Mechanism of Tryptophan Indole-Lyase: Insights from Pre-Steady-State Kinetics and Substrate and Solvent Isotope Effects

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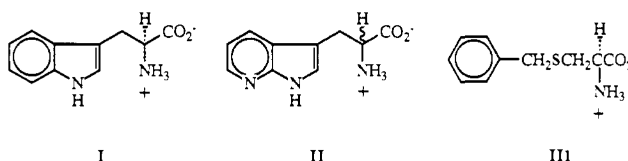
Abstract: We have examined the pre-steady-state kinetics of the reaction of *Escherichia coli* tryptophan indole-lyase with L-tryptophan, 7-aza-DL-tryptophan, and *S*-benzyl-L-cysteine. L-Tryptophan and 7-aza-DL-tryptophan exhibit three relaxations when the reactions are monitored at 506 nm. With L-tryptophan, α-deuteriation results in an estimated isotope effect of 3.6 on the first phase, while ^2H_2O produces apparent isotope effects of 2.5 and 2.7 on the second and third phases, respectively. On the basis of the substrate and solvent isotope effects and the effects of aza substitution, we assign these three processes to (1) deprotonation of the α-carbon, (2) an enzyme conformational change, and (3) indole tautomerization. In contrast, *S*-benzyl-L-cysteine exhibits only one catalytically competent relaxation, monitored at 512 nm. The intrinsic isotope effect for the reaction of α-[^{2}H]-*S*-benzyl-L-cysteine is estimated to be 7.9. α-Proton abstraction is 10–100-fold faster than catalytic turnover in these reactions; thus, tautomerization of the indole ring of L-tryptophan may be partially rate-determining.

Indolenines (3*H*-indoles) are postulated intermediates in electrophilic substitution reactions at the 3-position of indoles.[1] We have recently studied the mechanism of hydrolysis of 2-haloindoles to determine whether indolenines are reactive intermediates, as had been proposed.[2] We found that the hydrolysis of 2-haloindole-3-propionic acids occurs with rate-determining intramolecular proton transfer to form the reactive indoleninium intermediate, since a solvent isotope effect (k_{H_2O}/k_{D_2O}) of 3.9 was observed.[3] Indolenine intermediates have also been proposed in the catalytic mechanisms of tryptophan synthase and tryptophan indole-lyase;[4] this latter enzyme catalyzes the reversible hydrolytic cleavage of L-tryptophan to indole, pyruvic acid, and NH_3. Our previous results have provided strong support for indolenine intermediates, due to the potent inhibition of these enzymes by the reaction intermediate analogues oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan.[5] Inhibition of these enzymes by the diastereomers of 2,3-dihydro-L-tryptophan is stereospecific, providing further evidence for indolenine intermediates in these reactions.[6]

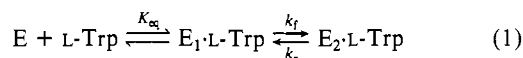
The proposed catalytic mechanisms of tryptophan synthase, tryptophan indole-lyase, and related pyridoxal phosphate dependent enzymes require formation of α-carbanionic (quinonoid) intermediates, which exhibit strong, sharp absorption peaks ($\epsilon \geq 10^4$) at $\sim$500 nm, from the substrate–pyridoxal phosphate complexes.[4,7] In the case of tryptophan indole-lyase, this peak is observed at 506 nm under steady-state turnover conditions.[8] Thus, the formation of this intermediate should be easily monitored in a stopped-flow pre-steady-state kinetic experiment. Indeed, the formation of the dead-end quinonoid complexes from tryptophan indole-lyase and quasi-substrates, L-alanine and L-ethionine, have been previously examined using stopped-flow methods by June et al.[9] We have been examining the pre-steady-state kinetics of the reaction of tryptophan indole-lyase with L-tryptophan (I), 7-aza-DL-tryptophan (II), and *S*-benzyl-L-cysteine (III). The examination of the effects of substrate and solvent deuteriation



allows us to make reasonable mechanistic assignments to the kinetic processes observed.

Results

L-Tryptophan. When *Escherichia coli* tryptophan indole-lyase[10] is mixed with L-tryptophan, the resultant reaction curve at 506 nm (Figure 1A) requires a minimum of three exponential terms to obtain a reasonable fit (compare residuals for two- and three-exponential fits in Figure 1B). The fast phase shows a hyperbolic dependence on [L-Trp], while the second and third phases are independent of [L-Trp] in the concentration range studied (1–10 mM), as can be seen in Figure 2. A hyperbolic dependence of a relaxation on the concentration of a reactant or ligand implies that there is a rapid equilibrium binding step, followed by a slow transformation (eq 1).[11] The expression for



$$E + \text{L-Trp} \overset{K_{eq}}{\rightleftharpoons} E_1\cdot\text{L-Trp} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} E_2\cdot\text{L-Trp} \qquad (1)$$

k_{obsd} as a function of [L-Trp] is given by eq 2, where k_f is the rate

$$1/\tau_1 = k_{obsd} = k_f[\text{L-Trp}]/(K_{eq} + [\text{L-Trp}]) + k_r \qquad (2)$$

of formation of the quinonoid intermediate and k_r is the rate of reprotonation.[11] Analysis of the data shown in Figure 2 by nonlinear least-squares fitting on eq 2 gives $k_f = 940 \pm 190$ s^{-1} and $k_r = 75 \pm 17$ s^{-1}, with $K_{eq} = 11.6 \pm 4.3$ mM. When α-[^{2}H]-L-tryptophan containing >98% deuterium is reacted, the first phase is significantly slowed (Figure 1A), the second phase is not significantly affected, and there is a small but reproducible effect on $1/\tau^3$ (compare open circles and open squares, Figure 2). Due to the lower absorbance at 506 nm and to the slower rate of formation of the quinonoid species, it was not possible to obtain reliable values of $1/\tau_1$ at low concentrations of α-[^{2}H]-L-tryptophan. On the basis of the data obtained at 6 and 10 mM, and assuming that the values of K_{eq} and k_r are identical for deuteriated and normal L-tryptophan, it is possible to estimate the intrinsic isotope effect for deprotonation of L-tryptophan as 3.6 ± 1.2. It should be noted that in previous steady-state kinetic experiments,

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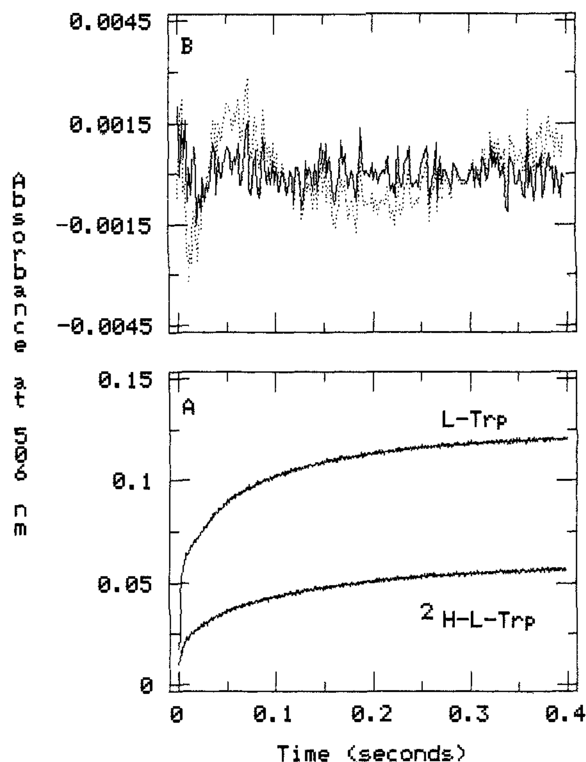

Figure 1. A: Progress curves in the reaction of 10 mM L-tryptophan and α-[^{2}H]-L-tryptophan with 8 μN tryptophan indole-lyase, followed at 506 nm. B: Residuals for the best two- (dotted line) and three-exponential (solid line) fits are presented.

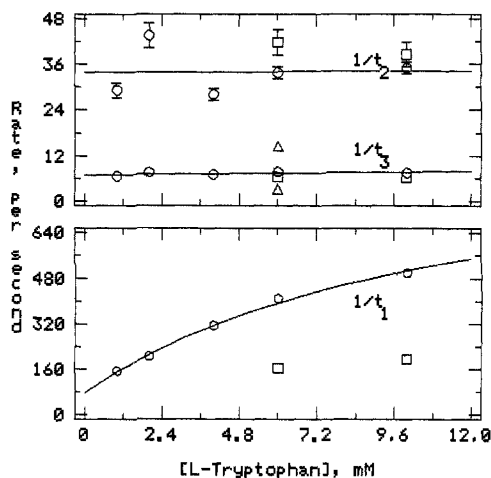

Figure 2. Dependence of the observed rate constants on L-tryptophan concentration. L-Tryptophan, open circles; α-[^{2}H]-L-tryptophan, open squares; L-Tryptophan in ^2H_2O, open triangles.

we observed $^DV = 2.5$ and $^D(V/K) = 2.8$.[12] When the reaction is performed with L-tryptophan in 99% ^2H_2O, the fast phase is unaffected, while $1/\tau_2$ and $1/\tau_3$ are dramatically slowed. These relaxations exhibit average values of 34 ± 5.5 s^{-1} and 7.3 ± 0.5 s^{-1} in H_2O. A solvent isotope effect of 2.5 ± 0.4 is observed for $1/\tau_2$, and $1/\tau_3$ shows an isotope effect of 2.7 ± 0.2 (see open triangles, Figure 2). No further changes were observed in a multiple kinetic isotope effect experiment (α-[^{2}H]-L-tryptophan in ^2H_2O).

The absorbance increase at 506 nm measured in static experiments ($\epsilon = 7.5 \times 10^3$ M^{-1} cm^{-1}) is identical with that observed in the first 500 ms in the pre-steady-state kinetic experiments. Furthermore, all three relaxations in the pre-steady-state are faster than steady-state turnover ($k_{cat} = 6.0$ s^{-1})[12] and are therefore "catalytically competent." At 10 mM L-tryptophan, the relative amplitudes of the three phases are 43.6 ± 0.7%, 18.0 ± 0.7%, and 38.4 ± 0.6%, respectively. On the basis of the steady-state absorbance at 506 nm, and using an estimated maximum ϵ for the quinonoid intermediate[9] of 4.0×10^4, we estimate that these three enzyme intermediates contribute 8.3%, 3.4%, and 7.2% of the steady-state total.

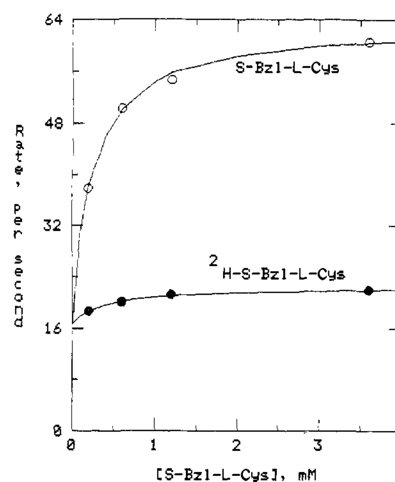

Figure 3. Dependence of the rate constant of the fast relaxation on the concentration of *S*-benzyl-L-cysteine (open circles) and α-[^{2}H]-*S*-benzyl-L-cysteine (filled circles).

7-Aza-DL-tryptophan. Preliminary studies with 7-aza-DL-tryptophan indicate that it is a very slow substrate, with $k_{cat} = 0.03$ s^{-1} and $K_m = 2.2$ mM. The very slow reaction is due to the 7-aza-DL-tryptophan and not a small amount of a reactive contaminant, since the K_I for competitive inhibition of tryptophan indole-lyase by 7-aza-DL-tryptophan, 2.2 mM, is identical with the observed K_m. Addition of 7-aza-DL-tryptophan to solutions of tryptophan indole-lyase also results in a absorption peak at 506 nm ($\epsilon = 1.74 \times 10^4$ M^{-1} cm^{-1}). When 20 mM 7-aza-DL-tryptophan is mixed with tryptophan indole-lyase under the conditions described in Figure 1, three relaxations are also observed, with apparent values of 138 ± 7, 34 ± 1, and 0.32 ± 0.01 s^{-1}.

***S*-Benzyl-L-cysteine.** *S*-Benzyl-L-cysteine is an excellent substrate for tryptophan indole-lyase,[13] with $k_{cat} = 5.2$ s^{-1}. When *S*-benzyl-L-cysteine is mixed with tryptophan indole-lyase, an intense peak at 512 nm results ($\epsilon = 2.6 \times 10^4$ M^{-1} cm^{-1}). When *S*-benzyl-L-cysteine is mixed with tryptophan in the stopped-flow instrument, there is a lag of 5–10 ms, followed by a rapid exponential process which accounts for ~60% of the total absorbance change. This rapid phase is concentration dependent and sensitive to substrate deuteriation (Figure 3), but is insensitive to ^2H_2O (data not shown). Fitting of the data in Figure 3 to eq 2 (as described above) allows us to evaluate the forward and reverse rate constants for both normal and deuteriated *S*-benzyl-L-cysteine. For *S*-benzyl-L-cysteine, $k_f = 47.6 \pm 4.7$ s^{-1} and $k_r = 15.7 \pm 5.0$ s^{-1}; for the deuteriated compound, $k_f = 6.0 \pm 1$ s^{-1} and $k_r = 16.7 \pm 1.2$ s^{-1}. The intrinsic isotope effect on the α-proton abstraction can be calculated from the ratio 47.6/6.0 = 7.9 ± 1.5.

With *S*-benzyl-L-cysteine, slower relaxations are also observed, with $k_{obsd} = 2.5$ s^{-1} (13% of the total amplitude) and 0.2 s^{-1} (27% of the total amplitude). Since these relaxations are slower than k_{cat}, they cannot be catalytically competent, but probably represent slow formation of abortive complexes. Thus, there appears to be only one essential step in the elimination of phenylmethanethiol from *S*-benzyl-L-cysteine catalyzed by tryptophan indole-lyase.

Discussion

The results reported by Vederas et al.,[14] which demonstrate that degradation of α-[^{2}H]-L-tryptophan by tryptophan indole-lyase produces indole enriched in deuterium at the 3-position (~10%), require that indolenine formation occurs subsequent to the for-

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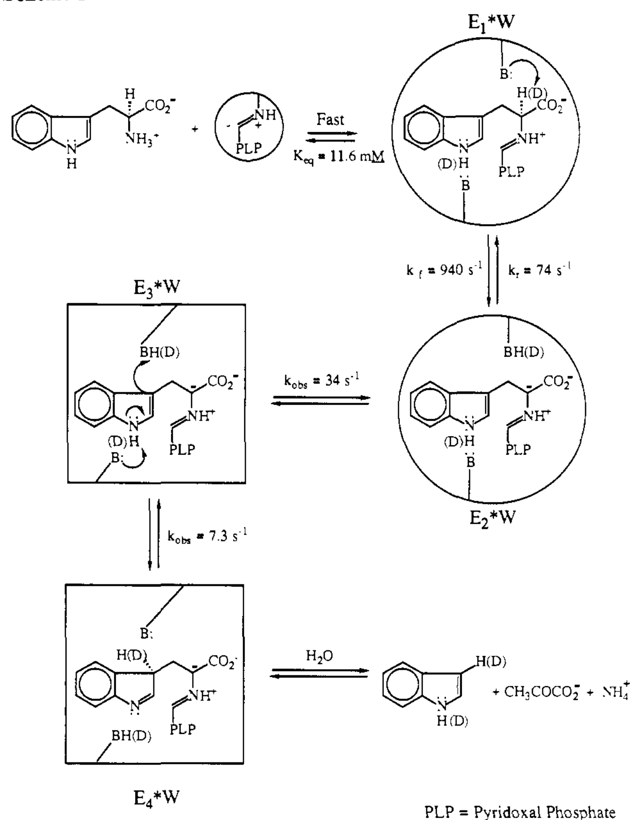
Scheme I



mation of the quinonoid intermediate. Thus, the minimal mechanism for the reaction catalyzed by tryptophan indole-lyase requires initial formation of the external aldimine of L-tryptophan with pyridoxal phosphate. Subsequent removal of the α-hydrogen forms the quinonoid intermediate which absorbs at 506 nm. Tautomerization of the indole ring would then give an intermediate which also absorbs at 506 nm. This reasoning predicts that the pre-steady-state kinetic progress curves followed at 506 nm should exhibit at least two relaxations, provided the rates of α-proton abstraction and indole tautomerization are sufficiently different. The data presented herein demonstrate that a minimum of three steps is required in the mechanism.

In our previous studies of the steady-state pH dependence of the reaction of L-tryptophan, we obtained evidence for two essential basic groups.[12] The base with pK_a = 7.6 is responsible for abstraction of the α-proton, while a base with pK_a = 6.0 interacts with the indole ring.[12] These results, together with the pre-steady-state kinetic data, allow us to propose the mechanism presented in Scheme I. The first relaxation process corresponds to formation of the quinonoid intermediate, E_2*W, from the external aldimine, E_1*W. This is consistent with the primary isotope effect observed with α-[^{2}H]-L-tryptophan and with the observed hyperbolic dependence of $1/\tau_1$ on L-tryptophan concentration. The rate constant for formation of the quinonoid intermediate from L-tryptophan (940 s^{-1}) is 20-fold that for S-benzyl-L-cysteine (47.6 s^{-1}), although both compounds are comparable substrates based on steady-state kinetic parameters. Thus, the structure of the leaving group has a dramatic influence on the reactivity of the substrate α-CH bond. Indeed, the rate constant for quinonoid formation from a nonreactive quasi-substrate, L-Ethionine (25.7 s^{-1}), is considerably slower.[9] Comparison of the rate constants and the isotope effects for reaction of L-tryptophan (3.6) and S-benzyl-L-cysteine (7.9) suggests that an earlier transition state obtains for the former. Abstraction of the α-proton of L-serine by the related enzyme, tryptophan synthase, exhibits a kinetic deuterium isotope effect of 4.6.[15] In addition, model transamination reactions catalyzed by pyridoxal phosphate were reported to exhibit kinetic isotope effects (k_H/k_D) of 3–7.[16]

The second relaxation process ($1/\tau_2$) is independent of L-tryptophan concentration, is unaffected by deuterium substitution on L-tryptophan, and thus may represent a conformational change of the quinonoid intermediate to give E_3*W. If this is the case, the solvent isotope effect observed on $1/\tau_2$ suggests that the enzyme conformational change is coupled to an ionization. There is ample precedent for solvent isotope effects on conformational changes of proteins in ^2H_2O.[17] The solvent isotope effects we observed in our previous steady-state kinetic studies with L-tryptophan are ^{D_2O}V = 3.8 and $^{D_2O}(V/K)$ = 2.8.[12] However, no solvent isotope effect on steady-state kinetics is seen with the nonphysiological substrate S-methyl-L-cysteine.[12] It is possible that the enzyme undergoes a conformational change to bring the acid catalyst from the α-carbon into proximity of the indole ring for subsequent proton transfer (Scheme I).

The slowest process ($1/\tau_3$) is independent of L-tryptophan concentration and is sensitive to solvent isotopic substitution. This suggests that $1/\tau_3$ represents the tautomerization of the indole to the reactive indolenine E_4*W, since this reaction would be affected by deuteriation of the indole NH. Since the α-proton is transferred to C-3 of the indole,[14] one might expect to see an isotope effect on this step with α-[^{2}H]-L-tryptophan. Although there is a consistent slight decrease in $1/\tau_3$ with α-[^{2}H]-L-tryptophan (see Figure 2), the apparent isotope effect is only 1.22 ± 0.05, consistent with the low deuterium transfer.[14] These data suggest that this base in Scheme I is polyprotic, probably a lysine ϵ-amino group. The stereochemistry at C-3 of the indolenine in E_4*W is presumed to be R, based on our previous studies of inhibitor stereospecificity.[6] Further support for the assignment of $1/\tau_3$ to the indole tautomerization is provided by our studies with the very slow substrate 7-aza-DL-tryptophan. Compared with indoles, the electron-withdrawing effect of the pyridine nitrogen dramatically reduces the reactivity of C-3 of the pyrrole ring of azaindoles with electrophiles.[18] Thus, the ring protonation step should be retarded with 7-azatryptophan, while the other steps should be relatively unaltered. As expected, the slow step, $1/\tau_3$, is reduced 23-fold with 7-aza-DL-tryptophan compared with L-tryptophan, while $1/\tau_2$ is unaffected and $1/\tau_1$ is reduced several-fold.

In addition, we have examined an alternate substrate, S-benzyl-L-cysteine. The mechanism for elimination of phenylmethanethiolate anion is greatly simplified, since protonation of the leaving group is unnecessary. We anticipated a single relaxation in the pre-steady-state experiments with this substrate, and our results indicate that there is only one catalytically competent process. Taken together, these results strongly support our assignment of the slowest relaxation in the pre-steady-state with L-tryptophan to the tautomerization of the indole ring. This tautomerization may occur via concerted "push–pull" general-acid–base catalysis, as shown in Scheme I. This conclusion is consistent with the results of our studies of the steady-state pH dependence and multiple kinetic isotope effects of tryptophan indole-lyase.[12] It should be noted that the rate of $1/\tau_3$, 7.3 s^{-1}, is in good agreement with the steady-state turnover number, k_{cat} = 6.0 s^{-1}, measured under the conditions of the pre-steady-state measurements.[12] Indole tautomerization may therefore be one of the rate-determining steps in the catalytic mechanism of tryptophan indole-lyase. It is likely that the subsequent carbon–carbon bond cleavage step occurs at a comparable rate, since the quinonoid intermediates accumulate. Experiments are now in progress to determine the contribution of carbon–carbon bond cleavage to this reaction.

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Conclusions

1. The elimination of indole from L-tryptophan catalyzed by tryptophan indole-lyase from *E. coli* requires both a protein conformational change as well as tautomerization of the indole ring, subsequent to α-carbanion formation.

2. Elimination of phenylmethanethiol from *S*-benzyl-L-cysteine requires only α-carbanion formation.

3. Deprotonation of the α-C–H of good substrates occurs much faster than steady-state turnover; estimated intrinsic isotope effects for α-proton abstraction are 3.6 for L-tryptophan and 7.9 for *S*-benzyl-L-cysteine.

Experimental Section

Materials. Tryptophan indole-lyase was purified from *E. coli* JM101 containing the *tnaA* gene on plasmid pMD6. This strain of *E. coli* produces >50% of the soluble protein as tryptophan indole-lyase when induced with 0.1% tryptophan. The enzyme was purified to homogeneity (single peak on reverse-phase HPLC analysis in 0.1% trifluoroacetic acid/acetonitrile) by hydrophobic interaction chromatography on Sepharose CL-4B[19] and exhibited a specific activity of 45 μmol/min mg with *S*-(*o*-nitrophenyl)-L-cysteine at pH 8 and 25 °C.[20] Catalytic turnover with L-tryptophan, 7-aza-DL-tryptophan, and *S*-benzyl-L-cysteine was measured by the coupled assay with lactate dehydrogenase and NADH at 340 nm.[21] Tryptophan synthase $\alpha_2\beta_2$ complex from *E. coli* was a generous gift from Dr. Edith Wilson Miles of the National Institutes of Health. α-[^{2}H]-L-Tryptophan was prepared by the reaction of *S*-methyl-L-cysteine and indole catalyzed by tryptophan indole-lyase in ^2H_2O, as previously described.[12] The α-[^{2}H]-L-tryptophan, isolated and crystallized in 70% yield, contained greater than 98% ^{2}H at the α-position by ^{1}H NMR analysis on a Bruker 250-MHz spectrometer. α-[^{2}H]-*S*-Benzyl-L-cysteine was prepared from phenylmethanethiol and L-serine by using tryptophan synthase[22] in ^2H_2O and contained greater than 98% ^{2}H at the α-position by ^{1}H NMR analysis. L-Tryptophan, 7-aza-DL-tryptophan, and *S*-benzyl-L-cysteine were obtained from United States Biochemical Corp. and were recrystallized from H_2O prior to use in kinetic experiments. Solutions of L-tryptophan and α-[^{2}H]-L-tryptophan were standardized from the absorbance at 278 nm ($\epsilon = 5.55 \times 10^3$ M^{-1} cm^{-1}).[23]

Instrumentation. The stopped-flow spectrophotometer is a custom-built instrument consisting of a stopped-flow mixing device from Kinetics Instruments, a converted Cary 14 UV/visible spectrophotometer (On Line Instruments Inc.) for the light source, and a Zenith Z-185 minicomputer equipped with hardware and software from On Line Instruments for data capture and analysis. The performance of the stopped-flow instrument was evaluated with the DCIP–ascorbic acid reaction. The rates obtained are linear up to at least 880 s^{-1} while the dead time is estimated at 1 ms, with the procedure of Nakatani and Hiromi.[24]

Reactions. Solutions of tryptophan indole-lyase in 20 mM potassium phosphate buffer, pH 8.0, containing 0.16 M KCl and 5 mM 2-mercaptoethanol, were mixed with varying amounts of L-tryptophan, 7-aza-DL-tryptophan, or *S*-benzyl-L-cysteine. The temperature of the stopped-flow instrument was maintained at 25 ± 0.5 °C by circulation of 30% ethylene glycol from an external water bath. Protein concentrations were determined from $A_{280} = 7.95$ for a 1% solution of apoenzyme[8] or by the dye-binding assay of Bradford.[25] The active subunit molecular weight was assumed to be 5.2×10^4 kDa.[26] Final enzyme concentrations were $(0.5–1) \times 10^{-5}$ N after mixing. For experiments in ^2H_2O, all solutions were prepared with 99.8% ^2H_2O (Aldrich, Gold Label), and the enzyme was exchanged by passage through a short desalting column equilibrated with ^2H_2O buffer. Typically, 800 data points were collected over a time period of 400 ms, with a photomultiplier time constant of 0.1 ms, and a 2-nm slit width. From four to six traces at each concentration were accumulated and averaged, and a blank trace obtained with buffer under identical conditions was subtracted. Curve fitting was then performed with a program utilizing a Levenberg–Marquardt algorithim. Quality of fit was evaluated from analysis of residuals (Figure 1B) and Durbin–Watson values.[27]

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Registry No. I, 73-22-3; II, 7303-50-6; III, 3054-01-1; $^2H_2$, 7782-39-0; tryptophan indole-lyase, 9024-00-4.

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