Binding of Phenol and Analogues to Alanine Complexes of Tyrosine Phenol-Lyase from *Citrobacter freundii*: Implications for the Mechanisms of α,β-Elimination and Alanine Racemization†

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ABSTRACT: We have examined the interaction of *Citrobacter freundii* tyrosine phenol-lyase with both L- and D-alanine. This enzyme catalyzes the racemization of alanine as a side reaction, in addition to the physiological β-elimination of L-tyrosine to give phenol and ammonium pyruvate. The steady-state kinetic parameters for alanine racemization, k_{cat} and K_m, for D-alanine are 0.008 s^{-1} and 32 mM, respectively, while those for L-alanine are 0.03 s^{-1} and 11 mM. Incubation of tyrosine phenol-lyase with either L- or D-alanine forms a quinonoid complex that exhibits a strong peak at 500 nm. The presence of K^+ increases the intensity of the 500-nm absorption with L-alanine, but decreases the intensity of the peak with D-alanine. Rate constants for the formation of these quinonoid intermediates and the effects of phenol and analogues on the reaction with either L- or D-alanine have been studied by rapid-scanning and single-wavelength stopped-flow spectrophotometry. Phenol binds to all the intermediates of tyrosine phenol-lyase with L- and D-alanine, but most strongly to the external aldimine complex, resulting in a decrease in the absorbance at 500 nm at equilibrium. Pyridine *N*-oxide binds selectively to the quinonoid complex of alanine, and thus causes an increase in the absorbance at 500 nm at equilibrium. 4-Hydroxypyridine causes a decrease in absorbance at 500 nm during the fast phase, but an increase in absorbance at 502 nm in a subsequent slow relaxation. From these studies of the effects of phenol and analogues on the reaction of tyrosine phenol-lyase with alanine, we have found there is a common quinonoid intermediate in the reactions of both L-alanine and D-alanine. The formation of this intermediate is the rate-determining step in the racemization reaction, as the rate constants for its formation (0.01 s^{-1} from D-alanine and 0.034 s^{-1} from L-alanine) are in good agreement with the steady-state k_{cat} values. The implications of these studies for the mechanisms of the β-elimination and alanine racemization reactions are discussed.

Tyrosine phenol-lyase (deaminating) (EC 4.1.99.2) is a pyridoxal 5′-phosphate (PLP)[1] dependent enzyme which catalyzes the β-elimination of L-tyrosine to produce phenol and ammonium pyruvate (eq 1). In addition to L-tyrosine,

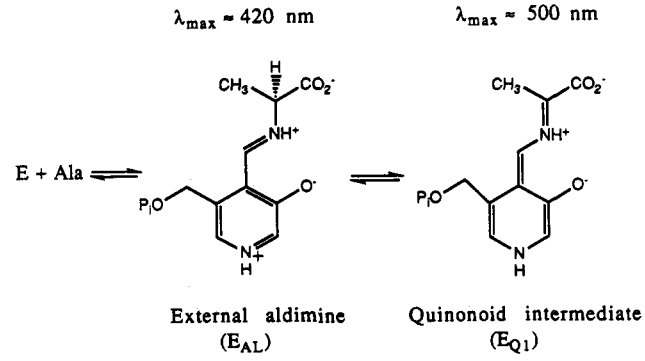
$$\text{L-tyrosine} + H_2O \rightleftharpoons \text{phenol} + \text{ammonium pyruvate} \quad (1)$$

L- and D-serine, *S*-methyl-L-cysteine, and β-chloro-L-alanine act as substrates in the α,β-elimination, with the formation of ammonium pyruvate (Kumagai et al., 1970a). The enzyme also catalyzes the reverse reaction of α,β-elimination (Yamada et al., 1972; Enei et al., 1972a), and the β-replacement reaction between the substrates for α,β-elimination and phenol derivatives (eq 2) (Kumagai et al., 1969; Ueno et al., 1970). In

$$\text{L-serine} + \text{phenol} \rightarrow \text{L-tyrosine} + H_2O \quad (2)$$

addition, the enzyme catalyzes the racemization of alanine, which cannot be a substrate for α,β-elimination or β-replacement reactions, since it does not contain a suitable leaving group on the β-carbon (Kumagai et al., 1970b). Addition of either L-alanine or D-alanine to tyrosine phenol-lyase results

Scheme I



in the appearance of an absorption band near 500 nm, which has been assigned to a quinonoid intermediate (E_{Q1} in Scheme I) (Kumagai et al., 1975). It has been demonstrated by stopped-flow kinetic methods that the formation of this quinonoid intermediate from L- or D-alanine occurs by a two-step mechanism for tyrosine phenol-lyase from *Citrobacter intermedius* (Scheme I) (Muro et al., 1978). However, these previous experiments provided no information about the mechanism of the alanine racemization reaction. Other PLP-dependent enzymes have been found to catalyze racemization as a side reaction. For example, tryptophan synthase $\alpha_2\beta_2$ complex from *Escherichia coli* racemizes tryptophan slowly (Miles et al., 1986). Serine hydroxymethyltransferase also exhibits an alanine racemase side activity (Shostak & Schrich,

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; SOPC, *S*-(*o*-nitrophenyl)-L-cysteine; LDH, lactate dehydrogenase; GPT, glutamate pyruvate transaminase; DAAO, D-amino acid oxidase.

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1988). Tyrosine phenol-lyase has been reported to have other unusual stereochemical properties. Although the reversal of the α,β-elimination reaction is highly stereoselective for formation of L-tyrosine, it has been reported that either L-tyrosine or D-tyrosine can serve as a substrate for α,β-elimination (Kumagai et al., 1970a). The β-substitution reaction with either D- or L-serine was reported to form L-tyrosine (Enei et al., 1972b). More recently, it was reported that D-tyrosine is not a substrate for the α,β-elimination reaction (Demidkina et al., 1984). For this reason, we have been carefully examining the stereochemical properties of tyrosine phenol-lyase reactions.

We have previously found that the external aldimine and quinonoid intermediates in the reaction of L-alanine with *E. coli* tryptophan indole-lyase can bind indole and analogues, and the intensity of the quinonoid absorption peak is altered by these ligands (Phillips, 1991). In the present study, we have examined the effects of phenol and analogues 4-hydroxypyridine and pyridine *N*-oxide on the steady-state kinetics and the pre-steady-state kinetics of the reactions of L- or D-alanine with tyrosine phenol-lyase. These results demonstrate the detection of distinct quinonoid intermediates and the first measurement of all of the rate constants for alanine racemization catalyzed by tyrosine phenol-lyase. These results also provide a foundation for further studies on the α,β-elimination reaction.

MATERIALS AND METHODS

Instrumentation. Single-wavelength stopped-flow kinetic measurements were performed using a Kinetics Instruments stopped-flow mixer with a modified Cary 14 (On-Line Instruments, Inc., Bogart, GA) as the light source. Data collection was performed on a Zenith Z-286 computer using hardware and software provided by On-Line Instruments, Inc. Rapid-scanning stopped-flow measurements were obtained with a photodiode array detector from Princeton Applied Research as previously described (Phillips, 1991). Steady-state kinetic measurements and static UV/vis spectra were obtained on a Gilford Response II UV/vis spectrophotometer equipped with a thermoelectric cell block. Circular dichroism was measured with a Jasco J500C spectropolarimeter.

Materials. Tyrosine phenol-lyase was purified from *Citrobacter freundii* cells as described previously (Phillips et al., 1987). Enzyme concentrations were estimated from the $A_{278}^{1\%}$ = 8.37 (Muro et al., 1978) assuming a subunit molecular weight of 51 kDa (Antson et al., 1993). The lactate dehydrogenase (LDH) from rabbit muscle and glutamate pyruvate transaminase (GPT) from porcine heart used in the coupled assays were purchased from United States Biochemical Co. (USB). D-Alanine, L-alanine, PLP, and NADH were also obtained from USB. The D-amino acid oxidase (DAAO) from porcine kidney was from ICN Biochemicals. The 4-hydroxypyridine and pyridine *N*-oxide were obtained from Aldrich, while phenol was from the J.T. Baker Chemical Co. All other reagents and chemicals obtained from commercially available sources were of the highest quality available. Chiral purities of L-alanine and D-alanine were determined by using the coupled enzyme assay systems as described by Badet et al. (1984). Chiral purities of 99.5% for L-alanine and 99.8% for D-alanine were obtained.

Enzyme Assay. Routine activity assays were performed with *S*-(*o*-nitrophenyl)-L-cysteine (SOPC) (Phillips, 1987), measuring the decrease in absorbance at 370 nm ($\Delta\epsilon = -1.86 \times 10^3$ M^{-1} cm^{-1}) in 50 mM potassium phosphate, pH 8.0, at 25 °C. The racemization reaction was assayed spectrophotometrically with either L-alanine or D-alanine as substrate. Production of D-alanine from L-alanine was followed using the same enzyme coupling system as described by Badet et al. (1984). A standard assay mixture contained 50 mM potassium phosphate (pH 8.0), 0.1 mg of LDH, 0.04 mg of DAAO, 0.2 mM NADH, 0.5 mg of TPL, and various concentrations of L-alanine (from 2 to 240 mM) in a final volume of 0.6 mL. Pyruvate produced by DAAO from D-alanine was reduced by LDH with NADH. The decrease in absorbance at 340 nm ($\Delta\epsilon = -6.22 \times 10^3$ M^{-1} cm^{-1}) was measured vs time.

Production of L-alanine from D-alanine was followed in an assay mixture (0.6 mL) containing 50 mM potassium phosphate buffer (pH 8.0), 0.1 mg of LDH, 0.02 mg of GPT, 0.2 mM NADH, 1.5 mM α-ketoglutarate, and various concentrations of D-alanine (from 2 to 240 mM). The reaction was initiated by adding 0.5 mg of tyrosine phenol-lyase, and the decrease in absorbance at 340 nm was measured vs time. Values of k_{cat} and K_m were obtained by fitting the data to the Michaelis–Menten equation (eq 3), using a nonlinear least-

$$v = k_{cat}[E][S]/(K_m + [S]) \quad (3)$$

squares program (Enzfitter) from Elsevier Biosoft. We were not able to obtain kinetic data using a coupled assay with L-alanine dehydrogenase. This appears to be due to inhibition of L-alanine dehydrogenase by the high concentrations of D-alanine required, because of the high K_m value for tyrosine phenol-lyase in the racemization reaction.

Spectral Titration. The absorbance of solutions of tyrosine phenol-lyase (all in a total volume of 0.4 mL) that contained 12 μM tyrosine phenol-lyase, 50 mM potassium phosphate buffer (pH 8.0), 0 or 2 mM phenol or analogues, and various concentrations of D- or L-alanine was measured at 500 nm. The concentration dependence of the absorbance at 500 nm was fit to eq 4 by using Enzfitter.

$$A = A_{max}[S]/(K_d + [S]) \quad (4)$$

Rapid Reactions. Prior to performing the rapid kinetic experiments, the stock enzyme was incubated with 0.5 mM PLP for 1 h at 30 °C and then separated from excess PLP on a short desalting column (Excellulose, Pierce) equilibrated with 50 mM phosphate buffer, pH 8.0. The rapid-scanning stopped-flow kinetic measurements were performed at room temperature, which was approximately 25 °C, while the single-wavelength stopped-flow kinetic measurements were performed at 25 °C, controlled by a circulating water bath. For the reactions with L- or D-alanine, the enzyme solutions were mixed with solutions of alanine in the same buffer. In the case of the binding reactions of phenol and analogues, both the enzyme solution and the phenol, 4-hydroxypyridine, or pyridine *N*-oxide solution contained 0.4 M L- or D-alanine, and control experiments without aromatic ligands were performed to ensure that no relaxation artifacts were observed due to concentration differences. 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. The quality of fit was judged by analysis of the residuals and by the Durbin–Watson value (Durbin & Watson, 1970).

RESULTS

Static Spectroscopic Studies. Upon mixing tyrosine phenol-lyase with L- or D-alanine, an increase in absorbance at about 500 nm is observed; this band has been ascribed to a quinonoid

Table I: Steady-State Kinetic Parameters for L-Alanine and D-Alanine at 25 °C

substrate	A_{max}[a]	K_d (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} M^{-1})
L-alanine	0.47	16	0.032	11	2.9
L-alanine + 0.25 mM phenol	0.33	7	0.012	4	3.0
D-alanine	0.35	33	0.008	32	0.3
D-alanine + 0.25 mM phenol	0.09	9	0.002	8	0.3

[a] The protein concentration is 26.4 μM.

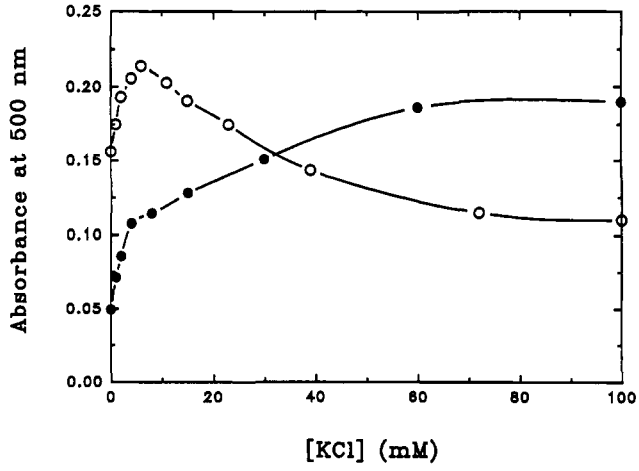


FIGURE 1: Effect of K^+ on the absorbance at 500 nm of tyrosine phenol-lyase complexes with L-alanine (filled circles) and D-alanine (open circles) in 50 mM triethanolamine phosphate buffer, pH 8.0, at 25 °C. The concentrations of alanine and tyrosine phenol-lyase are 0.5 M and 10 μM, respectively.

carbanionic intermediate (Kumagai et al., 1975). This absorbance peak at 500 nm remains constant for several hours. We measured the absorbance at 500 nm in the presence of increasing L- or D-alanine concentrations, and the data were fit to eq 3. This analysis results in a dissociation constant of 16 mM for L-alanine, and a dissociation constant of 33 mM for D-alanine (Table I). These measurements were performed in the presence of high [K^+]. Previously, it was reported that K^+ is required for the formation of the 500-nm peak with tyrosine phenol-lyase and L-alanine (Myagkikh & Demidkina, 1988). We confirmed this effect of K^+ on the L-alanine complex (Figure 1). However, the effects of K^+ on the D-alanine complex are very different (Figure 1). In the absence of K^+, D-alanine forms a strong 500-nm peak, which first increases and then decreases in intensity as [K^+] increases.

When phenol was added to the solutions of tyrosine phenol-lyase, along with L-alanine or D-alanine, the intensity of the quinonoid absorbance peak at 500 nm was decreased, compared with the spectra obtained without phenol (Figure 2, curves 1 and 2, and Table I). The dissociation constant for L-alanine, calculated from the absorbance measurements at 500 nm, is 7 mM in the presence of 0.25 mM phenol, while the dissociation constant for D-alanine is 9 mM in the presence of 0.25 mM phenol (Table I). Other phenol analogues were tested: 3-fluorophenol and 4-fluorophenol exhibited spectral changes similar to those produced by phenol, while aniline and pyridine showed almost no effect on the absorbance. Thus, these effects are not due to nonspecific effects of these compounds acting as solvents. However, 4-hydroxypyridine and pyridine *N*-oxide were found to cause an increase in the intensity of the quinonoid absorbance peak (Figure 2, curves 3 and 4).

Steady-State Kinetic Studies of Alanine Racemase Activity. The k_{cat} and K_m values for D-alanine are 0.008 s^{-1} and 32 mM, while those for L-alanine are 0.03 s^{-1} and 11 mM (Table I). In the presence of phenol, both k_{cat} and K_m for L-alanine or D-alanine decrease, but the ratio k_{cat}/K_m does not change significantly (Table I). This implies that phenol is an uncompetitive inhibitor of tyrosine phenol-lyase in the racemization reaction of alanine, indicating that phenol only binds to the enzyme–alanine complex. According to the Haldane equation, $(k_{cat}/K_m)_L/(k_{cat}/K_m)_D = K_{eq} = 1$. However, we found this ratio to be 10 (Table I). This finding suggests that L-alanine and D-alanine bind to different enzyme conformations (see Discussion).

Rapid Kinetic Studies with L- *and* D-*Alanine.* Single-wavelength stopped-flow kinetic studies of the reaction of tyrosine phenol-lyase with various concentrations of L-alanine were performed at 500 nm. The time courses of the reactions

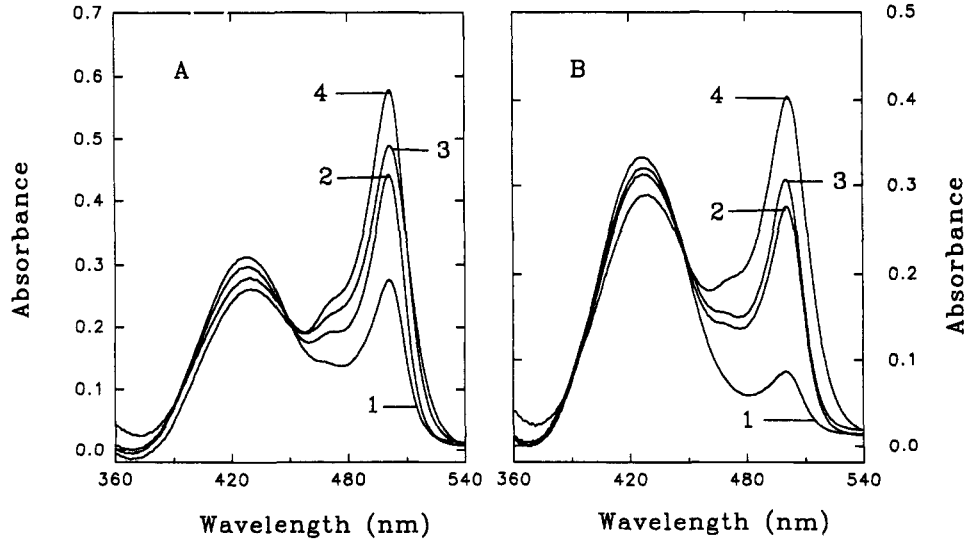
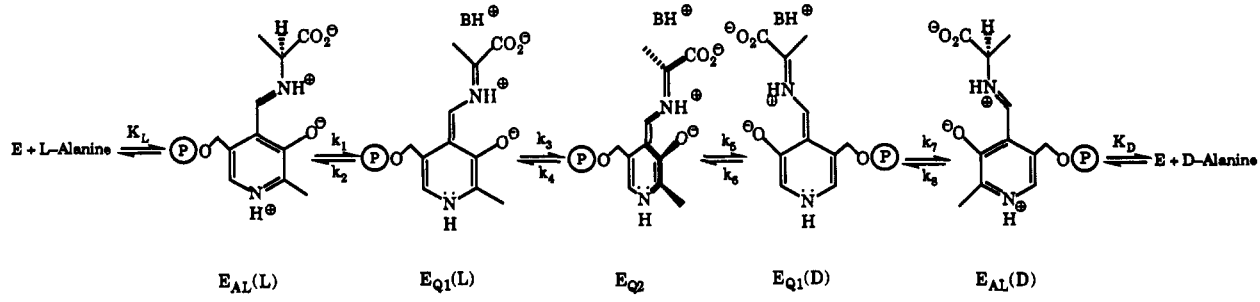


FIGURE 2: Absorption spectra of tyrosine phenol-lyase, with alanine in 50 mM potassium phosphate buffer, pH 8.0, at 25 °C: (A) curve 1, TPL, 26.4 μM with 0.4 M L-alanine in the presence of 0.25 mM phenol; curve 2, TPL, 26.4 μM with 0.4 M L-alanine; curve 3, TPL, 26.4 μM with 0.4 M L-alanine in the presence of 50 mM pyridine *N*-oxide; curve 4, TPL, 26.4 μM with 0.4 M L-alanine in the presence of 7 mM 4-hydroxypyridine; (B) curve 1, TPL, 26.4 μM with 0.4 M D-alanine in the presence of 0.25 mM phenol; curve 2, TPL, 26.4 μM with 0.4 M D-alanine; curve 3, TPL, 26.4 μM with 0.4 M D-alanine in the presence of 50 mM pyridine *N*-oxide; curve 4, TPL, 26.4 μM with 0.4 M D-alanine in the presence of 7 mM 4-hydroxypyridine.

Scheme II



at low L-alanine concentrations fit well to single exponentials, although a second slow exponential process with low amplitude can be observed at higher concentrations. The rate constant, k_{obs}, of the first phase was found to increase in a hyperbolic manner with the L-alanine concentration. The rates of the second phase are not affected by changes in concentration, and have an average value of 0.7 ± 0.05 s^{-1}. These results are similar to those reported previously by Muro et al. (1978), who concluded that the reaction of tyrosine phenol-lyase with L-alanine consists of at least three elementary steps, as shown in Scheme II, formation of $E_{AL}(L)$, $E_{Q1}(L)$, and E_{Q2}. The initial binding step is too fast to be measured by the stopped-flow method, and does not result in any absorbance change at 500 nm. The relaxation time for the first phase observed at 500 nm can be expressed by eq 5. Fitting of the

$$1/\tau = k_1\{[L]/(K_L + [L])\} + k_2 \quad (5)$$

concentration dependence data to eq 5 gives k_1 = 1.81 ± 0.06 s^{-1} for deprotonation, k_2 = 0.37 ± 0.07 s^{-1} for reprotonation, and the value of the binding constant K_L = 80 ± 1 mM for external aldimine formation from L-alanine. Thus, the dissociation constant for the quinonoid complex of L-alanine $K_d = K_L(k_2/k_1)$ = 16.3 mM, which is in good agreement with the value of 16 mM obtained in the static titration and the K_m for the alanine racemization reaction (11 mM) (Table I). The value of 0.7 s^{-1} should correspond to the sum of the rate constants in the third step ($k_3 + k_4$).

The reaction of tyrosine phenol-lyase with D-alanine was also measured by stopped-flow experiments at 500 nm. The results were found to be similar to those obtained for the reaction with L-alanine. However, there was a time lag before the absorbance increase at 500 nm began. The time lag was found to decrease with increasing concentration of D-alanine, and a time lag of 0.1 s was observed after mixing the enzyme solution with 0.32 M D-alanine. The time courses of the subsequent absorbance increase at 500 nm fit well to a single-exponential process. The rate constants were found to increase in a hyperbolic manner with increasing D-alanine concentration. Fitting the concentration dependence data to eq 5 gives k_1 = 0.75 ± 0.01 s^{-1} for deprotonation of the external aldimine, k_2 = 0.29 ± 0.01 s^{-1} for reprotonation of the quinonoid intermediate, and an equilibrium constant for external aldimine formation from D-alanine, K_D = 90.1 ± 6.3 mM. The estimated dissociation constant for D-alanine, $K_d = K_D(k_2/k_1)$ = 34.8 mM, is in good agreement with the value of 33 mM obtained in the static titration experiments and the K_m for D-alanine in the racemization reaction (32 mM) (Table I). The mechanism of this reaction is similar to that for L-alanine and is shown in Scheme II, starting from the right side.

Rapid Kinetic Studies of Phenol Binding. The binding of phenol to the complex of tyrosine phenol-lyase with L-alanine

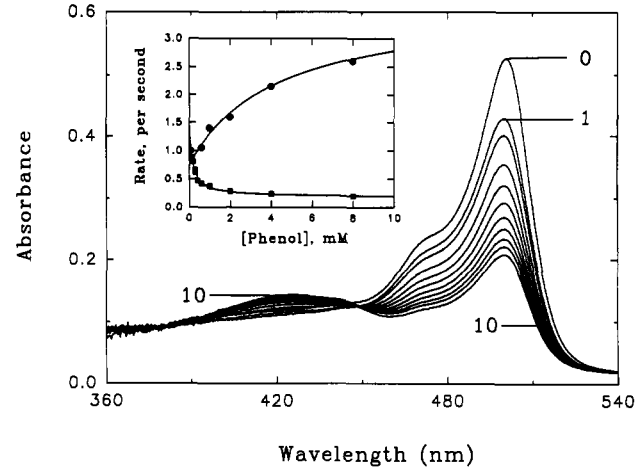
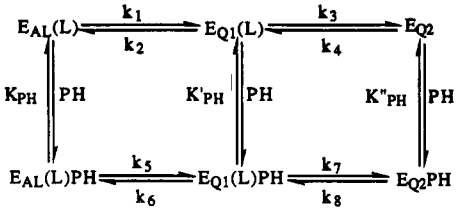


FIGURE 3: Rapid-scanning data for the reaction of the tyrosine phenol-lyase–L-alanine complex (18.2 μM) with 5 mM phenol. Scans were taken at 0.16-s intervals, beginning at 0.02 s (curve 1). Curve 0 is the spectrum of the initial enzyme complex without phenol present. The inset shows the dependence of the relaxations of the tyrosin phenol-lyase–L-alanine complex on the concentration of phenol for the fast phase (filled circles) and the slow phase (filled squares).

was examined by rapid-scanning stopped-flow spectrophotometry, with the results shown in Figure 3. There is an initial decrease in the absorbance at 500 nm that is completed before the first scan is collected (compare curves 0 and 1 in Figure 3), indicating that rapid binding of phenol to the tyrosine phenol-lyase complexes with L-alanine results in a perturbation of the absorption intensity. Subsequently, there is a slow relaxation, resulting in a further decrease in the 500-nm peak and concomitant increase at 422 nm, with a good isosbestic point at about 446 nm, demonstrating that there is direct interconversion between these external aldimine and quinonoid complexes. The rapid-scanning studies of the reaction of phenol with the complex of tyrosine phenol-lyase and D-alanine gave similar results. We next performed single-wavelength stopped-flow studies at 500 nm. The progress curves obtained for the reaction of the enzyme–L-alanine complex with high phenol concentrations are distinctly biphasic; however, at low phenol concentrations, only a single-exponential process is observed. The rate constant for the first phase (with high amplitude) decreases with increasing phenol concentration (Figure 3 inset, filled squares). In contrast, the rate constant for the second phase (with low amplitude) increases with phenol concentration (Figure 3 inset, filled circles). The mechanism of this reaction is considered to be a bicyclic system, as shown in Scheme III. This mechanism is characterized by five relaxation times, since it includes six states. Three of the relaxations are associated with the three phenol binding steps, and they are too rapid to be measured by our stopped-flow methods (see above). The fourth relaxation time refers to the

Scheme III[a]



[a] $E_{AL}(L)$ = external aldimine; $E_{Q1}(L)$ = the first quinonid complex of L-alanine; E_{Q2} = the second quinonoid complex; PH = phenol or analogue.

reaction $E_{AL}(L) \rightleftharpoons E_{Q1}(L)$ and $E_{AL}(L)PH \rightleftharpoons E_{Q1}(L)PH$. The rate equation for this relaxation time should be given by eq 6 (Bernasconi, 1986). The experimental values of $1/\tau_4$

$$1/\tau_4 = k_1\{K_{PH}/(K_{PH} + [PH])\} + k_2\{K'_{PH}/(K'_{PH} + [PH])\} + k_5\{[PH]/(K_{PH} + [PH])\} + k_6\{[PH]/(K'_{PH} + [PH])\} \quad (6)$$

decrease with increasing phenol concentration (Figure 3 inset, filled squares), indicating that k_5 and k_6 are very small. Thus, eq 6 can be simplified to eq 7. Fitting the data in Figure 3

$$1/\tau_4 \approx k_1\{K_{PH}/(K_{PH} + [PH])\} + k_2\{K'_{PH}/(K'_{PH} + [PH])\} \quad (7)$$

(inset, filled squares) to eq 7 gives $k_1 = 1.6 \pm 0.2$ s^{-1} and $k_2 = 0.3 \pm 0.05$ s^{-1}, in good agreement with the values of 1.81 and 0.37 s^{-1} obtained in the reaction of tyrosine phenol-lyase with L-alanine. This analysis also gives values of $K_{PH} = 0.11 \pm 0.04$ mM for binding of phenol to the external aldimine and $K'_{PH} = 85 \pm 7$ mM for binding of phenol to the first quinonoid intermediate; this remarkable 773-fold difference in affinity (3.9 kcal/mol at 25 °C) indicates a significant structural change in the active site must occur upon formation of the quinonoid intermediate. The binding constant of $E_{AL}(L)$ for phenol is similar to the K_m for phenol in the tyrosine synthesis reaction (0.2 mM) (Phillips et al., 1988).

The fifth relaxation time is due to the relaxation between $E_{Q1}(L) \rightleftharpoons E_{Q2}$ and $E_{Q1}(L)PH \rightleftharpoons E_{Q2}PH$. From the results of single-wavelength stopped-flow studies of the reaction of the enzyme–L-alanine complex with 4-hydroxypyridine (see below), $k_3 = 0.034$ s^{-1}; furthermore, it may be assumed on chemical grounds that k_7 is also very small. With these assumptions, the derivation of the fifth relaxation time is now straightforward. By using the methods described by Bernasconi (1976), the rate equation is given by eq 8. Fitting the

$$1/\tau_5 = k_8\{[PH]/(K''_{PH} + [PH])\} + k_4\{K''_{PH}/(K''_{PH} + [PH])\} \quad (8)$$

data in Figure 3 (inset, filled circles) to eq 8 gives $k_4 = 0.74 \pm 0.1$ s^{-1} (intercept in Figure 3 inset), in good agreement with the value (0.7 s^{-1}) which was obtained directly from the single-wavelength stopped-flow experiments with tyrosine phenol-lyase and L-alanine. The mechanism (Scheme III) predicts that the plateau value of $1/\tau_5$ at infinite phenol concentration, $k_8 = 2.9 \pm 0.5$ s^{-1}, is that for $E_{Q2}PH \rightarrow E_{Q1}(L)PH$. The binding constant K''_{PH} for phenol binding to E_{Q2}, from fitting the data in Figure 3 (inset, filled circles) to eq 8, is 4.2 ± 0.2 mM.

Single-wavelength stopped-flow kinetic studies of the reaction of phenol with the D-alanine complex of tyrosine phenol-lyase gave similar results. The mechanism of this reaction was considered to be the same as that of L-alanine.

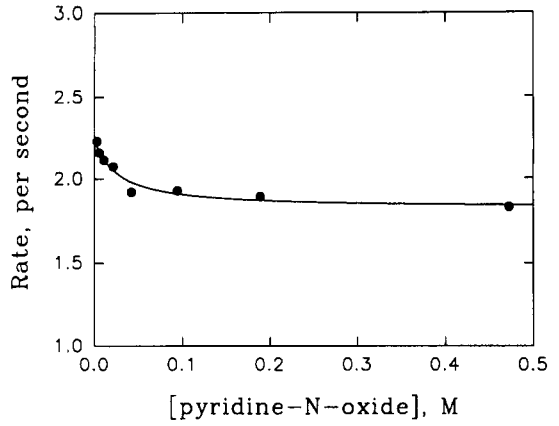


FIGURE 4: Dependence of the relaxations of the tyrosine phenol-lyase–L-alanine complex on the concentration of pyridine *N*-oxide.

Fitting the data to eq 7 gives the rate constant for $E_{AL}(D) \rightarrow E_{Q1}(D)$, $k_1 = 0.7 \pm 0.04$ s^{-1}, and the rate constant for $E_{Q1}(D) \rightarrow E_{AL}(D)$, $k_2 = 0.3 \pm 0.02$ s^{-1}, in good agreement with the values of 0.75 and 0.29 s^{-1}, obtained directly in the reaction with D-alanine. It also gives the binding constant for phenol binding to $E_{AL}(D)$, $K_{PH} = 1.1 \pm 0.2$ mM, and the binding constant for phenol binding to $E_{Q1}(D)$, $K'_{PH} = 88 \pm 9$ mM. It is interesting that phenol binds about 10-fold more strongly to the external aldimine complex of L-alanine than to that of D-alanine. Fitting the data for D-alanine to eq 8 gives the rate constant for $E_{Q1}(D) \rightarrow E_{Q2}$, $k_3 = 0.4 \pm 0.05$ s^{-1}, and that for $E_{Q2}PH \rightarrow E_{Q1}(D)PH$, $k_8 = 1.34 \pm 0.05$ s^{-1}. In contrast to the results for external aldimine binding, the binding constant for phenol binding to E_{Q2}, $K''_{PH} = 4.1 \pm 0.1$ mM, which is very close to the value of 4.2 mM obtained from the reaction with L-alanine. This suggests that the second quinonoid intermediate formed from L-alanine is similar to that formed from D-alanine.

Rapid Kinetic Studies of Pyridine N-Oxide Binding. In contrast to phenol, mixing of pyridine *N*-oxide with the L- or D-alanine complexes of tyrosine phenol-lyase results in an increase in absorbance at 500 nm (Figure 2). Single-wavelength studies were performed at 500 nm to clarify the mechanism of this reaction. The time courses fit well to a single-exponential process, and the rate constant decreases with increasing pyridine *N*-oxide concentration (Figure 4). This behavior is diagnostic for a mechanism where pyridine *N*-oxide binds only to the quinonoid intermediate(s) $E_{Q1}(L)$ and E_{Q2} in Scheme III. The relaxation time between $E_{AL}(L)$ and $E_{Q1}(L)$ can be expressed in eq 9. Fitting the data in

$$1/\tau = k_1 + k_2\{K'_{PH}/(K'_{PH} + [L])\} \quad (9)$$

Figure 4 to eq 9 gives $k_1 = 1.82 \pm 0.06$ s^{-1} and $k_2 = 0.4 \pm 0.07$ s^{-1}, in excellent agreement with the directly obtained values of 1.81 and 0.37 s^{-1} (see above). The binding constant for pyridine *N*-oxide to $E_{Q1}(L)$ $K'_{PH} = 27 \pm 5$ mM. The first quinonoid complex with bound pyridine *N*-oxide must also have an absorption maximum at 500 nm, so an increase in absorbance at 500 nm was observed. The relaxation between the first and second quinonoid complexes is undetectable. This may be due to negligible absorbance differences between these complexes with pyridine *N*-oxide, or to similar binding constants of pyridine *N*-oxide for both quinonoid complexes. Single-wavelength kinetic studies of the reaction of pyridine *N*-oxide with the enzyme-D-alanine complex gave similar results (data not shown).

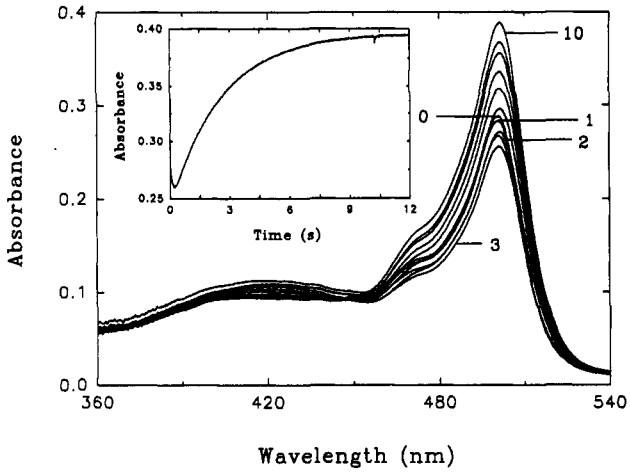


FIGURE 5: Rapid-scanning data for the reactions of the tyrosine phenol-lyase with L-alanine in the presence of 4-hydroxypyridine. The solution was 12 μM enzyme, 0.5 M L-alanine, and 0.4 mM 4-hydroxypyridine. Scans were taken at 0.02 (curve 1), 0.12 (curve 2), 0.22 (curve 3), 0.72 (curve 4), 1.52 (curve 5), 2.52 (curve 6), 3.52 (curve 7), 4.52 (curve 8), 5.92 (curve 9), and 12.02 s (curve 10). Curve 0 is the spectrum of the initial enzyme complex without 4-hydroxypyridine present. The inset shows the progress curve at 500 nm.

Rapid Kinetic Studies of 4-Hydroxypyridine Binding. The binding of 4-hydroxypyridine[2] to the complex of tyrosine phenol-lyase with L-alanine was examined by rapid-scanning stopped-flow spectrophotometry, with the results shown in Figure 5. In the fast phase, there is a decrease in the absorbance at 500 nm (Figure 5, curves 0–3). Subsequently, there is a slow relaxation, resulting in an increase in intensity of the quinonoid peak, and a concomitant red shift in the peak position from 501 to 502 nm. There is also a decrease in the intensity of the 422-nm peak, and the absorbance increase at 502 nm exhibits a reasonable isosbestic point at 444 nm with the external aldimine peak at 422 nm during the slow phase. Single-wavelength stopped-flow measurements of this reaction at various concentrations of 4-hydroxypyridine were performed at 500 nm. As expected, the progress curves of the reaction at 500 nm are distinctly biphasic, as shown in Figure 5 (inset). These data can also be explained by the mechanism shown in Scheme III. The fast phase of 4-hydroxypyridine binding exhibits a linear dependence of the rate constant on the 4-hydroxypyridine concentration (Figure 6A), with a slope of 1.65×10^4 M^{-1} s^{-1}. This suggests that this phase is due to the binding processes in Scheme III; however, the binding is considerably slower than expected for a diffusion-controlled process. This may be due to preferential binding of the minor hydroxypyridine tautomer. Since K_{eq} between the keto and enol forms of 4-hydroxypyridine has been reported as 2200 (Meislicb, 1962), the true second-order rate constant for its binding would be 3.6×10^7 M^{-1} s^{-1}, which is within an order of magnitude of diffusion control. The amplitude of the fast phase is also concentration dependent (Figure 6A), decreasing with concentration up to about 15 mM, but showing a small *increase* in absorbance at 30 mM. This complex behavior could be due to differential effects of the keto and enol tautomers on the absorbance of the quinonoid intermediate upon binding, or to subunit interactions in the complex (Antson et al., 1993).

[2] 4-Hydroxypyridine exists in equilibrium with its 4-pyridone tautomer. "4-Hydroxypyridine" is used herein to designate the compound without implication of a specific tautomeric form, unless the tautomer is explicitly stated.

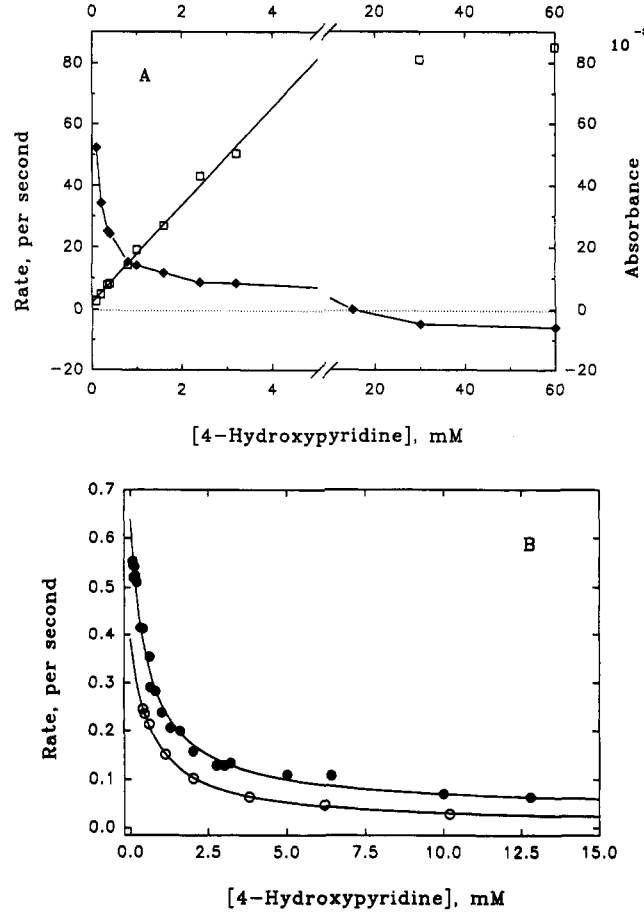


FIGURE 6: (A) Dependence of the relaxations (open squares) and amplitudes (filled diamonds) of the tyrosine phenol-lyase–L-alanine complex (15 μM) on the concentration of 4-hydroxypyridine for the fast phase. (B) Dependence of relaxations of the tyrosine phenol-lyase–L-alanine complex (15 μM) (filled circles) and the tyrosine phenol-lyase–D-alanine complex (15 μM) (open circles) on the concentration of 4-hydroxypyridine for the slow phase.

The slow phase in the reaction with 4-hydroxypyridine exhibits a decrease in the rate constant with increasing 4-hydroxypyridine concentration (Figure 6B, filled circles). This behavior is diagnostic for a mechanism which involves a slow interconversion, followed by a rapid binding process. Since our data from circular dichroism (see below) indicate that the major species at equilibrium is $E_{Q2}PH$, this relaxation must involve the interconversion of E_{Q1} and E_{Q2} and the binding to E_{Q2}. Thus, the rate law for this reaction is given by eq 10.

$$1/\tau = k_3 + k_4\{K''_{PH}/K''_{PH} + [HP]\} \qquad (10)$$

Fitting of the data in Figure 6B (filled circles) to eq 10 gives $k_3 = 0.034 \pm 0.002$ s^{-1} and $k_4 = 0.6 \pm 0.02$ s^{-1}. The sum of these two rate constants ($k_3 + k_4 = 0.634$ s^{-1}) is very close to the value for the second relaxation (0.7 s^{-1}) which was obtained from the single-wavelength experiments with tyrosine phenol-lyase and L-alanine. These results also give a binding constant $K''_{PH} = 0.57 \pm 0.02$ mM for 4-hydroxypyridine binding to the second quinonoid complex.

The reaction of the enzyme–D-alanine complex with 4-hydroxypyridine gave similar results. Figure 6B (open circles) shows the concentration dependence of the rate constant for the slow phase. Although no evidence for the existence of a second quinonoid intermediate (E_{Q2}) was obtained in the direct reaction of tyrosine phenol-lyase with D-alanine [above and Muro et al. (1978)], these experiments

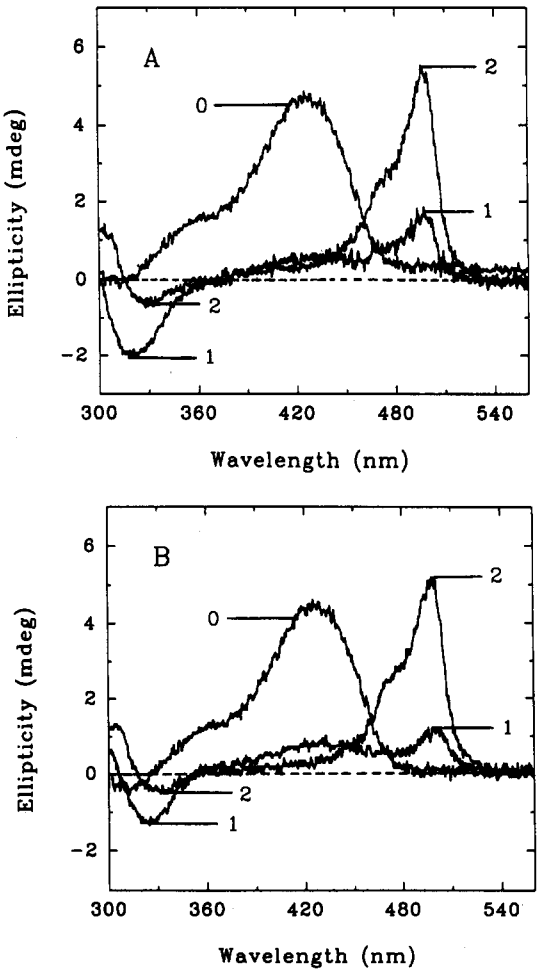


FIGURE 7: Circular dichroism spectra of tyrosine phenol-lyase and tyrosine phenol-lyase complexes with L-alanine and D-alanine in the absence and presence of 4-hydroxypyridine: (A) curve 0, CD spectrum of 15 μM enzyme; curve 1, CD spectrum of the enzyme–L-alanine complex (0.5 M L-alanine and 15 μM enzyme); curve 2, CD spectrum of the enzyme–L-alanine complex (0.5 M L-alanine and 15 μM enzyme) in the presence of 70 mM 4-hydroxypyridine; (B) curve 0, CD spectrum of 15 μM enzyme; curve 1, CD spectrum of the enzyme–D-alanine complex (0.5 M D-alanine and 15 μM enzyme); curve 2, CD spectrum of the enzyme–D-alanine complex (0.5 M D-alanine and 15 μM enzyme) in the presence of 70 mM 4-hydroxypyridine.

clearly show evidence for E_{Q2}. The rate constants calculated from fitting the data in Figure 6B to eq 10 are $k_3 = 0.01 \pm 0.002$ s^{-1} and $k_4 = 0.38 \pm 0.003$ s^{-1}, and the binding constant $K''_{PH} = 0.6 \pm 0.04$ mM for 4-hydroxypyridine binding to the second quinonoid complex. This binding constant is almost identical to that obtained in the corresponding studies with L-alanine. This provides additional support that the E_{Q2} complexes formed from either L-alanine or D-alanine are identical.

Circular Dichroism. Tyrosine phenol-lyase exhibits a positive CD band at 420 nm (Figure 7, curve 0) which is due to the PLP bound to Lys-257 as an internal aldimine. The addition of a saturating concentration of L-alanine (0.5 M) results in a positive CD band at 500 nm and a negative band at 323 nm (Figure 7A, curve 1). Obviously, the positive CD band at 500 nm can be ascribed to the E_{Q1} complex. The value of the apparent extinction coefficient difference ($\Delta\epsilon_{app}$) at 500 nm is 3.8 M^{-1} cm^{-1}. Myagkikh and Demidkina (1985) reported similar results in studies using tyrosine phenol-lyase from *C. intermedius*. The addition of a saturating concentration of D-alanine also results in a positive CD band at 500 nm and a negative CD band at 323 nm (Figure 7B, curve 1).

Table II: Summary of Optical Constants at 500 nm for Tyrosine Phenol-Lyase Complexes with Alanine

substrate[a]	ϵ_{app}[b] $\times 10^3$ (M^{-1} cm^{-1})	$\Delta\epsilon_{app}$[c] (M^{-1} cm^{-1})	anisotropy factor $\times 10^{-4}$
L-alanine	16.7	3.8	2.3
D-alanine	9.7	2.6	2.7
L-alanine + 4-hydroxypyridine	26.8	11.5	4.3
D-alanine + 4-hydroxypyridine	24.3	10.7	4.4

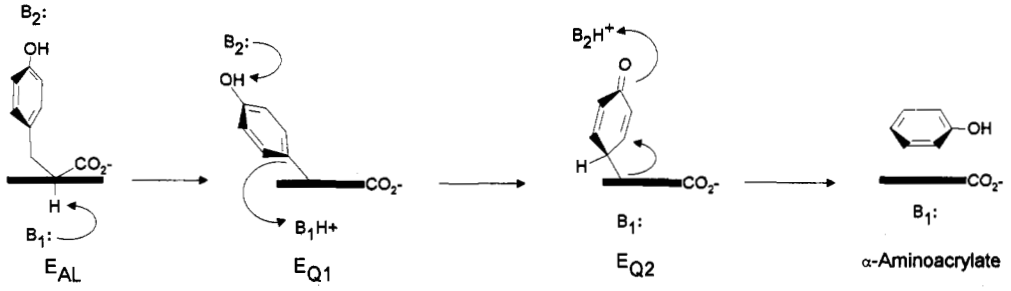
[a] The concentrations of alanine and 4-hydroxypyridine are 0.5 M and 70 mM, respectively. [b] All these apparent extinction coefficients were based on the absorbance change, A, at 500 nm obtained from the static spectrophotometric measurement: $\epsilon_{app} = (A/E)L$, where E is the concentration of tyrosine phenol-lyase (15×10^{-6} M) and L is the light path length (1 cm). [c] All these apparent extinction coefficient differences were based on the ellipticity change at 500 nm obtained from the static spectrophotometric measurement as shown in Figure 7: $\Delta\epsilon_{app} = (\Psi/3300)EL$ where Ψ is the ellipticity (mdeg), E is the concentration of tyrosine phenol-lyase (15×10^{-6} M), and L is the light path length (1 cm).

The value of $\Delta\epsilon_{app}$ (2.6 M^{-1} cm^{-1}) for the E_{Q1}(D) complex is smaller than that of E_{Q1}(L). The addition of a saturating concentration of 4-hydroxypyridine (70 mM) to the enzyme–L-alanine complex or the enzyme–D-alanine complex gave identical CD spectra (compare curve 2 in Figure 7A and curve 2 in Figure 7B), with much larger $\Delta\epsilon_{app}$ values and anisotropy factors (Table II). This result is also consistent with our postulate that the second quinonoid complexes formed from either L-alanine or D-alanine are identical. Table II summarizes the values of apparent extinction coefficients (ϵ_{app}), calculated from absorbance spectra, apparent extinction coefficient differences ($\Delta\epsilon_{app}$), calculated from CD spectra, and anisotropy factors for quinonoid complexes.

DISCUSSION

β-Elimination Reactions. Muro et al. (1978) reported stopped-flow kinetic studies of the reaction of tyrosine phenol-lyase from *Escherichia (Citrobacter) intermediates* with L- or D-alanine. They demonstrated that the formation of the first quinonoid intermediate was via a two-step mechanism, and they also detected a second quinonoid intermediate in the reaction with L-alanine, but not in the reaction with D-alanine. We have repeated these experiments with tyrosine phenol-lyase from *C. freundii*, and we have obtained similar results. Kazarinoff and Snell (1980) reported that indole and other small aromatic compounds bind to complexes of *E. coli* tryptophan indole-lyase with L-alanine and other amino acids with small side chains. Previously, we studied the effects of indole and benzimidazole on the reaction of *E. coli* tryptophan indole-lyase with L-alanine and substrates by stopped-flow kinetic methods (Phillips, 1991). These experiments demonstrated that indole binds to both external aldimine and quinonoid complexes of tryptophan indole-lyase, while benzimidazole binds selectively to the quinonoid intermediate of the tryptophan indole-lyase–L-alanine complex, resulting in an increase in absorbance at 502 nm. In the present study, we have examined the effects of phenol and analogues, pyridine *N*-oxide and 4-hydroxypyridine, on the reaction of tyrosine phenol-lyase with L-alanine or D-alanine by stopped-flow kinetic methods. Our results demonstrate that the effects of phenol on the reaction of tyrosine phenol-lyase with L-alanine are similar to those of indole on the reaction of tryptophan indole-lyase with L-alanine, while the effects of 4-hydroxypyridine on the reaction of tyrosine phenol-lyase with L-alanine are similar to those of benzimidazole on the reaction of tryptophan indole-lyase with L-alanine. Our kinetic data

Scheme IV



demonstrate that phenol binds to all the intermediates in the reaction of tyrosine phenol-lyase with alanine, but most strongly to the external aldimine complex (E_{AL}), resulting in a decrease in the absorbance at 500 nm at equilibrium (Figures 2 and 3). The structure of 4-hydroxypyridine is isoelectronic to that of phenol, but position 1 is a nitrogen atom. Our data demonstrate that 4-hydroxypyridine binds strongly and specifically to the second quinonoid complex (E_{Q2}) of alanine with tyrosine phenol-lyase, and thus causes an increase in the absorbance at 502 nm by increasing the amount of E_{Q2} present at equilibrium.

In the course of these experiments, we discovered a dramatic difference in the affinity of phenol for the the L-alanine external aldimine (0.11 mM) and the first quinonoid (85 mM) complexes. These results suggest that a structural reorganization occrs in the aromatic side chain binding site upon formation of the first quinonoid intermediate. The mechanism proposed for the α,β-elimination reaction (Kiick & Phillips, 1988; Faleev et al., 1988) suggests that there are two quinonoid intermediates, with the phenol ring undergoing a 90° reorientation, beginning perpendicular to the PLP–π system, but becoming parallel after C–C bond cleavage (Scheme IV). The data presented herein are the first experimental demonstration of conformational changes in tyrosine phenol-lyase complexes with phenol and amino acids. It was our expectation that phenol and analogues would bind to the alanine complexes in the site occupied by the phenol ring of bound tyrosine. However, recent X-ray crystallographic measurements of the holotyrosine phenol-lyase–L-alanine–4-hydroxypyridine complex show the 4-hydroxypyridine ring is bound 8 Å from the alanine (A. A. Antson, personal communication), and thus is in a position which is unlikely to be the catalytic site. The hydrogen bonds formed between the 4-hydroxypyridine O-4 and the amide NH of Val-337 and between the N-1 and the amide carbonyl of Ala-187 suggest that it is bound as the keto tautomer (A. A. Antson, personal communication).

Racemase Reaction. Previous investigators have proposed that racemization of amino acids by PLP-dependent racemases occurs by proton removal and addition to opposite faces of the PLP ring in the quinonoid complex (Floss & Vederas, 1982). Proton removal and addition can occur by a two-base mechanism (Cardinale & Abeles, 1968), or a single-base "swinging door" mechanism (Henderson & Johnson, 1976). Tyrosine phenol-lyase has been reported to catalyze racemization of alanine (Kumagai et al., 1969) as a slow nonphysiological side reaction. These side reactions are commonly observed in PLP enzymes, and often provide interesting mechanistic and stereochemical information about the enzyme active site structure. The demonstration of internal return of the α-hydrogen in the conversion of L- to D-alanine catalyzed by tyrosine phenol-lyase suggests that this enzyme racemizes by a single-base mechanism (Palcic et al., 1987). The amino

Table III: Pre-Steady-State Kinetic Parameters for Alanine Racemization by Tyrosine Phenol-Lyase

parameter[a]	value	parameter[a]	value
K_L	80 mM	k_4	0.60 s^{-1}
K_D	90 mM	k_5	0.30 s^{-1}
k_1	1.81 s^{-1}	k_6	0.01 s^{-1}
k_2	0.37 s^{-1}	k_7	0.29 s^{-1}
k_3	0.03 s^{-1}	k_8	0.75 s^{-1}

[a] Parameter as defined in Scheme II.

acid and PLP components in the quinonoid complex would have the same structure whether the complex was formed from either L-alanine or D-alanine. However, there would be two orientations of the quinonoid complex in the single-base "swinging door" mechanism, which might be expected to exhibit different optical properties. We have found that the anisotropy factor of the complex E_{Q1}(L) (2.3×10^{-4}) is close to that of the complex E_{Q1}(D) (2.7×10^{-4}) (Figure 7 and Table II). However, when 4-hydroxypyridine is added to complexes of either D- or L-alanine, there is a large increase in both the $\Delta\epsilon$ and anistropy factor for this quinonoid complex (Figure 7 and Table II), indicating that it is in a distinct environment. We have observed that both the enzyme–L-alanine complex and the enzyme–D-alanine complex have a negative CD band at 323 nm. The position of these bands suggests that they may be due to the formation of *gem*-diamine or ketimine complexes. The intensity of these bands is decreased by addition of 4-hydroxypyridine, and there is a red shift to about 330 nm.

It was found that the binding of phenol to the external aldimine of L-alanine (K_{PH} = 0.11 mM) was stronger than that of D-alanine (K_{PH} = 1.1 mM). We have also confirmed the report of Muro et al. (1978) that the rate constant for formation of the quinonoid complex from L-alanine (1.81 s^{-1}) is faster than that from D-alanine (0.7 s^{-1}). The steady-state kinetic parameters K_m and k_{cat} are also different (Table I). Furthermore, the effect of K^+ on the intensity of the quinonoid peak at 500 nm is very different (Figure 1). These differences imply that there are either separate binding sites for L- and D-alanine on the enzyme surface or distinct enzyme conformations which preferentially bind or react with each enantiomer. The 10-fold difference in the k_{cat}/K_m values for L- and D-alanine (Table I) clearly violates the Haldane relationship that $(k_{cat}/K_m)_L/(k_{cat}/K_m)_D = 1$ for a racemization reaction. Taken together with our other data, we believe this clearly shows evidence for distinct enzyme conformations which interact with L- and D-alanine, and thus the conformational equilibrium constant $K_{eq} = 10$.

The racemization of alanine should contain at least six steps, as shown in Scheme II, and the rate constants for all of the steps are summarized in Table III. Obviously, the formation of the second quinonoid intermediate, E_{Q2}, is very slow, and

is the rate-determining step in racemization. These rate constants (0.034 s^{-1} for L-alanine and 0.01 s^{-1} for D-alanine) are in good agreement with the steady-state k_{cat} values (L → D, 0.04 s^{-1}, and D → L, 0.008 s^{-1}, as shown in Table I). In a single-base mechanism, the base must move relative to the substrate or the substrate relative to the base during the catalytic process. Several experiments (Ivanov & Karpeisky, 1969; Vederras et al., 1979; Ford et al., 1980) have demonstrated similar cofactor motions in several PLP enzymes. It has been postulated that in racemases the base handling the α-H is relatively fixed, and the PLP–substrate complex pivots to expose different faces to the base, with rotation of 180°. Therefore, on the basis of the single-base swinging door mechanism, the formation of the central quinonoid intermediate (E_{Q2}) may be due to the planar PLP–alanine "door" in the "halfway" position, rotated by 90° (Scheme II). It should be noted that we cannot rule out that tyrosine phenol-lyase catalyzes racemization by a two-base mechanism. The α,β-elimination reaction catalyzed by *C. freundii* tyrosine phenol-lyase exhibits two pK_a's in the V/K profile, with an average value of 7.82 (Kiick & Phillips, 1988). Shostak and Schrich (1988) have demonstrated that a mutant serine hydroxymethyltransferase can catalyze alanine racemization by a two-base mechanism. They suggested that the internal return of protons between the two alanine isomers was due to a proton shuttle system between the two bases. Previously, we studied the pH dependence of the steady-state kinetic K_i value for L-alanine (Kiick & Phillips, 1988), which showed a dependence on the basic form of an enzyme group, with only one pK_a of 7.57. This base is likely to be the Lys-257, as is the case for the structurally related aspartate aminotransferase (Toney & Kirsch, 1993). It is possible that Lys-256 could serve as a second base for the α,β-elimination reaction. However, in the case of tryptophan indole-lyase, replacement of the sequentially homologous Lys-269 by arginine resulted in a mutant with about 10% activity (Phillips et al., 1991). It has been suggested, on the basis of chemical modification with diethyl pyrocarbonate, that a histidine residue is an essential base in the reaction of tyrosine phenol-lyase (Kumagai et al., 1975). However, the only histidine in the active site is His-343 (Antson et al., 1993), and a mutant of tyrosine phenol-lyase with this histidine replaced by alanine exhibits high activity (H. Chen and R. Phillips, unpublished observations).

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