Mechanistic Deductions from Multiple Kinetic and Solvent Deuterium Isotope Effects and pH Studies of Pyridoxal Phosphate Dependent Carbon–Carbon Lyases: *Escherichia coli* Tryptophan Indole-Lyase†

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ABSTRACT: Analysis of the pH dependence of the kinetic parameters and competitive inhibitor K_i values for tryptophan indole-lyase suggests two enzymic groups must be unprotonated in order to facilitate binding and catalysis of tryptophan. The V/K for tryptophan and the pK_i for oxindolyl-L-alanine, a putative transition state analogue and competitive inhibitor, decrease below two pK values of 7.6 and 6.0, while the K_i for L-alanine, also a competitive inhibitor, is 3300-fold larger (20 mM) than that for oxindolyl-L-alanine and increases below a single pK of 7.6. A single pK of 7.6 is also observed in the V/K profile for the alternate substrate, *S*-methyl-L-cysteine. Therefore, the enzymic group with a pK of 7.6 is responsible for proton abstraction at the 2-position of tryptophan, while the enzymic group with a pK of 6.0 interacts with the indole portion of tryptophan and probably catalyzes formation of the indolenine tautomer of tryptophan (in concert with proton transfer to C-3 of indole from the group with pK 7.6) to facilitate carbon–carbon bond cleavage and elimination of indole. The pH variation of the primary deuterium isotope effects for proton abstraction at the 2-position of tryptophan (^{D}V = 2.5 and $^{D}(V/K_{trp})$ = 2.8) are pH independent, while the V_{max} for tryptophan or *S*-methyl-L-cysteine is the same and also pH independent. Thus, substrates bind only to the correctly protonated form of the enzyme. Further, tryptophan is not sticky, and the pK values observed in both V/K profiles are the correct ones. Both of the D_2O solvent kinetic isotope effects (^{D_2O}V = 3.8 and $^{D_2O}(V/K_{trp})$ = 2.8) are pH independent. However, in D_2O, V/K_{trp} decreases below two pK values of 8.1 and 6.5, and the +0.5 ΔpK_a indicates that neither of the enzyme groups required for catalysis is a sulfhydryl residue. The primary deuterium isotope effects on V and V/K for tryptophan decrease to 1.25 and 1.82, respectively, when determined in D_2O. There is no observable D_2O solvent isotope effect on V or V/K for *S*-methyl-L-cysteine. Thus, internal aldimine formation and aminoacrylate transimination and hydrolysis do not appear to be solvent sensitive, while proton abstraction at the 2-position of the substrate is partially rate-limiting.

Tryptophan indole-lyase (deaminating)[1] catalyzes a pyridoxal phosphate dependent elimination reaction to form indole, pyruvate, and ammonia from L-tryptophan (Miles, 1986). Although this enzyme is widely distributed in *Enterobacteria* (Snell, 1975), the enzyme from *Escherichia coli* has been most extensively studied. Mechanistic studies of this reaction have suggested that the indolenine tautomer of tryptophan is an intermediate and may play an important role in activating the carbon–carbon bond to facilitate elimination of indole (Davis & Metzler, 1972; Phillips et al., 1984, 1985). A similar mechanism has been postulated in the preceding paper by Kiick and Phillips (1988) for *Erwinia herbicola* and *Citrobacter freundii* tyrosine phenol-lyase. These enzymes, which also catalyze a PLP-dependent elimination reaction, require activation of a carbon–carbon bond to facilitate the elimination of phenol.

The reaction catalyzed by tryptophan indole-lyase has the requirement for multiple proton transfers and also the internal transfer of the proton at the 2-position of the substrate to the indole ring (Vederas et al., 1978). This suggests proton abstraction must take place prior to tautomerization. As such, multiple heavy atom and solvent deuterium isotope effects make an excellent probe of this reaction mechanism. Recently, Blanchard and Cleland (1980) and Hermes et al. (1982) have shown that meaningful mechanistic information can be obtained from double-label isotope effect studies. And, in fact, a stepwise vs concerted mechanism can be distinguished for the enzymatic reaction being studied (Hermes et al., 1984; Rendina et al., 1985; Grissom et al., 1987). However, this has been accomplished for only a very few enzymes (Cleland, 1987).

To date, this study is one of the few applications of the theory advanced by Cleland (1985, 1987) to simultaneously determine deuterium and D_2O solvent isotope effects in a given system. O'Leary et al. (1981) studied the PLP-dependent enzyme glutamate decarboxylase in a similar manner by determining the ^{13}C isotope effect in both H_2O and D_2O. These investigators concluded Schiff base interchange and decarboxylation are both solvent sensitive and partially rate-limiting. This paper reports the findings from a multiple isotope effect

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[1] This enzyme is also referred to as tryptophanase (EC 4.1.99.1).

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and pH study of the kinetic parameters for *E. coli* tryptophan indole-lyase. In addition, the pH dependence of the K_i values for competitive inhibitors is also reported. The results of this study provide support for the mechanism previously proposed by Phillips et al. (1984) that requires two catalytic bases. In conclusion, a catalytic mechanism for tryptophan indole-lyase is proposed that is consistent with all the data from this and from previous studies of tryptophan indole-lyase (Phillips et al., 1984, 1985).

Experimental Procedures

Chemicals and Enzymes. The L-tryptophan, L-alanine, and pyridoxal phosphate used in this study were obtained from Sigma. Synthesis of oxindolyl-L-alanine was accomplished by the method of Savige and Fontana (1980) as previously described (Phillips et al., 1984). The *S*-(*o*-nitrophenyl)-L-cysteine was prepared by the reaction of L-cysteine and 2-fluoro-1-nitrobenzene (Phillips, 1988). The DTT[2] and NADH were purchased from Boehringer Mnnheim. The D_2O and KOD (99 atom % D) were obtained from Cambridge Isotopes. Both *S*-methyl-L-cysteine and indole (Gold Label, 99%) were purchased from Aldrich. The Good's buffers were from Research Organics, and all other reagents and chemicals obtained from commercially available sources were of the highest quality available.

Tryptophan indole-lyase from *E. coli* B/lt7-A was purified by hydrophobic interaction chromatography (Phillips et al., 1987). The enzyme preparation used in this study was greater than 95% pure on the basis of PAGE and reverse-phase HPLC. The enzyme exhibited specific activities of 7 units/mg with L-tryptophan and 50 units/mg with *S*-(*o*-nitrophenyl)-L-cysteine as the substrate at pH 8 and 25 °C. The enzyme (M_r 208 000) binds 4 mol of PLP/mol of enzyme (Deeley & Yanofsky, 1981). The protein concentration of the purified enzyme was determined from absorbance at 278 nm (Morino & Snell, 1967). The LDH used in the coupled assays was purchased as a lyophilized powder from Sigma (rabbit muscle) or as a solution in 50% glycerol, pH 6.5, from Boehringer Mannhein (hog muscle).

Preparation of [2-^2H]-L-Tryptophan. To K_2HPO_4 (321 mg), KH_2PO_4 (31 mg), *S*-methyl-L-cysteine (133 mg), PLP (0.6 mg), and indole (46 mg) were added 20 mL of D_2O and 0.007 mL of 2-mercaptoethanol. The pD of the solution was 8. To this solution was added 0.1 mL of *E. coli* tryptophan indole-lyase (6.4 units). The solution was stoppered with a serum cap and stirred at room temperature. After 2 days, the pH was adjusted to 5 with glacial acetic acid, and the solution was then filtered through a Celite pad and evaporated in vacuo. The residue was dissolved in 6 mL of water and extracted with 2 × 6 mL of toluene. The aqueous layer was evaporated in vacuo and taken up in 5 mL of water, and the residual tryptophan was purified by preparative HPLC on a Dynamax 21 × 250 mm C_{18} column, eluting at 4 mL/min with 50 mM triethylammonium acetate, pH 5, containing 25% methanol. The tryptophan-containing fractions were pooled and evaporated in vacuo. The residue was then taken up in 1 mL of methanol, and the amino acid was precipitated with 3 mL of ether. A light cream solid was obtained in 40% yield (32.1 mg). This material was essentially pure as determined by its UV spectrum and TLC analysis. A single UV and ninhydrin-positive spot on reverse-phase plates (Analtech) was eluted with 50 mM sodium acetate at pH 6, 25% methanol. A ^1H NMR analysis obtained on a JEOL FX 270-MHz spectrometer of the deuteriated compound indicated >99% incorporation of deuterium at the 2-carbon.

Substrate Calibration. The concentrations of L-tryptophan-2-(H,D) were determined and standardized from measurements of their UV spectra by using an extinction coefficient at 278 nm of 5.55 mM^{-1} cm^{-1} (Morton, 1975). The concentrations from several determinations were in agreement within 1%.

Initial Velocity Studies. Tryptophan indole-lyase was assayed in an identical manner using the same enzyme coupling system as described in the previous paper (Kiick & Phillips, 1988). A typical assay contained 0.1 M buffer (see below, pL Studies), 0.2 M KCl, 1 mM DTT, 0.2 mM NADH, 100 units/mL LDH, and variable concentrations of L-tryptophan.

Velocity as a function of enzyme concentration was determined at pH 6.4, 7.8, and 9.4 when either L-tryptophan or *S*-methyl-L-cysteine was used as the substrate and also at the highest inhibitor concentration used in obtaining the inhibition patterns. In all cases, the activity per milliliter determined equally reflected the rate of tryptophan indole-lyase. Furthermore, no NADH oxidase activity was detectable in the enzyme preparation.

pL Studies. Determination of V and V/K for L-tryptophan and *S*-methyl-L-cysteine and the K_i for inhibitors vs either L-tryptophan or *S*-methyl-L-cysteine were determined in an identical manner as described in the previous paper (Kiick & Phillips, 1988). The pL measurements and ranges of buffers used in H_2O to obtain the pL profiles were the same as described by Kiick and Phillips (1988) in the previous paper. The same buffers were used in the D_2O solvent isotope effect studies but at a range 0.5 pH unit higher than in H_2O. This higher range was used because of the equilibrium isotope effect on the acid dissociation constant of the buffers (Schowen, 1977).

Data Analysis. Data were analyzed accordingly, and a description of the equations utilized to fit the data and definition of the appropriate constants are given in the previous paper (Kiick & Phillips, 1988).

Results

pH Dependence of the Kinetic Parameters. The pH dependence of the kinetic parameters for *E. coli* tryptophan indole-lyase was determined, and the results are shown in Figure 1 (filled circles). The maximum velocity is pH independent from pH 5.9 to 9.4. The V/K for tryptophan decreases below two pK values of 7.6 ± 0.09 and 6.0 ± 0.2. The pH-independent value of these parameters is $V/E_t = 6\ s^{-1}$ and $V/(K_{trp}E_t) = 2.0 \times 10^4\ M^{-1}\ s^{-1}$.

Tryptophan indole-lyase has been shown to act on various *S*-alkyl-L-cysteines (Snell, 1975). Although the V with these compounds is comparable to that with tryptophan, the K_m is not. In order to further explore the reasons for this difference, the pH dependence of the kinetic parameters for *S*-methyl-L-cysteine were obtained (Figure 2). The maximum velocity is pH independent from pH 5.9 to 8.9, while V/K_{SMC} decreases below a single pK of 7.6 ± 0.06. The pH-independent values of these parameters are $V/E_t = 5.0\ s^{-1}$ and $V/(K_{SMC}E_t) = 3.3 \times 10^2\ M^{-1}\ s^{-1}$.

pH Dependence of Inhibitors. The pH dependence of the dissociation constants for L-alanine and oxindolyl-L-alanine were determined. From the inhibition patterns obtained over the pH range 5.9–9.0, it was determined that inhibition by both inhibitors vs either tryptophan or *S*-methyl-L-cysteine is competitive (data not shown). The pK_i profile for oxindolyl-L-alanine inhibition is shown in Figure 3 (open circles). The

[2] Abbreviations: DTT, dithiothreitol; LDH, lactate dehydrogenase; PAGE, polyacrylamide gel electrophoresis; PLP, pyridoxal phosphate; SMC, *S*-methyl-L-cysteine; TPL, tyrosine phenol-lyase; WNL, tryptophan indole-lyase.

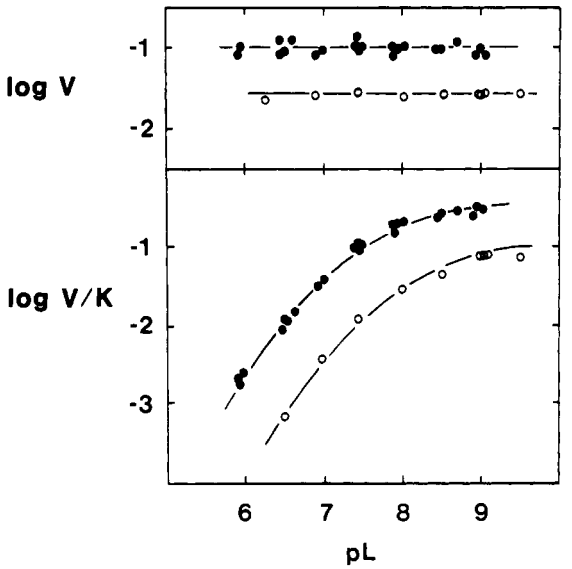

FIGURE 1: pL dependence of the kinetic parameters for *E. coli* tryptophan indole-lyase. The filled circles represent data obtained in H_2O. The open circles represent data obtained in D_2O. The points are the experimentally determined values from a fit of the data to the Michaelis–Menton equation, while the curves for V/K are from a fit of the resulting values using the appropriate equation as described by Kiick and Phillips (1988). For V, the lines represent the average value of the parameter.

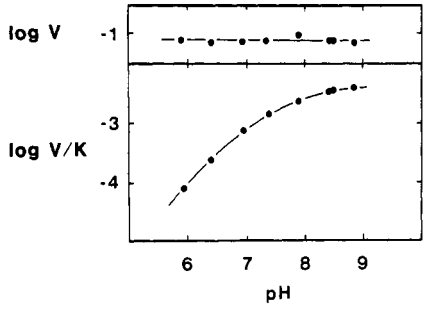

FIGURE 2: pH dependence of the kinetic parameters for *E. coli* tryptophan indole-lyase with the alternate substrate *S*-methyl-L-cysteine. The points are the experimentally determined values from a fit of the data to the Michaelis–Menton equation, while the curve for V/K_{SMC} is from a fit of the resulting values to the appropriate equation as described by Kiick and Phillips (1988). For V, the line represents the average value of the parameter.

K_{is} for oxindolyl-L-alanine increases below two pK values of 7.6 ± 0.07 and 6.3 ± 0.1, while the pH-independent value of the K_{is} is 6.0 ± 0.1 μM. This value is in good agreement with the previously reported value of 2.5 μM (Phillips et al., 1984). On the other hand, the K_{is} for L-alanine (Figure 3, filled circles) increases below a pK of 7.7 ± 0.1 and shows no evidence of a second pK in the pH range 6–6.5. The pH-independent value of the K_{is} for L-alanine is 20 ± 3 mM.

pH Variation of Deuterium Isotope Effects. By use of the direct comparison method, the primary deuterium isotope effects for abstraction of the proton at the 2-position of tryptophan were measured as a function of pH. Both ^{D}V and $^{D}(V/K)$ are pH independent over the pH range 5.9–9.0. The average pH-independent values from a fit of the data to the appropriate equation (Kiick & Phillips, 1988) are ^{D}V = 2.5 ± 0.3 and $^{D}(V/K)$ = 2.8 ± 0.3. The value of the parameters as a function of pH are listed in Table I.

pL Variation of Solvent Isotope Effects. The solvent isotope effects were measured as a function of pL, and the results are shown in Figure 1 (open circles). The maximum velocity is pL independent from pH 6.2 to 9.4 in D_2O as well as in H_2O. However, V/K_{trp} decreases below two pK values of 8.1 ± 0.1 and 6.5 ± 0.2 in D_2O. Consequently, as can be seen in Figure 1, ^{D_2O}V is pL independent, while $^{D_2O}(V/K)$ is pL dependent over this range. Also, at pL 9.1 the solvent isotope effects are ^{D_2O}V = 3.8 ± 0.2 and $^{D_2O}(V/K)$ = 2.8 ± 0.1.

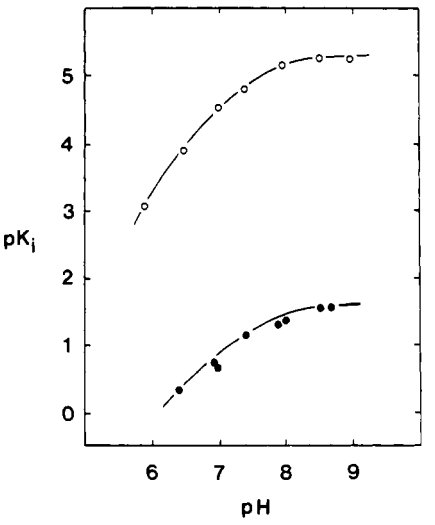

FIGURE 3: pH dependence of inhibitor dissociation constants for *E. coli* tryptophan indole-lyase. The open circles represent data obtained for the binding of oxindolyl-L-alanine, while the filled circles represent data obtained for the binding of L-alanine. In all cases, tryptophan was varied ca. an order of magnitude around its K_m at fixed levels (ca. 0, 0.5, and 2 K_i) of the inhibitor. The inhibition patterns were fitted to the equation that describes competitive inhibition (Cleland, 1979), and the resulting $1/K_{is}$ values were fitted to the appropriate equations as described by Kiick and Phillips (1988) to obtain the curves shown.

Table I: Primary Deuterium Isotope Effects[a] as a Function of pH for *E. coli* Tryptophan Indole-Lyase

pH	^{D}V	$^{D}(V/K_{trp})$
5.9	2.3 ± 0.3	2.9 ± 0.3
6.4	3.0 ± 0.3	2.8 ± 0.2
7.1	2.0 ± 0.5	3.0 ± 0.3
7.9	2.6 ± 0.1	2.4 ± 0.1
8.4	2.7 ± 0.3	2.8 ± 0.4
9.1	2.5 ± 0.1	2.8 ± 0.2

[a] The values listed for ^{D}V and $^{D}(V/K)$ are from a fit of the data to the appropriate isotope effect equation (Kiick & Phillips, 1988).

There is no observable solvent isotope effect at pL 9.1 on either V or V/K when *S*-methyl-L-cysteine is utilized as the substrate. When plotted, the data were virtually superimposable and gave identical values for V and V/K when fit to the appropriate equations (Kiick & Phillips, 1988).

Multiple Isotope Effects. For *E. coli* tryptophan indole-lyase, from a direct comparison experiment at pL 9.1, the primary deuterium isotope effect was determined in the presence of H_2O and D_2O (data not shown). In H_2O, ^{D}V = 2.5 ± 0.1 and $^{D}(V/K_{trp})$ = 2.8 ± 0.1, while these values decrease to 1.25 ± 0.01 and 1.82 ± 0.01, respectively, in D_2O.

Discussion

pH Dependence of Kinetic Parameters. Tryptophan indole-lyase and tyrosine phenol-lyase both catalyze the same type of reaction, elimination of indole from tryptophan or phenol from tyrosine, with the subsequent formation of pyruvate and ammonia. As shown for *C. freundii* tyrosine phenol-lyase from the pH dependence of the kinetic parameters (Kiick & Phillips, 1988), a V profile that is pH independent is diagnostic of a protonation mechanism in which substrate binds only to the correctly protonated form of the enzyme. In such a mechanism, true pK values for catalytic and binding groups will only be observed in the V/K profile (Cleland,

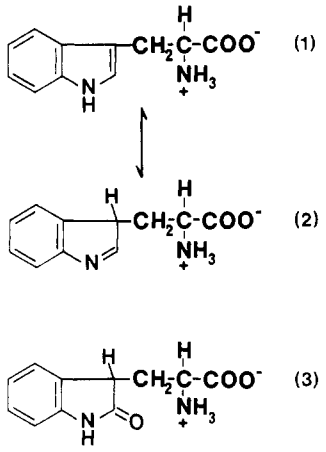

FIGURE 4: Chemical structures of tryptophan (1), the indolenine tautomer of tryptophan (2), and oxindolyl-L-alanine (3).

1977). For *E. coli* tryptophan indole-lyase, the V profiles for both L-tryptophan (Figure 1) and *S*-methyl-L-cysteine (Figure 2) are pH independent. Thus, it appears that these substrates bind only to the correctly protonated form of the enzyme and the pK values of 6.0 (observed only in the V/K_{trp} profile) and 7.6 (observed in both V/K profiles) are the correct ones, and this will be further shown below.

Since neither substrate has any titratable moieties in the range of pH measured, the two observed pK values reflect enzymic groups that must be deprotonated in order for tryptophan to bind and undergo catalysis. However, there is a requirement for only one enzymic group (pK of 7.6) to be deprotonated for the binding and catalysis of *S*-methyl-L-cysteine. As with tyrosine phenol-lyase (Kiick & Phillips, 1988), this group probably abstracts the proton at the 2-position of the substrate with subsequent quinonoid formation prior to elimination of methylmercaptan.

The turnover number for tryptophan indole-lyase is essentially the same when either *S*-methyl-L-cysteine or tryptophan is the substrate. However, the K_m values, as well as the pH dependence of V/K for the substrates are quite different. As reflected in the V/K profiles, *S*-methyl-L-cysteine does not interact with the enzymic group with a pK of 6.0. As a result of the loss of this interaction with the indole-binding pocket, the K_m for *S*-methyl-L-cysteine is 75-fold larger than for tryptophan. Presumably, this interaction helps to orient the substrate properly in the active site. However, once *S*-methyl-L-cysteine is bound, it can undergo catalysis at the same rate as tryptophan. This is not surprising since methylmercaptan is a good leaving group and should undergo elimination quite readily once the quinonoid is formed. More will be said about the catalytic role of the enzymic group with a pK of 6.0 below.

Interpretation of Inhibition Data. The compound oxindolyl-L-alanine (Figure 4) has been shown to bind as a competitive inhibitor to *E. coli* tryptophan indole-lyase and also tryptophan synthase (Phillips et al., 1984). This compound is a structural analogue of the indolenine tautomer of tryptophan (Figure 4), having tetrahedral geometry at C-3 of the indole ring. Thus, if the indolenine tautomer of tryptophan is formed as an intermediate in the tryptophan indole-lyase reaction, it follows that oxindolyl-L-alanine should bind tightly to this enzyme, since enzymes preferentially stabilize reaction intermediates (Pauling, 1946; Wolfenden, 1969). The data (Figure 3) show that oxindolyl-L-alanine has a K_i that is 40-fold tighter than the K_m for tryptophan, suggesting that the indolenine tautomer is indeed an intermediate as previously postulated (Miles, 1986).

The pH dependence of the K_i for oxindolyl-L-alanine shows the same pK values observed in the V/K_{trp} profile. The binding of L-alanine, on the other hand, shows a requirement for only the group with the pK of 7.6 to be unprotonated. As with *S*-methyl-L-cysteine, the requirement of the group with a pK of 6.0 is lost, and the K_i for alanine is 3300-fold higher than for oxindolyl-L-alanine. A direct comparison of the $\Delta G°$ values, calculated from the K_i values for these inhibitors, shows that interaction of the ring portion of oxindolyl-L-alanine with the indole-binding site on the enzyme is worth ca. 4.8 kcal/mol. This binding energy is lost when *S*-methyl-L-cysteine is the substrate. The acid–base chemistry of this reaction suggests the role of the enzymic group with a pK of 6 is to abstract the ring nitrogen proton and allow electrons to flow out to the C-3 carbon of indole, facilitating the formation and stabilization of the indolenine tautomer of tryptophan. When this enzymic group is protonated, it prevents binding of tryptophan. This role is certainly consistent with its observed protonation state. Thus, the function of this group is similar to that of the enzymic group for tyrosine phenol-lyase that abstracts the proton from the hydroxyl group of the phenolic ring of tyrosine facilitating formation of the cyclohexadienone (Kiick & Phillips, 1988).

Interpretation of Isotope Effects. The invariance of the deuterium isotope effects as a function of pH is consistent with a mechanism whereby substrate binds only to the correctly protonated form of the enzyme. Also, for most enzyme-catalyzed reactions that involve proton transfers, ^{D}V and/or $^{D}(V/K)$ will not be equal to the intrinsic isotope effect for proton transfer because non isotope-dependent steps are often partially rate-limiting (Cook & Cleland, 1981a; Cleland, 1982). The tryptophan indole-lyase deuterium isotope effects for proton abstraction at the 2-position of tryptophan and transfer to C-3 of indole (Vederas et al., 1978; Snell, 1975) are 2.5 and 2.8, respectively, for ^{D}V and $^{D}(V/K)$. The size of the isotope effects suggests these proton-transfer steps are at least partially rate-limiting for this reaction.

The chemistry of this reaction suggests a number of steps have the potential to be solvent-mediated. Therefore, the D_2O solvent isotope effects for tryptophan indole-lyase were determined. The kinetic isotope effects are pH independent, while the solvent isotope effect on the acid dissociation constant for the enzymic groups reflected in the pL profiles are in the range expected ($\Delta pK_a = 0.5$) if the groups are simple carboxylic or ammonium acids (Schowen & Schowen, 1982; Schowen, 1978). These data effectively rule out the possibility that a sulfhydryl residue may be involved in the acid–base chemistry (Cleland, 1987). Photooxidation of tryptophan indole-lyase was reported to result in irreversible inactivation concomitant with the loss of one histidine residue (Nihira et al., 1979), suggesting the enzymic group that transfers the proton from the 2-position of tryptophan to C-3 of indole is possibly a histidine residue.

The relative size of the kinetic solvent isotope effects ($^{D_2O}V = 3.5$, $^{D_2O}(V/K_{trp}) = 2.8$) suggests that some solvent-mediated step(s) is (are) partially rate-limiting for tryptophan indole-lyase. Given the number of possible solvent-sensitive steps, it would seem difficult at the outset to try to determine which step(s) in the overall catalytic mechanism for this enzyme is (are) indeed solvent sensitive. As discussed above, the data for *S*-methyl-L-cysteine show that it is just as competent a substrate as tryptophan once it is oriented properly in the active site. Although there is a significant loss of energy because *S*-methyl-L-cysteine cannot interact with the indole-binding pocket of the enzyme, this is presumably offset by the fact that

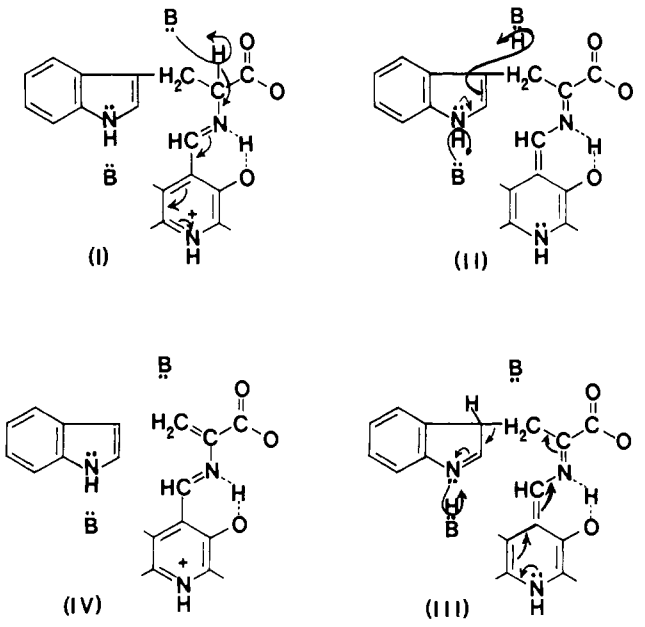

FIGURE 5: Chemical mechanism for the reaction catalyzed by *E. coli* tryptophan indole-lyase.

methylmercaptan is a good leaving group. Thus, the V_{max} for both of these substrates is the same. It should also be noted that the two substrates must undergo exactly the same chemistry with regard to external aldimine and quinonoid formation, transimination, and aminoacrylate hydrolysis. However, there is no observable D_2O solvent isotope effect on either V or V/K for *S*-methyl-L-cysteine, while there are substantial isotope effects with tryptophan. If we assume there are no changes in the rates of external aldimine and quinonoid formation, as well as transimination and aminoacrylate hydrolysis, regardless of the substrate, one can begin to rule out a number of steps that could be solvent sensitive. None of the product release steps, external aldimine formation, transimination, or aminoacrylate hydrolysis, can be solvent sensitive. Thus, quinonoid formation through elimination of indole are the only steps that could be solvent sensitive and can account for the observed D_2O solvent isotope effects with tryptophan. One can further narrow down the possibilities.

Multiple isotope effect studies are an excellent probe to determine the degree of concertedness for a reaction (Cleland, 1982). As previously stated, both ^{D}V and $^{D}(V/K_{trp})$ decrease in D_2O. Therefore, proton abstraction and quinonoid formation are not solvent sensitive, and indolenine tautomerization and/or elimination of indole are the only possible solvent-dependent steps that account for the observed isotope effects.

Chemical Mechanism. Similar to tyrosine phenol-lyase (Kiick & Phillips, 1988), the following acid–base chemistry is proposed for tryptophan indole-lyase once the external aldimine is formed (Figure 5, I). The enzyme group with a pK of 7.6 abstracts the proton at the 2-position of the substrate with electron flow into the pyridine ring, forming a stable quinonoid (Figure 5, II). For tyrosine phenol-lyase, the proton on the hydroxyl group must be abstracted for cyclohexadienone formation (Kiick & Phillips, 1988) and probably requires ca. 20 kcal/mol since the aromaticity of the benzene ring is lost (Badger, 1969). However, on the basis of a comparison of empirical resonance energies of indole and indene, the indolenine is only ca. 7 kcal/mol higher in energy than indole (Badger, 1969). Thus, the enzymic group with a pK of 6.0 abstracts the ring nitrogen proton of tryptophan as the lone pair of electrons on nitrogen collapse into the ring, and the C-3 carbon of indole develops sufficient sp^3 character and abstracts the proton from the other enzyme base (pK = 7.6) to form the indolenine tautomer of tryptophan. Once the indolenine tautomer is formed (Figure 5, III), elimination of indole can take place, electron flow is out of the pyridine ring to form aminoacrylate, and the enzymic group (pK = 6.0) donates its proton to indole (Figure 5, IV). The aminoacrylate can then undergo transimination and hydrolysis as previously described (Miles, 1987).

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Chemical Modification of Acyl-CoA:Cholesterol *O*-Acyltransferase. 1. Identification of Acyl-CoA:Cholesterol *O*-Acyltransferase Subtypes by Differential Diethyl Pyrocarbonate Sensitivity†

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ABSTRACT: Acyl-CoA:cholesterol *O*-acyltransferase (EC 2.3.1.26) (ACAT) catalyzes the intracellular synthesis of cholesteryl esters from cholesterol and fatty acyl-CoA at neutral pH. Despite the probable pathophysiologic role of ACAT in vascular cholesteryl ester accumulation during atherogenesis, its mechanism of action and its regulation remain to be elucidated because the enzyme polypeptide has never been identified or purified. Present chemical modification results identify two distinct tissue types of ACAT, based on marked differences in reactivity of an active-site histidine residue toward diethyl pyrocarbonate (DEP) and acetic anhydride. The apparent K_i of the DEP-sensitive ACAT subtype, typified by aortic ACAT, was 40 μM, but the apparent K_i of the DEP-resistant ACAT subtype, typified by liver ACAT, was 1500 μM, indicating a 38-fold difference in sensitivity to DEP. Apparent K_i's of aortic and liver ACAT for inhibition by acetic anhydride were also discordant (less than 500 μM and greater than 5 mM, respectively). On the basis of the reversibility of inhibition by hydroxylamine, a neutral pK_a for maximal modification, and acetic anhydride protection against DEP inactivation, DEP and acetic anhydride appear to modify a common histidine residue. Oleoyl-CoA provided partial protection against inactivation by DEP and acetic anhydride, suggesting that the modified histidine is at or near the active site of ACAT. Systematic investigation of ACAT activity from 14 different organs confirmed the existence of 2 subtypes of ACAT on the basis of their different reactivities toward DEP and acetic anhydride. These studies not only implicate a histidine in the catalytic mechanism of ACAT but also provide the first structural basis for differentiating ACAT subtypes and are potentially useful for identifying the ACAT polypeptide.

Acyl-CoA:cholesterol *O*-acyltransferase (ACAT)[1] (EC 2.3.1.26) catalyzes the synthesis of cholesteryl esters from fatty acyl-CoA and cholesterol at neutral pH and is the major cholesterol esterifying activity in vascular tissue under physiological conditions (Spector et al., 1979). Up to 50-fold increases and decreases in ACAT activity parallel induction and regression, respectively, of experimental atherosclerotic lesions (St. Clair et al., 1970; St. Clair, 1983), indicating that ACAT activity regulation is pathophysiologically related to cholesteryl ester accumulation during atherogenesis. Thus, ACAT activity represents a critical control point for vascular intracellular cholesteryl ester metabolism potentially subject to regulation by atherogenic or protective influences.

In other organ systems, ACAT activity is also important for intracellular cholesterol esterification. For example, intestinal ACAT is postulated to partly mediate cholesterol absorption (Suckling & Stange, 1985; Heider et al., 1983), and hepatic ACAT may modulate the cholesteryl ester composition of VLDL (Drevon et al., 1980). In adrenal gland and ovary, cholesteryl esters formed by ACAT are substrates stored for ultimate use in the synthesis of steroid hormones, and ACAT activity is regulated reciprocally with steroid hormone synthesis (Civen et al., 1984; Tavani et al., 1982). Thus, ACAT activity regulation, known to be important in atherogenesis, may also have pathophysiological relevance in cholesterol metabolism in other organs.

Despite its recognized importance, little knowledge exists about the structure or regulation of ACAT. This deficit primarily reflects the lack of identification of the ACAT polypeptide, an integral and very hydrophobic microsomal membrane protein which has proved extremely difficulty to purify. Major efforts in this area have employed exacting preparative methods (Doolittle & Chang, 1982a) which have failed to yield significantly purified enzyme. Postulated mechanisms for the marked increases and decreases in enzyme activity in vascular tissue and intestine during cholesterol feeding and deprivation include variations in cellular free

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[1] Abbreviations: ACAT, acyl-CoA:Cholesterol *O*-acyltransferase; DEP, diethyl pyrocarbonate; BME, 2-mercaptoethanol; ATP, adenosine 5′-triphosphate; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; BHT, butylated hydroxytoluene.

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