Mechanistic Deductions from Kinetic Isotope Effects and pH Studies of Pyridoxal Phosphate Dependent Carbon–Carbon Lyases: *Erwinia herbicola* and *Citrobacter freundii* Tyrosine Phenol-Lyase†

Dennis M. Kiick*

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Robert S. Phillips

School of Chemical Sciences, Departments of Chemistry and Biochemistry, University of Georgia, Athens, Georgia 30602

Received March 17, 1988; Revised Manuscript Received May 31, 1988

ABSTRACT: The pH dependence of the kinetic parameters and primary deuterium isotope effects have been determined for tyrosine phenol-lyase from both *Erwinia herbicola* and *Citrobacter freundii*. The primary deuterium isotope effects indicate that proton abstraction from the 2-position of the substrate is partially rate-limiting for both enzymes. The *C. freundii* enzyme primary deuterium isotope effects [$^{D}V = 3.5$ and $^{D}(V/K_{tyr}) = 2.5$] are pH independent, indicating that tyrosine is not sticky (i.e., does not dissociate slower than it reacts to give products). Since V_{max} for both tyrosine and the alternate substrate *S*-methyl-L-cysteine is also pH independent, substrate binds only to the correctly protonated form of the enzyme. For the *E. herbicola* enzyme, both V_{max} and V/K for tyrosine or *S*-methyl-L-cysteine are pH dependent, as well as both ^{D}V and $^{D}(V/K_{tyr})$. Thus, while both the protonated and unprotonated enzyme can bind substrate, and may be interconverted directly, only the unprotonated Michaelis complex is catalytically competent. At pH 9.5, $^{D}V = 2.5$ and $^{D}(V/K_{tyr}) = 1.5$. However, at pH 6.4 the isotope effect on both parameters is equal to 4.1. From these data, the forward commitment factor ($c_f = 5.2$) and catalytic ratio ($c_{vf} = 1.1$) for tyrosine and *S*-methyl-L-cysteine ($c_f = 2.2$, $c_{vf} = 24$) are calculated. Also, the Michaelis complex partition ratio (c_f/c_{vf}) for substrate and products is calculated to be 4.7 for tyrosine and 0.1 for *S*-methyl-L-cysteine. The chemical mechanism of these enzymes appears to require two bases, one of which (p*K* ca. 7.6–7.8) abstracts the proton from the 2-position of the substrate to form a quinonoid intermediate and the second of which (p*K* ca. 8.0–8.2), acting in concert with proton transfer from the first group to C-1 of the phenolic ring, abstracts the substrate hydroxyl group to facilitate cyclohexadienone formation and subsequent elimination of phenol. Thus, although both of these enzymes catalyze the same reaction and have essentially the same acid–base chemistry, their abilities to bind substrates differ quite markedly.

The carbon–carbon lyase tyrosine phenol-lyase (deaminating) (EC 4.1.99.2) catalyzes a pyridoxal phosphate dependent elimination reaction:

$$H_2O + \text{L-tyrosine} \rightleftharpoons \text{phenol} + \text{pyruvate} + \text{ammonia} \quad (1)$$

The enzyme is found in a variety of enterobacteria (Enei et al., 1972). However, only the enzymes from *Erwinia herbicola* (Kumagai et al., 1972) and *Citrobacter freundii* (Kumagai et al., 1970b) have been studied in any detail. This enzyme, as well as tryptophan indole-lyase, differs from other PLP-dependent[1] lyases catalyzing elimination reactions in that formally unactivated carbon–carbon bonds are broken (Miles, 1986). The reaction is intriguing because of the multiple proton transfers in the catalytic mechanism and because of the internal transfer of a proton from the 2-position of the substrate to the leaving phenolic ring (Faleev et al., 1983). This implies quinonoid formation must take place prior to tautomerization of the phenol ring to cyclohexadienone (Sawada et al., 1975) and subsequent elimination of phenol.

A chemical mechanism for these reactions has been postulated and previously described (Sawada et al., 1975; Walsh, 1979; Miles, 1986). However, there has been no in-depth steady-state kinetic analysis of either of the above-mentioned enzymes to corroborate the postulated mechanism. This study presents a detailed comparative investigation of the pH variation of the primary deuterium isotope effects for proton abstraction at the 2-position of the substrate and pH dependence of the steady-state kinetic parameters and K_i values for competitive inhibitors for tyrosine phenol-lyase from *E. herbicola* and *C. freundii*. These data suggest that although the enzymes from different organisms both catalyze the same reaction and appear to have a requirement for two enzymic groups to be unprotonated in order to facilitate catalysis, the effect of protonation of these groups on reactant binding differs. In a companion paper (Kiick & Phillips, 1988), similar

† This research was supported by a National Institutes of Health postdoctoral fellowship (GM10857) to D.M.K., a grant from the National Institutes of Health (GM18938) to W. W. Cleland, and a Bristol-Meyers grant of Research Corp. to R.S.P. Acknowledgment is also made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, and the University of Georgia Research Foundation for support of this research.

* Address correspondence to this author at the Department of Biochemistry, College of Medicine, University of Tennessee, Memphis, 800 Madison Ave., Memphis, TN 38163.

[1] Abbreviations: Ches, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; LDH, lactate dehydrogenase; Mes, 2-(*N*-morpholino)-ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; SMC, *S*-methyl-L-cysteine; PLP, pyridoxal phosphate; Taps, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; TPL, tyrosine phenol-lyase.

0006-2960/88/0427-7333$01.50/0

data for tryptophan indole-lyase are presented that further bring into focus the overall catalytic mechanism for these carbon–carbon lyases.

Experimental Procedures

Chemicals and Enzymes. Tyrosine phenol-lyase from *E. herbicola* (ATCC 21434) was purified as previously described (Phillips, 1987). The enzyme preparation exhibited a specific activity of 0.4 unit/mg with L-tyrosine as substrate at pH 8 and 25 °C. The enzyme (M_r 259000) binds 2 mol of PLP/mol of enzyme (Kumagai et al., 1972). The protein concentration of this preparation was estimated by the method of Bradford (1976). The tyrosine phenol-lyase from *C. freundii*[2] (ATCC 29063) was obtained from bacteria grown in a medium containing 0.1% L-tyrosine. The enzyme (M_r 170000), which also binds 2 mol of PLP/mol of enzyme (Kumagai et al., 1972), was purified by DEAE-cellulose chromatography followed by hydrophobic interaction chromatography on octyl-Sepharose CL-4B (Phillips et al., 1987). This preparation exhibited a specific activity of about 1.5 units/mg with L-tyrosine at pH 8 and 25 °C. 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 Mannheim (hog muscle).

The compounds L-tyrosine, L-alanine, L-phenylalanine, and pyridoxal phosphate were from Sigma. The NADH and DTT were purchased from Boehringer Mannheim. The D_2O (99 atom % D) was obtained from Sigma. The *S*-methyl-L-cysteine was purchased from Aldrich, while phenol was from the J. T. Baker Chemical Co. 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.

Preparation of [2-^2H]-L-Tyrosine. To K_2HPO_4 (314 mg), KH_2PO_4 (29 mg), *S*-methyl-L-cysteine (135 mg), PLP (0.5 mg), and phenol (60 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. herbicola* TPL (1.0 unit). The solution was stoppered with a serum cap and stirred at room temperature for 3 days, during which time the tyrosine produced precipitated. The reaction mixture was then acidified by careful addition of 1 mL of 12 N HCl, and the precipitated protein was removed by filtration through a Celite pad. The filtrate was evaporated in vacuo to dryness, and the residue was dissolved in 5 mL of water, adjusting the pH to 5 with solid $NaHCO_3$. After standing overnight at 4 °C, the precipitate was collected and recrystallized from hot water. This resulted in 36.9 mg of silky white needles, representing a 32% yield of [2–^{2}H]-L-tyrosine. 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. A ^{1}H NMR analysis obtained on a Bruker 250-MHz spectrometer of the deuteriated compound indicated >99% incorporation of deuterium at the 2-carbon.

Substrate Calibration. The concentrations of L-tyrosine-2-(H,D) were determined and standardized from measurements of their UV spectra by using an extinction coefficient (0.1 N HCl) at 275 nm of 1.34 mM^{-1} cm^{-1} (Morton, 1975). The concentrations from several determinations were in agreement within 1%.

Initial Velocity Studies. Tyrosine phenol-lyase was assayed spectrophotometrically on a Beckman DU monochromator and a Gilford OD converter, using a Leeds-Northrop chart recorder with multispeed drive. All assays were carried out at 25 °C, and reaction rates were measured by coupling the pyruvate produced to the oxidation of NADH in the presence of excess LDH (Cleland, 1977; Morino & Snell, 1970). The temperature was maintained with a circulating water bath with the capacity to heat and cool the thermospacers of the Beckman DU. Reaction cuvettes were 1 cm in path length and 1 or 3 mL in volume. Assay temperatures were routinely monitored with a YSI telethermometer while the cuvette was still in the cell compartment. A typical assay contained 0.1 M buffer (see pH Studies below), 0.2 M KCl, 1 mM DTT, 0.05 mM PLP, 0.2 mM NADH, 100 units/mL LDH, and variable concentrations of L-tyrosine. The reaction was initiated by the addition of enzyme, and the disappearance of NADH was monitored at 340 nm, E_{340} = 6.22 mM^{-1} cm^{-1}.

For both the *C. freundii* and *E. herbicola* enzyme systems, velocity as a function of enzyme concentration was determined at pH 6.5, 7.5, and 9.0 when either L-tyrosine 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 tyrosine phenol-lyase. No NADH oxidase activity was present in the *C. freundii* preparation. There was, however, a finite amount (no greater than 18% at the lowest concentration of substrate) of NADH oxidase activity in the *E. herbicola* preparation. This background rate was subtracted by obtaining the rate in the absence of L-tyrosine under all conditions assayed. All corrected double-reciprocal plots were linear.

pH Studies. Determination of V and V/K for L-tyrosine and *S*-methyl-L-cysteine was carried out by varying the levels of the desired substrate and obtaining the initial velocity. The K_i for inhibitors vs either L-tyrosine or *S*-methyl-L-cysteine were determined by varying the concentrations of substrate at several different inhibitor concentrations (including zero). Individual experiments will be discussed in further detail under results. All assays reflected initial velocity conditions with less than 10% of the limiting reactant utilized over the time course of the reaction. The pH ranges of the buffers were as follows: Mes, 5.5–6.5; KP_i, 6.5–7.5; Hepes, 7.0–8.0; Taps, 8.0–9.0; Ches, 9.0–10.0. All buffers were titrated to the appropriate pH with KOH. In all cases, sufficient overlaps were obtained when buffers were changed so that correction could be made for spurious buffer effects (Blanchard, 1984). The pH of the reaction mixture was measured with a Beckman ψ 21 pH meter with a combined microelectrode before and after sufficient data were collected for determination of initial velocities. Negligible pH changes were observed before and after reaction.

Data Analysis. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations. Data were analyzed by using the appropriate rate equations and whenever possible by using the FORTRAN programs of Cleland (1979). Individual saturation curves used to obtain the pH profiles were fitted by using

$$v = \frac{VA}{K_a + A} \tag{2}$$

Data for linear competitive inhibition were fitted by using

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \tag{3}$$

In eq 2 and 3, A is the reactant concentration, I is the inhibitor

[2] A culture of this organism was originally deposited with the American Type Culture Collection (ATCC) as *Aeromonas phenologenes*. It has subsequently been reclassified as *Citrobacter freundii* (synonymous with *Citrobacter intermedius*) (*ATCC Catalog*, 16th ed., 1985).

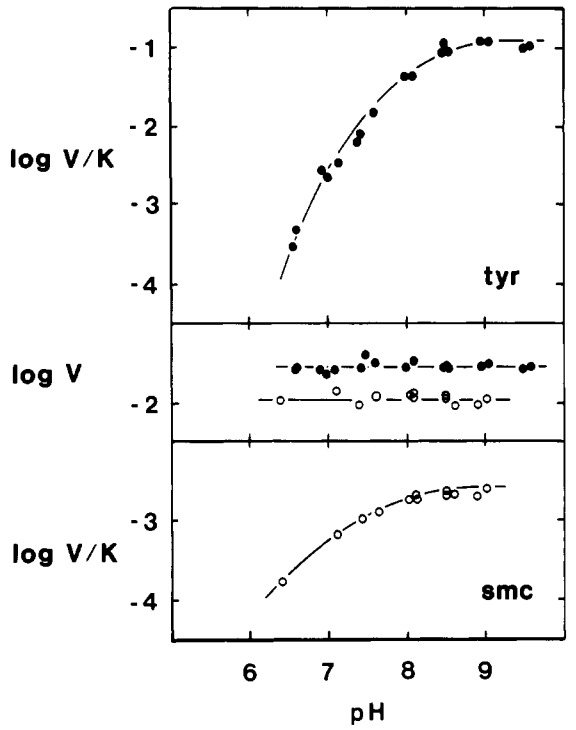


FIGURE 1: pH dependence of the kinetic parameters for *C. freundii* tyrosine phenol-lyase. The points are the experimentally determined values from a fit of the data to eq 2, while the curve for V/K_{SMC} (open circles) is from a fit of the resulting values using eq 4. The curve for V/K_{tyr} is from a fit of the data using eq 5. For both V profiles (open circles, SMC; filled circles, Tyr), the lines represent the average value of the parameter.

concentration, K_a is the Michaelis constant, and K_{is} is the slope inhibition constant. Data for pH profiles that decreased with a slope of 1 or 2 at low pH were fitted to eq 4 and 5, respectively.

$$\log Y = \log \frac{C}{1 + H/K_1} \quad (4)$$

$$\log Y = \log \frac{C}{1 + H/K_1 + H^2/K_1K_2} \quad (5)$$

In eq 4 and 5, K_1 and K_2 represent the dissociation constants for enzyme groups, Y is the value of the parameter observed as a function of pH, and C is the pH-independent value of Y. Initial velocities obtained by varying the concentration of L-tyrosine-2-(H,D) were fitted by using eq 6 and 7. These

$$v = \frac{VA}{K_a(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \quad (6)$$

$$v = \frac{VA}{(K_a + A)(1 + F_iE_{V/K})} \quad (7)$$

equations assume independent isotope effects on V and V/K (eq 6) or equal effects on both V and V/K (eq 7). In eq 6 and 7, F_i is the fraction of deuterium label in the substrate, while E_V and $E_{V/K}$ are the isotope effect minus one for the respective parameters. In all cases, the best fit of the data was chosen on the basis of the lowest values of the standard errors of the fitted parameters and the lowest value of σ (Cleland, 1979).

RESULTS

pH Dependence of the Kinetic Parameters. The pH dependence of the kinetic parameters for *C. freundii* tyrosine phenol-lyase is shown in Figure 1. The maximum velocity

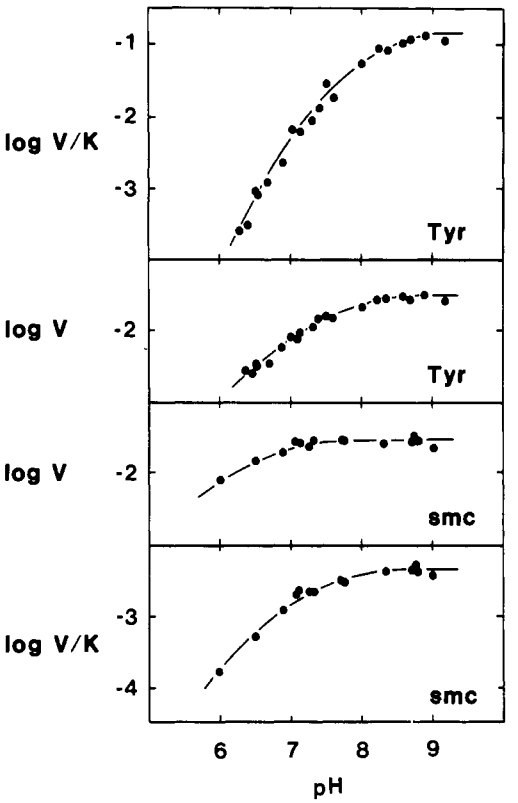


FIGURE 2: pH dependence of the kinetic parameters for *E. herbicola* tyrosine phenol-lyase. The points are the experimentally determined values from a fit of the data to eq 2, while the *S*-methyl-L-cysteine V and V/K profiles and V_{tyr} are from a fit of the resulting values using eq 4. The curve for V/K_{tyr} is from a fit of the data using eq 5.

is pH independent from pH 6.9 to 9.6. The V/K for tyrosine decreases below two pK values. From a fit of the data to eq 5, an average of the two values is 7.82 ± 0.06. The pH-independent value of these parameters is $V/E_t = 2\ s^{-1}$ and $V/(K_{tyr}E_t) = 8.5 \times 10^3\ M^{-1}\ s^{-1}$. In contrast, the pH dependences of the kinetic parameters with tyrosine as the substrate for the *E. herbicola* enzyme are quite different (Figure 2). Both V and V/K are pH dependent, with V decreasing below a pK of 7.41 ± 0.07 and V/K decreasing below two pK values of 7.0 ± 0.2 and 8.2 ± 0.2. The pH-independent values of these parameters are $V/E_t = 0.9\ s^{-1}$ and $V/(K_{tyr}E_t) = 4.5 \times 10^3\ M^{-1}\ s^{-1}$.

Both tyrosine phenol-lyases can also use *s*-methyl-L-cysteine as a substrate, and the pH dependences of the kinetic parameters for the *C. freundii* enzyme with this substrate are shown in Figure 1. The maximum velocity is pH independent from pH 6.3 to 9.0, while V/K_{SMC} decreases below a single pK of 7.63 ± 0.08. The pH-independent values of these parameters are $V/E_t = 1\ s^{-1}$ and $V/(K_{SMC}E_t) = 2.0 \times 10^2\ M^{-1}\ s^{-1}$. On the other hand, for the *E. herbicola* enzyme, the kinetic parameters for *S*-methyl-L-cysteine are both pH dependent (Figure 2). The V/K for *S*-methyl-L-cysteine decreases below a pK value of 7.3 ± 0.1, while V decreases below a pK of 6.4 ± 0.1. The pH-independent values of these parameters are $V/E_t = 0.9\ s^{-1}$ and $V/(K_{SMC}E_t) = 1.4 \times 10^2\ M^{-1}\ s^{-1}$.

pH Dependence of Inhibitors. The pH dependence of the dissociation constants for L-alanine and L-phenylalanine were determined for both enzymes. From the inhibition patterns obtained over the pH range of 6.0–9.0, it was shown that inhibition by both inhibitors vs either tyrosine or *S*-methyl-L-cysteine for both enzymes is competitive (data not shown). The pK_i profiles for both L-alanine and L-phenylalanine inhibition for the *E. herbicola* enzyme are shown in Figure 3A.

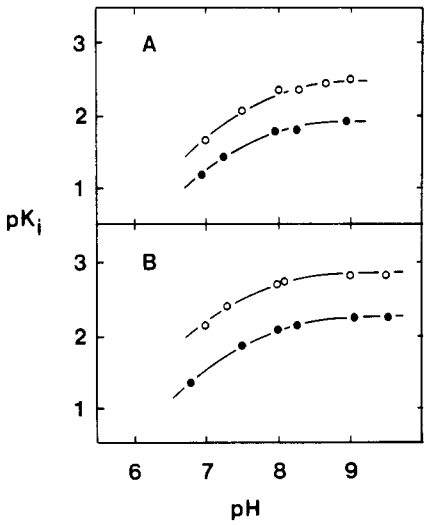


FIGURE 3: pH dependence of inhibitor dissociation constants. (A) Inhibition of *E. herbicola* tyrosine phenol-lyase. The open circles represent data obtained for the binding of L-phenylalanine, while the filled circles represent data obtained for the binding of L-alanine. (B) Inhibition of *C. freundii* tyrosine phenol-lyase. The open circles represent data obtained for the binding of L-phenylalanine, while the filled circles represent data obtained for the binding of L-alanine. In all cases tyrosine 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 fit to eq 3, and the resulting values of $1/K_{is}$ were fitted to eq 4 to obtain the curves shown.

The K_{is} values for both inhibitors increase below the same pK (7.7 ± 0.1 for L-alanine and 7.8 ± 0.1 for L-phenylalanine). The pH-independent value of the K_{is} for L-phenylalanine is 2.8 ± 0.3 mM and for L-alanine is 10 ± 1 mM. These values are in good agreement with the previously reported K_i values of 2 mM for L-phenylalanine and 9.5 mM for L-alanine (Kumagai et al., 1972). For the *C. freundii* enzyme, L-alanine and L-phenylalanine dissociation constants also decrease below the same pK (Figure 3B) of 7.65 ± 0.02 for L-alanine and 7.57 ± 0.03 for L-phenylalanine. The pH-independent value of the K_{is} for L-phenylalanine is 1.49 ± 0.05 mM and for L-alanine is 5.9 ± 0.2 mM, which agrees well with the previously reported K_i value for L-alanine of 6.5 mM (Kumagai et al., 1970b).

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 tyrosine were measured as a function of pH. For the *E. herbicola* enzyme, both ^DV and $^D(V/K)$ are pH dependent. At pH 9.5, the isotope effects are ^DV = 2.5 ± 0.2 and $^D(V/K)$ = 1.5 ± 0.3. Both of these effects increase and become essentially equal at pH 6.5, ^DV = 4.1 ± 0.3 and $^D(V/K)$ = 4.1 ± 0.3. The values of the parameters as a function of pH are listed in Table I. In contrast, the *C. freundii* enzyme displays deuterium isotope effects of ^DV = 3.5 ± 0.4 and $^D(V/K)$ = 2.5 ± 0.4 that are pH independent. These parameters as a function of pH are also listed in Table I.

Table I: Primary Deuterium Isotope Effects[a] as a Function of pH for Tyrosine Phenol-Lyase

enzyme	pH	^DV	$^D(V/K_{tyr})$
E. herbicola	6.4[b]	4.1 ± 0.3	4.1 ± 0.3
	6.7	3.9 ± 0.1	3.5 ● 0.1
	7.0	3.9 ± 0.3	3.5 ± 0.4
	7.5	3.6 ± 0.2	2.6 ± 0.3
	8.6	2.9 ± 0.2	2.1 ± 0.5
	8.9	2.9 ± 0.3	1.9 ± 0.4
	9.1	2.5 ± 0.2	1.5 ± 0.3
C. freundii	6.6	3.3 ± 0.1	2.8 ± 0.3
	7.4	3.5 ± 0.2	2.2 ± 0.1
	8.3	3.7 ± 0.4	2.5 ± 0.4
	9.5	3.4 ● 0.2	2.5 ± 0.3

[a] Except where noted, the values for ^DV and $^D(V/K)$ are from a fit of the data to eq 6. [b] The values for ^DV and $^D(V/K)$ are from a fit of the data to eq 7.

Discussion

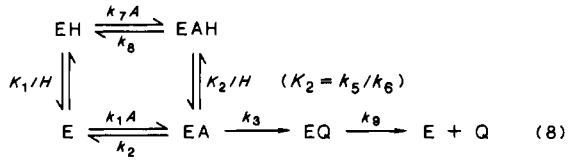
Protonation Mechanism for Citrobacter Enzyme. When either tyrosine or *S*-methyl-L-cysteine is the substrate, the V profile for this enzyme is pH independent. This shows that the substrate binds only to the correctly protonated form of the enzyme. In such a mechanism, the pK values for catalytic and binding groups displayed in the V/K profile will be the correct ones (Cleland, 1977). For tyrosine, the V/K decreases below an average of two pK values calculated to be 7.8. A single pK of 7.6 is observed in the V/K profile for *S*-methyl-L-cysteine. Since neither substrate has any ionizable groups in the range of pH measured, the observed pK values reflect enzyme groups that must be deprotonated in order to permit binding of substrate. Moreover, since the pH profiles do not level off at low pH, a mechanism where both EA and EAH can be converted to products can be ruled out (Cook & Cleland, 1981b). Only the EA complex is catalytically competent.

Since the variable substrate concentration is extrapolated to zero in determining a competitive inhibitor dissociation constant, true pK values are observed for the pH dependence of K_i values for competitive inhibitors (Cleland, 1977). Both of the pK_i profiles for the competitive inhibitors phenylalanine and alanine display a true pK of 7.6. Since only the group with a pK of 7.6 is observed in the V/K profile for *S*-methyl-L-cysteine, this group is probably responsible for abstraction of the proton at the 2-position of the substrate. For tryptophan indole-lyase, which has a similar chemical mechanism [see the following paper, Kiick & Phillips (1988)], June et al. (1981) have shown that dead-end quinonoid complexes can be formed from L-alanine and L-ethionine. This requires that the enzyme be able to abstract the proton from the 2-position of the pseudosubstrate to facilitate quinonoid formation.

Knowing that the observed pK values in the V/K profiles are the correct ones allows one to calculate the value of the other pK in the V/K_{tyr} profile as 8.0. Since this pK of 8.0 is observed only in the V/K profile for tyrosine and not in the pK_i profile for phenylalanine or alanine, this enzyme group must be interacting directly with the hydroxyl moiety of tyrosine. Most likely this group abstracts the hydroxyl proton to permit the phenol ring to tautomerize to the cyclohexadienone, a postulated reaction intermediate (Sawada et al., 1975). More will be said below of this interaction and the pK assignments.

Protonation Mechanism for Erwinia Enzyme. In this system the V for tyrosine decreases below a pK of 7.4, while two pK values of 7.0 and 8.0 are observed in the V/K profile. On the other hand, the V for *S*-methyl-L-cysteine decreases below a pK of 6.4, and V/K decreases below a single pK of 7.3. As stated above, neither substrate has any ionizable groups in the range of pH measured. Thus, the observed pK values reflect enzyme groups that must be deprotonated in order to facilitate catalysis. Since neither V nor V/K appears to level off at low pH, only the unprotonated Michaelis complex is catalytically active. Therefore, we can write the following protonation mechanism in which substrate can bind to the protonated and unprotonated enzyme and EA and EAH

may be interconverted directly:



(8)

All chemical interconversions are included in k_3, and dissociation of all products is included in k_9. In this system, k_9 is pH and isotope independent, while k_3 is the only isotope-dependent step. The distribution between EA and EAH will vary with pH and thus make the rate of formation of EQ pH dependent. Thus, V will appear to be pH dependent [for a further discussion of this mechanism, see Cook and Cleland, (1981a)].

If $k_5 = k_6 = k_7 = k_8 = 0$, the above mechanism reduces to one in which substrate binds only to the correctly protonated form of the enzyme. This is the case observed for the *Citrobacter* enzyme. However, the *Erwinia* system must have either a finite k_5 and k_6 or k_7 and k_8 to account for the pH dependence observed in the V profile. The easiest way to distinguish between these three cases is from the pH variation of the isotope effects, as will be discussed further below.

The pK_i profiles for both of the competitive inhibitors, phenylalanine and alanine, display a single pK of 7.8. Most probably, as in the *Citrobacter* system (pK = 7.6), this is the group that abstracts the proton from the 2-position of the substrate. However, pK assignments of the observed pK values in the V/K_{tyr} profile for the *Erwinia* enzyme are more difficult due to the stickiness of the substrates. The isotope effect data will allow us to make the proper assignments.

Deuterium Isotope Effects. For the *Citrobacter* system, the deuterium isotope effects in Table I on V and V/K for abstraction of the proton at the 2-position of tyrosine are pH independent and equal to 3.5 and 2.5, respectively. These data are consistent with a mechanism for *C. freundii* tyrosine phenol-lyase in which substrate binds only to the correctly protonated form of the enzyme. Proton abstraction is at least partially rate-determining in this system.

For the *Erwinia* system, the deuterium isotope effects on both V and V/K (Table I) are pH dependent and become equal at pH 6.4. As shown by Cook and Cleland (1981a) for the above protonation mechanism, if $k_5 = k_6 = 0$ and/or $k_7 = k_8 = 0$, $^D(V/K)$ will be pH independent. This is clearly not the case for the *Erwinia* enzyme. Thus, by use of mechanism 8 for our analysis, at pH 9.5 (the plateau region of the V and V/K profiles) the following equations apply (Cook & Cleland, 1981a):

$$V/K = \frac{k_1 k_3}{k_2 + k_3} \tag{9}$$

$$V = \frac{k_3 k_9}{k_3 + k_9} \tag{10}$$

$$^D(V/K) = \frac{^Dk_3 + k_3/k_2}{1 + k_3/k_2} \tag{11}$$

$$^DV = \frac{^Dk_3 + k_3/k_9}{1 + k_3/k_9} \tag{12}$$

However, at pH 6.4 and below, which is 1.4 pH units below the pK for the group that abstracts the proton at the 2-position of the substrate, the above equations reduce to the following:

$$V/K = \frac{k_1 k_3 K_1}{k_2 H} \tag{13}$$

$$V = \frac{k_3 K_2}{H} \tag{14}$$

Also, at this pH $^D(V/K) = {}^DV = {}^Dk_3$. As a result, this mechanism predicts the kinetic parameters and the isotope effects to be pH dependent, with expression of the full isotope effect below the pK for the group that abstracts the proton. The data for *E. herbicola* obviously conform to this mechanism.

By substituting values of ^DV and $^D(V/K)$ determined at pH 6.4 into eq 11 and 12, one can calculate both the forward commitment factor ($c_f = k_3/k_2 = 5.2$) and the catalytic ratio ($c_{vf} = k_3/k_9 = 1.1$) for *E. herbicola* tyrosine phenol-lyase. Dividing c_f by c_{vf} yields $k_9/k_2 = 4.7$, the partition ratio for substrate and products from the Michaelis complex. Therefore, we conclude that the off-rate for tyrosine from the binary complex is 4.7 times slower than product release at pH 9.5 and thus limits the overall reaction.

Knowledge of the above commitments allows one to make the proper pK assignments in the V/K_{tyr} profile, which could not have been done otherwise. From the pK_i profiles we know the enzyme group that abstracts the proton has a pK of 7.8. This pK will be perturbed to lower pH by log $(1 + c_f)$ in the V/K profile and log $(1 + c_{vf})$ in the V profile. In the V_{tyr} profile, this pK should be perturbed 0.32 pH unit from 7.8 to 7.48. The experimental value of 7.4 is in good agreement with this prediction. For the V/K_{tyr} profile, the pK should be perturbed 0.8 pH unit from 7.8 to 7.0, and this agrees exactly with the experimental value. Thus, the group with a pK of 8.0 in the V/K_{tyr} profile interacts with the hydroxyl group of tyrosine, presumably in the same way as in the *Citrobacter* system. Moreover, since this group does not appear in the V profile, this enzymic group must be in the correct protonation state in order for tyrosine to bind to the enzyme.

For *S*-methyl-L-cysteine, only one enzymic group is reflected in the pH profiles. These data further confirm that the group with a pK of 8.0 in the V/K_{tyr} profile is interacting with the hydroxyl moiety of tyrosine, and the group with a pK of 7.8 abstracts the proton at the 2-position of the substrate. Since we know the group in the pK_i profiles is responsible for proton abstraction, we can calculate the commitments for *S*-methyl-L-cysteine from the displacement of the pK values in the V and V/K profiles. When *S*-methyl-L-cysteine is the substrate, the commitments for *E. herbicola* tyrosine phenol-lyase are 2.2 for c_f and 24 for c_{vf}, while the Michaelis complex partition ratio $(k_9/k_2) = 0.1$.

Chemical Mechanism. It is generally accepted that with most pyridoxal phosphate dependent enzymes some enzyme residue, usually the ϵ-amino group of a lysine, forms a Schiff base with the C-4′ aldehyde carbon of PLP (Martinez-Carrion, 1986). Upon binding of substrate, a *gem*-diamine is formed when nucleophilic attack by the 2-amino group of the substrate takes place at the C-4′ position of PLP (Snell & Di Mari, 1970; Davis & Metzler, 1972). The *gem*-diamine then collapses to form the external aldimine and free lysine (Figure 4, I). This may be accompanied by rotation of the coenzyme (Floss & Vederas, 1982), as is the case for aspartate aminotransferase (Arnone et al., 1985; Kirsch et al., 1984).

Following transimination, the enzyme group with a pK of 7.6 for the *Citrobacter* system and 7.8 for the *Erwinia* system[3]

[3] As shown in the pH profiles, this group must be unprotonated in order to facilitate binding. This is in agreement with its proposed role as a general base.

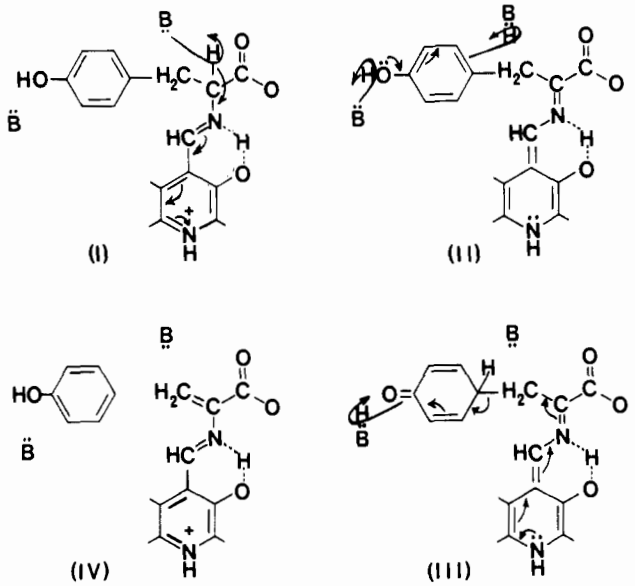


FIGURE 4: Chemical mechanism for the tyrosine phenol-lyase reaction.

abstracts the proton at the 2-position of the substrate to form a stabile quinonoid (Figure 4, II). Kumagai et al. (1970a, 1975) have observed an absorption band at ca. 500 nm upon binding of L-alanine, L-tyrosine, and various tyrosine analogues including L-phenylalanine (Kumagai et al., 1972) to the *Erwinia* enzyme and have ascribed this to the quinonoid intermediate (Miles, 1986). Similar results have been obtained with the *Citrobacter* enzyme (Nagasawa et al., 1981). The fact that this pK of 7.6 is observed in the pK_i profiles for both L-alanine and L-phenylalanine further supports this role, while the relative size of the isotope effects for proton abstraction argue that this step is partially rate-limiting. Moreover, chemical modification of the enzyme with diethyl pyrocarbonate and studies with the modified enzyme suggest the enzymic group modified is a histidine residue and this group is responsible for proton abstraction at the 2-position of the substrate (Kumagai et al., 1975).

Activation of the carbon–carbon bond (C-3 and C-4 of the substrate) can now take place. The group with a pK of 8.2 for the *Erwinia* system and 8.0 for the *Citrobacter* system,[3] abstracts the hydroxyl proton facilitating electron movement to C-4 of the substrate where the group with a pK of 7.6–7.8 donates its proton to permit formation of the cyclohexadienone tautomer of tyrosine (Figure 4, III). In support of this, Faleev et al. (1983) have shown that the proton at the 2-position of the substrate is transferred to the leaving phenolic ring. Therefore, proton abstraction and cyclohexadienone formation must take place prior to elimination to form phenol and the enzyme-bound aminoacrylate (Figure 4, IV). Release of phenol, hydrolysis, and transimination can now take place as have been previously described (Miles, 1986).

ACKNOWLEDGMENTS

We thank Professor W. W. Cleland for his support during the course of these studies and also for his many discussions and insightful comments on the manuscript.

REFERENCES

Arnone, A., Rodgers, P. H., Hyde, C. C., Briley, P. D., Metzler, C. M., & Metzler, D. M. (1985) in *Transaminases* (Christian, P. J., & Metzler, D. E., Eds.) pp 138–155, Wiley, New York.

Blanchard, J. S. (1984) *Methods Enzymol. 104*, 404.

Bradford, M. (1976) *Anal. Biochem. 72*, 248.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273.

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

Cleland, W. W. (1982) *CRC Crit. Rev. Biochem. 13*, 385.

Cook, P. F., & Cleland, W. W. (1981a) *Biochemistry 20*, 1797.

Cook, P. F., & Cleland, W. W. (1981b) *Biochemistry 20*, 1805.

Enei, H., Matsui, H., Yamashita, K., Okumura, S., & Yamada, H. (1972) *Agric. Biol. Chem. 36*, 1861.

Faleev, N. G., Lyubarev, A. E., Martinkova, N. S., & Belikov, V. M. (1983) *Enzyme Microb. Technol. 5*, 219.

Floss, H. G., & Vederas, J. C. (1982) in *Stereochemistry* (Tamm, C., Ed.) pp 161–199, Elsevier North-Holland, Amsterdam.

June, D. S., Suelter, C. H., & Dye, J. L. (1981) *Biochemistry 20*, 2714.

Kiick, D. M., & Phillips, R. S. (1988) *Biochemistry* (following paper in this issue).

Kirsch, J. F., Eichele, G., Ford, G. C., Vincent, M. G., Jansonius, J. N., Gehring, H., & Christen, P. J. (1984) *J. Mol. Biol. 174*, 497.

Kumagai, H. J., Kashima, N., & Yamada, H. J. (1970a) *Biochem. Biophys. Res. Commun. 39*, 796.

Kumagai, H. J., Yamada, H. J., Matsui, H., Ohkishi, H., & Ogata, K. (1970b) *J. Biol. Chem. 245*, 1767.

Kumagai, H. J., Yamada, H. J., Matsui, H., Ohkishi, H., & Ogata, K. (1970c) *J. Biol. Chem. 245*, 1773.

Kumagai, H. J., Kashima, N., Torii, H., Yamada, H. J., Enei, H., & Okumura, S. (1972) *Agric. Biol. Chem. 36*, 472.

Kumagai, H. J., Utagawa, T., & Yamada, H. J. (1975) *J. Biol. Chem. 250*, 1661.

Martinez-Carrion, M. (1986) *Coenzymes Cofactors 1*, 1.

Miles, E. W. (1986) *Coenzymes Cofactors 1*, 253.

Morino, Y., & Snell, E. E. (1970) *Methods Enzymol. 17A*, 439.

Morton, R. A. (1975) *Biochemical Spectroscopy*, Vol. 1, p 184, Halsted, New York.

Nagasawa, T., Utagawa, T., Goto, J., Kim, C.-J., Tani, Y., Kumagai, H., & Yamada, H. (1981) *Eur. J. Biochem. 117*, 33.

Phillips, R. S., von Tersch, R. L., Miles, E. W., & Ahmed, S. A. (1987) *Biochemistry 26*, 4163 (Abstract).

Sawada, S., Kumagi, H., Yamada, H., & Hill, R. K. (1975) *J. Am. Chem. Soc. 97*, 4334.

Snell, E. E., & Di Mari, S. J. (1970) *Enzymes (3rd Ed.) 2*, 335.

Walsh, C. (1979) *Enzymatic Reaction Mechanisms*, Freeman, San Francisco.