Reaction of Indole and Analogues with Amino Acid Complexes of *Escherichia coli* Tryptophan Indole-Lyase: Detection of a New Reaction Intermediate by Rapid-Scanning Stopped-Flow Spectrophotometry†

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ABSTRACT: The effects of indole and analogues on the reaction of *Escherichia coli* tryptophan indole-lyase (tryptophanase) with amino acid substrates and quasisubstrates have been studied by rapid-scanning and single-wavelength stopped-flow spectrophotometry. Indole binds rapidly (within the dead time of the stopped-flow instrument) to both the external aldimine and quinonoid complexes with L-alanine, and the absorbance of the quinonoid intermediate decreases in a subsequent slow relaxation. Indoline binds preferentially to the external aldimine complex with L-alanine, while benzimidazole binds selectively to the quinonoid complex of L-alanine. Indole and indoline do not significantly affect the spectrum of the quinonoid intermediates formed in the reaction of the enzyme with *S*-alkyl-L-cysteines, but benzimidazole causes a rapid decrease in the quinonoid peak at 512 nm and the appearance of a new peak at 345 nm. Benzimidazole also causes a rapid decrease in the quinonoid peak at 505 nm formed in the reaction with L-tryptophan and the appearance of a new absorbance peak at 345 nm. Furthermore, addition of benzimidazole to solutions of enzyme, potassium pyruvate, and ammonium chloride results in the formation of a similar absorption peak at 340 nm. This complex reacts rapidly with indole to form a quinonoid intermediate very similar to that formed from L-tryptophan. This new intermediate is formed faster than catalytic turnover (k_{cat} = 6.8 s^{-1}) and may be an α-aminoacrylate intermediate bound as a *gem*-diamine.

Tryptophan indole-lyase[1] (tryptophanase; EC 4.1.99.1) from enteric bacteria is a pyridoxal 5′-phosphate (PLP)[2] dependent enzyme which catalyzes the hydrolytic cleavage of L-tryptophan to yield indole, pyruvate, and ammonium (eq 1). The

$$\text{L-tryptophan} + H_2O \rightleftharpoons \text{indole} + \text{pyruvate} + \text{ammonium} \quad (1)$$

enzyme from *Escherichia coli* has been studied most extensively (Snell, 1975). This enzymatic β-elimination of a carbon leaving group is mechanistically interesting, since the indole ring must tautomerize in order for the elimination reaction to proceed (Davis & Metzler, 1972; Phillips et al., 1984, 1985). In support of this postulate, we demonstrated that analogues of the proposed indolenine intermediate are potent and stereospecific inhibitors of tryptophan indole-lyase as well as tryptophan synthase (Phillips et al., 1984, 1985). Recently, we have studied the steady-state (Kiick & Phillips, 1988) and pre-steady-state kinetics (Phillips, 1989) of the reaction of tryptophan indole-lyase with L-tryptophan and other substrates. These results demonstrated that a basic group with a pK_a of 7.8 is required for formation of the quinonoid α-carbanion and that the deprotonation is rate-limiting for quinonoid complex formation. More recently, we have used rapid-scanning stopped-flow spectrophotometry to study the mechanism of substrate analogue binding to tryptophan indole-lyase, which demonstrated that the heterocyclic NH is critical for rapid formation of aldimine, *gem*-diamine, and quinonoid intermediates (Phillips et al., 1990). However, these experiments provided no information about the subsequent elimination reaction. In the present study, we have examined the effects of indole and benzimidazole on the pre-steady-state kinetics of the reaction of tryptophan indole-lyase with the substrates *S*-alkyl-L-cysteines and L-tryptophan and a quasisubstrate, L-alanine. These results demonstrate the first direct measurement of the breakdown of the quinonoid intermediates in the reaction of substrates with tryptophan indole-lyase and provide a foundation for further studies directed toward the effects of variation in leaving group structure on the chemical steps in the β-elimination reaction.

Materials and Methods

Chemicals. L-Tryptophan, *S*-ethyl-L-cysteine, and *S*-benzyl-L-cysteine were purchased from U.S. Biochemical Corp. and were recrystallized from water or aqueous ethanol before use. *S*-Methyl-L-cysteine was purchased from ICN. Lactate dehydrogenase (from rabbit muscle, lyophilized) and NADH, disodium salt, were also obtained from U.S. Biochemical Corp. Indole (gold label; 99+%) and benzimidazole were obtained from Aldrich Chemical Co.; benzimidazole was recrystallized from hot water, after treatment with charcoal, before use. Indoline was from Chemalog and was distilled before use. *S*-(*o*-Nitrophenyl)-L-cysteine (SOPC) for enzyme assays was prepared as previously described (Phillips et al., 1989).

Enzyme and Assays. Tryptophan indole-lyase was purified as previously described (Phillips & Gollnick, 1989) from cells of *E. coli* JM101 containing plasmid pMD6, with the *tna*A gene under natural regulation (Deeley & Yanofsky, 1981). Routine activity assays were performed with SOPC (Suelter et al., 1975), following the decrease in absorbance at 370 nm (ϵ = −1860 M^{-1} cm^{-1}) in 0.1 M potassium phosphate, pH 8.0, at 25 °C. The activity of other substrates was measured with

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[1] Tryptophan indole-lyase (deaminating) (EC 4.1.99.1) is more commonly known by the trivial name of tryptophanase.

[2] Abbreviations: PLP, pyridoxal 5′-phosphate; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine.

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the lactate dehydrogenase coupled assay (Morino & Snell, 1970), following the decrease in absorbance at 340 nm (ϵ = −6220 M^{-1} cm^{-1}) in 0.1 M potassium phosphate, pH 8.0, at 25 °C. Enzyme concentrations were estimated from the 278-nm absorbance of the holoenzyme [$A^{1\%}$ = 9.19 (Phillips & Gollnick, 1989)].

Kinetic Measurements. Steady-state kinetic measurements were performed at 25 °C as described previously (Phillips & Gollnick, 1989). Prior to performing the rapid kinetic 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 room temperature, which was approximately 25 °C. Generally, the enzyme solutions were mixed with solutions of the amino acid substrates in the same buffer, with and without indole, indoline, or benzimidazole (all usually at 5 mM final concentration). In the case of the reactions with L-alanine, both the enzyme solution and the indole, indoline, or benzimidazole solution contained 0.25 M L-alanine, and control experiments without aromatic ligands were performed to ensure that no relaxation artifacts were observed due to concentration differences. Concentration dependencies for the effects of indole, indoline, and benzimidazole on the L-alanine complex were fit to either eq 2 (hyperbolic increase) or eq 3 (hyperbolic decrease) by

$$k_{obs} = k_f[L]/(K_d + [L]) + k_r \quad (2)$$

$$k_{obs} = k_f K_d/(K_d + [L]) + k_r \quad (3)$$

using a nonlinear least-squares program, ENZFITTER, from Elsevier Biosoft, where k_f is the rate constant for the forward reaction, k_r is the rate constant for the reverse reaction, and K_d is the apparent equilibrium constant for the rapid binding equilibrium (Strickland & Massey, 1975). Steady-state kinetic parameters were determined by using the programs of Cleland (1970), HYPER for K_m and k_{cat} determination and UNCOMP for uncompetitive inhibition by benzimidazole.

Instrumentation. Static spectra and steady-state kinetic measurements were performed on a Gilford Response II UV/vis spectrophotometer, equipped with a Peltier-type thermoelectric cell block for temperature control. The instrument used for single-wavelength stopped-flow measurements has been described previously (Phillips, 1989). Rapid-scanning experiments were performed by using a Kinetics Instruments stopped-flow mixer, passing the lamp output from a 150-W quartz–halogen lamp through a fiber optic light guide into the observation cell (20-mm path length), and focusing the exit beam onto the entrance slit of a Jarrell-Ash spectrograph. The spectra were collected by an EG&G Princeton Applied Research Model 1451 512-element photodiode array, using a Model 1461 detector interface, with a 1462 detector controller and a 14-bit analog to digital converter. Spectra were collected with an acquisition time of either 10 or 15 ms. The raw data were transmitted to a Zenith Z-286 personal computer for handling, storage, and analysis, by using the PCOMA software supplied by Princeton Applied Research.

RESULTS

L-Alanine Complex. Indole binds to the complex of tryptophan indole-lyase with L-alanine, resulting in a change in the relative amounts of external aldimine and quinonoid intermediates (Kazarinoff & Snell, 1980). We have reexamined this reaction using rapid-scanning stopped-flow spectrophotometry, with the results shown in Figure 1A. There is a rapid decrease in the absorbance at 501 nm, which is completed

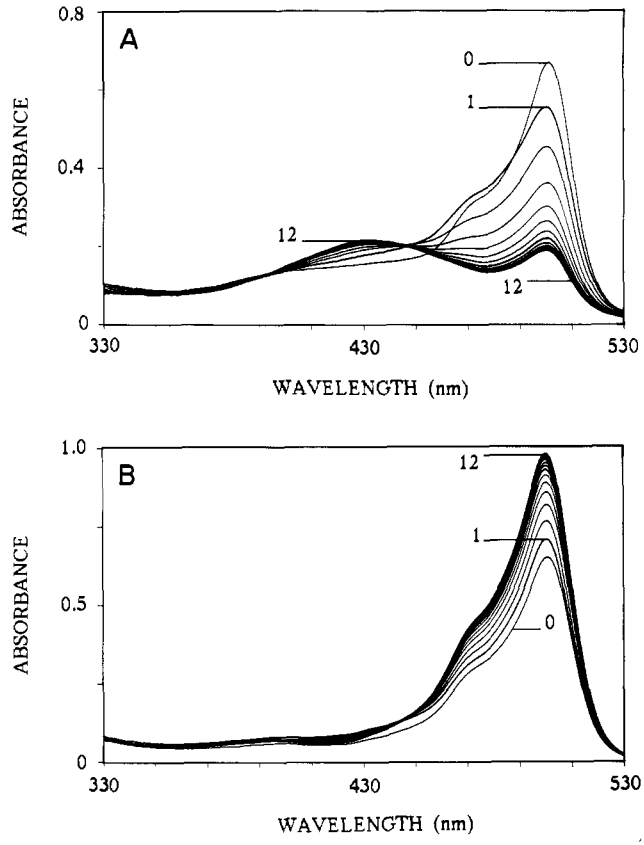


FIGURE 1: Rapid-scanning data for the reactions of the tryptophan indole-lyase complex with L-alanine. (A) Reaction of the L-alanine complex (17.2 μM) with 5 mM indole. Scans were taken at 0.015 s (curve 1), 0.0925 s (curve 2), 0.17 s (curve 3), 0.2475 s (curve 4), 0.325 s (curve 4), 0.4025 s (curve 5), 0.48 s (curve 6), 0.5575 s (curve 7), 0.635 s (curve 8), 0.7125 s (curve 9), 0.790 s (curve 10), 0.8675 s (curve 11), and 0.945 s (curve 12). Curve 0 is the spectrum of the enzyme complex without indole present. (B) Reaction of the L-alanine complex (17.2 μM) with 5 mM benzimidazole. Scans were taken at 0.015 s (curve 1), 0.39 s (curve 2), 0.765 s (curve 3), 1.14 s (curve 4), 1.515 s (curve 5), 1.890 s (curve 6), 2.265 s (curve 7), 2.640 s (curve 8), 3.015 s (curve 9), 3.390 s (curve 10), 3.765 s (curve 11), and 4.140 s (curve 12). Curve 0 is the spectrum of the enzyme complex without benzimidazole present.

before the first scan is collected (compare curves 0 and 1 in Figure 1A). Subsequently, there is a slow relaxation resulting in a further decrease in the 501-nm peak and concommitant increase at 420 nm, with a good isosbestic point at about 446 nm, indicating that there are no other kinetically important intermediates. Other indole analogues were tested; indazole, indoline, and benzo[*b*]thiophene exhibited spectral changes similar to those produced by indole. However, benzimidazole was found to cause an increase in the intensity of the quinonoid peak, as shown in Figure 1B. In contrast to indole, benzimidazole causes a rapid initial increase in absorbance, which is completed before the first scan (compare curves 0 and 1 in Figure 1B), followed by a slower relaxation between the 501-nm peak and the 420-nm peak, with a good isosbestic point at about 446 nm.

The mechanism of the binding of indole, indoline, and benzimidazole was then examined by single-wavelength stopped-flow methods. In agreement with the rapid-scanning data, when the enzyme–L-alanine complex is mixed with equimolar L-alanine containing indole, there is a rapid decrease in absorbance at 501 nm which is completed in the dead time (ca. 1 ms) of the stopped-flow instrument. Thus, the binding of indole to the enzyme–L-alanine complex is evidently extremely rapid, with an estimated rate constant of $\geq 6.9 \times 10^6$

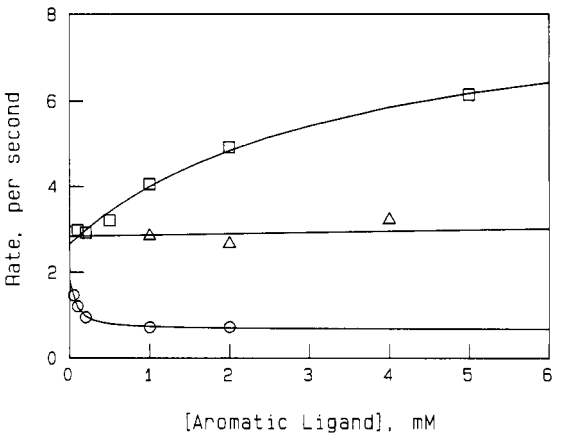
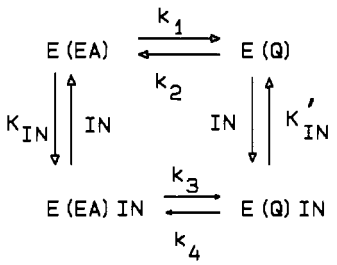


FIGURE 2: Dependence of the relaxations of the L-alanine complex on the concentration of indole or benzimidazole. Open squares: Effect of indole. The line is the calculated curve for fitting of the data to eq 2, with the parameter values given in the text. Open circles: Effect of benzimidazole. The line is the calculated curve for fitting of the data to eq 3, with the parameter values given in the text. Open triangles: Effect of indoline.

Scheme I[a]



[a] E(EA) = external aldimine of L-alanine. E(Q) = quinonoid complex of L-alanine. IN = indole.

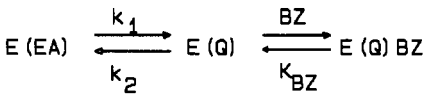
M^{-1} s^{-1}, and is likely to be diffusion-controlled.[3] The subsequent relaxation of the indole–L-alanine complex fits well to a single exponential process, although sometimes slightly better fits were obtained by adding a second exponential process, which usually was less than 10% of the amplitude of the first. The rate constant, k_{obs}, was found to increase in a hyperbolic manner with changing [indole], as shown in Figure 2 (open squares). These data are consistent with a mechanism involving rapid binding of indole to both the quinonoid complex and the external aldimine, followed by a slower relaxation between the complexes, shown in Scheme I. The rate law expected for this mechanism is shown in eq 4 (Bernasconi,

$$1/\tau = k_{obs} = k_1\{K_I/([In] + K_I)\} + k_2\{K_I'/([In] + K_I')\} + k_3\{[In]/([In] + K_I)\} + k_4\{[In]/([In] + K_I')\} \quad (4)$$

1986). The intercept at [indole] = 0 is equal to the rate of interconversion of the external aldimine and quinonoid complexes ($k_1 + k_2$), which can be measured independently by mixing the enzyme with L-alanine and following the absorbance increase at 501 nm. This rate constant was reported by June et al. (1980) to be 2.8 $s1^{-1}$, and we recently reported a very similar value (2.7 s^{-1}) (Phillips & Gollnick, 1989), in excellent agreement with the intercept in Figure 2 (2.6 s^{-1}). This mechanism predicts that the plateau value of k_{obs} at infinite [indole], 5.9 s^{-1}, is that for interconversion of the

[3] The rate constant is estimated by using the dead time of the stopped-flow instrument (ca. 1 ms) as an upper limit for the $t_{1/2}$ for the indole binding reaction at 0.1 mM indole, which was the lowest concentration studied. Thus: $k \geq 0.693/(1 \times 10^{-3}\ s)(1 \times 10^{-4}\ M) \geq 6.93 \times 10^6\ M^{-1}\ s^{-1}$.

Scheme II[a]



[a] E(EA) = external aldimine of L-alanine. E(Q) = quinonoid complex of L-alanine. BZ = benzimidazole.

Table I: Steady-State and Pre-Steady-State Kinetic Parameters for *S*-Alkyl-L-cysteines

R	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	k_f (s^{-1})	k_r (s^{-1})	K_d (M)
Me	6	3.0×10^2	43	24	0.22
Et	6	9.0×10^3	60	15	0.017
Bzl	5.2	8.0×10^4	48	16	0.0003

quinonoid and external aldimine complexes with bound indole ($k_3 + k_4$). The binding constant for indole, from fitting of the data in Figure 2 to eq 2, is 3.4 mM; however, this apparent constant is a composite of binding constants for both the external aldimine (K_{IN}) and quinonoid complexes (K_{IN}'):

$$K_{app} = K_{IN}K_{IN}'/(K_{IN} + K_{IN}') \quad (5)$$

The progress curves of the reaction of the enzyme–L-alanine complex with indoline at 501 nm are distinctly biphasic, with similar amplitudes for both phases. In contrast to the results obtained with indole, the rate constant of the fast process is unaffected by [indoline] and exhibits an average value of 2.9 s^{-1} (Figure 2, open triangles). The second phase increases in rate with increasing [indoline], with an apparent binding constant for indoline of 4.95 mM from fitting to eq 2 (data not shown). Furthermore, indoline does not cause any immediate absorbance change at 501 nm upon mixing. Thus, these data are consistent with the binding of indoline exclusively to the external aldimine in the fast phase, which represents the relaxation of the free external aldimine and quinonoid complexes. The slow phase may be an isomerization of the external aldimine–indoline complex.

The reaction of the enzyme–L-alanine complex with benzimidazole also exhibits biphasic kinetics, with the fast phase accounting from 80–90% of the absorbance change. In contrast to the results obtained with indole and indoline, the rate constant for the fast phase decreases with increasing [benzimidazole] (Figure 2, open circles). This behavior is diagnostic for a mechanism in which a slow relaxation of the free enzyme is followed by rapid ligand binding (Bernasconi, 1986). Thus, these data imply that benzimidazole binds only to the L-alanine quinonoid complex, but not to the external aldimine (Scheme II). As with the indole system, the intercept should give the value for the relaxation of the external aldimine and quinonoid complexes of L-alanine ($k_1 + k_2$). The calculated value, 1.8 s^{-1}, is in reasonable agreement with the expected value of 2.7 s^{-1}. The plateau value at high [benzimidazole], 0.8 s^{-1}, is equal to k_1, the rate constant for deprotonation of the external aldimine of L-alanine. The binding of benzimidazole to the L-alanine quinonoid complex is much stronger than that of indole or indoline, since the data fit to eq 3 with an apparent K_d of 40 μM.

S-Alkyl-L-cysteines. *S*-Alkyl-L-cysteines are good substrates for tryptophan indole-lyase (Morino & Snell, 1967; Watanabe & Snell, 1977) and exhibit prominent quinonoid bands absorbing at about 512 nm under steady-state conditions (Morino & Snell, 1967; Watanabe & Snell, 1977; Phillips, 1989), as shown in Figure 3A. We have previously reported steady-state kinetic parameters for *S*-methyl-L-cysteine (Kiick & Phillips, 1988) and pre-steady-state kinetic parameters for *S*-benzyl-L-cysteine (Phillips, 1989). We have now measured the

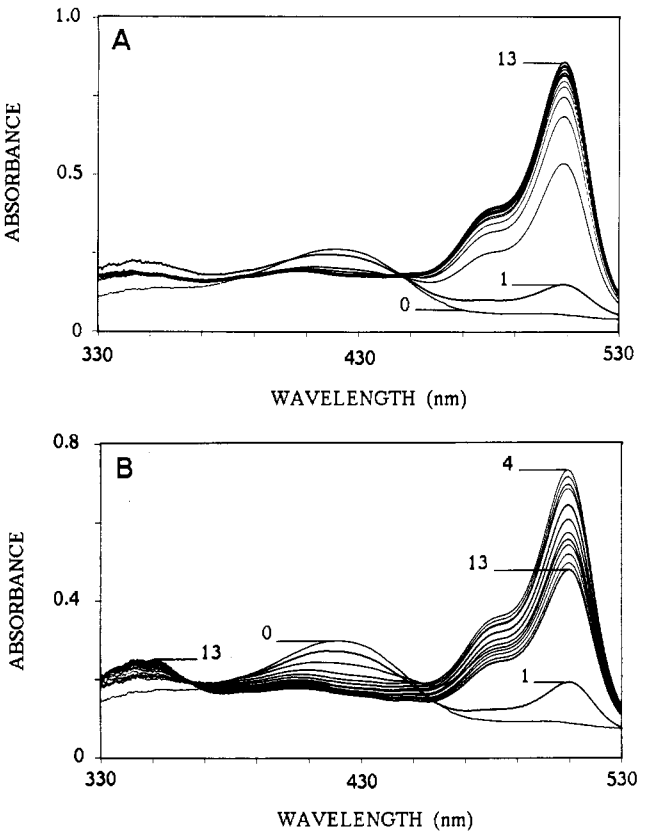


FIGURE 3: (A) Tryptophan indole-lyase (20.9 μM) was mixed with 20 mM *S*-ethyl-L-cysteine. Scans were collected at 0.010 s (curve 1), 0.0310 s (curve 2), 0.0520 s (curve 3), 0.0730 s (curve 4), 0.0940 s (curve 5), 0.1150 s (curve 6), 0.1360 s (curve 7), 0.1570 s (curve 8), 0.178 s (curve 9), 0.1990 s (curve 10), 0.2200 s (curve 11), 0.2410 s (curve 12), and 0.2620 s (curve 13). Curve 0 is the spectrum of the enzyme solution without *S*-ethyl-L-cysteine. (B) Tryptophan indole-lyase (20.9 μM) was mixed with 20 mM *S*-ethyl-L-cysteine and 5 mM benzimidazole. Scans were collected at the same times as in (A).

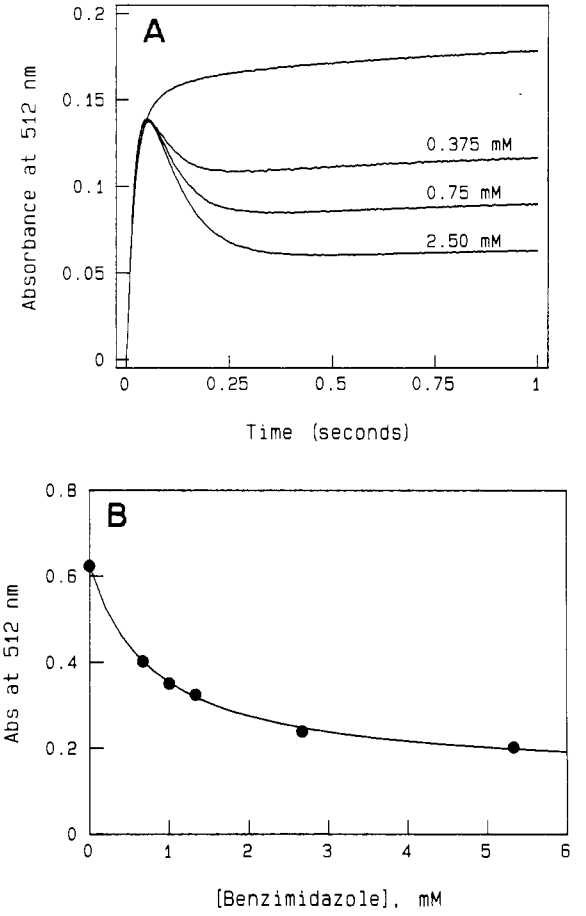


FIGURE 4: (A) Progress curves at 512 nm in the reaction of tryptophan indole-lyase with 20 mM *S*-ethyl-L-cysteine and varying amounts of benzimidazole, as indicated above the respective curves. Two hundred data points were collected for each curve, with a time constant of 1 ms and 2-nm bandwidth. (B) Replot of the steady-state absorbance at 512 nm of the tryptophan indole-lyase complex in the presence of varying amounts of benzimidazole. The solid line is the calculated fit to the equation: $A_{obs} = A_{max}\{K_d/(K_d + [\text{benzimidazole}])\} + A_{min}$, where A_{max} is the absorbance in the absence of benzimidazole and A_{min} is the value in the presence of an infinite concentration of benzimidazole. The value of K_d from these data is 0.74 mM.

steady-state and pre-steady-state kinetic parameters for *S*-methyl-, *S*-ethyl-, and *S*-benzyl-L-cysteines (Table I). The size of the side chain does not have any significant effect on k_{cat} or the rate constants for α-deprotonation (k_f) or reprotonation (k_r) but is reflected in enhanced equilibria (K_d) for external aldimine formation (Table I). The large decrease in the steady-state k_{cat}/K_m for the lower homologues (Table I) is apparently due primarily to the weaker binding constants for external aldimine formation. In static spectroscopic experiments, the presence of indole, indazole, and indoline were found to have no significant effect on the intensity of the quinonoid peak at 512 nm, but benzimidazole significantly decreases the intensity of the *S*-ethyl- and *S*-benzyl-L-cysteine quinonoid complexes. Rapid-scanning studies of this reaction show that the quinonoid peak forms rapidly (Figure 3A) and then slowly decays to a lower steady-state level in the presence of benzimidazole (Figure 3B). A new peak, absorbing at 345 nm, forms concommitant with the decline at 512 nm, with a good isosbestic point at 362 nm, indicating that the two species interconvert directly. The spectra in Figure 3 were obtained in buffer containing 0.2 M K^+; similar results were obtained in buffer containing 0.2 M NH_4^+ and Rb^+. However, in the presence of 0.2 M Na^+, the rate of formation and the intensity of the 345-nm peak were dramatically reduced (data not shown). This result is consistent with the known requirement of tryptophan indole-lyase (K^+, NH_4^+, and Rb^+, but not Na^+) for monovalent cations for catalytic activity (Högberg-Raibaud et al., 1975; Suelter & Snell, 1977).

Single-wavelength experiments were then performed at 512 nm to determine the rate constant for the decay of the quinonoid intermediate and the effect of [benzimidazole] on the rate. The rate constant for the decay of the quinonoid intermediate derived from *S*-ethyl-L-cysteine (6 s^{-1}) is unaffected by variation in [benzimidazole], but the amplitude is concentration dependent, as shown in Figure 4A. A replot of the absorbance change against [benzimidazole] exhibits saturation, with an apparent K_d of 0.81 ± 0.06 mM (Figure 4B). Benzimidazole was examined as an inhibitor of the steady-state β-elimination reaction of *S*-ethyl-L-cysteine, and it was found to be an uncompetitive inhibitor of pyruvate formation, with a K_i of 0.74 ± 0.05 mM (data not shown). This is the inhibition pattern expected if benzimidazole binds only to a form of the enzyme–substrate complex, and not to the free enzyme (Cleland, 1964). Data very similar to those presented in Figures 3 and 4 were obtained with *S*-benzyl-L-cysteine. However, the decay of the quinonoid intermediate derived from *S*-benzyl-L-cysteine in the presence of benzimidazole is somewhat faster, with a rate constant of 10 s^{-1}.

L-Tryptophan. When tryptophan indole-lyase is mixed with L-tryptophan, a quinonoid peak at 505 nm forms (Figure 5A); this absorbance increase is triphasic (Phillips, 1989). Rapid-scanning spectra of the reaction with 10 mM L-tryptophan

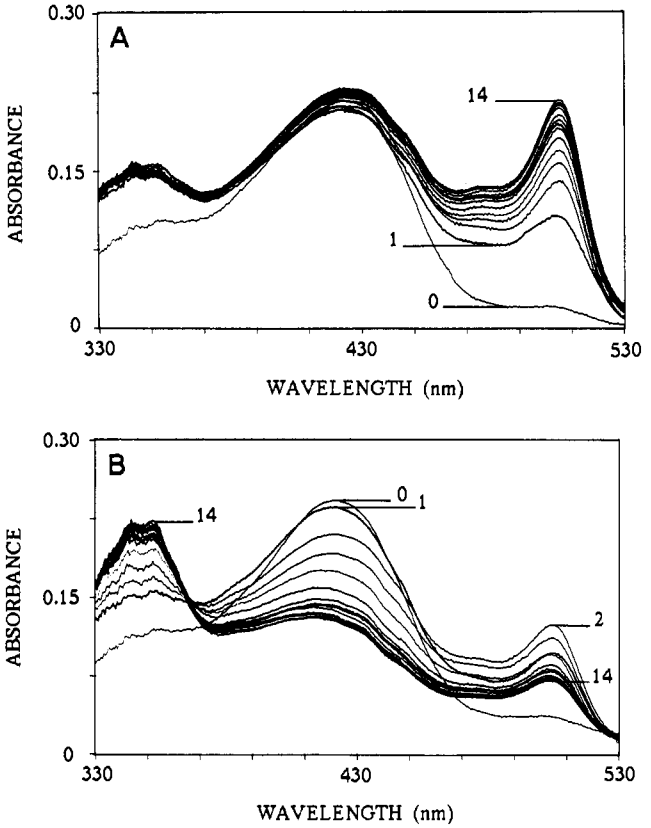


FIGURE 5: (A) Reaction of tryptophan indole-lyase (25.5 μM) with 10 mM L-tryptophan. Scans were collected at 0.0100 s (curve 1), 0.0205 s (curve 2), 0.0310 s (curve 3), 0.0415 s (curve 4), 0.0520 s (curve 5), 0.0625 s (curve 6), 0.0730 s (curve 7), 0.0835 s (curve 8), 0.0940 s (curve 9), 0.1045 s (curve 10), 0.1150 s (curve 11), 0.1255 s (curve 12), 0.1360 (curve 13), and 0.1465 s (curve 14). Curve 0 is the spectrum of the enzyme in the absence of L-tryptophan. (B) Reaction of tryptophan indole-lyase (25.5 μM) with 10 mM L-tryptophan and 5 mM benzimidazole. Scans were collected at the same times as in (A).

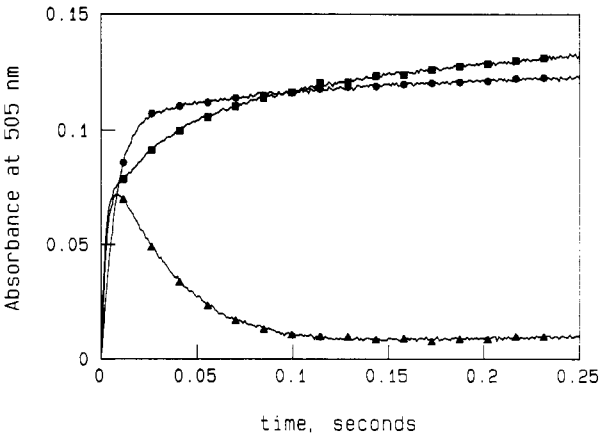


FIGURE 6: Time courses at 505 nm for the reaction of tryptophan indole-lyase with L-tryptophan, and with indole and benzimidazole. A total of 256 data points were collected for each curve, with a time constant of 0.1 ms and 2-nm bandwidth. Squares: Reaction with 10 mM L-tryptophan. Triangles: Reaction with 10 mM L-tryptophan and 5 mM benzimidazole. Circles: Reaction with 10 mM L-tryptophan and 5 mM indole.

are consistent with our previous conclusion that the second phase of quinonoid complex formation is a conversion of the first quinonoid species, since there is no isosbestic point of the 505-nm intermediate with the aldimine peak at 425 nm (Figure 5A). The absorbance at 425 nm decreases initially and then increases along with the second phase of formation of the quinonoid band. When tryptophan indole-lyase is mixed with 10 mM L-tryptophan and 5 mM benzimidazole, the quinonoid peak at 505 nm forms rapidly (first two spectra in Figure 5B) and then diminishes, concommitant with formation of a new peak at about 345 nm (Figure 5B) and with a good isosbestic point at 365 nm. The 425-nm peak continues to decrease as the 345-nm peak increases. In contrast, the presence of 5 mM indole results in only slight differences in the rapid-scanning spectra (data not shown), most notably, smaller absorbance increases below 450 nm.

Single-wavelength studies were then performed at 505 and 345 nm to obtain quantitative data on the effects of benzimidazole and indole. The decrease at 505 nm (Figure 6, triangles) and the increase at 345 nm (data not shown) produced by benzimidazole exhibit identical rate constants (32 s^{-1}), which are also the same as the second phase in the reaction with L-tryptophan (Figure 6, squares). When the time course of the reaction with L-tryptophan was measured in the presence of indole, it was found that the presence of indole results in an increase in the rate of the second phase of quinonoid intermediate formation, without significantly affecting the steady-state amplitude (Figure 6, circles). Although the data presented in Figure 6 were obtained in the presence of 5 mM indole, this effect of indole is also observed at stoichiometric [indole], indicating strong and specific binding to the enzyme intermediate. It should be noted that indole is a potent inhibitor of the steady-state β-elimination reaction catalyzed by tryptophan indole-lyase, with a reported K_i of 10 μM (Kazarinoff & Snell, 1980).

Reverse Reaction. Addition of tryptophan indole-lyase to solutions of indole, potassium pyruvate, and ammonium chloride results in synthesis of L-tryptophan (Watanabe & Snell, 1972; Nakazawa et al., 1972). Under these conditions, a quinonoid intermediate with a peak at 505 nm is observed, but it is less prominent than that seen in the direction of L-tryptophan degradation. We observe similar results in static spectra of solutions of enzyme, potassium pyruvate, ammonium chloride, and indole (Figure 7A, solid line with squares). However, when benzimidazole is added to this solution in place of indole, a peak at 340 nm is formed, which is stable for at least an hour (Figure 7A, dotted line with circles). When the enzyme complex with pyruvate and ammonium ions and benzimidazole is mixed with indole, a prominent quinonoid peak at 505 nm is formed within 10 ms, as shown in Figure 7B (solid line). The intensity of this quinonoid peak decays rapidly to a much lower value within 60 ms. In contrast, the enzyme complex with pyruvate and ammonium alone forms a very weak quinonoid peak under these conditions (Figure 7B, dotted line). Thus, these results suggest that the enzyme complex of pyruvate and ammonium to which benzimidazole binds can react rapidly with indole to form a covalent bond.

Discussion

Kazarinoff and Snell (1980) reported that indole and other small aromatic compounds (skatole, toluene) bind to complexes of tryptophan indole-lyase with amino acids with small side chains (glycine, L-alanine, β-cyanoalanine, and α-aminobutyrate), but not those with larger side chains (L-ethionine). In contrast, Kazarinoff and Snell (1980) could not detect binding of indole to the free enzyme; thus, they concluded that the indole binding site is inaccessable unless an amino acid in bound. We have examined the binding of indole and analogues to the L-alanine complex of tryptophan indole-lyase by rapid-scanning and single-wavelength stopped-flow kinetic techniques. Our data demonstrate that the binding of indole and benzimidazole to the quinonoid and external aldimine

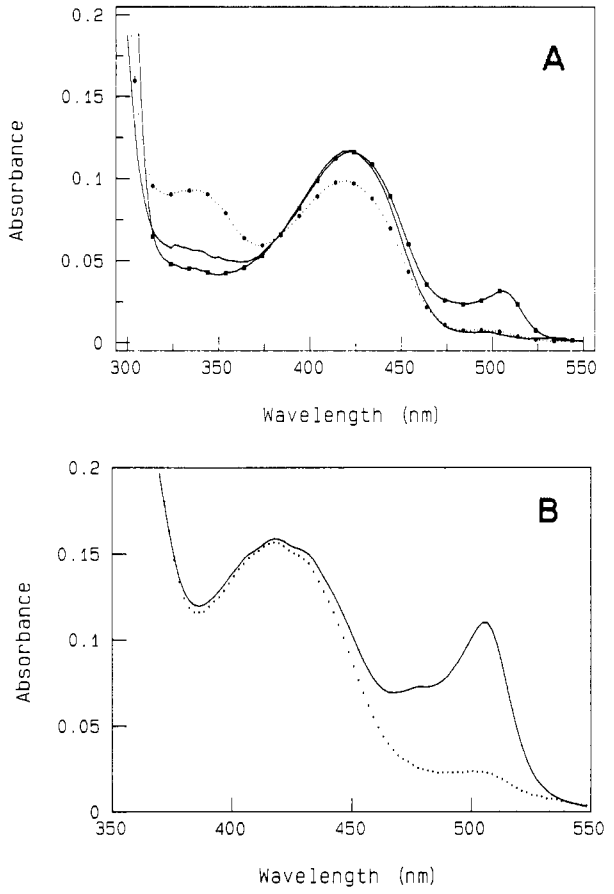
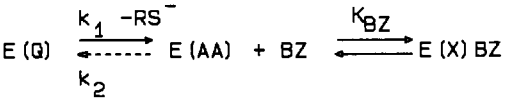


FIGURE 7: (A) Spectra of 19.5 μM tryptophan indole-lyase in 0.1 M triethanolammonium phosphate, pH 8, in the presence of 20 mM potassium pyruvate and 0.2 M NH_4Cl. Solid line: No further addition. Solid line with filled squares: Addition of 1 mM indole. Dotted line with filled circles: Addition of 5 mM benzimidazole. (B) Transient spectra of 24.0 μM tryptophan indole-lyase in 0.1 M triethanolammonium phosphate, pH 8, containing 20 mM potassium pyruvate and 0.2 M NH_4Cl. Solid line: Spectrum 10 ms after mixing enzyme in the buffer containing 5 mM benzimidazole with the buffer containing 5 mM indole (final concentration). Dotted line: Spectrum 10 ms after mixing enzyme in the buffer with buffer containing 5 mM indole.

complexes of L-alanine is very rapid and that subsequent relaxation between the enzyme complexes is slow. It is interesting that indole binds to both intermediates, but most strongly to the external aldimine complex of L-alanine, as previously found by Kazarinoff and Snell (1980), while benzimidazole, an isoelectronic and isosteric analogue, binds strongly and specifically to the quinonoid complex of L-alanine. These results suggest that the binding or release of indole will not be a rate-determining step in the reaction of L-tryptophan catalyzed by the enzyme.

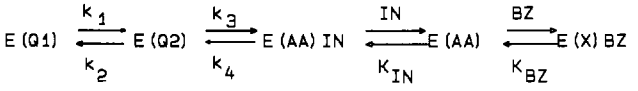
Indole does not significantly affect the spectra of the *S*-ethyl- or *S*-benzyl-L-cysteine quinonoid intermediates with tryptophan indole-lyase, even though L-tryptophan is synthesized under similar conditions. This implies that the steps occurring after the quinonoid intermediate are very fast and that the breakdown of the quinonoid intermediate (i.e., the β-elimination of the thiolate ion) must be rate-determining in the reaction of these substrates. Further support for this conclusion is provided by our finding that benzimidazole binds to a subsequent intermediate, allowing direct observation of the quinonoid intermediate decay (6–10 s^{-1}). These rate constants are in good agreement with the steady-state k_{cat} values for the *S*-alkyl-L-cysteines (Table I). The mechanism for elimination of the thiolate anion from these quinonoid intermediates is

Scheme III[a]



[a] E(Q) = quinonoid intermediate. E(AA) = aminoacrylate intermediate. E(X) = 345-nm intermediate. BZ = benzimidazole.

Scheme IV[a]



[a] E(Q1) = first quinonoid intermediate. E(Q2) = second quinonoid intermediate. E(AA) = aminoacrylate intermediate. E(X) = 345-nm intermediate. IN = indole.

simple (Scheme III), since no activation of the leaving group is required. Because these eliminations of *S*-alkyl-L-cysteines are not readily reversible ($k_2 \approx 0$) (Watanabe & Snell, 1977), the relaxation should be given by

$$1/\tau = k_1 \quad (6)$$

This equation predicts that [benzimidazole] will have no effect on the rate of quinonoid intermediate decay, as observed (Figure 4A).

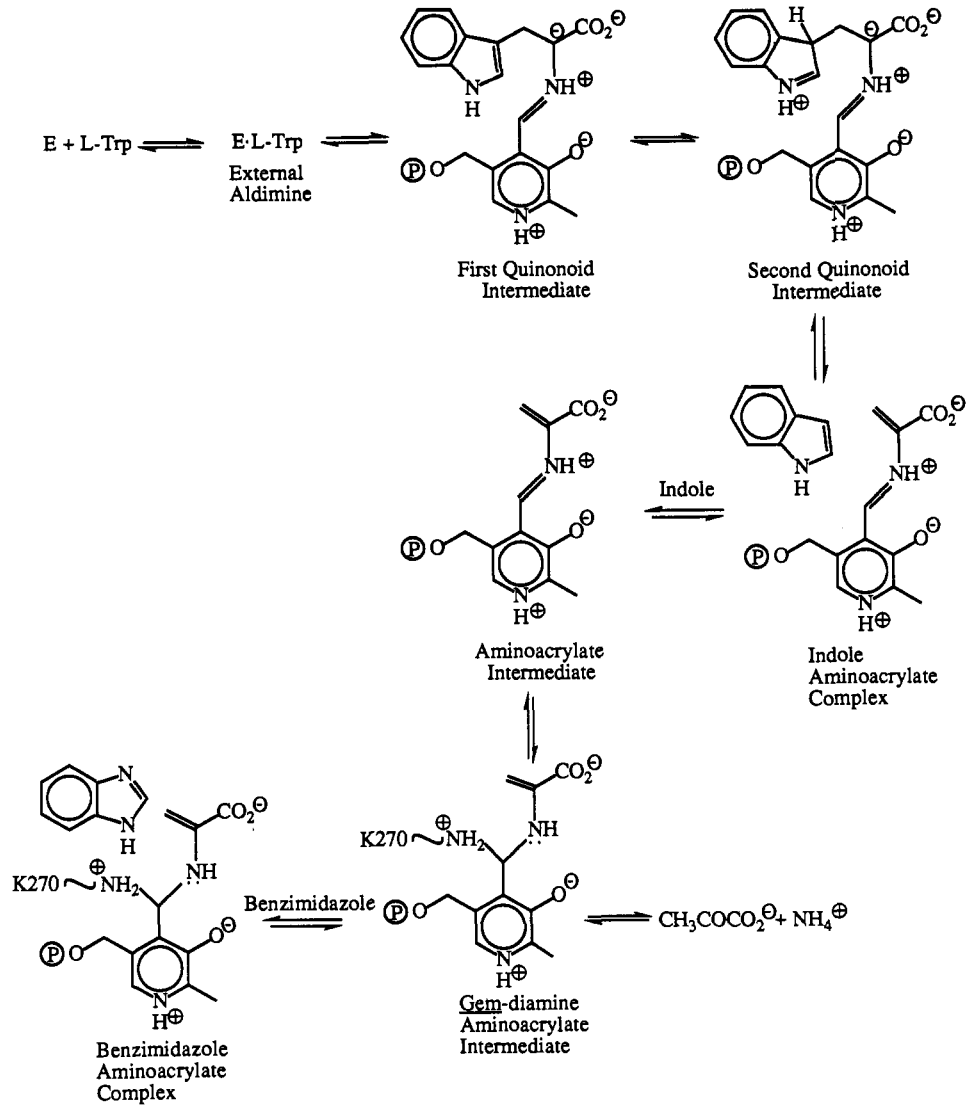
Benzimidazole has a similar effect on the reaction of L-tryptophan; however, the decline of the quinonoid peak occurs with a significantly faster rate constant (32 s^{-1}) than do those derived from the *S*-alkyl-L-cysteines. The formation of the quinonoid intermediate in the reaction with L-tryptophan occurs in three phases (Phillips, 1989). The decrease in absorbance in the presence of benzimidazole is identical in rate with that for the second phase previously observed (Phillips, 1989). On the basis of solvent and substrate primary kinetic isotope effects, we demonstrated that the first phase of quinonoid intermediate formation is α-deprotonation and we suggested that the second phase in the pre steady state is a conformational change of the enzyme to allow subsequent proton transfer to the indole ring. However, the current data suggest that the second phase may be the elimination of indole. Why is there an increase in absorbance at 505 nm, presumably due to the formation of a quinonoid intermediate, if this process is the indole elimination? This is possible if the protein conformational change, indole tautomerization, and the C–C bond cleavage are tightly coupled and the internal equilibrium between the second quinonoid intermediate and the α-aminoacrylate intermediate lies in favor of the activated tryptophan intermediate (Scheme IV). The relaxation equations for this coupled system are complex and are most conveniently expressed as the sum and product of the following two predicted relaxations (Bernasconi, 1986):

$$1/\tau_1 + 1/\tau_2 = k_1 + k_2 + k_3 + k_4([\text{indole}]/(K_{In} + [\text{indole}])) \quad (7)$$

$$(1/\tau_1)(1/\tau_2) = k_1k_3 + (k_1 + k_2)(k_4)\{[\text{indole}]/(K_{In} + [\text{indole}])\} \quad (8)$$

The rapid binding of benzimidazole would then displace the position of the equilibrium to the intermediate after C–C bond cleavage, and the terms containing [indole] would be eliminated. However, the relaxation rates for the decrease in absorbance with benzimidazole and the increase in absorbance without benzimidazole would be similar when [indole] is negligible, as in the initial stage of the reaction with L-tryptophan. These equations predict that the rate constant for the second phase of quinonoid intermediate formation should be *increased* by addition of indole. Indeed, we have found that

Scheme V



this is the case (see Figure 6). Thus, we believe that these data are the first direct observation of the elimination event in a tryptophan indole-lyase β-elimination reaction. The use of benzimidazole should allow us to determine substituent effects on the β-elimination reaction without interference from prior binding or subsequent product release steps.

In the light of these new results, the role of the third phase in the pre steady state (Phillips, 1989) is unclear. However, since the second phase appears to be too fast (32 s^{-1}) to be rate-determining in the steady-state reaction [k_{cat} = 6.8 s^{-1} (Kiick & Phillips, 1988; Phillips & Gollnick, 1989)], it is possible that the third phase is the second relaxation ($1/\tau_2$) predicted for the coupled reaction between the first quinonoid intermediate and the elimination reaction (Scheme IV).

What is the structure of the 345-nm intermediate to which benzimidazole binds? The rapid formation of this intermediate and the effect of indole on its formation are consistent with a catalytic role, rather than an abortive complex. The product expected after the elimination of indole is the PLP–Schiff's base of α-aminoacrylate. However, this species should exhibit a broad absorption maximum at about 455 nm, on the basis of the spectrum of a transient intermediate seen in the reaction of D-serine dehydratase with D-serine (Schnakertz et al., 1979). We do observe a significant absorbance increase below 450 nm during the second phase of quinonoid intermediate formation with L-tryptophan (Figure 5A); addition of indole greatly diminishes this absorbance increase below 450 nm. The spectrum formed from L-serine and the $\alpha_2\beta_2$ complex of tryptophan synthase exhibits a complex absorption spectrum, with an absorption peak at about 350 nm and significant absorbance extending beyond 525 nm (Drewe & Dunn, 1985). Reaction of this latter complex of tryptophan synthase with sodium borohydride resulted in the formation of (phosphopyridoxyl)alanine (Miles et al., 1982), which suggests that this species is either the α-aminoacrylate complex or is in rapid equilibrium with it. Furthermore, this complex of tryptophan synthase reacts rapidly with nucleophiles such as indoline and aniline to form quinonoid intermediates (Roy et al., 1988). Benzimidazole also binds tightly to the 350-nm intermediate of tryptophan synthase but does not react to form a covalent intermediate (Roy et al., 1988). Although benzimidazole can react readily with electrophiles on the sp^2 lone pair of the N, it exhibits very low π-nucleophilicity and thus is precluded from reaction with the α-aminoacrylate intermediate by stereoelectronic considerations (Roy et al., 1988). It is therefore unlikely that the intermediate that we have observed herein is formed by covalent reaction of benzimidazole. Our finding that a similar spectrum can be formed from tryptophan indole-lyase, pyruvate, and ammonium in the presence of benzimidazole, and the high reactivity of this complex with indole to form a quinonoid intermediate, further supports assignment to an α-aminoacrylate intermediate. If the 345-nm peak

formed in the reaction of tryptophan indole-lyase is an α-aminoacrylate intermediate, the low wavelength might be due to a different tautomeric form than D-serine dehydratase. Alternatively, the peak position at 345 nm is consistent with a tetrahedral C-4′ of the PLP moiety, possibly the *gem*-diamine of the α-aminoacrylate and the ε-amino group of Lys-270 (Scheme V). It is reasonable to expect that a *gem*-diamine structure should exist as a transient intermediate between the α-aminoacrylate Schiff's base and iminopyruvate, which is the likely reaction product (Vederas et al., 1978; Hillebrand et al., 1979). Further studies are in progress to conclusively establish the structure of this new intermediate in the reaction of tryptophan indole-lyase.

ACKNOWLEDGMENTS

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Registry No. Tryptophan indole-lyase, 9024-00-4; indole, 120-72-9; L-alanine, 56-41-7; indoline, 496-15-1; benzo[*b*]thiophene, 95-15-8; indazole, 271-44-3; benzimidazole, 51-17-2; *S*-methyl-L-cysteine, 1187-84-4; *S*-ethyl-L-cysteine, 2629-59-6; *S*-benzyl-L-cysteine, 3054-01-1; L-tryptophan, 73-22-3; potassium pyruvate, 4151-33-1; ammonium chloride, 12125-02-9.

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