Effects of α-Deuteration and of Aza and Thia Analogs of L-Tryptophan on Formation of Intermediates in the Reaction of *Escherichia coli* Tryptophan Indole-lyase†

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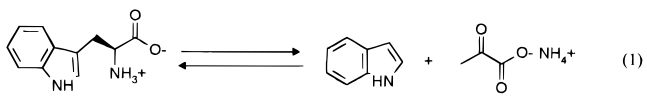
ABSTRACT: Tryptophan indole-lyase catalyzes the hydrolytic cleavage of L-tryptophan to indole and ammonium pyruvate. After the enzyme is mixed with L-tryptophan in the rapid-scanning stopped-flow spectrophotometer, there is an absorbance increase at 505 nm in the pre-steady state attributed to formation of a quinonoid intermediate, which occurs in at least three consecutive first-order phases. Reaction with [α-^{2}H]-L-tryptophan results in significant primary kinetic isotope effects on the first two phases, and there is a significant isotope effect on the amplitude of the absorbance increase in the second phase. This result suggests that proton transfer to carbon to form the indolenine intermediate is relatively slow and is probably at least partially rate-determining. Reaction of L-tryptophan in the presence of benzimidazole results in a rapid increase in absorbance in the first phase, followed by a decrease in absorbance in the second phase, with rate constants very similar to those observed without benzimidazole. We have also examined aza and thia analogs of L-tryptophan, with the benzene ring of the indole replaced by pyridine or thiophene. Both 4,5-thiatryptophan and 6,7-thiatryptophan form quinonoid intermediates in the reaction with tryptophan indole-lyase; however, 6,7-thiatryptophan is a better substrate (k_{cat}/K_m = 32% of L-trp) for tryptophan indole-lyase than is 4,5-thiatryptophan (k_{cat}/K_m = 4% of L-trp). Benzimidazole affects the pre-steady-state reaction of 6,7-thiatryptophan in a way similar to L-tryptophan, while benzimidazole does not affect the pre-steady-state reaction of 4,5-thiatryptophan. 4-Aza-, 5-aza-, 6-aza-, and 7-aza-L-tryptophan are all very slow substrates (k_{cat} < 1% of L-trp) for *Escherichia coli* tryptophan indole-lyase. β-Indazolyl-L-alanine is a relatively good substrate and exhibits a quinonoid intermediate in its reaction with tryptophan indole-lyase. 6-Aza- and 7-azatryptophan accumulate quinonoid intermediates in the reaction with tryptophan indole-lyase, whereas 4-aza- and 5-azatryptophans do not significantly accumulate quinonoid intermediates, and these latter compounds exhibit very high K_m values. Addition of benzimidazole does not change the rapid-scanning stopped-flow spectra of 6-aza- and 7-azatryptophan. This suggests that the rate-determining step in the reaction changes depending on the position and type of heteroatom substitution. For 6-aza- and 7-azatryptophan, the very slow rates of elimination may be due to slow C-protonation of the azaindole, while for 4,5-thiatryptophan, the elimination of thienopyrrole is probably slow. Of all analogs examined, 6,7-thiatryptophan is most similar to tryptophan in its reaction with *E. coli* tryptophan indole-lyase.

Tryptophan indole-lyase (tryptophanase, EC 4.1.99.1) is a pyridoxal 5′-phosphate- (PLP-)[1] dependent enzyme that catalyzes the hydrolytic elimination of L-tryptophan to yield indole and ammonium pyruvate (eq 1). Tryptophan indole-lyase[2] has been identified in a number of bacteria (Snell, 1975), but the enzymes from *Escherichia coli* (Deeley & Yanofsky, 1981) and *Proteus vulgaris* (Kamath & Yanofsky, 1992) have been cloned and studied most extensively. In addition to the physiological reaction, tryptophan indole-lyase can also catalyze the β-elimination of a number of amino acids with suitable leaving groups, including *S*-alkyl-L-cysteines (Watanabe & Snell, 1978), S-(*o*-nitrophenyl)-L-cysteine (Suelter et al., 1976), β-chloro-L-alanine (Watanabe & Snell, 1978), *O*-methyl and *O*-benzyl-L-serine (Watanabe & Snell, 1978), and *O*-benzoyl-L-serine (Phillips, 1987). The chemical mechanism of this enzyme has been of continuing interest, since it catalyzes the elimination of a formally unactivated carbon leaving group. We demonstrated that an indolenine tautomer of tryptophan is an intermediate, based on the potent inhibition of tryptophan indole-lyase by "transition-state analogs", oxindolyl-L-alanine and 2,3-dihy-



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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine; SVD, single-value decomposition.

[2] This enzyme is commonly referred to by the trivial name of tryptophanase.

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dro-L-tryptophan (Phillips *et al.*, 1984, 1985). More recently, using single-wavelength and rapid-scanning stopped-flow spectrophotometric techniques, we found that the pre-steady state of the reaction with L-tryptophan followed at 505 nm exhibits three phases, the second of which is affected by the presence of benzimidazole and thus was proposed to be an α-aminoacrylate intermediate (Phillips, 1989, 1991). We have also shown that there is a "burst" of indole formed in the pre-steady state of the reaction with a rate constant comparable to that of formation of the proposed aminoacrylate intermediate (Lee & Phillips, 1995). Recently, we have prepared a series of aza and thia analogues of L-tryptophan using tryptophan synthase (Sloan & Phillips, 1992; Phillips *et al.*, 1995), and we have now examined their reactions with *E. coli* tryptophan indole-lyase by steady-state and rapid-scanning stopped-flow kinetic methods. In addition, we have examined the effects of [α-^{2}H]-L-tryptophan on the reaction by rapid-scanning stopped-flow kinetics. The results of these studies and their implications for the reaction mechanism are reported below.

EXPERIMENTAL PROCEDURES

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. Benzimidazole was purchased from Aldrich Chemical Co. and was recrystallized from hot water, after treatment with charcoal, before use. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) for enzyme assays was prepared from L-cysteine and 2-fluoronitrobenzene as previously described (Phillips *et al.*, 1989). Aza-L-tryptophans, *β*-indazolyl-L-alanine, and thia-L-tryptophans were prepared from L-serine and the corresponding azaindoles and thiaindoles using *Salmonella typhimurium* tryptophan synthase, as described elsewhere (Sloan & Phillips, 1992; Phillips *et al.*, 1995). *β*-Indazolyl-L-alanine was prepared from indazole and L-serine using *S. typhimurium* tryptophan synthase by the method of Tanaka *et al*. (1986).

Enzyme and Assays. Tryptophan indole-lyase was purified from cells of *E. coli* JM101 containing plasmid pMD6, with the *E. coli tna*A gene under natural regulation, as described, except that the Sepharose treatment was performed in a column rather than batchwise (Phillips & Gollnick, 1989). Routine activity assays were performed with SOPC in 0.1 M potassium phosphate, pH 8.0, at 25 °C, following the absorbance decrease at 370 nm ($\Delta\epsilon = -1860$ M^{-1} cm^{-1}) (Suelter *et al.*, 1976). The activities of other substrates were measured with the lactate dehydrogenase coupled assay, following the decrease in absorbance at 340 nm ($\Delta\epsilon = -6220$ M^{-1} cm^{-1}) in 0.1 M potassium phosphate, pH 8.0, at 25 °C (Morino & Snell, 1970). Enzyme concentrations were estimated from the absorbance of the holoenzyme at 278 nm ($A^{1\%} = 9.19$) (Phillips & Gollnick, 1989), using a subunit molecular mass of 52 kDa (Deeley & Yanofsky, 1981).

Steady-State Kinetic Measurements. Steady-state kinetic measurements were performed at 25 °C as described previously (Kiick & Phillips, 1988) using a Gilford Response or a Cary 1 UV/Vis spectrophotometer equipped with a thermoelectric cell block. Steady-state kinetic data were analyzed by using the compiled FORTRAN programs of Cleland (1979) as well as by using Enzfitter (Elsevier) to fit the data to eq 2. The standard errors on calculated steady state kinetic parameters reported in Table 1 were generally less than 10%.

$$v_{obs} = \frac{k_{cat}[E]_0[S]}{K_m + [S]} \quad (2)$$

Pre-Steady-State Kinetic Measurements. Single-wavelength stopped-flow kinetic measurements and rapid-scanning experiments were performed using an RSM spectrophotometer and a stopped-flow compartment with a 18-mm path length observation cell from Olis, Inc. This instrument has a mixing dead time of about 2 ms; thus, a reaction with a rate constant of 350 s^{-1} will lose about half its amplitude during mixing. Rapid-scanning measurements were performed at 1 kHz, while single-wavelength measurements for the concentration dependencies (Figure 7) were collected at 4 kHz. Some of the single-wavelength measurements were performed on a Kinetics Instruments stopped-flow mixer with a modified Cary 14 UV/Vis spectrophotometer (Olis), and preliminary rapid-scanning experiments were performed with a diode array detector from EG&G Princeton Applied Research, as previously described (Phillips, 1991). Prior to 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 (PD-10, Pharmacia) equilibrated with 0.02 M potassium phosphate, pH 8.0, and 0.16 M KCl, and the reactions were performed in the same buffer. The stopped-flow kinetic measurements were performed at 25 °C with the stopped-flow compartment thermostated by an external water bath. Generally, the enzyme solutions, with or without 10 mM benzimidazole, were mixed with solutions of the tryptophan derivatives in the same buffer. Time courses at selected wavelengths were analyzed by fitting with the SIFIT or LMFIT programs (Olis, Inc.), which can fit up to three exponentials with amplitudes and an offset, where A_t is the absorbance at time t, a_i is the amplitude of each phase, k_i is the rate constant for each phase, and c is the final absorbance, if non-zero (eq 3). Quality of fit was judged by analysis of

$$A_t = \sum_{i=1}^{3} a_i e^{-k_i t} + c \quad (3)$$

the residuals and by the Durbin−Watson value (Durbin & Watson, 1970). The concentration dependencies of relaxations were fit to a hyperbolic equation for first-order reactions preceded by a rapid binding equilibrium (Strickland *et al.*, 1975) (eq 4), where k_f is the rate constant for the forward reaction and k_r is the rate constant for the reverse reaction. The fitting to eq 4 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. Robust global analysis of the spectra was performed with the Global Fit program of I. B. C. Matheson, provided by Olis, Inc. (Matheson, 1990; Maeder & Zuberbuhler, 1990). Both single-wavelength fits at several wavelengths and global fits were performed on all sets of data, and the results were judged to be valid when identical values of rate constants were obtained.

Chart 1: Structures of Tryptophan Analogs Used in This Study

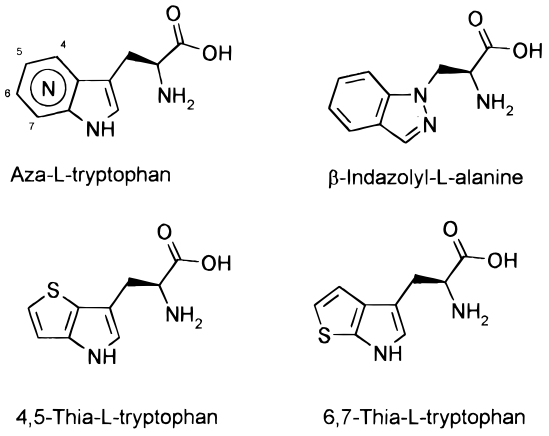


Table 1: Steady-State Kinetic Parameters for Reaction of Aza- and Thiatryptophans with *E. coli* Tryptophan Indole-lyase

substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
L-tryptophan[a]	4.0	0.15	2.7×10^4
4-aza-L-tryptophan	0.06	20.4	2.9
5-aza-L-tryptophan	not determined	>6.4	1.3
6-aza-L-tryptophan	0.015	1.2	12.5
7-aza-L-tryptophan	0.044	5	8.7
indazolyl-L-tryptophan	0.54	0.2	2.7×10^3
4,5-thia-L-tryptophan	0.11	0.095	1.2×10^3
6,7-thia-L-tryptophan	0.84	0.098	8.6×10^3

[a] Data from Lee and Phillips (1995).

$$k_{obs} = 1/\tau = \frac{k_f[L]}{K_d + [L]} + k_r \quad (4)$$

RESULTS

Steady-State Kinetics. All of the aza and thia analogs of L-tryptophan examined in this study (Chart 1) were found to be substrates for *E. coli* tryptophan indole-lyase (Table 1), as determined by the coupled assay for pyruvic acid using lactate dehydrogenase and NADH. However, there are some dramatic differences in the reactivity of the azatryptophans and thiatryptophans, depending on the atom type and position of substitution. The values of k_{cat}/K_m for all of the azatryptophans which contain a pyridine ring are reduced by about 4 orders of magnitude compared with L-tryptophan. In contrast, the reactivity of β-indazolyl-L-alanine is reduced only 10-fold (Table 1). The K_m value is very high for 4-azatryptophan, and for 5-azatryptophan the observed rate was linear up to the highest concentration possible because of solubility (6.4 mM). Thus, it was only possible to determine k_{cat}/K_m for 5-azatryptophan. In contrast, the thiatryptophans are much better substrates than the azatryptophans, with k_{cat}/K_m values reduced only about 3-fold for 6,7-thiatryptophan and 22-fold for 4,5-thia-L-tryptophan (Table 1) compared to L-tryptophan. It is also noteworthy that the values of K_m for the thiatryptophans are about half that for L-tryptophan.

Pre-Steady-State Kinetics. L-*Tryptophan.* The pre-steady state of the reaction of *E. coli* tryptophan indole-lyase with L-tryptophan is complex (Phillips, 1989, 1991). There is formation of a quinonoid intermediate absorbing at 505 nm, the progress of which requires at least three exponentials in the first 500 ms in order to obtain a suitable fit to eq 3 (Figure 1C). The first phase exhibits a rapid increase at 505 nm, with little change in the 420- and 340-nm peaks, as can be seen in Figure 1A, spectra 1−3, and more clearly in the SVD spectra in Figure 1B, solid line and short dashed line. Previously, we demonstrated that k_{obs} for the first phase is affected by the concentration of L-tryptophan (Phillips, 1989)

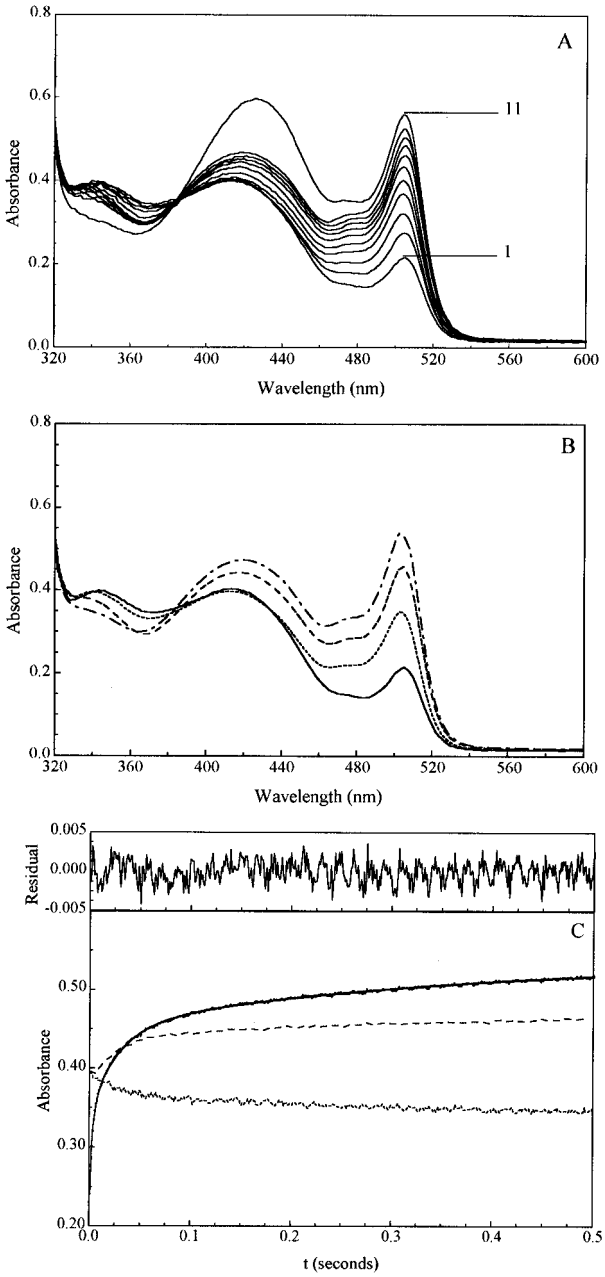


FIGURE 1: Reaction of *E. coli* tryptophan indole-lyase (2 mg/mL; 38.5 μM) with 10 mM L-tryptophan. (A) Selected spectra from the set of 1000 spectra collected during the reaction. Spectra shown are 1, 0.001 s; 2, 0.003 s; 3, 0.005 s; 4, 0.010 s; 5, 0.020 s; 6, 0.040 s; 7, 0.080 s; 8, 0.160 s; 9, 0.320 s; 10, 0.640 s, and 11, steady state (approximately 1 min after the start of the reaction). (B) Single-value decomposition (SVD) spectra of reaction intermediates from global analysis of the first 512 spectra in the reaction. Solid line, initial spectrum; short dashes, first intermediate; long dashes, second intermediate; short and long dashes, third intermediate. (C) Time courses for the reaction taken at 346 nm (dotted line), 421 nm (dashed line), and 505 nm (solid line) The time course at 505 nm is overlaid with points calculated from the fitting to eq 3 for a three-exponential process with the rate constants given in the text. The residuals for the experimental and calculated data points are shown above.

according to eq 4. Fitting of k_{obs} to eq 4 gave a calculated deprotonation rate constant, k_f, of 760 s^{-1} and reprotonation rate constant, k_r, of 60 s^{-1} (Phillips, 1989) (Table 1). The observed rate constant for the fast phase in the present data, 377 ± 14 s^{-1} at 10 mM L-tryptophan, is in good agreement with our previous results. During the second and third phases, there is a further increase in the peak at 505 nm, concomitant with an absorbance increase at 420 nm and a decrease in the 340-nm peak (Figure 1A, spectra 4−10, and Figure 1B, long dashes), with a good isosbestic point at about 385 nm. The time courses for the reaction at 505 nm (solid line), 421 nm (dashed line), and 346 nm (dotted line) are shown in Figure 1C. The rate constants for the second and third phases of absorbance increase at 505 nm are independent of [L-tryptophan], exhibiting rate constants of 37.7 ± 0.5 s^{-1} and 2.3 ± 0.1 s^{-1}, respectively, in good agreement with our previous results (Phillips, 1989, 1991; Lee & Phillips, 1995). We have shown by rapid chemical quench experiments that a "burst" of indole is formed during the second phase of the reaction (Lee & Phillips, 1995), with a rate constant of about 30 s^{-1}. Thus, the second phase of absorbance increase at 505 nm is correlated to the indole elimination step. At steady state (approximately 1 min after the start of the reaction), the absorbance at 505 nm (Figure 1A, spectrum 11) is only slightly higher than at 0.64 s, but the absorbance at 420 nm has increased significantly, possibly due to the buildup of indole and corresponding reversal of the reaction, leading to an increase in the concentration of L-tryptophan external aldimine. Indole is a strong product inhibitor of the enzyme, with a K_i value of 10 μM (Kazarinoff & Snell, 1980)

When L-tryptophan is replaced by [α-^{2}H]-L-tryptophan, the absorbance of the 505-nm peak in the pre-steady state is dramatically decreased (Figure 2A). Using single-wavelength stopped-flow measurements, we previously demonstrated that the first phase of absorbance increase is sensitive to isotopic substitution at C-α and exhibits an isotope effect with [α-^{2}H]-L-tryptophan of $^Hk/^Dk = 3.6$ (Phillips, 1989). In the present work, we found that the second phase of the reaction also exhibits a kinetic isotope effect of $^Hk/^Dk = 3.18 \pm 0.18$ with [α-^{2}H]-L-tryptophan.[3] Furthermore, the absorbance amplitude of the second phase is significantly reduced by deuteration ($^HA/^DA = 2.37 \pm 0.06$), as can be seen by comparison of the SVD spectra (Figures 1B and 2B, short dashes and long dashes). This kinetic isotope effect was not observed previously in single-wavelength studies of the reaction of tryptophan indole-lyase with L-tryptophan and [α-^{2}H]-L-tryptophan performed at 505 nm (Phillips, 1989), probably because of difficulties in accurate fitting of the second phase of the multiexponential data. The present data, obtained in the RSM instrument from Olis, has significantly improved signal/noise compared to the data obtained in the older instrument. The progress of the reaction of [α-^{2}H]-L-tryptophan monitored at 505 nm (solid line), 421 nm (dashed line), and 346 nm (dotted line) is shown in Figure 2C. Finally, it is noteworthy that the steady state spectrum of the solution (Figure 2A, curve 11) is essentially identical with that of either L-tryptophan or [α-^{2}H]-L-tryptophan.

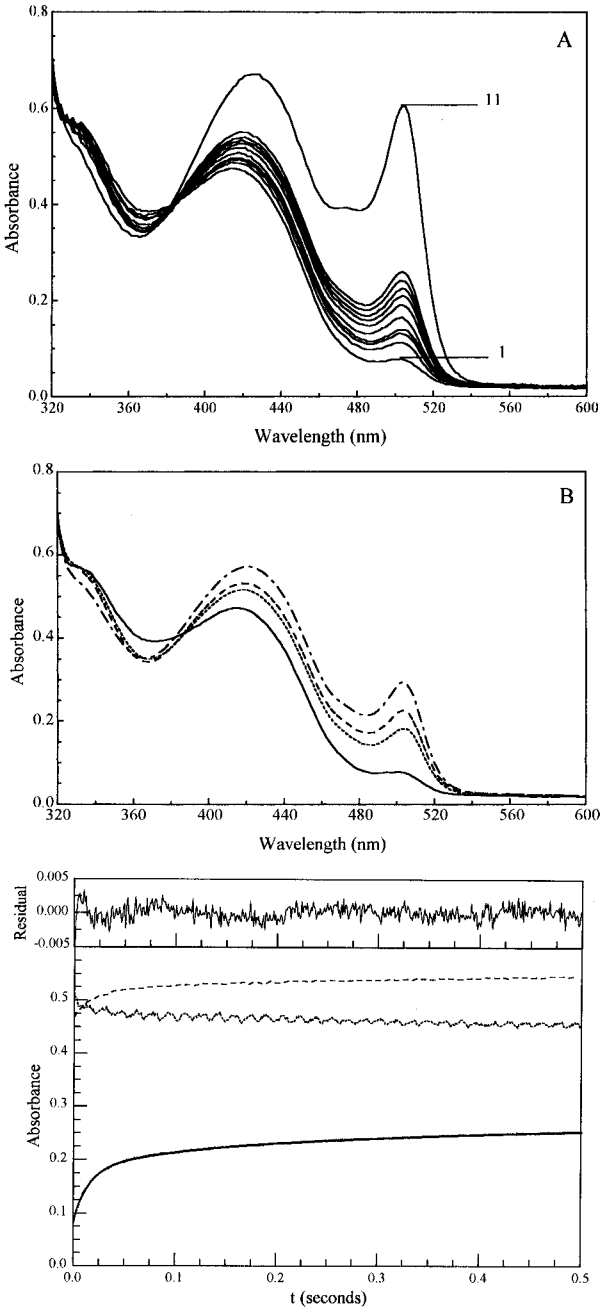


FIGURE 2: Reaction of *E. coli* tryptophan indole-lyase (2 mg/mL; 38.5 μM) with 10 mM [α-^{2}H]-L-tryptophan. Conditions for all panels are as described for Figure 1.

When 5 mM benzimidazole is included in the reaction of tryptophan indole-lyase with L-tryptophan, there is rapid formation of the 505-nm peak, with a rate constant of 351 ± 7 s^{-1}, followed by a decrease in absorbance, with concomitant formation of a prominent peak at 345 nm and with a good isosbestic point at 365 nm (Phillips, 1991) (Figure 3A,B, short dashes and long dashes). The time courses at 505 nm (solid line), 421 nm (dashed line), and 341 nm (dotted line) for the reaction of L-tryptophan with benzimidazole are shown in Figure 3C. The absorbance decrease at 421 nm and the absorbance increase at 341 nm occur at similar rate constants, 36.5 s^{-1} and 33.8 s^{-1}, respectively, as the decrease at 505 nm, 35.5 ± 0.3 s^{-1} (Figure 3C). Finally, the 345-nm peak slowly decreases and the 505-nm peak slowly increases in intensity, until the final steady-state spectrum (Figure 3A, curve 11) is very similar to that seen without benzimidazole present. The slowest

[3] Isotope effects are indicated using the nomenclature of Northrop (1975). $^Hk/^Dk$ is the kinetic deuterium isotope effect on a particular rate constant, while $^HA/^DA$ is the deuterium isotope effect on the phase amplitude absorbance of a particular step in the pre-steady state.

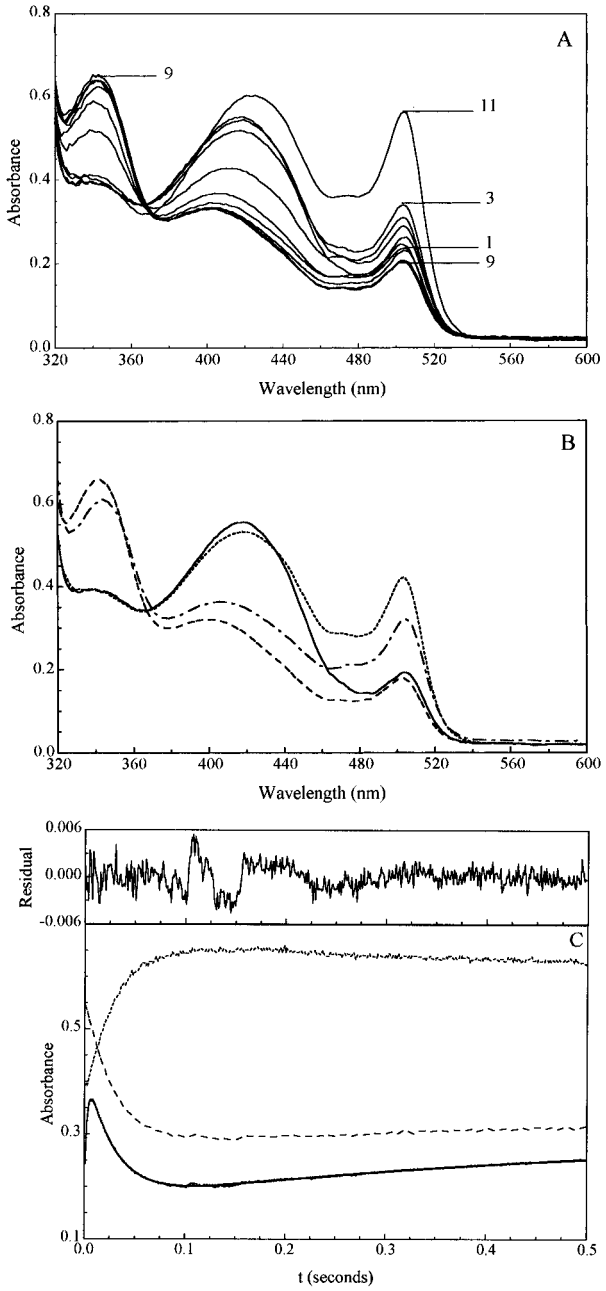


FIGURE 3: Reaction of *E. coli* tryptophan indole-lyase (2 mg/mL; 38.5 μM) with 10 mM L-tryptophan in the presence of 5 mM benzimidazole. (A) Selected spectra from the set of 1000 spectra collected during the reaction. Spectra 1−11 shown are as described for Figure 1. (B) Single-value decomposition (SVD) spectra of reaction intermediates from global analysis of spectra in the reaction. Spectra are as described for Figure 1. (C) Time courses for the reaction taken at 346 nm (dotted line), 421 nm (dashed line), and 505 nm (solid line). Conditions are as described for Figure 1.

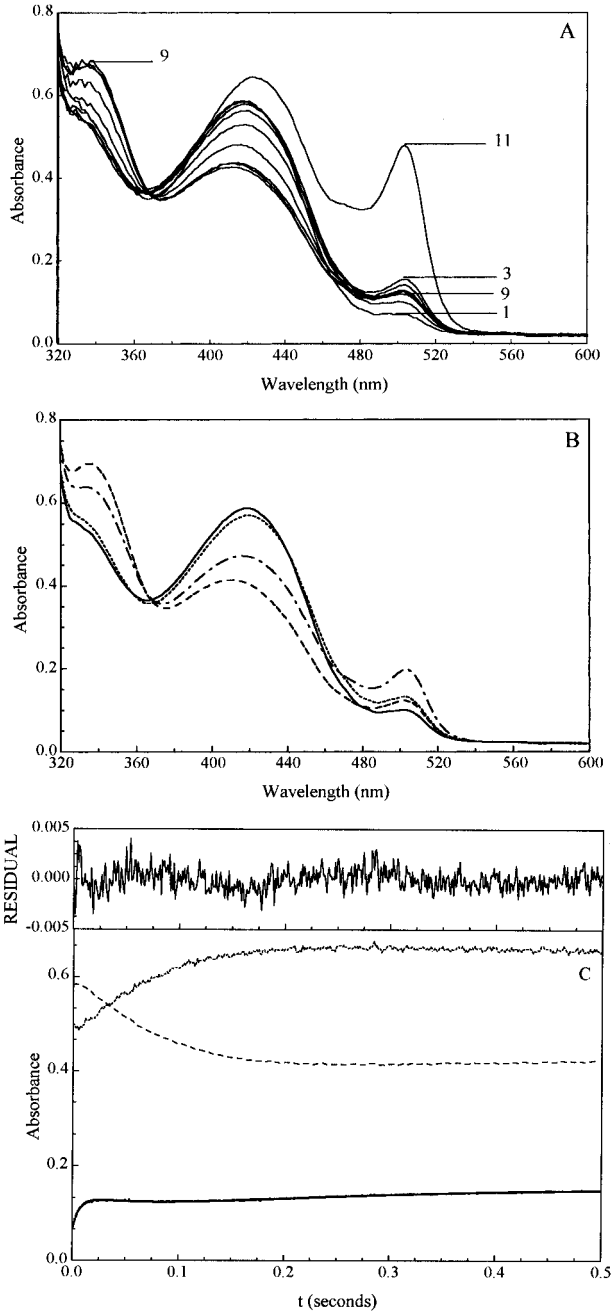


FIGURE 4: Reaction of *E. coli* tryptophan indole-lyase (2 mg/mL; 38.5 μM) with 10 mM [α-^{2}H]-L-tryptophan in the presence of 5 mM benzimidazole. (A) Selected spectra from the set of 1000 spectra collected during the reaction. Spectra 1−11 shown are as described for Figure 1. (B) Single-value decomposition (SVD) spectra of reaction intermediates from global analysis of spectra in the reaction. Spectra are as described for Figure 1. (C) Time courses for the reaction taken at 346 nm (dotted line), 421 nm (dashed line), and 505 nm (solid line). Conditions are as described for Figure 1.

phase of the absorbance increase at 505 nm exhibits a similar rate in the presence or absence of benzimidazole. When 5 mM benzimidazole is included in the reaction mixture with [α-^{2}H]-L-tryptophan, there is a small rapid increase in absorbance at 505 nm, but there is very little subsequent decrease in absorbance (Figure 4A,B, short dashes and long dashes). However, the formation of the peak at about 345 nm and the decrease in the 420 nm peak are still observed. The time courses for the reaction of [α-^{2}H]-L-tryptophan in the presence of 5 mM benzimidazole at 505 nm (solid line), 421 nm (dashed line), and 341 nm (dotted line) are shown in Figure 4C. The rate constants, 12.3 s^{-1} for the absorbance increase at 341 nm and 12.4 s^{-1} for the absorbance decrease at 421 nm, are significantly reduced compared to the corresponding reactions shown in Figure 3C. The calculated isotope effects are $^{H}k/^{D}k = 2.97 \pm 0.07$ for the reaction at 341 nm and 2.73 ± 0.04 for the reaction at 421 nm, in good agreement with the isotope effect on the second phase of the absorbance increase without benzimidazole. The final steady-state spectrum of the reaction mixture with [α-^{2}H]-L-tryptophan and benzimidazole (Figure 4A, curve 11) is similar to those of the other steady-state spectra (Figures 1−3, panels A, curves 11).

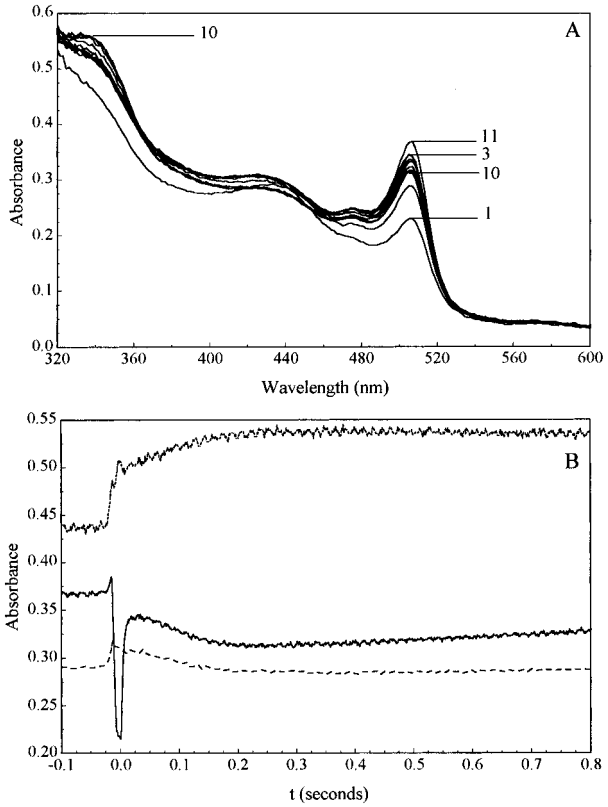


FIGURE 5: Reaction of *E. coli* tryptophan indole-lyase (1 mg/mL; 19.2 μM) with 10 mM 6,7-thiatryptophan in the presence of 5 mM benzimidazole. (A) Selected spectra from the set of 1000 spectra collected during the reaction. Spectra 1−11 shown are as described for Figure 1. (B) Time courses for the reaction taken at 346 nm (dotted line), 421 nm (dashed line), and 505 nm (solid line).

Thiatryptophans. The reaction of 4,5-thiatryptophan with tryptophan indole-lyase also exhibits rapid formation of a quinonoid intermediate absorbing at 505 nm, along with an increase and gradual red shift in the 420-nm peak (data not shown). The absorbance increase at 505 nm occurs in three phases, as observed for L-tryptophan, with rate constants comparable to those of L-tryptophan. The first phase of the absorbance increase at 505 nm is dependent on [4,5-thiatryptophan], as shown in Figure 6 (dotted line with filled hexagons). There is also a decrease in absorbance at 345 nm that parallels the increase at 421 nm. The steady-state spectrum is slightly higher at 420 and 505 nm. However, in contrast to L-tryptophan, the addition of benzimidazole does not result in any significant changes in the spectra. The time courses at 421 and 345 nm do not show the decrease and increase, respectively, seen in the reaction with L-tryptophan and benzimidazole.

For the reaction of 6,7-thiatryptophan, formation of a quinonoid intermediate absorbing at 505 nm is also seen (data not shown), the time course of which also requires three exponentials to fit, with rate constants similar to those for L-tryptophan and 4,5-thiatryptophan. In contrast to 4,5-thiatryptophan, however, addition of benzimidazole to the reaction of 6,7-thiatryptophan results in a decrease in absorbance at 505 nm similar to that seen with L-tryptophan and benzimidazole (Figure 5A) but with smaller amplitude and with a slower rate constant of 7 s^{-1}. There is a corresponding increase in absorbance at 346 nm (dotted line) and decrease at 421 nm (dashed line) (Figure 5B), analogous to the effects on the reaction of L-tryptophan. The apparent rate constants for the fast phase of the absorbance increase

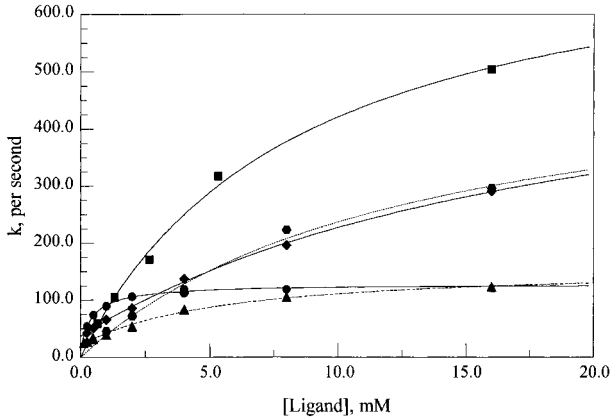


FIGURE 6: Rates of reaction of aza- and thiatryptophans with *E. coli* tryptophan indole-lyase. 4,5-Thiatryptophan, dotted line with filled hexagons; 6,7-thiatryptophan, solid line with filled squares; 7-azatryptophan, dashed line with filled triangles; 6-azatryptophan, solid line with filled diamonds; β-indazolyl-L-alanine, solid line with filled circles. The lines are the curves calculated using eq 4 and the parameter values are given in Table 2.

Table 2: Pre-Steady-State Kinetic Parameters for the Fast Phase of Reaction of *E. coli* Tryptophan Indole-lyase with Aza- and Thiatryptophans

substrate	k_f (s^{-1})	k_r (s^{-1})	K_d (mM)	k_f/K_d (M^{-1} s^{-1})
L-tryptophan	760[a]	60[a]	10	7.6×10^4
6-aza-L-tryptophan	140	20	5.4	2.6×10^4
7-aza-L-tryptophan	140	10	3.9	3.6×10^4
β-indazolyl-L-alanine	110	25	0.7	1.5×10^5
4,5-thia-L-tryptophan	480	~0	6.9	7.0×10^4
6,7-thia-L-tryptophan	765	~0	7.9	9.7×10^4

[a] k_f and k_r data from Phillips (1989).

at 505 nm are dependent on [6,7-thiatryptophan], as shown in Figure 6 (solid line with filled squares). Fitting of these data to eq 3 give the apparent rate constants for deprotonation, k_f, and reprotonation, k_r, at the C-α of the thiatryptophan Schiff bases reported in Table 2. The rate constant for deprotonation of 6,7-thiatryptophan, 765 s^{-1}, is essentially identical with that for L-tryptophan and faster than that of 4,5-thiatryptophan, 480 s^{-1} (Table 2). However, the rate constants for reprotonation of both thiatryptophan isomers appear to be slower than that of L-tryptophan, and neither set of data would give an adequate fit to eq 4 with a non-zero intercept term. Thus, although the rates of k_r are reported as zero in Table 2, they must be finite but too small to be determined in this experiment.

Azatryptophans. 4-Azatryptophan and 5-azatryptophan exhibited very minor changes in the spectrum of *E. coli* tryptophan indole-lyase when they were mixed in the rapid-scanning stopped-flow instrument with these substrates at 5 mM (data not shown). These results are consistent with the high K_m values observed in the steady-state measurements (Table 1). However, the reaction with 7-azatryptophan exhibits an intense peak at 498 nm, as we previously observed in single-wavelength measurements (Phillips, 1989). This absorbance increase is isosbestic with the decrease in absorbance of the 420-nm peak in the early part of the reaction. There is no significant change in absorbance of the spectra below 380 nm. The apparent rate constant for the absorbance increase at 498 nm is dependent on [7-azatryptophan], as shown in Figure 6 (dashed line with filled triangles). The calculated rate constant for deprotonation, k_f, is 140 s^{-1}, and the reprotonation rate constant, k_r, is 10

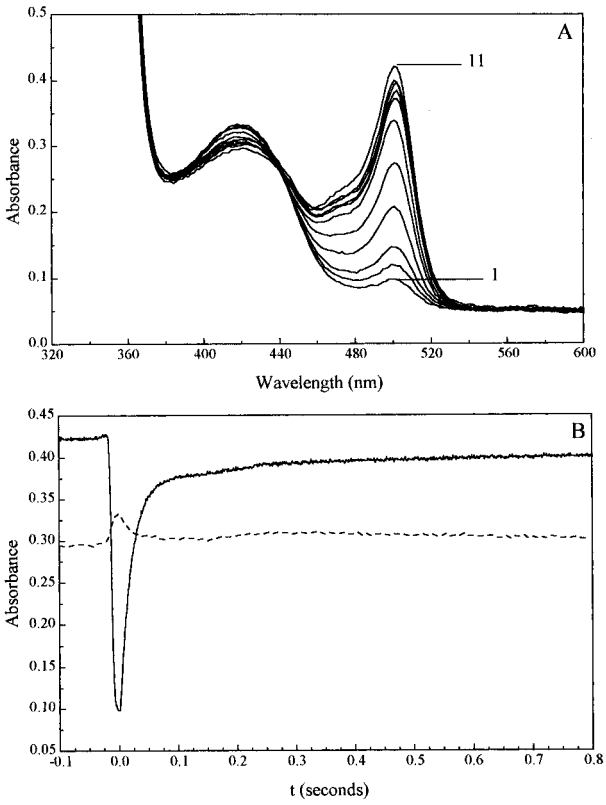


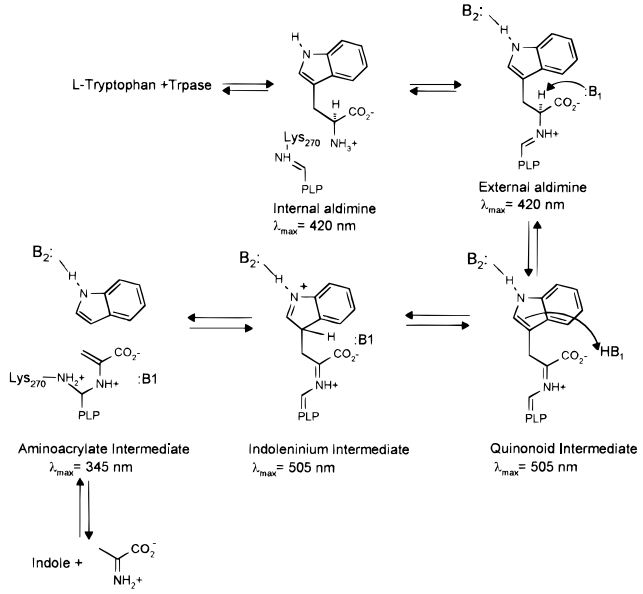
FIGURE 7: Reaction of *E. coli* tryptophan indole-lyase (1 mg/mL; 19.2 μM) with 10 mM 6-aza-L-tryptophan in the presence of 5 mM benzimidazole. (A) Selected spectra from the set of 1000 spectra collected during the reaction. Spectra 1−11 shown are as described for Figure 1. (B) Time courses for the reaction taken at 421 nm (dashed line) and 501 nm (solid line).

s^{-1} (Table 2). Addition of 5 mM benzimidazole to reaction mixtures of 7-azatryptophan does not result in a decrease in the peak at 498 nm; in fact, there is a small increase in the absorbance of the peak (data not shown). The time courses of the absorbance increases at 498, 421, and 346 nm for 7-azatryptophan with benzimidazole are very similar to those without benzimidazole present, and in neither case is there any change between the 0.64-s spectrum and the steady-state spectrum.

6-Azatryptophan also reacts to form a peak at 501 nm, along with a concomitant decrease in absorbance at about 420 nm. Because of the high absorbance of this substrate, it was not possible to observe the reaction below 360 nm. The apparent rate constant for the increase in absorbance at 501 nm is dependent on [6-azatryptophan], as shown in Figure 6 (solid line with filled diamonds). From these data, the calculated rate constant for deprotonation, k_f, is 140 s^{-1} and that for reprotonation, k_r, is 20 s^{-1}, which are considerably slower than those of L-tryptophan (Table 2). Addition of 5 mM benzimidazole to reactions of 6-azatryptophan had very little effect on the spectra (Figure 7A) or the time courses at 501 nm (solid line) and 421 nm (dashed line) (Figure 7B).

β-Indazolyl-L-alanine is an aza analog of tryptophan with a β-C−N bond rather than a C−C bond, so that the elimination is of a nitrogen leaving group instead of carbon. It also exhibits formation of a strong absorbance peak at 501 nm when reacted with tryptophan indole-lyase, although the absorbance changes elsewhere are rather modest (data not shown). The apparent rate constant for the fast phase

Scheme 1: Mechanism of Tryptophan Indole-lyase



measured at 501 nm is dependent on [β-indazolyl-L-alanine], as seen in Figure 6 (solid line with filled circles). The calculated rate constant for deprotonation is 110 s^{-1}, and the reprotonation rate constant is 25 s^{-1} (Table 2). The presence of 5 mM benzimidazole has no significant effect on the spectra or the time courses at 501, 421, and 346 nm in the reaction of β-indazolyl-L-alanine.

DISCUSSION

All of the aza and thia analogs of L-tryptophan examined in this study (Chart 1) are substrates for the β-elimination reaction catalyzed by tryptophan indole-lyase. However, there are large differences in the reactivity of the compounds depending on the position and type of heteroatom substitution. In general, the thiatryptophans are more similar to tryptophan in both their steady-state and pre-steady-state kinetic properties than are the azatryptophans. The mechanism for tryptophan indole-lyase, based on our present and previous studies, is presented in Scheme 1. After Michaelis complex formation with the internal aldimine, transaldimination occurs to give the external aldimine, followed by α-deprotonation by a base, B1, to give the quinonoid intermediate, which can be observed at about 500 nm in the rapid-scanning stopped-flow spectra. This latter intermediate is formed for all of these substrates with a rate constant at least 100-fold greater than the corresponding steady-state k_{cat} (Table 2) and thus can hardly be considered "rate-determining", yet there is a significant primary deuterium isotope effect on k_{cat} of 2.5 in the reaction of [α-^{2}H]-L-tryptophan (Kiick & Phillips, 1988). The present work may provide some insight into this seeming paradox. Both the amplitude ($^HA/^DA \cong 2.4$) and the rate constant ($^Hk/^Dk \cong 3.0$) for the second phase of quinonoid intermediate formation are affected by α-deuteration of L-tryptophan. This kinetic isotope effect is especially apparent in the comparison of the time courses at 421 and 346 nm of the reaction of L-tryptophan and [α-^{2}H]-L-tryptophan in the presence of benzimidazole (compare Figures 3C and 4C). Our rapid chemical quench experiments have demonstrated that there is a "burst" of indole produced in the pre-steady state, with a comparable rate constant (25−30 s^{-1}) to the second phase

of quinonoid intermediate formation (Lee & Phillips, 1995). This burst is enhanced in the presence of benzimidazole, consistent with the interpretation that benzimidazole binds noncovalently to the aminoacrylate intermediate subsequent to indole release. *The effect of added benzimidazole is to make indole release irreversible in the pre-steady state of the reaction.* No covalent reaction of benzimidazole with the α-aminoacrylate is expected, since stereoelectronic effects preclude the reaction of benzimidazole as a π-nucleophile (Roy *et al.*, 1988). The position of the absorbance peak of the α-aminoacrylate intermediate at 345 nm led us to propose (Phillips, 1991) that it is present as the *gem*-diamine rather than the Schiff base (Scheme 1). The present data suggest that the second phase of the reaction consists of both the proton transfer to C-3 of the indole ring to form the indolenine or indoleninium intermediate (Phillips *et al.*, 1984, 1985) (Scheme 1) as well as the subsequent elimination of indole with C−C cleavage to form the aminoacrylate intermediate, that may be kinetically coupled (Phillips, 1991). It should be noted that the partial internal transfer of label from [α-^{2}H]-L-tryptophan to the 3-position of indole product has been reported by Vederas *et al.* (1978). The magnitude of the primary isotope effect on this step (3.0) observed with [α-^{2}H]-L-tryptophan suggests that the internal proton transfer is much slower than the subsequent elimination and further suggests that the proximate acid is monoprotic. In this regard, it is interesting that we have found that Tyr71 is the general acid catalyst for phenol elimination in a mechanistically similar enzyme, tyrosine phenol-lyase (Chen *et al.*, 1995), and this tyrosine is conserved in the sequence of all tyrosine phenol-lyases and tryptophan indole-lyases (Chen & Phillips, 1995).

The isotope effect of 2.5 previously observed on k_{cat} with [α-^{2}H]-L-tryptophan (Kiick & Phillips, 1988) is thus probably related to the amplitude effect on the second phase, which reduces the steady-state concentration of the ketoquinonoid and aminoacrylate intermediates (Scheme 1). It is noteworthy that the steady-state rapid-scanning spectra are very similar for either L-tryptophan or [α-^{2}H]-L-tryptophan (Figures 1A and 2A, curves 11). This suggests that there is a slow "washout" of the isotope label as the reverse reaction becomes kinetically significant. The previous steady-state kinetic experiments were performed with much lower catalytic concentrations of enzyme, and thus the reverse reaction and label washout were not significant in the time frame of the initial rate measurements.

The 4-aza- and 5-azatryptophans exhibit poor binding and low reactivity in steady-state kinetic measurements. These are the most basic of the aza-substituted tryptophans, with pK_as of 7−8 for the pyridine ring, and thus are found in the N-protonated form at neutral pH. They show very small effects on the enzyme spectrum in the rapid-scanning stopped-flow spectrophotometer. It is possible that the rate-limiting step for the reaction of these compounds is formation of the external aldimine, since the k_{cat}/K_m values are extremely low for these compounds (Table 1). In contrast, 6-aza- and 7-azatryptophan bind readily and rapidly form a mixture of external aldimine and quinonoid intermediates, as can be seen from the rapid-scanning data in Figure 7. However, the k_{cat} values for these compounds are quite low. Furthermore, there is no effect of benzimidazole on the spectra of the azatryptophans. Since benzimidazole binds to the aminoacrylate intermediate (Phillips, 1991), it appears that there is no significant accumulation of the aminoacrylate intermediate in the reactions of 6-aza- and 7-azatryptophans. Hence, due to the electron-withdrawing effect of the pyridine ring, it is likely that proton transfer to C-3 of 6-aza- and 7-azatryptophan is very slow and may be rate-determining. In contrast, elimination of the azaindoles from the azaindoleninium intermediate should be fast, since the azaindoles are at least as good leaving groups as indole. β-Indazolyl-L-alanine is the only compound examined which reacts with C−N bond cleavage and thus does not require protonation on carbon for the elimination to occur. However, it is likely that N-1 protonation does occur to form a structure analogous to the indoleninium intermediate for efficient elimination of indazole, since the indazolyl anion would not be a good leaving group at pH values near neutrality.

The steady-state, rapid-scanning, and single-wavelength stopped-flow results for the thiatryptophans (Figure 5) are much more similar to those of tryptophan. However, 6,7-thiatryptophan shows a decrease in absorbance at 505 nm, similar to tryptophan but of lower magnitude and rate, when benzimidazole is present, while 4,5-thiatryptophan does not show any effect of added benzimidazole. Moreover, k_{cat} is significantly higher for 6,7-thiatryptophan than for 4,5-thiatryptophan. This suggests that the aminoacrylate intermediate does not significantly accumulate in the reaction of 4,5-thiatryptophan, while it does for 6,7-thiatryptophan. Accumulation of the aminoacrylate intermediate in the steady state for the reactions of L-tryptophan and 6,7-thiatryptophan suggests that the release of the second product, iminopyruvate (Vederas *et al.*, 1978; Hillebrand *et al.*, 1979), is partially rate-determining for these substrates. It is unlikely that there would be a large difference in the rates of protonation of the indole and thienopyrrole rings, since they are expected to exhibit comparable low pK_as [the pK_a of indole for protonation at C-3 in solution is −2.5 (Gut & Wirz, 1994)]. Similarly, the rate of iminopyruvate release from the aminoacrylate intermediate should be identical for all the substrates examined. Thus, it appears most likely that there is a difference in the rates of thienopyrrole elimination in the two thiatryptophan isomers. The electron-rich thienopyrroles should form more stable indoleninoid intermediates than indole, and hence, the elimination rate may be reduced, apparently more so for the 4,5-isomer than the 6,7-isomer. Previously, we examined the steady-state and pre-steady-state reactions of a wide range of tryptophans substituted in the benzene ring (Lee & Phillips, 1995). We found that a number of these derivatives exhibit steady-state k_{cat} values comparable to the second phase of quinonid intermediate formation in the pre-steady state, and these show little effect upon addition of benzimidazole. We concluded that these substituted tryptophans probably exhibit rate-determining indole elimination. Hence, it is likely that the rate-determining step for the reaction of 4,5-thiatryptophan is thienopyrrole elimination.

Conclusions. Deuterium isotope effects in the pre-steady state on the second phase of reaction of L-tryptophan suggest that internal proton transfer from C-α to C-3 of the substrate indole ring is relatively slow and at least partially rate-determining. For 6-aza- and 7-azatryptophan, the very slow rates of elimination may be due to very slow C-protonation of the azaindole rings, while for 4,5-thiatryptophan, the elimination of thienopyrrole product is probably slow. Of all the analogs examined in the present study, 6,7-thiatryp-

tophan is most similar to tryptophan in its reaction with *E. coli* tryptophan indole-lyase.

SUPPORTING INFORMATION AVAILABLE

Eight figures, showing spectra and time courses for reaction of *E. coli* tryptophan indole-lyase with 4,5-thiatryptophan, 7-azatryptophan, and β-indazolyl-L-alanine (in the presence and absence of benzimidazole) and with 6,7-thiatryptophan and 6-azatryptophan (10 pages). Ordering information is given on any current masthead page.

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