} (s^{-1})	
Wild-type	5.1×10^5	44	3.0×10^4	6.8	0.53 ± 0.17
W330F	1.2×10^6	46	4.3×10^4	6.7	1.43 ± 0.22
W248F	8.7×10^5	40	3.0×10^4	5.4	1.32 ± 0.18

Table II

Spectroscopic properties of tryptophan indole-lyase and mutants

Enzyme	$A_{278}^{1\%}$	ϵ_{278}[a]	$\Delta\epsilon_{278}$[b]	ϵ_{420}	$\Delta\epsilon_{420}$[c] (CD)
Wild-type	9.19[d]	4.8×10^4	–	4.5×10^3	+10.6
W330F	7.64	4.0×10^4	8×10^3	4.3×10^3	+9.5
W248F	7.61	4.0×10^4	8×10^3	4.0×10^3	+10.0

[a] Based on an approximate subunit molecular weight of 5.2×10^4
[b] Difference between the wild-type and the respective mutants
[c] From the circular dichroism spectra
[d] From [14]

3.3. *Pyrodixal-5'-phosphate titrations*

Tryptophan indole-lyase which has been treated with ozone to convert the tryptophan residues to N-formylkynurenine was reported to exhibit weaker binding of PLP [12]. We performed titrations of all three apoenzymes by measuring the absorbance change at 430 nm, as described by Kazarinoff and Snell [23]. The wild-type enzyme binds PLP with a K_d value of about 0.5 μM at pH 7.0 and 37°C, in good agreement with the value of 0.6 μM reported by Kazarinoff and Snell at pH 8.0 and 25°C [23] (Table I). The W330F and W248F mutant enzymes exhibit slightly higher K_d values of about 1.3 μM (Table I). Thus, the replacement of either of the tryptophan residues with phenylalanine does not significantly affect the binding of PLP to the enzyme, while ozonolysis increases the K_d for PLP to 18.6 μM [12]. These results suggest that the decreased binding of PLP to ozonized enzyme may be due to reaction of other residues. The blue shift in the PLP absorption maximum for the W330F mutant upon incubation at 4°C indicates that the Trp-330 can influence the PLP binding site, even though the affinity for PLP in the mutant is only slightly affected. Incubation at 4°C does not appear to affect the quaternary structure of the enzyme, since HPLC gel filtration did not show any difference in the elution pattern of enzyme at 4°C and after incubation at 37°C.

3.4. *Fluorescence spectra*

The wild-type enzyme exhibits a typical protein fluorescence emission at 335 nm, arising from the tryptophan residues, and a weaker emission at about 500 nm, from the PLP [4]. The emission spectra of the W248F and W330F mutant forms were examined, for comparison with the wild-type enzyme (Fig. 1). Both mutant enzymes exhibit lower intensity than the wild-

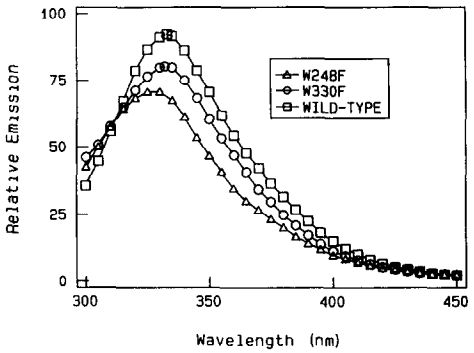

Fig. 1. Fluorescence spectra of tryptophan indole-lyase and mutant forms. The solutions contained 50 μg/ml protein in 0.1 M potassium phosphate buffer, pH 8.0. The spectra have been corrected for the solvent Raman band by subtraction of the solvent baseline.

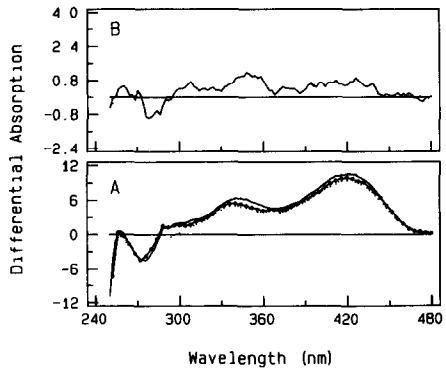

Fig. 2. (A) Circular dichroism spectra of tryptophan indole-lyase and mutant forms. Wild-type (——); W248F (○—○); W330F (···). The spectra were obtained at 1 mg/ml protein in 0.1 M potassium phosphate, pH 8.0. Eight spectra were accumulated and averaged for each sample. The spectra are plotted in units of $\Delta\epsilon$, $M^{-1} \cdot cm^{-1}$. (B) Difference spectra obtained by subtraction of the spectra of the mutant enzymes from the wild-type enzyme. (Solid line) Wild-type-W248F; (dotted line) wild-type-W330F.

type enzyme; however, the peak for the W248F form is blue-shifted to 325 nm. This observation suggests that Trp-330 is deeply buried in a non-polar environment [24], and that the major contribution to the emission of the wild-type enzyme is from Trp-248. Both mutant enzymes exhibit emission peaks at about 500 nm which are not significantly different than the wild-type enzyme.

3.5. *Circular dichroism*

Tryptophan indole-lyase exhibits positive CD bands at 420 nm and 337 nm due to the PLP bound to the enzyme [25]. The CD spectra of the W330F and W248F mutant forms are very similar to that of the wild-type enzyme in this region, with slight blue shifts observed in the 337 nm band (Fig. 2A). In contrast, the W330F mutant enzyme exhibits a dramatic difference in the 280 nm region, with a marked increase in the intensity of the negative band arising from the aromatic amino acid side chains (Fig. 2A). The difference CD spectra are presented in Fig. 2B. There is a strong positive band at 280 nm in the difference spectrum generated by subtracting the W330F enzyme spectrum from that of the wild-type enzyme. Thus, Trp-330 must be rigidly held in a strongly asymmetric environment. This result, together with fluorescence data, indicates that Trp-330 is deeply buried in the protein interior. In contrast, the difference spectrum generated from the spectrum of the W248 enzyme exhibits only a weak negative band at 280 nm (Fig. 2B).

3.6. *Reaction with* N-*bromosuccinimide*

N-Bromosuccinimide (NBS) reacts readily with exposed tryptophan residues in proteins [26,27]. The wild-type, W330F, and W248F apoenzymes are nearly completely inactivated (1% or less residual activity) with 1 mM NBS. This result implies that the inactivation by NBS must be unrelated to modification of tryptophan. This conclusion is consistent with a lack of absorbance change at 280 nm upon incubation of wild-type apoenzyme with NBS (data not shown). Thus, the tryptophan residues in tryptophan indole-lyase must not be readily accessible to NBS, and the inactivation by NBS may be related to that recently reported with chloramine-T, which modifies a methionine residue [11], or to oxidation of cysteine SH groups.

4. CONCLUSIONS

Our results demonstrate that neither Trp-248 nor Trp-330 is catalytically essential for tryptophan indole-lyase from *E. coli*, in contrast to conclusions from previous ozonolysis experiments. These results demonstrate the advantage of site-directed mutagenesis over chemical modification for the study of functional residues in enzymes. Our results have demonstrated that the two tryptophan residues reside in quite different environments, and that Trp-330 influences the spectrum of the PLP cofactor and the ability of the enzyme to react with an amino acid quasi-substrate, L-alanine. These results suggest that replacement of Trp-330 with phenylalanine may have subtle effects on the conformation of tryptophan indole-lyase.

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