Mechanism of binding of substrate analogues to tryptophan indole-lyase: Studies using rapid-scanning and single-wavelength stopped-flow spectrophotometry

ARTICLE *in* BIOCHEMISTRY · OCTOBER 1990

Impact Factor: 3.02 · DOI: 10.1021/bi00489a016 · Source: PubMed

4 AUTHORS, INCLUDING:

Robert Stephen Phillips
University of Georgia
217 PUBLICATIONS **3,767** CITATIONS
SEE PROFILE

Michael F Dunn
University of California, Riverside
129 PUBLICATIONS **3,204** CITATIONS
SEE PROFILE

Mechanism of Binding of Substrate Analogues to Tryptophan Indole-Lyase: Studies Using Rapid-Scanning and Single-Wavelength Stopped-Flow Spectrophotometry†

Robert S. Phillips,*,‡ Sheri L. Bender,‡ Peter Brzovic,§ and Michael F. Dunn§

Departments of Chemistry and Biochemistry, School of Chemical Sciences, University of Georgia, Athens, Georgia 30602, and Department of Biochemistry, University of California at Riverside, Riverside, California 92521

Received March 16, 1990; Revised Manuscript Received May 16, 1990

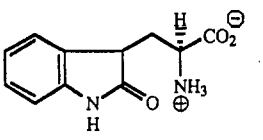
ABSTRACT: We have examined the binding of oxindolyl-L-alanine, (3*R*)-2,3-dihydro-L-tryptophan, L-homophenylalanine, and N^1-methyl-L-tryptophan to tryptophan indole-lyase (tryptophanase) from *Escherichia coli* by using rapid-scanning and single-wavelength stopped-flow kinetic techniques. Rate constants for the reactions were determined by fitting the concentration dependencies of relaxations to either linear (pseudo-first-order) or hyperbolic (rapid second-order followed by slow first-order) equations. The reaction with oxindolyl-L-alanine forms a quinonoid intermediate that exhibits a strong peak at 506 nm. This species is formed more rapidly than with the other analogues (84.5 s^{-1}) and is reprotonated very slowly (0.2 s^{-1}). Reaction with L-homophenylalanine also forms a quinonoid intermediate with a strong peak at 508 nm, but the rate constant for its formation is slower (6.9 s^{-1}). The reaction with L-homophenylalanine exhibits a transient intermediate absorbing at about 340 nm that decays at the same rate as the quinonoid peak forms and that may be a *gem*-diamine. Tryptophan indole-lyase reacts with (3*R*)-2,3-dihydro-L-tryptophan much more slowly to form a moderately intense quinonoid peak at 510 nm, and a transient intermediate absorbing at about 350 nm is also formed. The species formed in the reaction of N^1-methyl-L-tryptophan exhibits a peak at 425 nm and a very weak quinonoid absorption peak at 506 nm, which is formed at <4 s^{-1}. These results demonstrate that the structure of the aromatic rings of amino acids can have significant effects on both the rates of formation and the equilibrium concentrations of intermediates in the reaction of tryptophan indole-lyase and suggest that the indole NH is important for optimal substrate binding and catalysis.

Tryptophan indole-lyase (tryptophanase)[1] is a pyridoxal phosphate (PLP)[2] dependent enzyme that catalyzes the hydrolytic cleavage of L-tryptophan to indole, pyruvate, and ammonium ion:

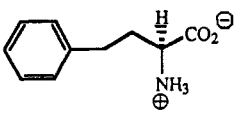
L-tryptophan + H_2O ⇌ indole + pyruvate + ammonium

The distinctive spectroscopic properties of the PLP and amino acid complexes with tryptophan indole-lyase have been extensively studied in order to understand the precise structures of reaction intermediates (Morino & Snell, 1967; June et al., 1981).

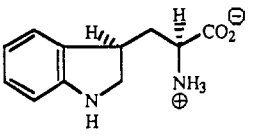
The tryptophan indole-lyase catalyzed cleavage of the carbon–carbon bond requires tautomerization of the indole ring of the tryptophan substrate to an indolenine derivative (Davis & Metzler, 1972). The demonstration that analogues of the proposed reaction intermediate, oxindolyl-L-alanine (I) and 2,3-dihydro-L-tryptophan (II), are potent inhibitors provides experimental support for the proposed reaction mechanism (Phillips et al., 1984, 1985). We have recently examined the interaction of the $\alpha_2\beta_2$ complex of *Escherichia coli* tryptophan synthase with these inhibitors by rapid-scanning and single-wavelength stopped-flow spectrophotometry (Roy et al., 1988).



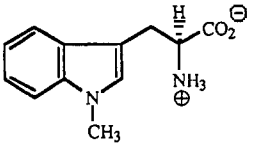
I



II



III



IV

The results of these studies demonstrated that these potent inhibitors bind to tryptophan synthase preferentially as *gem*-diamine complexes. We have now studied the interactions of tryptophan indole-lyase with oxindolyl-L-alanine (I), L-homophenylalanine (II), (3*R*)-2,3-dihydro-L-tryptophan (III), and N^1-methyl-L-tryptophan (IV) in order to gain an understanding of the role of structural features in the rates and equilibria of intermediate formation. The results of these studies show that the indole NH is important for rapid formation of the external aldimine and quinonoid intermediates and that deprotonation is not the rate-limiting step in the slow formation of the quinonoid intermediate from (3*R*)-2,3-dihydro-L-tryptophan.

† This research was partially supported by a grant from the National Institutes of Health (GM 42588-01) to R.S.P. and by a National Science Foundation grant (NSF/DMB-87-03697) to M.F.D. The Kinetics Instruments stopped-flow mixer and the OLIS Cary 14 conversion were purchased with a Bristol-Myers grant of Research Corporation to R.S.P., with matching funds from the University of Georgia Research Foundation.

‡ University of Georgia.
§ University of California at Riverside.

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

[2] Abbreviations: PLP, pyridoxal 5′-phosphate.

0006-2960/90/0429-8608$02.50/0

Materials and Methods

Instrumentation. Single-wavelength stopped-flow kinetic measurements were performed by using a Kinetics Instruments stopped-flow mixer with a modified Cary 14 (On-Line Instruments, Inc.) as the light source. Data collection was performed on a Zenith Z-158 computer using hardware and software provided by On-Line Instruments, Inc. Rapid-scanning stopped-flow measurements were obtained on a modified Durrum Model D110 stopped-flow instrument interfaced with a Princeton Applied Research 1463 controller and 1214 photodiode array detector (Dunn et al., 1982; Koerber et al., 1983). Steady-state kinetic measurements were obtained on a Gilford Response UV/vis spectrophotometer equipped with a thermoelectric block.

Materials. Tryptophan indole-lyase was obtained from *E. coli* JM101 containing plasmid pMD6, which has an insert including the *tna*A gene from *E. coli* K-12 and which overproduces the enzyme upon induction with L-tryptophan (Deeley & Yanofsky, 1981). The cells were grown and the enzyme was purified as described elsewhere (Phillips & Gollnick, 1989). The specific activity of the purified enzyme was 46–50 units/mg, measured with *S*-(*o*-nitrophenyl)-L-cysteine as substrate at pH 8.0 and 25 °C (Suelter et al., 1976), and the enzyme was a single band by polyacrylamide gel electrophoresis. Enzyme concentrations were estimated from the $A^{1\%}_{278} = 9.19$ (Phillips & Gollnick, 1989), assuming a subunit molecular weight of 5.2×10^4 Da (Deeley & Yanofsky, 1981).

Oxindolyl-L-alanine (Phillips et al., 1984), (3*R*)-2,3-dihydro-L-tryptophan (Phillips et al., 1985), and [α-^{2}H]-(3*R*)-2,3-dihydro-L-tryptophan (Roy et al., 1988) were prepared as described. L-Homophenylalanine (L-2-amino-4-phenylbutanoic acid) was obtained from United States Biochemical Corp. N^1-Methyl-L-tryptophan was prepared by alkylation of the disodium salt of L-tryptophan in liquid NH_3, as described by Yamada et al. (1965).

[α-^{2}H]-L-Homophenylalanine was prepared by the tryptophan indole-lyase catalyzed exchange of the α-proton in D_2O. To 40 mL of 99.8% [^{2}H]H_2O (Aldrich) were added 642 mg of K_2HPO_4, 62 mg of KH_2PO_4, 1.2 mg of PLP, 14 μL of 2-mercaptoethanol, 40 mg of L-homophenylalanine, and 0.2 mL of tryptophan indole-lyase (15.8 mg/mL; 145 units). The solution was tightly sealed and incubated at 37 °C; an aliquot was transferred to an NMR tube to monitor the progress of the exchange reaction. After two days, exchange of the α-proton was judged complete by NMR, and the reaction was acidified to pH 5 with glacial acetic acid. After filtration through Celite, the solution was concentrated in vacuo and stored at 4 °C. The [α-^{2}H]-L-homophenylalanine was obtained as crystalline plates in two crops (total, 35 mg). ^{1}H NMR of the isolated [α-^{2}H]-L-homophenylalanine indicated that the α-proton was >98% exchanged.

Methods. Single-wavelength transients were analyzed by fitting with the SIFIT program (On Line Instruments, Inc.), which can fit up to three exponentials and an offset. Quality of fit was judged by analysis of the residuals and by the Durbin–Watson value (Durbin & Watson, 1970). The concentration dependence of relaxations was fit to either a linear equation for pseudo-first order reactions (eq 1) or a hyperbolic

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

equation for first-order reactions preceded by a rapid binding equilibrium (Strickland et al., 1975) (eq 2), where k_f is the

$$1/\tau_{obs} = k_f[L]/(K_{eq} + [L]) + k_r \quad (2)$$

rate constant for the forward reaction and k_r is the rate constant for the reverse reaction. K_I values were estimated from eq 3, where the parameters were obtained from fitting to eq 2. The fitting was performed by using a nonlinear least-

$$K_I = K_{eq}^*(k_r/k_f) \quad (3)$$

squares program (Enzfitter) from Elsevier Biosoft. Steady-state kinetic data were analyzed by using the compiled FORTRAN programs of Cleland (1970), as adapted for personal computers by C. B. Grissom, and steady-state K_I values were calculated from eq 4.

$$v = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_I}\right) + [S]} \quad (4)$$

Results

Oxindolyl-L-alanine (I). Rapid-scanning studies of the reaction of tryptophan indole-lyase with oxindolyl-L-alanine (1 mM) are characterized by a decrease at 425 nm and an increase at 506 nm (Figure 1). Similar static spectra were recently reported by Zakomyrdina et al. (1988). There is an immediate increase in intensity and a red shift in the peak position from 420 to 425 nm, which is completed in the first scan. Thus, we conclude that the external aldimine formation from oxindolyl-L-alanine is fast. Furthermore, there is an isosbestic point between the 425-nm peak and the rapid phase of the increase at 506 nm, indicating that these two species directly interconvert. Single-wavelength measurements were performed at 506 nm at various concentrations of oxindolyl-L-alanine. The progress curves required a minimum of two exponentials to obtain an adequate fit; at high concentrations, a third phase of low amplitude and very slow rate was also observed. The first phase shows a strong concentration dependence (Figure 2). Fitting of these data to eq 2 (see Methods) gives k_f for deprotonation of 84.6 ± 0.1 s^{-1} while the rate constant for reprotonation of the quinonoid anion, k_r, is 0.2 ± 0.15. The curvature of the concentration dependence gives an apparent equilibrium constant for external aldimine formation of 4.85 ± 0.30 mM. These constants predict an overall binding constant of 11.5 μM, in reasonable agreement with previously determined K_I values of 2.5 μM (Phillips et al., 1984) and 5 μM (Kiick & Phillips, 1988). The slower phases show a similar increase in rate with increasing concentrations of oxindolyl-L-alanine. At 2 mM oxindolyl-L-alanine, the rate constants for the second and third phases are 4.25 and 0.5 s^{-1}.

L-Homophenylalanine (II). Mixing of 2 mM L-homophenylalanine with tryptophan indole-lyase results in a remarkably complex series of scans (Figure 3). The 420-nm peak diminishes, a new peak at 340 nm appears transiently, and finally an intense quinonoid peak at 508 nm is observed. There is no isosbestic point between the 420-nm and 508-nm peaks; thus, the 420-nm intermediate does not react directly to give the quinonoid complex. Single-wavelength studies were performed at 508, 420, and 340 nm to clarify the mechanism of this reaction. The apparent first-order rate constants at 1 mM L-homophenylalanine are 38.7 s^{-1} (decrease at 420 nm, fast phase), 31.7 s^{-1} (increase at 340 nm), 5.2 s^{-1} (decrease at 340 nm), and 5.4 s^{-1} (increase at 508 nm, fast phase). The data obtained at 508 nm are biphasic, and the fast phase shows a hyperbolic dependence on the concentration of L-homophenylalanine. From fitting of these data to eq 2, k_f for quinonoid formation is calculated to be 6.9 ± 0.5 s^{-1}, and k_r for reprotonation is 1.2 ± 0.2 s^{-1}, with a binding constant (K_{eq}) of 0.64 ± 0.15 mM. These constants predict a K_I for homo-

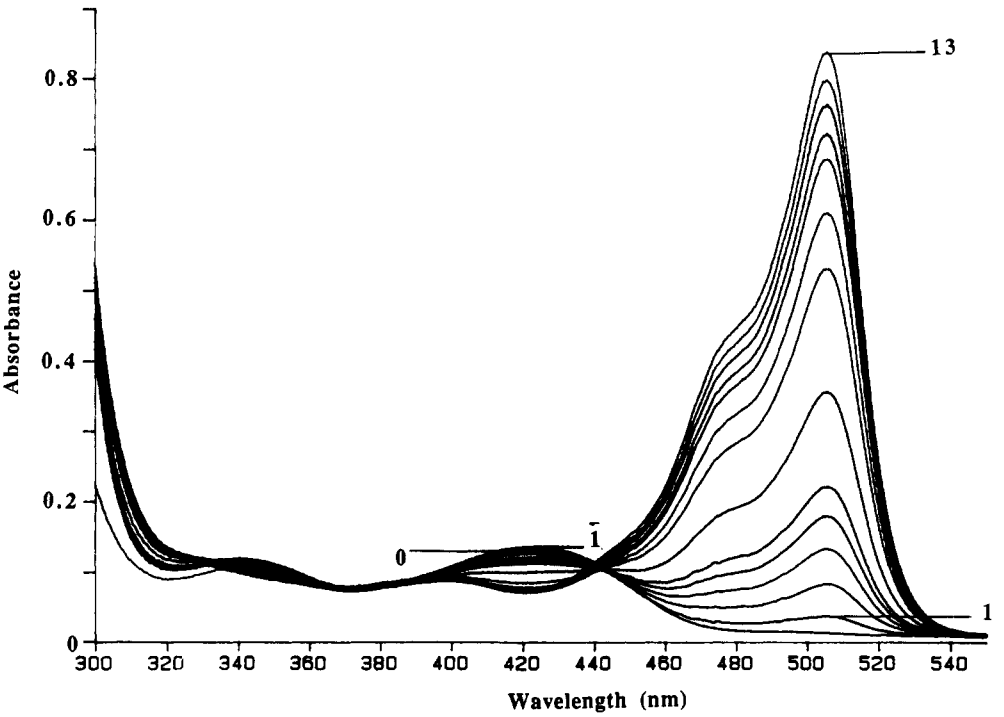


FIGURE 1: Rapid-scanning spectra for the reaction of 20 μM tryptophan indole-lyase with 1 mM oxindolyl-L-alanine in 0.02 M potassium phosphate buffer, pH 8.0, containing 0.16 M KCl and 5 mM mercaptoethanol. Scans were collected at 8.54 ms (curve 1), 17.08 ms (curve 2), 25.62 ms (curve 3), 34.16 ms (curve 4), 42.70 ms (curve 5), 76.86 ms (curve 6), 145.18 ms (curve 7), 213.50 ms (curve 8), 384.30 ms (curve 9), 640.50 ms (curve 10), 1.281 s (curve 11), 2.220 s (curve 12), and 4.27 s (curve 13). Curve 0 is a spectrum of the enzyme in the absence of oxindolyl-L-alanine.

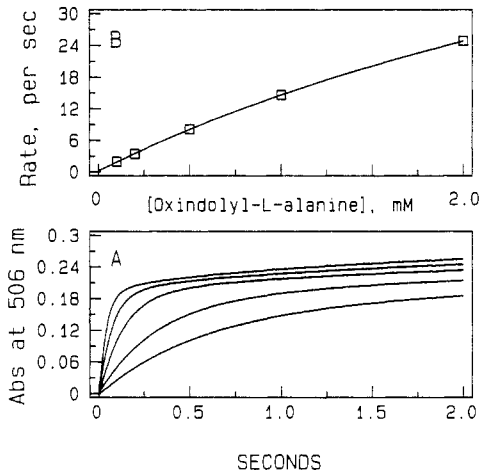


FIGURE 2: Concentration dependencies of single-wavelength stopped-flow measurements at 506 nm for the reaction of 5 μM tryptophan indole-lyase with oxindolyl-L-alanine. (A) Time courses with 0.1, 0.2, 0.5, 1.0, and 2.0 mM oxindolyl-L-alanine. (B) Rate constants for the fast phase plotted as a function of oxindolyl-L-alanine concentration. The solid line is the calculated fit to eq 2, with k_f = 84.6 s^{-1}, k_r = 0.2 s^{-1}, and K_{eq} = 4.85 mM.

phenylalanine of 110 μM, in reasonable agreement with experimental values (vide infra). The time courses at 340 and 420 nm also are biphasic. Analysis of the fast phases of these time courses gives apparent first-order rate constants with linear dependencies on the concentration of homophenylalanine, suggesting that these processes are second order. From the slope of these data, a second-order rate constant of 2.2×10^4 M^{-1} s^{-1} is obtained at either 340 or 420 nm (Figure 4), suggesting that the 340-nm species is formed from the 420-nm species. Furthermore, the rate constant for decomposition of the 340-nm species (the phase of decreasing absorbance) is identical with that found for formation of the 508-nm species. The slower phase observed at 420 nm also shows a linear dependence of k_{obs} on homophenylalanine concentration (Figure 4), suggesting that the two phases are due to two forms of the enzyme with different reactivities.

We next examined the primary deuterium kinetic isotope effect on the reaction of [α-^{2}H]-L-homophenylalanine. L-Homophenylalanine is a moderately potent competitive inhibitor of tryptophan indole-lyase, with a reported K_I of 53 μM (Watanabe & Snell, 1977). Repetition of these measurements gave a K_I value of 49 μM in our hands, in excellent agreement. However, for [α-^{2}H]-L-homophenylalanine, K_I was found to be 189 μM; thus, there is a large equilibrium isotope effect of 3.9 for inhibition of the enzyme by [α-^{2}H]-L-homophenylalanine. Furthermore, single-wavelength stopped-flow studies with [α-^{2}H]-L-homophenylalanine show that both the rate of decomposition of the 340-nm species and the rate of quinonoid complex formation at 508 nm are subject to a kinetic isotope effect (data not shown). The apparent rate constants for formation of the quinonoid intermediate from 1.75 mM L-homophenylalanine measured at 508 nm are ^Hk_f = 5.42 s^{-1} and ^Dk_f = 2.35 s^{-1}. Assuming that the equilibrium for external aldimine formation and k_r are unaffected by deuterium substitution, we calculate an intrinsic kinetic isotope effect on quinonoid complex formation, $^Hk/^Dk$ = 4.3, in good agreement with the equilibrium isotope effect for inhibition (vide supra). This value is comparable to the value measured with L-tryptophan of 3.6, but it is significantly less than the value of 7.9 observed with *S*-benzyl-L-cysteine (Phillips, 1989).

(3R)-2,3-Dihydro-L-tryptophan (III). In previous studies, it was found that (3*R*)-2,3-dihydro-L-tryptophan is a potent, slow-binding inhibitor of tryptophan indole-lyase (Phillips et al., 1984, 1985). Rapid-scanning measurements confirmed the slow rate of reaction and showed the formation of a rather broad quinonoid peak centered at 510 nm (Figure 5). In single-wavelength kinetic studies, the rate constant for appearance of the quinonoid peak at 0.05 mM was found to be 8×10^{-3} s^{-1}, a value comparable to the rate constant for the

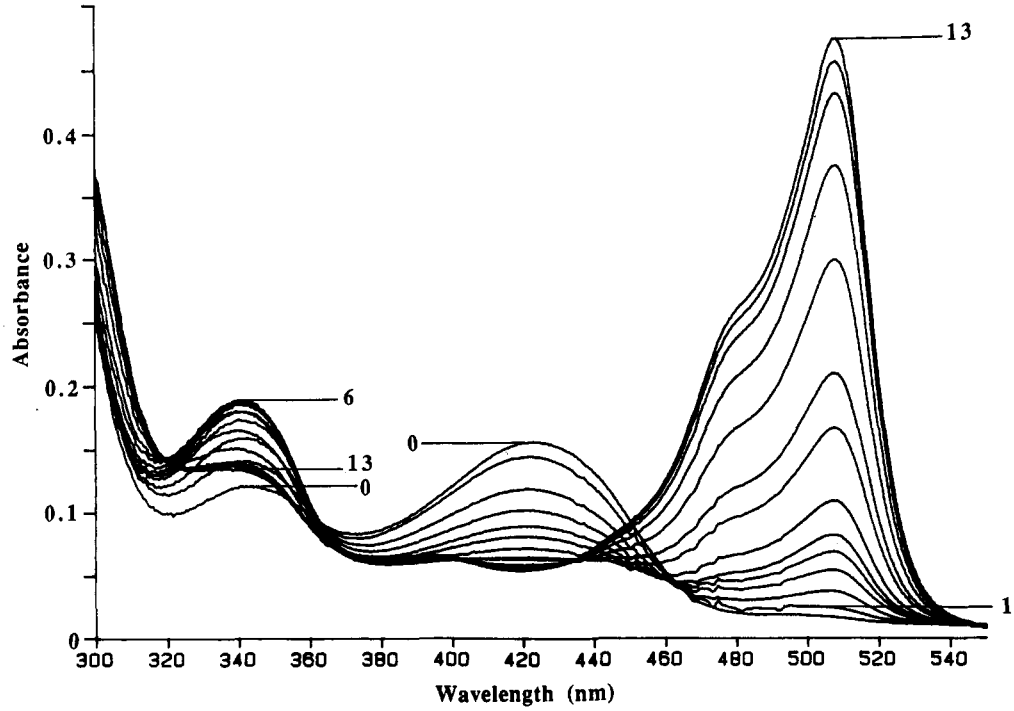


FIGURE 3: Rapid-scanning stopped-flow spectra for the reaction of 20 μM tryptophan indole-lyase with 2 mM L-homophenylalanine in 0.02 M potassium phosphate buffer, pH 8.0, containing 0.16 M KCl and 5 mM 2-mercaptoethanol. Scans were collected at 8.54 ms (curve 1), 17.08 ms (curve 2), 25.62 ms (curve 3), 34.16 ms (curve 4), 42.70 ms (curve 5), 59.78 ms (curve 6), 93.94 ms (curve 7), 128.10 ms (curve 8), 230.58 ms (curve 9), 384.30 ms (curve 10), 606.12 ms (curve 11), 1.025 s (curve 12), and 1.708 s (curve 13). Curve 0 is a spectrum of the enzyme in the absence of L-homophenylalanine.

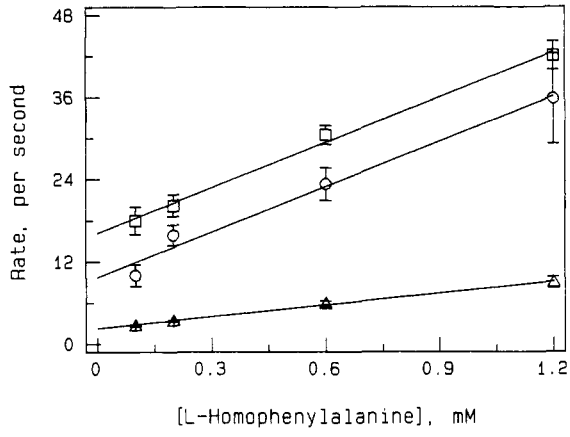


FIGURE 4: Concentration dependence of the absorbance increase at 340 (open circles) or absorbance decrease at 420 nm (open squares and triangles) for the reaction of L-homophenylalanine. The lines are calculated by using linear regression analysis, with $k_f = 2.2 \times 10^4$ M^{-1} s^{-1} and $k_r = 9.7$ s^{-1} for the 340-nm absorbance, $k_f = 2.2 \times 10^4$ M^{-1} s^{-1} and $k_r = 16.1$ s^{-1} for the fast phase at 420 nm, and $k_f = 5.7 \times 10^3$ M^{-1} s^{-1} and $k_r = 2.2$ s^{-1} for the slow phase at 420 nm.

onset of inhibition observed previously (Phillips et al., 1985). We examined the reaction of [α-^{2}H]-(3*R*)-2,3-dihydro-L-tryptophan to see if an isotope effect is observed. However, no isotope effect is detected either on the rate of quinonoid complex formation, on the steady-state kinetic inhibition constant, or on the rate of onset of inhibition (data not shown). Thus, in contrast to homophenylalanine, proton abstraction is not rate-determining for formation of the quinonoid complex with dihydrotryptophan. The rate of internal aldimine disappearance at 420 nm is similar to the rate of formation of the quinonoid complex, and there is a transient appearance and decay of bands at 350 nm concomitant with the decay of the 420-nm band and appearance of the 510-nm band. The increase at 320 nm also occurs concomitantly with the formation of the 510-nm peak; therefore, it is likely that these electronic transitions are associated with the same chemical species. There is a definite lag in the formation of the 510-nm absorption band, suggesting the processes at 420 and 510 nm are not tightly coupled or that formation of the quinonoid species is multiphasic.

N^1-Methyl-L-tryptophan (IV). N^1-Methyl-L-tryptophan has no detectable substrate activity with tryptophan indole-lyase and is a relatively weak competitive inhibitor, with a K_I value in the millimolar range (data not shown). Addition of N^1-methyl-L-tryptophan to solutions of tryptophan indole-lyase results in an absorption band at 506 nm that can be attributed to formation of a quinonoid species (data not shown) and a prominent peak at 425 nm that is probably due to formation of an external aldimine. However, the intensity of the quinonoid band produced in the reaction of N^1-methyl-L-tryptophan is much weaker than with the other analogues that we studied. The rate constant for formation of the 506-nm band is 24 $\pm$ 4 s^{-1} and is invariant with concentration of the ligand for the range 0.85–6.80 mM. If $k_f \ll k_r$, then eq 2 would simplify to $1/\tau \approx k_r$ and become concentration independent; thus, we conclude that α-proton abstraction for *N*-methyl-L-tryptophan is much slower than 24 s^{-1} and can be no greater than 4 s^{-1}.

Discussion

June et al. (1981) studied the reaction of the quasisubstrates L-alanine and L-ethionine with tryptophan indole-lyase by using single-wavelength stopped-flow methods. These quasisubstrates predominantly form quinonoid structures upon binding to the enzyme (June et al., 1981). June et al. (1981) demonstrated that the ketoenamine form of the internal aldimine, with a λ_{max} of 420 nm, is the form that directly reacts with the amino acids. We have examined the binding and covalent reaction mechanisms of four aromatic amino acid inhibitors of tryptophan indole-lyase that are structural analogues of L-tryptophan. Despite the significant similarities in structure,

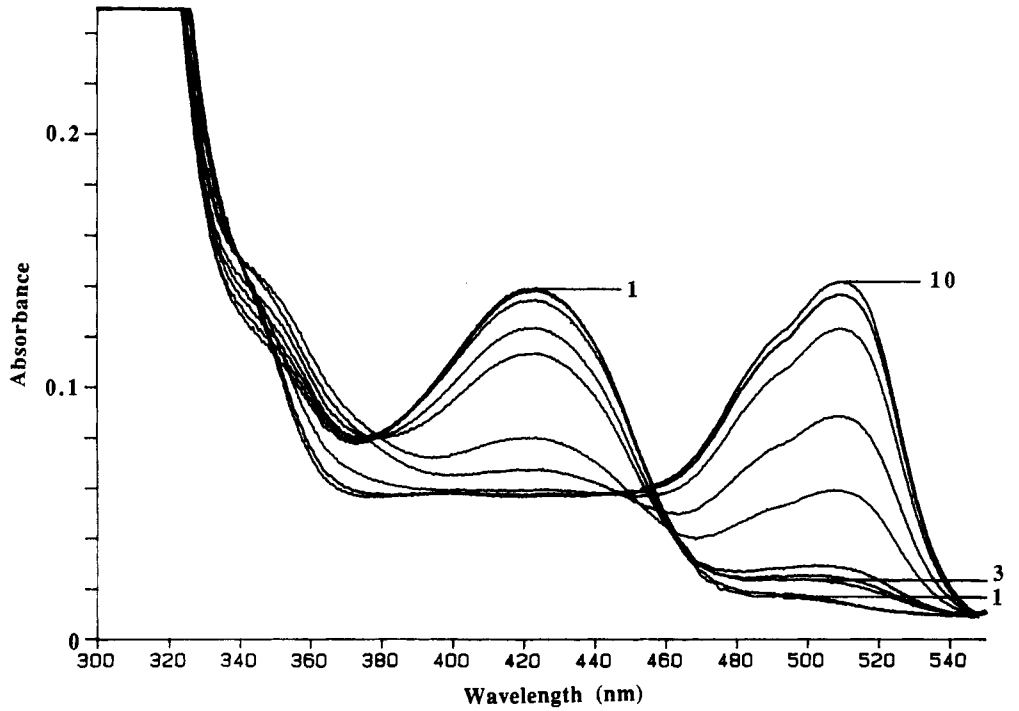
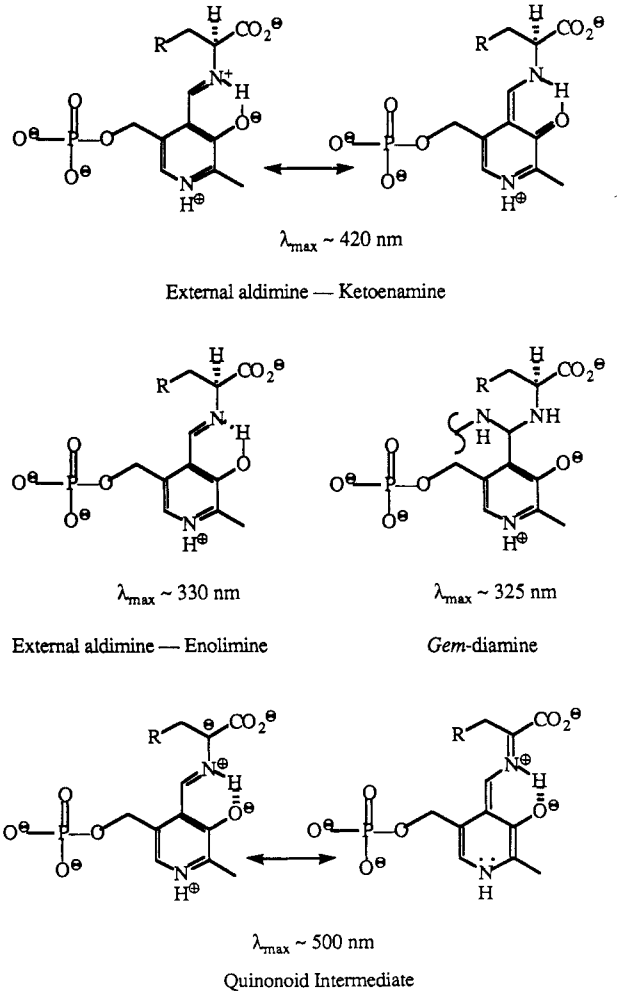


FIGURE 5: Rapid-scanning stopped-flow spectra obtained with 20 μM tryptophan indole-lyase and 1 mM (3*R*)-2,3-dihydro-L-tryptophan in 0.02 M potassium phosphate buffer, pH 8.0 containing 0.16 M KCl and 5 mM mercaptoethanol. Scans were collected at 8.54 ms (curve 1), 42.70 ms (curve 2), 170.80 ms (curve 3), 427.00 ms (curve 4), 683.20 ms (curve 5), 2.39 s (curve 6), 4.95 s (curve 7), 12.64 s (curve 8), 22.89 s (curve 9), and 43.38 s (curve 10).

there are great differences in the interactions of these analogues with the enzyme. All the inhibitors are shown to undergo covalent reaction to give equilibrating mixtures of external aldimine and quinonoid species. The quinonoid complexes are the dominant final products in the reactions of I and II, a species absorbing below 340 nm is the major product in the reaction of III, and an external aldimine is the major product in the reaction of IV. Oxindolyl-L-alanine (I) reacts rapidly (within the dead time of the stopped-flow instrument) to form an external aldimine that absorbs at 425 nm (Kallen, 1985), consistent with a PLP ring in the ketoenamine form (Scheme I). In contrast, L-homophenylalanine (II) reacts somewhat more slowly to form an intermediate absorbing at 340 nm, suggesting either a PLP ring in the enolimine form or in a *gem*-diamine structure (Kallen et al., 1985) (Scheme I).[3] Both oxindolyl-L-alanine and L-homophenylalanine react to give external aldimines that produce quinonoid structures absorbing strongly at 506–508 nm (Kallen et al., 1985) (Scheme I); however, the equilibrium constant (k_f/k_r) for the interconversion of the external aldimine and the quinonoid intermediate is much larger for oxindolyl-L-alanine (420) than for L-homophenylalanine (5.8). Although similar K_I values are observed for dihydrotryptophan (III) and oxindolyl-L-alanine (II), the rate constant for quinonoid complex formation with (3*R*)-2,3-dihydro-L-tryptophan (III) is several orders of magnitude slower. Furthermore, the absence of a primary kinetic isotope effect with [α-^{2}H]dihydrotryptophan shows that the transition state of the rate-determining step in quinonoid complex formation does not include deprotonation. The ab-

Scheme I

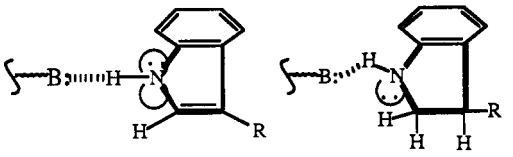


[3] It is not possible to rigorously assign the structure of the 340-nm intermediate at present. However, since the ketoenamines are generally the reactive forms of PLP Schiff bases, it is likely that the intermediate is a *gem*-diamine. This would be consistent with the observed kinetic behavior and isotope effects if the following mechanism is assumed, where K_2 is fast but unfavorable:

$$\text{E} + \text{L-homoPhe} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{E·L-homoPhe (}gem\text{-diamine)} \overset{K_2}{\rightleftharpoons}$$

$$\text{E·L-homoPhe (external aldimine)} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \text{E·L-homoPhe (quinonoid)}$$

Chart I



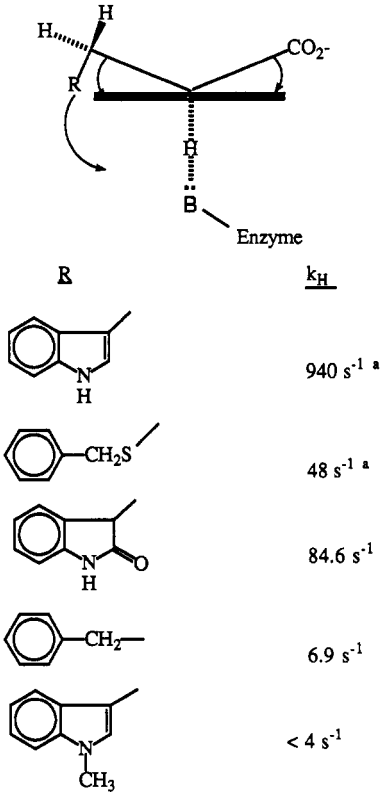
L-tryptophan (3R)-2,3-dihydro-L-tryptophan

sorbance changes at 420 nm and in the 320–360-nm region occur at the same rate as does the formation of the quinonoid species at 510 nm. Thus, with (3*R*)-2,3-dihydro-L-tryptophan, some early step (such as *gem*-diamine formation or breakdown) may be very slow, perhaps due to the sp^3 hybridization of the heterocylic nitrogen[4] (Chart I). This result is very similar to our previous findings on the reaction of (3*S*)-2,3-dihydro-L-tryptophan with tryptophan synthase (Roy et al., 1988). In contrast, oxindolyl-L-alanine (I) forms a stable *gem*-diamine with tryptophan synthase (Roy et al., 1988) but is predominantly bound as the quinonoid species with tryptophan indole-lyase (Figure 1). Taken together, these results suggest that the specific interactions at the indole subsite involving hydrogen bonding at the heterocylic NH and van der Waals contacts with the ring affect covalent bonding effects at the Schiff base linkage by providing initially important constraints on the allowed orientation of the reacting substrate. We propose that this recognition triggers protein conformational transitions obligatory for the formation and decay of the *gem*-diamine intermediate during interconversion of the internal and external aldimines.

We recently studied the pH dependence of the steady-state kinetic K_I value for oxindolyl-L-alanine (Kiick & Phillips, 1988). The binding of the inhibitor shows a dependence on the basic forms of two enzyme groups, with pK_a's of 6.0 and 7.6; similar pK_a's are observed in the V/K pH profile for the reaction of L-tryptophan (Kiick & Phillips, 1988). Since the pK_a of 6.0 is not observed in the pH dependence of K_I for L-alanine or in the pH dependence of V/K for *S*-methylcysteine, we concluded that the basic group with a pK_a of 6.0 must interact with the NH of oxindolyl-L-alanine and L-tryptophan (Kiick & Phillips, 1988). L-Homophenylalanine can be considered as an acyclic analogue of oxindolyl-L-alanine that is lacking the carboxamido bridge. Since the K_I for homophenylalanine is about 10-fold larger than that for oxindolyl-L-alanine, one might expect to see significant differences in binding or deprotonation rates. Indeed, there is a 12-fold increase in the rate constant for quinonoid complex formation from oxindolyl-L-alanine relative to homophenylalanine (Chart II). Thus, the presence of the carboxamido group strongly enhances the rate of quinonoid complex formation. Previously, we found that the rate of quinonoid complex formation from L-tryptophan (940 s^{-1}) is much faster than that from another good substrate, *S*-benzyl-L-cysteine (48 s^{-1}) (Phillips, 1989). The rate accelerations (12–20-fold) observed for both substrates (L-Trp) and inhibitors (oxindolyl-L-alanine) bearing a heterocylic NH are due to $\Delta\Delta G^{\ddagger}$ lowering of 1.47–1.77 kcal/mol. This finding suggests that hydrogen bonding of the aromatic ring NH is important for rapid deprotonation of the α-carbon. Indeed, the rate of deprotonation of N^1-methyl-L-tryptophan (IV) is very slow, and

[4] Since the indoline nitrogen is single-bonded, its formal hybridization should be sp^3; however, due to resonance interactions with the aromatic ring, it would likely be distorted from the ideal geometry for sp^3 toward the planar geometry expected for sp^2 hybridization.

Chart II: Effect of the Side Chain on α-Proton Abstraction by Tryptophan Indole-Lyase



[a] From Phillips (1989).

this compound exhibits no detectable substrate activity. In the case of (3*R*)-2,3-dihydro-L-tryptophan, the sp^3 hybridization of the indoline ring nitrogen[4] (Chart I) and the nonplanar ring structure may preclude formation of this H-bond, which would form in the plane of the aromatic ring for L-tryptophan and oxindolyl-L-alanine. However, since proton abstraction is not rate-determining in the formation of the quinonoid intermediate from dihydrotryptophan, we cannot estimate the rate constant for the deprotonation. The seminal studies by Dunathan demonstrated that the bond to be broken or formed must be oriented perpendicular to the plane of the PLP π-system (Dunathan, 1966; Dunathan et al., 1968). Since the C_α–C_β bond is moving in plane as the deprotonation proceeds, the aromatic rings are part of the transition-state motion, as shown in Chart II. Thus, our results demonstrate that changes in substrate analogue structure at centers distance from the point of the covalent bond transformation may have significant effects both on rates of intermediate formation and decomposition and on the preferred structures of intermediates in reaction pathways catalyzed by tryptophan indole-lyase. We propose that these structural effects are a manifestation of the tight linkage between the covalent transformation and (obligatory) protein conformation transitions during catalysis.

References

Cleland, W. W. (1979) *Methods Enzymol 63*, 103–138.

Davis, L., & Metzler, D. E. (1972) in *The Enzymes* (Boyer, P. D., Ed.) 3rd ed., Vol. 7, pp 33–74, Academic Press, New York.

Deeley, M. D, & Yanofsky, C. (1981) *J. Bacteriol. 147*, 787–796.

Dunathan, H. C. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55*, 712–716.

Dunathan, H. C., Davis, L., Kury, P. G., & Kaplan, M. (1968) *Biochemistry 7*, 4532–4537.

Dunn, M. F., Dietrich, H., MacGibbon, A. K. H., Koerber, S. C., & Zeppezauer, M. (1982) *Biochemistry 21*, 354-363.
Durbin, J., & Watson, G. S (1970) *Biometrika 37*, 409.
June, D. S., Suelter, C. H., & Dye, J. L. (1981) *Biochemistry 20*, 2707-2713.
Kallen, R. G., Korpela, T., Martell, A. E., Matsushima, Y., Metzler, C. M., Metzler, D. E., Morozov, Y. V., Ralston, I. M., Savin, F. A., Torchinsky, Y. M., & Ueno, H. (1985) *Transaminases* (Christen, P., & Metzler, D. E., Eds.) Vol. 2, pp 37-108, John Wiley and Sons, New York.
Kiick, D. M., & Phillips, R. S. (1988) *Biochemistry 27*, 7339-7344.
Koerber, S. C., MacGibbon, A. K. H., Dietrich, H., Zeppezauer, M., & Dunn, M. F. (1983) *Biochemistry 22*, 3424-3431.
Morino, Y., & Snell, E. E. (1967) *J. Biol. Chem. 242*, 2800-2809.
Phillips, R. S. (1989) *J. Am. Chem. Soc. 111*, 727-730.
Phillips, R. S., & Gollnick, P. D. (1989) *J. Biol. Chem. 264*, 10627-10632.
Phillips, R. S., Miles, E. W., & Cohen, L. A. (1984) *Biochemistry 23*, 6228-6234.
Phillips, R. S., Miles, E. W., & Cohen, L. A. (1985) *J. Biol. Chem. 260*, 14665-14670.
Roy, M., Miles, E. W., Phillips, R. S., & Dunn, M. F. (1988) *Biochemistry 27*, 8661-8669.
Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048.
Suelter, C. H., Wang, J., & Snell, E. E. (1976) *FEBS Lett. 66*, 230-232.
Watanabe, T., & Snell, E. E. (1977) *J. Biochem. (Tokyo) 82*, 733-745.
Yamada, S.-I., Shioiri, T., Itaya, T., Hara, T., & Matsueda, R. (1965) *Chem. Pharm. Bull. 13*, 88-93.
Zakomyrdina, L. N., Sakharova, I. S., & Torchinskii, Y. M. (1988) *Mol. Biol. (Engl. Transl.) 22*, 159-165.

Overexpression and Mutagenesis of the Catalytic Domain of Dihydrolipoamide Acetyltransferase from *Saccharomyces cerevisiae*†

Xiao-Da Niu,‡ James K. Stoops,§ and Lester J. Reed*,‡

Clayton Foundation Biochemical Institute and Department of Chemistry, The University of Texas at Austin, Austin, Texas 78712, and Department of Pathology and Laboratory Medicine, The University of Texas Health Science Center, Houston, Texas 77225

Received May 18, 1990; Revised Manuscript Received June 26, 1990

ABSTRACT: The inner core domain (residues ~221-454) of the dihydrolipoamide acetyltransferase component (E_2p) of the pyruvate dehydrogenase complex from *Saccharomyces cerevisiae* has been overexpressed in *Escherichia coli* strain JM105 via the expression vector pKK233-2. The truncated E_2p was purified to apparent homogeneity. It exhibited catalytic activity (acetyl transfer from [1-^{14}C]acetyl-CoA to dihydrolipoamide) very similar to that of wild-type E_2p. The appearance of the truncated and wild-type E_2p was also very similar, as observed by negative-stain electron microscopy, namely, a pentagonal dodecahedron. These findings demonstrate that the active site of E_2p from *S. cerevisiae* resides in the inner core domain, i.e., catalytic domain, and that this domain alone can undergo self-assembly. The purified truncated E_2p showed a tendency to aggregate. Aggregation was prevented by genetically engineered attachment of the interdomain linker segment (residues ~181-220) to the catalytic domain. All dihydrolipoamide acyltransferases contain the sequence His-Xaa-Xaa-Xaa-Asp-Gly near their carboxyl termini. By analogy with chloramphenicol acetyltransferase, the highly conserved His and Asp residues were postulated to be involved in the catalytic mechanism [Guest, J. R. (1987) *FEMS Microbiol. Lett. 44*, 417-422]. Substitution of the sole His residue in the *S. cerevisiae* truncated E_2p, His-427, by Asn or Ala by site-directed mutagenesis did not have a significant effect on the k_{cat} or K_m values of the truncated E_2p. However, the Asp-431 → Asn, Ala, or Glu substitutions resulted in a 16-, 24-, and 3.7-fold reduction, respectively, in k_{cat}, with little change in K_m values. These findings indicate that a His residue is not involved in the catalytic mechanism of E_2p from *S. cerevisiae* but that Asp-431 plays an important role. Whether this role is structural or catalytic remains to be established.

All dihydrolipoamide acyltransferases possess a unique multidomain structure (Reed & Hackert, 1990; Guest et al., 1989; Perham & Packman, 1989). The amino-terminal part of the polypeptide chain contains 1, 2, or 3 highly similar lipoyl domains, each of about 80 amino acid residues, in tandem array. The lipoyl domain (or domains) is followed by another structurally distinct segment that is involved in binding dihydrolipoamide dehydrogenase (E_3)[1] or the α-keto acid dehydrogenase (E_1), or both. These domains are linked to each other and to the carboxyl-terminal part of the polypeptide chain (inner core domain) by conformationally flexible seg-

†This work was supported by USPHS Grants GM06590 (L.J.R.) and GM39536 (J.K.S.) and by a grant from the Foundation for Research (L.J.R.).

‡The University of Texas at Austin.
§The University of Texas Health Science Center at Houston.

[1] Abbreviations: E_1, α-keto acid dehydrogenase or pyruvate dehydrogenase; E_2p, dihydrolipoamide acetyltransferase; E_3, dihydrolipoamide dehydrogenase; PCR, polymerase chain reaction; r, recombinant; IPTG, isopropyl β-thiogalactoside; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/90/0429-8614$02.50/0