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The Mechanism of *Escherichia coli* Tryptophan Indole-Lyase: Substituent Effects on Steady-State and Pre-Steady-State Kinetic Parameters for Aryl-Substituted Tryptophan Derivatives

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Abstract—We have examined the reaction of *Escherichia coli* tryptophan indole-lyase with fluoro, chloro, methyl and hydroxytryptophans using steady-state kinetics, rapid-scanning and single wavelength stopped-flow spectrophotometry, and rapid chemical quench methods. All of the 16 tryptophan derivatives examined are substrates for α,β-elimination catalyzed by tryptophan indole-lyase. The steady-state kinetic parameter, k_{cat}/K_m, did not show a consistent trend with the steric bulk of the substituent, but K_m increased for larger substituents. Rapid-scanning stopped-flow spectra show that all tryptophan analogues undergo covalent reaction with the pyridoxal-5'-phosphate cofactor to give equilibrating mixtures of external aldimine and quinonoid intermediates, but the relative amounts of each intermediate are strongly dependent on the nature and position of the substituent. The dissociation constants for external aldimine formation, K_d, obtained from single-wavelength stopped-flow experiments decreased for most substituted tryptophans, which suggests that part of the binding energy is derived from hydrophobic interactions between the enzyme and the indole ring of tryptophan. In contrast, the rate constants of quinonoid intermediate formation and reprotonation and of indole elimination were quite variable, depending on the position and the nature of the substituent. Overall, 6-substituted tryptophans have the most consistent reactivity, which indicates that there may be space in the enzyme active site near the 6-position. There is a good linear correlation between log (k_{cat}/K_m) and log (k_f/K_d) (apparent second order rate constant for quinonoid intermediate formation), with a slope of 0.66. This suggests that quinonoid intermediate formation contributes only about 66% of the activation energy for the reaction, and thus a later step in the reaction must be partially rate-limiting. Rapid chemical quench experiments demonstrate a 'burst' of indole in the reaction of L-tryptophan under single turnover conditions, confirming that a step subsequent to the elimination is partially rate-determining. In contrast, 5-methyl-L-tryptophan does not exhibit a significant 'burst', suggesting that 5-methylindole elimination is nearly completely rate-determining. These results support the proposed mechanism and demonstrate that there are significant effects of aryl substituents on the distribution of covalent intermediates and on the rate-determining step in the α,β-elimination reaction catalyzed by *E coli* tryptophan indole-lyase.

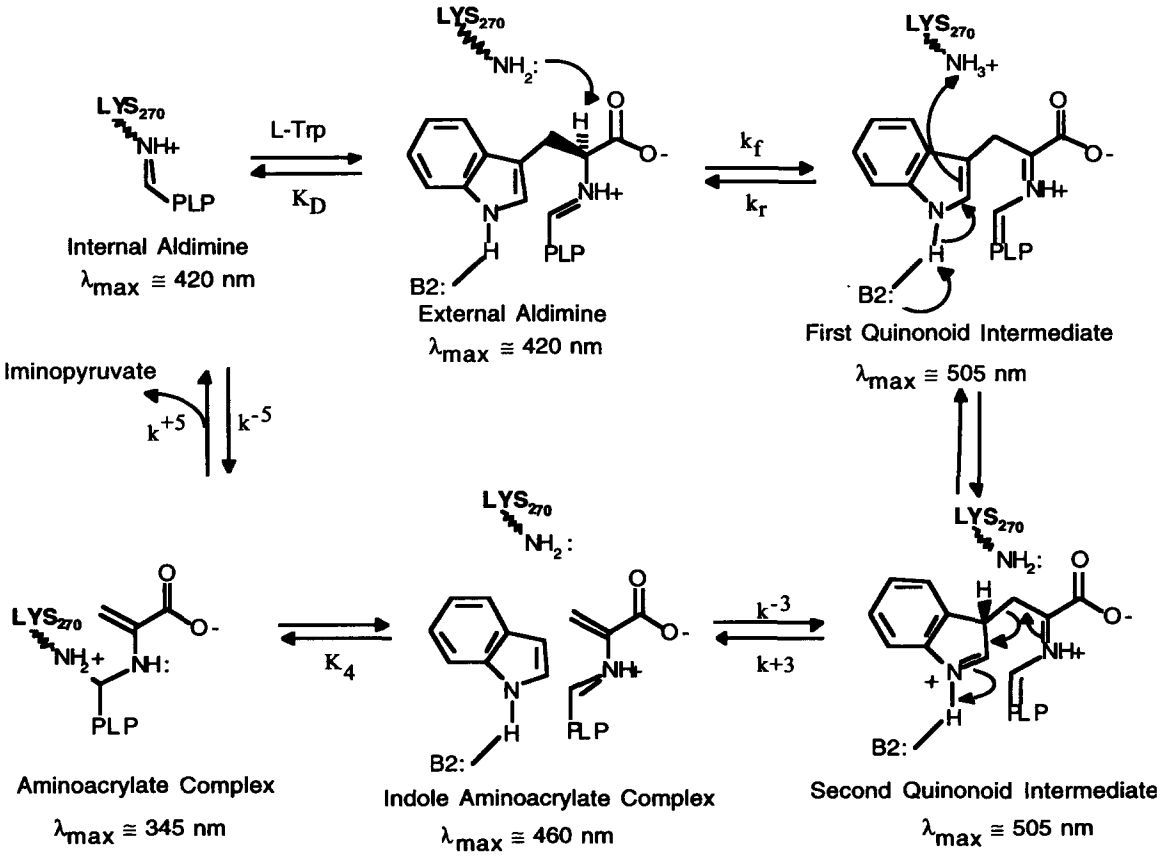
Introduction

Tryptophan indole-lyase (tryptophanase) [EC 4.1.99.1] is a pyridoxal-5'-phosphate (PLP) dependent enzyme which catalyzes the hydrolytic cleavage of L-tryptophan (1) to indole and ammonium pyruvate (Equation 1).

$$\text{L-Tryptophan} + H_2O \Leftrightarrow \text{Indole} + CH_3COCO_2^- + NH_4^+ \quad (1)$$

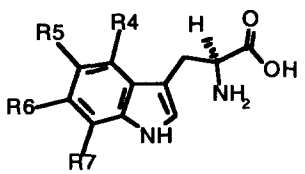
This enzyme also catalyzes α,β-elimination reactions, β-replacement reactions, and α-hydrogen exchange reactions *in vitro* with a variety of L-amino acids, including substituted tryptophans, serine, cysteine, S-alkylcysteines and S-arylcysteines, and other amino acids carrying appropriate leaving groups in the β-position.[1–3] The enzymatic α,β-elimination of a carbon leaving group is mechanistically interesting, since the indole ring has been proposed to tautomerize as shown in Scheme 1 to activate the ring for the β-elimination reaction. [4–6] In support of this theory, we have previously demonstrated that analogues of the postulated indolenine intermediate are potent and stereospecific inhibitors of tryptophan indole-lyase as well as the β-reaction of tryptophan synthase.[5,6]

We have studied the steady-state[7] and pre-steady-state kinetics[8] of the reaction of *Escherichia coli* tryptophan indole-lyase with L-tryptophan and other substrates. These results demonstrated that a basic group with pK_a of 7.6 (Scheme 1, probably Lys-270, which binds the PLP) is required for formation of the quinonoid intermediate. A second catalytic base ($\mathbf{B_2}$ in Scheme 1) with a pK_a of 6.0, interacts with the indole NH.[7] We have also used rapid-scanning stopped-flow spectrophotometry to study the mechanism of substrate analogue binding to tryptophan indole-lyase, demonstrating that a heterocyclic NH is critical for rapid formation of aldimine, *gem*-diamine, and quinonoid intermediates.[9] The same technique has been used to study the reaction of indole and analogues with amino acid complexes of the enzyme.[10] These results



Scheme 1. Proposed mechanism for tryptophan indole-lyase.

demonstrated the first direct measurement of the leaving group elimination from the quinonoid intermediates in the reaction of substrates with the enzyme, and provided a foundation for further studies directed towards the effects of variation in substrate structure on the elimination step. In order to extend our understanding of tryptophan indole-lyase, we have now systematically examined the reactivity of a series of tryptophan derivatives substituted in the benzenoid ring at positions 4, 5, 6 and 7 (Scheme 2), using both steady-state and pre-steady-state kinetic measurements. Previous studies[5,6,11–15] indicated a broad specificity for substituted tryptophans, but were limited in scope or utilized only steady-state measurements. Our results demonstrate that the structure of the substituent has significant effects on both the binding of tryptophan derivatives and the rates of intermediate formation in the reaction of tryptophan indole-lyase. Moreover, these data suggest that the rate-limiting step of the reaction may change for substituted tryptophans.



Scheme 2. Position of substitution of tryptophan derivatives.

Results

Steady-state kinetics

Since the studies by Hall *et al.* established that tryptophan indole-lyase acted on substituted tryptophans as well as tryptophan itself,[11] there have been a number of reports of the reaction of the enzyme with tryptophan derivatives.[5,6,8,9,12–15] However, most of the previous work, including our own, used a small number of tryptophan analogues. In the present work, all 16 tryptophan derivatives tested were found to be substrates for tryptophan indole-lyase, as summarized in Figure 1 and Table 1. Fluoro-L-tryptophans are the best substrates, followed by chloro-L-tryptophans, methyl-L-tryptophans and hydroxy-L-tryptophans, based on the comparison of values of k_{cat}/K_m for a series of tryptophan derivatives substituted in the same position of the indole nucleus. Among fluorinated tryptophans, substitution at the 4-position gives greater activity than at any other position (Fig. 1). In fact, 4-fluoro-L-tryptophan is more reactive than the physiological substrate, L-tryptophan, in terms of both k_{cat}/K_m and k_{cat}. Besides 4-fluoro-L-tryptophan, 5-fluoro, 4-chloro, 4-methyl and 5-hydroxy-L-tryptophan have turnover numbers as high or higher than L-tryptophan (Table 1). On the other hand, 6-substituted tryptophans have greater k_{cat}/K_m values than those substituted at the 4-position for chloro- and methyl-L-tryptophans, and 6-substituted tryptophans exhibit the most consistently

high reactivity (Fig. 1). In contrast, tryptophan analogues substituted in position 7 exhibit consistently lower values of k_{cat}/K_m. The hydroxytryptophans have the lowest k_{cat}/K_m values of all compounds tested, primarily due to their high K_m values (Table 1). The value of k_{cat} for L-tryptophan obtained in the present studies is slightly lower than found previously (6 s^{-1})[7]; however, the value of k_{cat}/K_m is in excellent agreement with our previous results (3.0×10^4 M^{-1} s^{-1}).[7]

Rapid kinetic studies

The results of the pre-steady-state measurements of tryptophan derivatives are summarized in Table 2.

4-Substituted tryptophans. When tryptophan indole-lyase is mixed with 4-substituted tryptophans, a peak at 505 nm forms; this absorbance increase is triphasic, as is also the case when L-tryptophan reacts[8,10]. The absorbance at 425 nm also decreases initially, and then increases along with the second phase of the absorbance increase at 505 nm, again like L-tryptophan. The steady-state absorbance at 505 nm of all 4-substituted tryptophans is less intense than that of L-tryptophan (Table 2). In fact, the absorbance changes at 505 nm are so small for 4-chloro, 4-methyl and 4-hydroxy-L-tryptophan that we cannot accurately determine the rate constants from single-wavelength stopped-flow experiments (Fig. 2A), since this requires fitting to the observed rate constants over a range of concentrations. For these analogues, significant absorbance is only observed at the highest concentrations (10 mM) of substrate.

Fitting the apparent rate constants obtained from the single-wavelength measurements of the reaction of 4-fluoro-L-tryptophan to Equation 4 gives a k_f value for deprotonation of 1200 s^{-1} (Table 2). This value is greater than that of L-tryptophan, and the rate constant for reprotonation of the quinonoid anion, k_r, is also faster than that of L-tryptophan (Table 2). The binding constant, K_d, was estimated to be the same (~5 mM) as those of other monofluoro-L-tryptophans. On the other hand, the rate constants for deprotonation of other 4-substituted tryptophans are slower than that of L-tryptophan (Table 2).

The rate constants, k^{+3}, for elimination of the substituted indoles were evaluated from the second phase of absorbance increase at 505 nm,[10] and

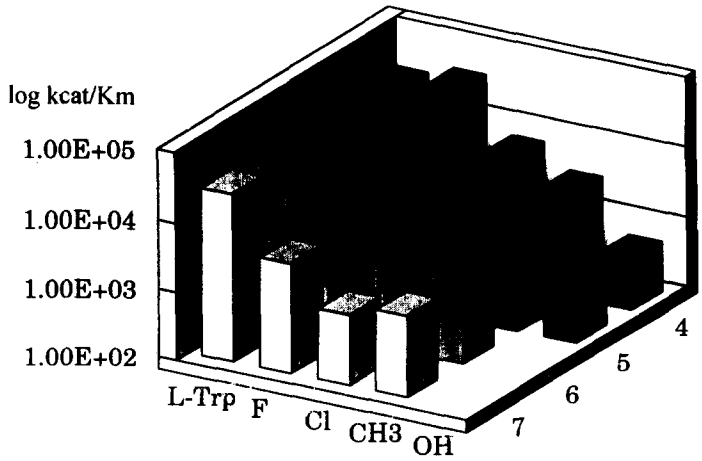


Figure 1. Comparison of k_{cat}/K_m values of substituted L-tryptophans with *E. coli* tryptophan indole-lyase.

Table 1. Steady-state kinetic parameters for substituted tryptophans

Substituent	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
L-Trp	0.15	4.0	2.7×10^4
4-F	0.16	6.2	3.8×10^4
5-F	0.20	4.9	2.4×10^4
6-F	0.16	3.4	2.1×10^4
7-F	0.52	2.2	4.1×10^3
4,7-diF	1.67	2.1	1.2×10^3
5,7-diF	0.38	2.2	6.4×10^3
4-Cl	1.5	9.1	6.1×10^3
5-Cl	0.20	2.0	1.0×10^4
6-Cl	0.10	1.8	1.8×10^4
7-Cl	1.0	1.1	1.1×10^3
4-CH_3	1.8	5.2	2.9×10^3
5-CH_3	0.72	1.7	2.4×10^3
6-CH_3	0.20	2.3	1.2×10^4
7-CH_3	1.4	2.2	1.6×10^3
4-OH	2.1	1.1	5.2×10^2
5-OH	9.0	4.4	4.9×10^2

Table 2. Pre-steady-state kinetic parameters for substituted tryptophans

Substituent	K_d (mM)	k_f (s^{-1})	k_f/K_d (M^{-1} s^{-1})	k_r (s^{-1})	k^{+3} (s^{-1})	Apparent ε_{505}[a] (M^{-1} cm^{-1})
L-Trp	13	770	5.9×10^4	60	21	7.7×10^3
4-F	5	1200	2.4×10^5	100	28	5.5×10^3
5-F	4.3	570	1.4×10^5	40	22	1.4×10^4
6-F	5.3	560	1.0×10^5	20	26	2.6×10^4
7-F	5.1	350	6.9×10^4	30	26	8.5×10^3
4,7-diF	3	310	1.0×10^5	30	13	5.5×10^3
5,7-diF	13	410	3.2×10^4	60	19	4.8×10^4
4-Cl	-	-	-	-	-	1.3×10^3
5-Cl	2.6	340	1.3×10^5	20	8	2.4×10^4
6-Cl	2.7	300	1.1×10^5	20	21	1.9×10^4
7-Cl	7.5	110	1.5×10^4	20	6	3.2×10^4
4-CH_3	-	-	-	-	-	1.9×10^3
5-CH_3	3	50	1.8×10^4	14	4	2.8×10^4
6-CH_3	3	230	7.8×10^4	12	23	1.0×10^4
7-CH_3	12	250	2.1×10^4	22	19	1.1×10^4
4-OH	-	-	-	-	-	2.1×10^3
5-OH	-	-	-	-	-	4.3×10^2

[a]Apparent extinction coefficients calculated from the maximum absorbance obtained in the rapid-scanning spectra with 23.5 μN enzyme and 10 mM of each compound.

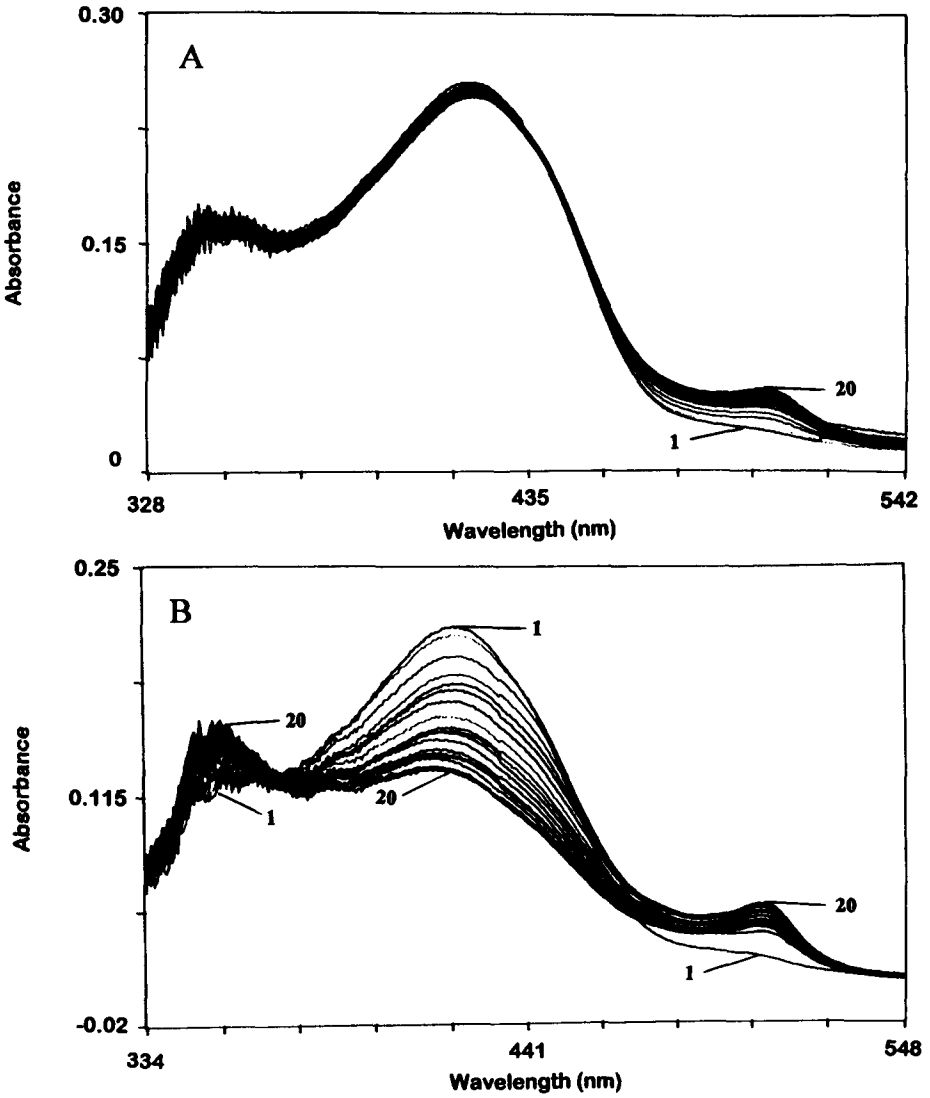


Figure 2. Rapid-scanning data from the reaction of tryptophan indole-lyase (23.5 μN) with (A) 4-hydroxytryptophan (10 mM) and (B) 4-methyltryptophan (10 mM) plus benzimidazole (5 mM). Scans were collected at 10 ms (curve 1), 20.5 ms (curve 2), 31 ms (curve 3), 41.5 ms (curve 4), 52 ms (curve 5), 62.5 ms (curve 6), 73 ms (curve 7), 83.5 ms (curve 8), 94 ms (curve 9), 104.5 ms (curve 10), 115 ms (curve 11), 125.5 ms (curve 12), 136 ms (curve 13), 146.5 ms (curve 14), 157 ms (curve 15), 167.5 ms (curve 16), 178 ms (curve 17), 188.5 ms (curve 18), 199 ms (curve 19) and 209.5 ms (curve 20).

confirmed from the decrease in absorbance at 505 nm produced in the presence of a kinetic trap, benzimidazole.[10] When 4-fluoro, 4-chloro and 4-methyl-L-tryptophan react with tryptophan indole-lyase in the presence of benzimidazole, the absorbance peak at 505 nm forms rapidly (Fig. 2b) and then diminishes, concomitant with the formation of another peak at 345 nm, and with a good isosbestic point at 365 nm. This behavior resembles that previously observed for L-tryptophan.[10] The rate constant obtained for the elimination of 4-fluoroindole from 4-fluoro-L-tryptophan was similar to that of L-tryptophan.

5-Substituted tryptophans. 5-Substituted tryptophans exhibit a variety of reactivities with tryptophan indole-lyase in pre-steady-state kinetics (Table 2) as well as steady-state kinetics (Table 1). Mixing of 5-fluoro-L-tryptophan with tryptophan indole-lyase results in a series of scans similar to those of L-tryptophan (data not shown), but with a more intense peak at 505 nm (Table 2). On the other hand, the reactions of 5-chloro and 5-methyl-L-tryptophan produced totally different rapid-scanning spectra (Fig. 3A). The intensity of the 505 nm peak produced with 5-methyl is much greater than with 5-fluoro-L-tryptophan, and there is an isosbestic point between the 425 nm peak and the rapid phase of the increase at 505 nm, indicating that these two species directly interconvert. These tryptophans do not show any significant increase at 425 nm concomitant with quinonoid intermediate formation, in contrast to L-tryptophan and the 4-substituted tryptophans. Interestingly, the reaction of 5-hydroxy-L-tryptophan exhibited very little change in spectrum from that of the resting enzyme (data not shown).

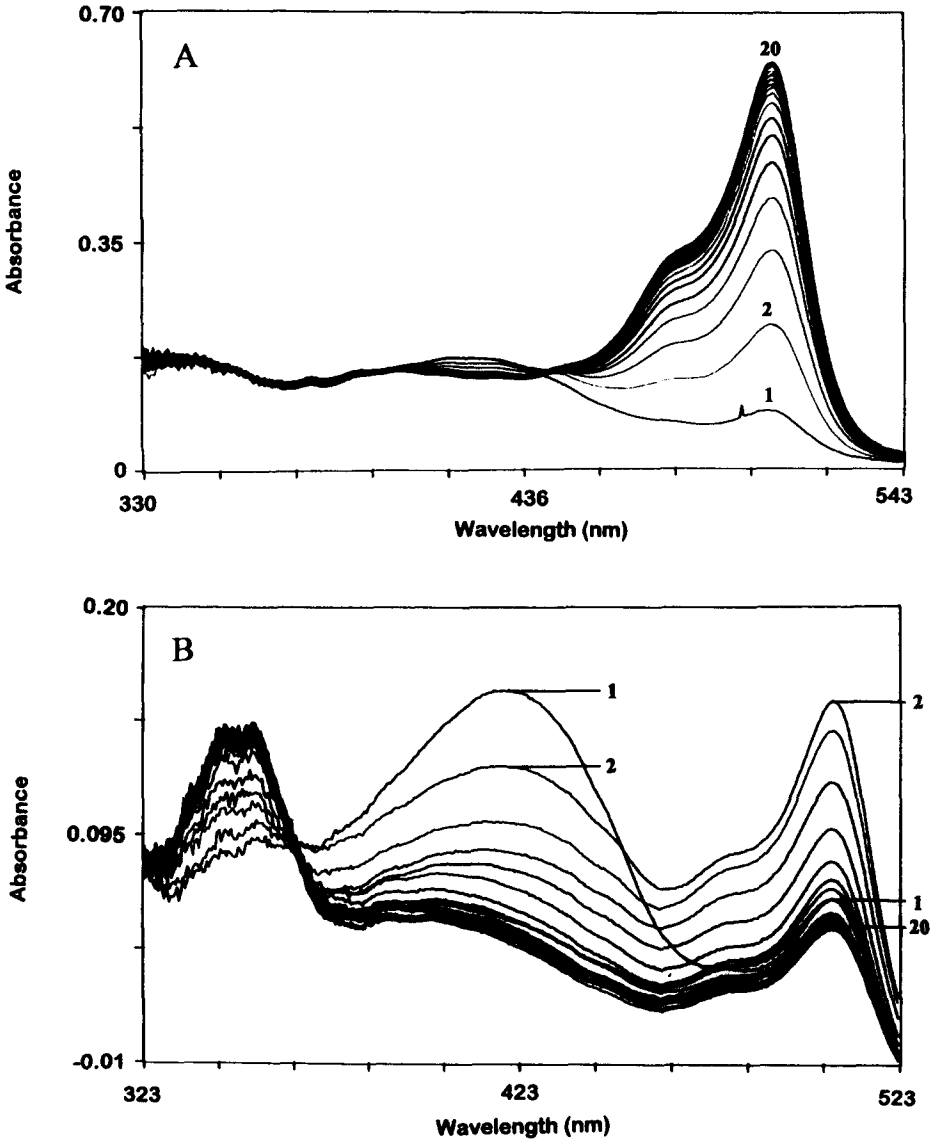


Figure 3. Rapid-scanning data from the reaction of the enzyme with (A) 5-methyltryptophan and (B) 5-fluorotryptophan plus benzimidazole. The scanning intervals and the concentrations of the tryptophan indole-lyase, tryptophan derivatives and benzimidazole were the same as those in Figure 2.

The rate constants for α-deprotonation (k_f) and reprotonation (k_r) decreased with the size of the indole ring substituent (F > Cl > CH_3), and a similar trend was observed for the binding constants (F > Cl ~ CH_3). Thus, the decrease in the steady-state k_{cat}/K_m for the large substituents is apparently due primarily to the reduced rates of formation of the quinonoid intermediates. The differences in the rapid-scanning spectra were clarified when the reactions of 5-substituted tryptophans were performed in the presence of benzimidazole (Fig. 3B). The rate constant, k^{+3}, for the elimination of 5-substituted indoles decreased with the size of the substituent group, and in the case of 5-methyl-L-tryptophan approached the value of k_{cat}, which suggests the rate limiting step for the reaction of tryptophan indole-lyase with this compound may be the elimination of 5-methylindole.

6-Substituted tryptophans. Unlike the 5-substituted tryptophans, the reactions of all 6-substituted tryptophans result in similar rapid-scanning spectra (Fig. 4A). Interestingly, the intensity of the absorbance peak at 505 nm is stronger with 6-fluoro than with 6-chloro and 6-methyl-L-tryptophan (Table 2). Quinonoid intermediate formation is fast for 6-fluoro, like the other fluorotryptophans, but is reduced for 6-chloro and 6-methyl-L-tryptophans. All 6-substituted tryptophans exhibit remarkably similar rates of reprotonation and elimination of the indole ring, while the K_d values are dependent on the size of the substituent. In the presence of benzimidazole, the resulting spectra exhibit peaks at 345, 435 and 505 nm. The peaks at 345 and 505 nm increase with time, but that at 435 nm decreases with time, and there is always an isosbestic point between the 345 and 505 nm bands (Fig. 4B). These are qualitatively similar to the spectra previously obtained with L-tryptophan in the presence of benzimidazole.[10]

7-Substituted tryptophans. Among the 7-substituted tryptophans, 7-fluoro, 4,7-difluoro and 7-methyl-L-

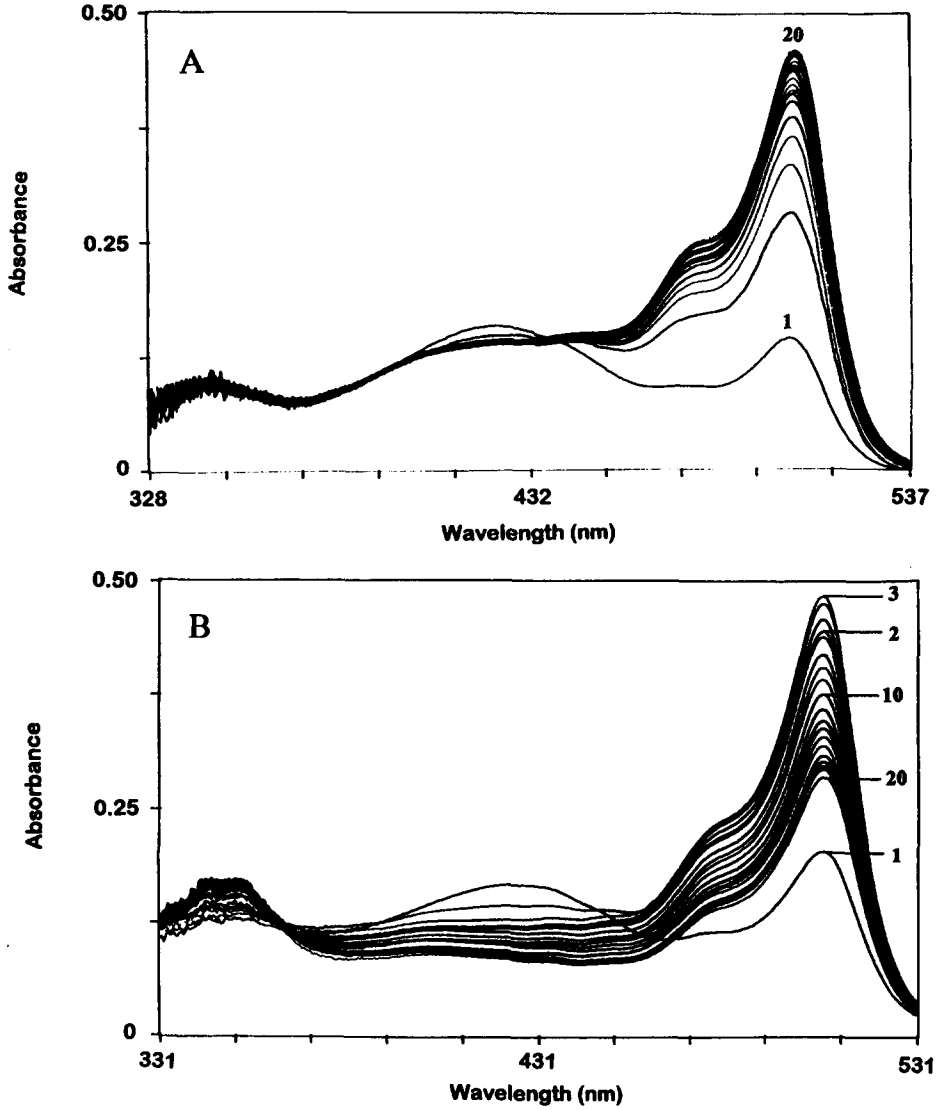


Figure 4. Rapid-scanning data from the reaction of the enzyme with (A) 6-chlorotryptophan (5 mM) and (B) 6-fluorotryptophan plus benzimidazole. The scanning intervals and the concentrations of the tryptophan indole-lyase, tryptophan derivatives and benzimidazole were the same as those in Figure 2.

tryptophan exhibit rapid-scanning spectra similar to those of L-tryptophan (data not shown). On the other hand, 5,7-difluoro and 7-chloro-L-tryptophan result in distinct rapid-scanning spectra in which the absorbance intensity at 505 is very strong (Fig. 5A and Table 2). As expected, the rate constants for α-deprotonation are about the same for 7-fluoro, 4,7-difluoro and 5,7-difluoro-L-tryptophans. Unlike the tryptophan derivatives substituted in other positions, 7-methyl-L-tryptophan reacts faster than 7-chloro for quinonoid intermediate formation.

When 7-fluoro, 4,7-difluoro and 7-methyl-L-tryptophan react with tryptophan indole-lyase in the presence of benzimidazole, the absorbance peak at 505 nm forms rapidly and then diminishes, concomitant with the formation of another peak at 345 nm, and with a good isosbestic point at 365 nm, as seen for L-tryptophan (data not shown). On the other hand, there is almost no difference in the spectra upon reaction with 5,7-difluoro and 7-chloro-L-tryptophan in the presence or absence of benzimidazole (Fig. 5B). The rate constant of elimination of the indole ring, k^{+3},' approaches the overall steady-state k_{cat} for 7-chloro-L-tryptophan (Table 1 and Table 2).

Chemical quench experiments. The results of chemical quench experiments are presented in Figure 6. In the reaction with L-tryptophan, a clear 'burst' of indole is observed, indicating that a step subsequent to the C–C bond cleavage is at least partially rate determining. The data were simulated with KINSIM[16] for the mechanism shown in Equation 2, using the parameters in Table 1 for the binding (K_d), α-deprotonation (k_f), protonation (k_r) and elimination (k^{+3}) steps, and 10 μM as the binding constant for indole (K_4). This is the reported value of the K_m for indole in the synthesis of L-tryptophan catalyzed by tryptophan indole-lyase.[17] These results give a rate constant of 300 s^{-1} for the reaction of indole with the α-aminoacrylate

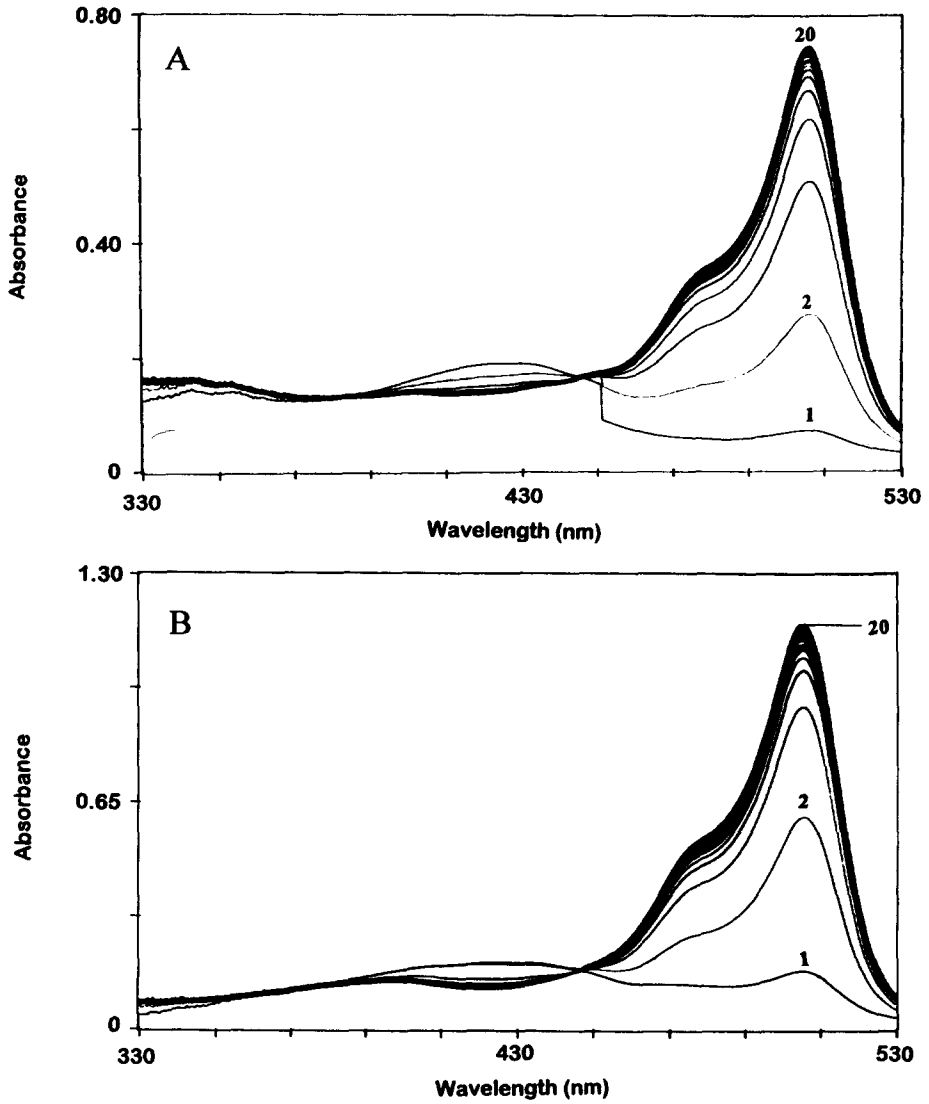


Figure 5. Rapid-scanning data from the reaction of the enzyme with (A) 7-chlorotryptophan and (B) 5,7-difluorotryptophan plus benzimidazole. The scanning intervals and the concentrations of the tryptophan indole-lyase, tryptophan derivatives and benzimidazole were the same as those in Figure 2.

intermediate (k^{-3}), 24 s^{-1} for the release of iminopyruvate (k^{+5}), and 1×10^4 M^{-1} s^{1} for the binding of iminopyruvate (k^{-5}). In the presence of 5 mM benzimidazole, the amplitude of the burst is increased (Fig. 6). KINSIM simulation gives adequate agreement with the benzimidazole quench data by including the binding of benzimidazole to EQ with a K_d of 0.5 mM[10] without changing the values of the other

$$E + A \underset{k_r}{\overset{K_d \; k_f}{=\!=}} EA \underset{k^{-3}}{\overset{k^{+3}}{=\!=}} EB = EPQ = EQ + P \underset{k^{-5}}{\overset{k^{+5}}{=\!=}} E + Q \qquad (2)$$

parameters (Fig. 6). A rapid quench experiment was also performed with 5-methyl-L-tryptophan, and the data show a dramatically reduced burst of indole (Fig. 6). The quench data for 5-methyl-L-tryptophan can be reasonably simulated by simply changing the value of the elimination step (k^{+3}) to 4 s^{-1} (Fig. 6), and holding the other parameters constant at the values given above.

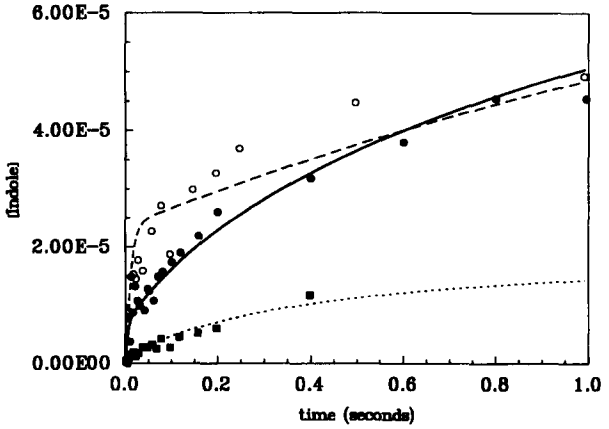


Figure 6. Data from chemical quench experiments. Filled circles: reaction of 10 mM L-tryptophan with 93.5 μN tryptophan indole-lyase. The line is the simulated progress curve from KINSIM. Open circles: reaction of 10 mM L-tryptophan with 93.5 μN tryptophan indole-lyase in the presence of 5 mM benzimidazole. The dashed line is the simulated progress curve from KINSIM. Filled squares: reaction of 10 mM 5-methyl-L-tryptophan with 112.5 μN tryptophan indole-lyase. The dotted line is the simulated progress curve from KINSIM.

Discussion

The proposed mechanism of action of tryptophan indole-lyase is illustrated in Scheme 1. In the absence of substrates, the pyridoxal-5'-phosphate (PLP) is bound to the ε-amino group of Lys-270[18] as a Schiff's base, or internal aldimine, which exhibit absorbance peaks at 337 and 420 nm, although the form which is reactive with substrates may absorb at 360 nm.[19] Binding of an amino acid results in the formation of an external aldimine, which also absorbs at about 420 nm. The base, with pK_a of 7.6, can then remove the α-proton to form the quinonoid intermediate,[7] which for tryptophan absorbs at 505 nm.[10] For elimination to occur, tautomerization of the indole ring must take place;[4-6] however, since this second quinonoid intermediate should also absorb at 505 nm, we have not attempted in this work to treat it as an independent step, and assume that the formation of both quinonoid intermediates is included in the k_f term. We have previously shown that the second phase of absorbance increase at 505 nm in the reaction of L-tryptophan can be assigned to the elimination of indole by the use of benzimidazole as a kinetic trap.[10] The initial α-aminoacrylate Schiff's base complex would be expected to exhibit an absorbance maximum at about 460 nm, based on the transient intermediate observed by Schnakertz and coworkers with D-serine dehydratase.[20] However, we observed an intermediate with an absorbance maximum at 345 nm, which was assigned to the *gem*-diamine complex of pyridoxal phosphate, lysine-270 and α-aminoacrylate.[10] This 345 nm intermediate reacts rapidly with indole to form a quinonoid intermediate, and thus exhibits the expected reactivity of a catalytically competent α-aminoacrylate intermediate.[10] Release of iminopyruvate[21] then regenerates the internal aldimine to complete the catalytic cycle.

We have previously studied the binding and covalent reaction mechanisms of four aromatic amino acid inhibitors of tryptophan indole-lyase that are structural analogues of L-tryptophan using rapid-scanning and single-wavelength stopped-flow kinetic techniques.[9] Despite the significant similarities in structure, there were great differences in the interactions of these analogues with the enzyme.[9] However, all of the analogues we examined at that time were unsubstituted on the benzene ring. In the present work, we have examined the reactivity of 16 tryptophan derivatives, substituted in the benzene ring with fluoro, chloro, methyl and hydroxy substituents, to evaluate the effect of the substitution position as well as the nature of the substituent on the kinetic parameters of the reaction with the enzyme. We have found that all these tryptophan analogues are substrates for α,β-elimination and undergo covalent reaction to give equilibrating mixtures of external aldimine and quinonoid intermediates (Scheme 1). However, there are dramatic differences in the amounts of quinonoid intermediates present in the steady-state; the apparent ε_{505} values for the tryptophan derivatives are listed in Table 2.

The steady-state kinetic parameter K_m (the Michaelis constant), increased with the size of the substituent. However, the dissociation constants, K_d, obtained from the single-wavelength stopped-flow kinetic experiments show the opposite trend, which suggests that part of the energy for the initial binding to form the external aldimine complex derives from hydrophobic interactions between the enzyme and the indole ring of tryptophan derivatives. This observation agrees with the conclusions from our previous studies with S-methyl, S-ethyl and S-benzyl-L-cysteines.[10] Furthermore, the hydroxytryptophans, which have a polar OH substituent, exhibit the highest K_m values (Table 1). It is interesting to note that the 4-substituted tryptophans, with the

exception of 4-hydroxy-L-tryptophan, have higher k_{cat} values than the physiological substrate, L-tryptophan. In this regard, we note that 4-chloro-L-tryptophan has been identified as a component of pea seed protein,[22] and thus may be a physiologically significant substrate.

The absorbance at 505 nm of the complexes of 4-substituted tryptophans is decreased, compared with those of the other analogues, which suggests that either the elimination of the indole ring is faster or that the α-deprotonation is slower, reducing the steady-state level of the quinonoid intermediates. For 4-fluoro-L-tryptophan, the former appears to be the case, even though the rate acceleration is not very large (Table 2). This compound has a very low K_m compared with other 4-substituted tryptophans, and thus it has the highest k_{cat}/K_m among tryptophan derivatives examined in this study. A possible explanation for the higher reactivity of 4-substituted tryptophans is that geometric distortion in the indole ring exists in 4-substituted tryptophans, as we have demonstrated by ^{13}C NMR.[23,24] The steric strain would be released with the elimination of the 4-substituted indole, and thus the formation of the indole will be favored compared with the other unstrained tryptophans. Furthermore, the distortion will increase with the size of the substituent,[23,24] which may make binding less favorable for 4-chloro and 4-methyl-L-tryptophan.

In constrast, all tryptophan derivatives substituted at the 7-position have reduced values of k_{cat}/K_m (Fig. 1). Also, 7-substituted tryptophans, with the exception of 7-fluoro, have high dissociation constants, K_d, which may indicate the presence of steric restriction for binding, possibly due to the presence of a catalytic group, **B2**, near the 7-position. Previously, we demonstrated that a basic catalytic group with pK_a of 6.0 interacts with substrates containing a heterocyclic NH group,[7] and that the heterocyclic NH is important for rapid α-deprotonation and reprotonation of substrates and analogues.[9] Presumably, the energy of the hydrogen bond is used to lower the activation energy for α-deprotonation, as the indole ring is part of the transition state motion.[9] The presence of bulky substituents at the other ring positions may alter the binding of the indole and disrupt the hydrogen bonding of the NH, thus reducing the rate of α-deprotonation. This effect is particularly striking on the reaction of 5-methyl-L-tryptophan, suggesting that there is limited space for bulky substituents near the 5-position of the bound indole. This steric effect may also explain the nonadditivity of reactivity for the difluorotryptophans compared to the monofluoro-tryptophans (Table 1 and Table 2).

Even though the binding constants were the same for 5- and 6-substituted tryptophans, 5-methyl and 6-methyl-L-tryptophan were totally different from each other both in steady-state and pre-steady-state kinetic parameters. Overall, 6-substituted tryptophans have the most consistently high reactivity (Fig. 1), which indicates there may be space around the 6-position of the bound indole in the aromatic binding pocket of the enzyme active site. It is of interest that a plot of the log (k_{cat}/K_m) values in Table 1 against the log (k_f/K_d) values (apparent rate constant for quinonoid intermediate formation) from Table 2 is reasonably linear, with a slope of 0.66 (Fig. 7). This suggests that quinonoid intermediate formation contributes only 66% of the total activation energy for the reaction, and thus a later step in the mechanism must be higher in energy. This conclusion is consistent with the results of rapid chemical quench experiments, which show a significant substoichiometric burst of indole (Fig. 6), indicating that for L-tryptophan both the indole elimination and the release of iminopyruvate[21] are slow and thus partially rate-determining. This is also consistent with the results of solvent isotope effect studies on the reaction of *E. coli* tryptophan indole-lyase with L-tryptophan.[25] However, for 5-methyl-L-tryptophan, the formation of 5-methylindole is considerably slower than iminopyruvate release, and 5-methylindole elimination is almost totally rate-determining. Thus, for 5-methyl-L-tryptophan, the rate constant for elimination of indole (4 s^{-1}) is very similar to k_{cat} (1.7 s^{-1}). This could be due to steric effects, as discussed above, or to the stabilization of the second quinonoid intermediate (Scheme 1) by electron donation from the methyl group. The rapid quench experiment confirms this conclusion, as the 5-methylindole burst in the 5-methyl-L-tryptophan reaction is dramatically reduced (Fig. 6). In addition to 5-methyl, 5-chloro and 7-chloro-L-tryptophan exhibit low values of k^{+3}, suggesting that indole elimination may also be rate-limiting. For all these compounds, the absorbance of the complexes at 505 nm is high (Table 2), indicating greater accumulation of the quinonoid intermediates (Figs 3A and 5A), probably due to their reduced rate of reaction. Although most of the substituted tryptophans examined exhibit the 345 nm α-aminoacrylate intermediate in the presence of benzimidazole (Figs 3B and 4B), these latter compounds and 5,7-difluoro-L-tryptophan (Fig. 5B) do not exhibit significant accumulation of this intermediate, as expected if the elimination step is now largely rate-determining. Indeed, the magnitude of the apparent ε_{505} of the complex of 5,7-difluorotryptophan ($\varepsilon_{505} = 4.8 \times 10^4$ M^{-1} cm^{-1}) is even greater than that of the complex of L-ethionine with tryptophan indole-lyase ($\varepsilon_{510} = 4.0 \times 10^4$ M^{-1} cm^{-1}).[26] Thus, the 5,7-difluoro-L-tryptophan complex with tryptophan indole-lyase must exist almost entirely in one or both of the quinonoid structures shown in Scheme 1.

The results of these experiments provide additional strong support for the mechanism presented in Scheme 1. Furthermore, these experiments demonstrate that although the steady-state kinetic parameters for the reactions of a wide range of aryl-substituted tryptophans with *E. coli* tryptophan indole-lyase are not very different, there are significant substituent effects on the internal distribution of covalent intermediates measured by pre-steady-state methods and on the rate-determining step.

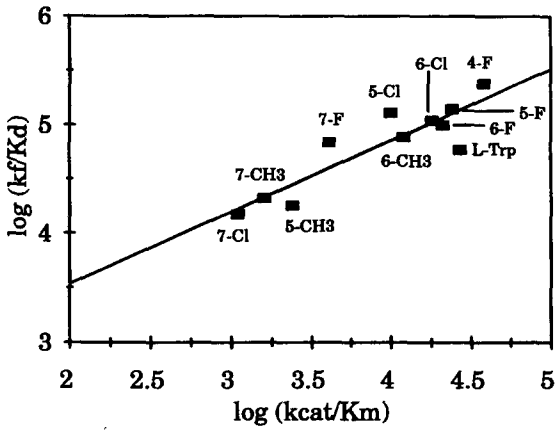


Figure 7. Plot of log (k_{cat}/K_m) against log (k_f/K_d). Values for the parameters were taken from Table 1 and Table 2, respectively. The line is determined by regression analysis, with a slope of 0.66.

Experimental

Materials

L-Tryptophan was purchased from U.S. Biochemical Corp. and was recrystallized from 50% aqueous ethanol before use. Lactate dehydrogenase (from rabbit muscle, lyophilized) and NADH, disodium salt, were also obtained from U.S. Biochemical Corp. 4-, 5- and 6-Fluoro, and 5- and 6-methyl-DL-tryptophans were obtained from Sigma Chemical Co. and were resolved by carboxypeptidase A-catalyzed hydrolysis of the *N*-trifluoroacetyl derivatives as previously described.[27] 4-Hydroxy-L-tryptophan was a generous gift from Dr Susan Cady (University of South Carolina). 4- and 7-Methyl-DL-tryptophans, used as a racemic mixture, and 5-hydroxy-L-tryptophan were also purchased from Sigma Chemical Co.; the concentrations of 4-methyl and 7-methyltryptophan were calculated based on the content of L-isomer. 7-Fluoro, 4,7-difluoro and 5,7-difluoro-DL-tryptophans were synthesized from the appropriate fluoroanilines, and were resolved as described in our previous publication.[27] Chlorotryptophans were prepared from chloroindoles and L-serine using tryptophan synthase as described.[28] Benzimidazole was purchased from Aldrich Chemical Co. and was recrystallized from hot water, after treatment with charcoal, before use. Indole (Gold label) and 5-methylindole were purchased from Aldrich. S-(*o*-Nitrophenyl)-L-cysteine (SOPC) for enzyme assays was prepared as previously described.[29]

Enzyme and assays

Tryptophan indole-lyase was purified as previously described[30] from cells of *E. coli* JM101 containing plasmid pMD6, with the *E. coli tna*A gene under natural regulation.[31] Routine activity assays were performed with SOPC[32] in 0.1 M potassium phosphate, pH 8.0, at 25 °C, following the absorbance decrease at 370 nm ($\Delta\varepsilon$ = −1860 M^{-1} cm^{-1}). The activities of other substrates were measured with the lactate dehydrogenase coupled assay, following the decrease in absorbance at 340 nm ($\Delta\varepsilon$ = −6220 M^{-1} cm^{-1}) in 0.1 M potassium phosphate, pH 8.0, at 25 °C.[33] Enzyme concentrations were estimated from the absorbance of the holoenzyme at 278 nm ($A^{1\%}$ = 9.19).[30]

Steady-state kinetic measurements

Steady-state kinetic measurements were performed at 25 °C as described previously.[30] Steady-state kinetic measurements were obtained on a Gilford Response UV-Vis spectrophotometer equipped with a thermoelectric cell block. Steady-state kinetic data were analyzed by using the compiled FORTRAN programs of Cleland[34] as well as by using ENZFITTER. The standard errors on calculated steady-state kinetic parameters reported in Table 1 were generally less than 10%.

Pre-steady-state kinetic measurements

Single-wavelength stopped-flow kinetic measure-ments and rapid-scanning experiments were performed using a Kinetics Instruments stopped-flow mixer with a modified Cary 14 (OLIS) for single wavelength measurements and a diode array detector from EG&G Princeton Applied Research for rapid-scanning measurements as previously described.[9,10] Prior to performing the rapid kinetics experiments, the stock enzyme was incubated with 0.5 mM PLP for 1 h at 37 °C and then separated from excess PLP on a short desalting column (Excellulose, Pierce) equilibrated with 0.02 M potassium phosphate, pH 8.0, and 0.16 M KCl. The stopped-flow kinetic measurements were performed at 25 °C with the stopped-flow compartment thermostatted by an external water bath. Generally, the enzyme solutions were mixed with solutions of the tryptophan derivatives in the same buffer, with or without 5 mM benzimidazole. Single-wavelength transients were analyzed by averaging the traces from 3–5 separate reactions, followed by fitting with the SIFIT or LMFIT programs (On Line Instruments, Inc.), which can fit up to three exponentials and an offset. Quality of fit was judged by analysis of the residuals and by the Durbin–Watson value.[35] The concentration dependencies of relaxations were fit to either a linear equation for pseudo-first order reactions (Equation 3) or a hyperbolic equation for first-order reactions preceded by a rapid binding equilibrium[36] (Equation 4), where k_f is the rate constant for the forward reaction and k_r is the rate constant for the reverse reaction (see Scheme 1). The fitting was performed using a nonlinear least-squares program (ENZFITTER) from Elsevier Biosoft. The standard errors on the computed parameters were generally 10% or less of the calculated values reported in Table 2.

$$1/\tau_{obs} = k_f\,[L] + k_r \qquad (3)$$

$$1/\tau_{obs} = k_f\,[L]/(K_d + [L]) + k_r \qquad (4)$$

Chemical quench experiments

Rapid chemical quench experiments were performed using a preparative quench apparatus from Kintek Instruments. Enzyme (12 mg mL^{-1}) solution (40 μL) in 0.02 M potassium phosphate, pH 7.8, 0.16 M KCl was mixed with 40 μL of 20 mM L-tryptophan or 5-methyl-L-tryptophan and quenched after various times (5 ms to 1 s) with 0.5 M $HClO_4$. All solutions were adjusted to a final volume of 350 μL with 0.5 M $HClO_4$, and 300 μL was added to 300 μL of freshly prepared Erlich's reagent.[37] After 30 min of incubation, the absorbance at 571 nm was measured. Standards were prepared from blank solutions containing 0.5 to 4 nmol of indole or 5-methylindole, and a standard curve was prepared. The quench data were then simulated using KINSIM[16] with the parameters obtained from the single-wavelength stopped-flow experiments.

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