Stereochemistry and Mechanism of Reactions Catalyzed by Tyrosine Phenol-Lyase from *Escherichia intermedia**

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Dedicated to Professor Helmut Simon on the occasion of his 60th birthday

Stereochemical studies on tyrosine phenol-lyase from *Escherichia intermedia* have shown that the α,β-elimination reactions of L-serine and D- and L-tyrosine proceed with retention of configuration at C-β. Stereospecifically β-tritiated L-serine is slowly racemized at C-β. Deuterium from the α-position of L-tyrosine is partially transferred to C-4 of the phenol formed when the α,β-elimination reaction is carried out in H_2O, although no transfer of α-^1H in ^2H_2O was seen. The result favors tautomerization of the *p*-hydroxyphenyl to a cyclohexadienonyl moiety prior to carbon-carbon bond cleavage. In the conversion of L- to D-alanine catalyzed by tyrosine phenol-lyase, some α-hydrogen recycling is observed, pointing to a single-base racemization mechanism. Attempts to demonstrate cofactor motion during racemization by $NaBH_4$ reduction of [^3H]PLP-enzyme: D- and L-alanine complexes failed, but showed that, as in other PLP enzymes, the holoenzyme is reduced preferentially from the *Re* face with respect to C-4′ of PLP and enzyme-substrate complexes preferentially from the *Si* face.

Tyrosine phenol-lyase (E.C. 4.1.99.2) is a pyridoxal phosphate (PLP) containing enzyme that catalyzes the α,β-elimination of phenol from tyrosine to give pyruvate and ammonia [1]. The reaction is reversible and the enzyme is characterized by a broad substrate specificity. It will also catalyze α,β-elimination reactions of D-tyrosine and several other β-substituted D- and L-amino acids, as well as β-replacement reactions of L-tyrosine, S-methyl-L-cysteine and some related amino acids. The crystalline enzyme also can racemize amino acids, including alanine, although at a slower rate [2].

Extensive stereochemical studies of PLP enzymes have shown that, with few exceptions, reactions occur on only one face of a relatively planar PLP-substrate complex, frequently mediated by a single catalytic base group which is involved in multiple proton transfer steps along the reaction coordinate [3, 4]. The other face of the PLP-substrate complex tends to be more shielded. Remarkably, the "exposed face" of the PLP-substrate (or PLP-substrate analog) complex always seems to be the same in an absolute sense, *i.e.,* always the *Si* face relative to C-4′ of the cofactor, in all the enzymes studied, suggesting a common evolutionary origin of these proteins [5, 6]. Stereochemical studies on tyrosine phenol-lyase were of particular interest in view of the unusual promiscuity this enzyme exhibits toward L- and D-amino acids. The steric course of several β-replacement reactions catalyzed by this enzyme has been determined in earlier work by Sawada *et al.* [7] and Fuganti *et al.* [8]. They were found to proceed with retention of configuration of the β-carbon atom, conforming to the patterns seen in all the other PLP-catalyzed nucleophilic β-replacement reactions studied [3, 4]. In this paper we report in detail on further stereochemical and mechanistic studies with this enzyme. Some of the results have been previously communicated in preliminary form [9, 10].

Abbreviations: PLP, pyridoxal phosphate; PM, pyridoxamine.

Experimental Procedures

Materials and methods

Tyrosine phenol-lyase was isolated from *Escherichia intermedia* A21 grown on tyrosine-enriched media, as previously described [1]. The specific activity of the enzyme preparation was 0.85 IU/mg protein based on the α,β-elimination of phenol from L-tyrosine. Acetyl-CoA: D-amino acid α-N-acetyltransferase was purified 2,000-fold from yeast [11] and had a specific activity of 0.6 IU/mg. Porcine heart aspartate aminotransferase was obtained from Sigma Chemical Co. and converted to the apoenzyme form by a published procedure [12]. Lactate dehydrogenase was obtained from Sigma or P−L Biochemicals, hog kidney acylase I from Sigma.

Samples of $(2S,3R)$- and $(2S,3S)$-[3-³H]serine were prepared as previously described [13] and were mixed with L-[U-¹⁴C]serine (NEN Corp.). These samples were chirally pure at C-3 (>99%) as determined by enzymatic conversion to tryptophan and then to indolymycin by incubation with *Streptomyces griseus* ATCC 12648 [13]. Samples of $(2S,3R)$-[3-²H] tyrosine and $(2S,3S)(2R,3R)$-[2,3-²H₂] tyrosine, synthesized by the method of Kirby and Michael [14], were available from earlier stereochemical studies on tyrosine phenol-lyase [7]. L-[2-²H] Tyrosine was prepared by enzymatic exchange of L-tyrosine with tyrosine phenol-lyase in D₂O. N-Acetyl-D,L-[2-²H]alanine was prepared by reaction of D,L-alanine with acetic anhydride in deuterated glacial acetic acid (CH₃COOD, 98% ²H) [15]. L-[2-²H]Alanine was prepared from N-acetyl-D,L-[2-²H]alanine by enzymatic resolution with hog kidney acylase I [15]. The deuterium content of the L-[2-²H]alanine was $82 \pm 1\%$ as determined by chemical ionization mass spectrometry. The enantiomeric purity was assessed by mixing with D-alanine (L/D = 5/1) and incubation with acetyl CoA and D-amino acid acetyltransferase. Chemical ionization mass spectral analysis of the product N-acetyl-D-alanine indicated that the starting L-[2-²H]alanine contained no more than $0.35 \pm 0.06\%$ of the deuterated D-isomer. Racemic [4′-³H]pyridoxamine (PM) was prepared by exchange with [³H]H₂O (NEN Corp.) [16] and [4′-³H]pyridoxal phosphate by a modification [17] of the method of Stock *et al.* [18]. Final purification of [4′-³H]PLP was achieved by chromatography on a Dowex AG 1 × 8 column (acetate form); however, PLP eluted at considerably higher acetic acid

concentration (5.7 M) than reported in ref. [17]. Pyridoxyl-D,L-alanine and ε-pyridoxyl-L-lysine were synthesized as previously described [19].

Paper chromatograpy

Descending paper chromatography was carried out on Whatman No. 3 MM paper using the following solvent systems: System A, ethanol/conc. NH₄OH/water, 80:4:16 (R_f values: lactate 0.55); System B, *n*-butanol/88% formic acid/water, 2:1:1 (N-acetyl-D-alanine, 0.83); System C, *t*-butanol/acetone/water/acetic acid, 40:35:20:5 (pyridoxyl-alanine, 0.38; ε-pyridoxyllysine, 0.07; pyridoxamine, 0.42; pyridoxal, 0.72); System D, isopropanol/water/acetic acid, 70:25:10 (pyridoxylalanine, 0.48; ε-pyridoxyllysine, 0.34; pyridoxamine, 0.52); System E, 95% ethanol/water, 70:30 (ε-pyridoxyllysine 0.22).

Instrumental methods

Radioactivity on paper chromatograms was located using a Packard model 7201 radiochromatogram scanner. Radioactivity of compounds in solution was determined by scintillation counting as previously described [13, 19]. Nuclear magnetic resonance spectra were recorded on JEOL PTF-100, JEOL FX-60 or Nicolet NTC-360 spectrometers. Chemical ionization mass spectra were measured on a Finnigan 4023 mass spectrometer (reagent gas: isobutane).

Stereochemistry of α,β-elimination of serine and tyrosine

The reaction mixture contained in 0.25 ml ²H₂O: potassium phosphate, pH 8.3, 25 µmol; $(2S,3R)$- or $(2S,3S)$-[U-¹⁴C,3-³H]serine, <0.1 µmol; tyrosine phenol-lyase, 0.18 IU; pyridoxal phosphate 0.5 µmol; NADH, 1 µmol; and lactate dehydrogenase, 5 IU. Following incubation at 30 °C for 3 h, lithium lactate, 20 µmol, was added as a carrier and the lactate isolated by paper chromatography in system A. In the first incubation, conversion of serine to lactate was only about 15%. The residual serine was reisolated and incubated again in essentially the same manner. The lactate samples derived from these four incubations were oxidized to acetate [20] for configurational analysis of the methyl group by the method of Cornforth [21], Arigoni [22] and coworkers using the procedure described in ref. [23].

Reaction mixtures for determinations of the stereochemical course of the α,β-elimination of phenol from tyrosine contained in 1.0 ml: (2S,3R)-[3-^{2}H]tyrosine, 6.6 μmol, or (2S,3S)(2R,3R)-[2,3-^{2}H$_2$]tyrosine, 5.5 μmol; potassium phosphate, pH 8.3, 50 μmol; dithiothreitol, 1.0 μmol; pyridoxal phosphate, 0.1 μmol; NADH, 10 μmol; lactate dehydrogenase, 25 IU, tyrosine phenol-lyase, 0.28 IU; 100 mCi [^{3}H]H$_2$O. After incubation for 2 h at 30°C, the reaction was terminated by immersion in a boiling water bath. Precipitated protein was removed by centrifugation, and following removal of [^{3}H]H$_2$O by lyophilization, lactate was isolated by paper chromatography in system A and oxidized to acetate for configurational analysis.

Hydrogen transfer from C-2 of tyrosine to phenol

Incubation mixtures contained in 25 ml ^{2}H$_2$O or H$_2$O, respectively: L-tyrosine or L-[2-^{2}H]tyrosine, 83 μmol; potassium phosphate, pH 8.3, 1.25 mmol; dithiothreitol, 2.5 μmol; pyridoxyl phosphate, 0.25 μmol; EDTA, 1.25 μmol; and tyrosine phenol-lyase, 0.9 IU. Incubations were carried out at 30°C overnight and quenched by the addition of 5 ml 6 N HCl. Following the removal of precipitated protein by centrifugation, the supernatant solution was extracted with methylene chloride. Final purification of phenol for subsequent ^{1}H NMR analysis was achieved by sublimation.

In another experiment, L-[2,3-^{2}H$_2$]tyrosine, 220 μmol was incubated in 50 ml potassium phosphate buffer, pH 8.3, containing tyrosine phenol-lyase, 6 IU; pyridoxal phosphate, 25 μmol; dithiothreitol, 50 μmol; EDTA, 20 μmol; NADH, 300 μmol; and lactate dehydrogenase, 180 IU. Protein was removed by ultrafiltration, the filtrate adjusted to pH 5.0, extracted with methylene chloride and carefully concentrated *in vacuo* to yield crude phenol which was purified by sublimation for ^{1}H NMR analysis.

Internal return of H-α in the racemization of alanine

Reactions for the tyrosine phenol-lyase-catalyzed conversion of L-[2-^{2}H]alanine in H$_2$O or unlabeled L-alanine in ^{2}H$_2$O were carried out under essentially single turnover conditions by *in situ* coupling of formation of the product D-alanine to further conver-

sion into N-acetyl-D-alanine with D-amino acid acetyltransferase. Reaction mixtures contained in 4 ml H$_2$O or ^{2}H$_2$O (98% ^{2}H): L-[2-^{2}H]alanine (82% ^{2}H) or unlabeled L-alanine, 300 μmol; potassium phosphate, pH 7.4, 1.6 mmol; tyrosine phenol-lyase, 2.4 IU; pyridoxal phosphate, 12 μmol; acetyl-CoA, 27 μmol; D-amino acid N-acetyltransferase, 2.9 IU. After 36 h incubation at 35°C, reactions were terminated by acidifying the solution to pH 4−5 with 1 N HCl. N-Acetyl-D-alanine was isolated by passage through a column of Dowex 50 H$^+$ (2.5 × 15 cm), paper chromatography of the effluent in solvent system B and ion-exchange chromatography on Dowex 1-X8, acetate form (1 × 15 cm column), with an acetic acid gradient (300 ml 0.5 N acetic acid and 300 ml 2.0 N acetic acid). Following repeated evaporation and dissolution in H$_2$O the product was analyzed by high field ^{1}H NMR spectroscopy (360 MHz) and by chemical ionization mass spectrometry, collecting at least four sets of spectra for each data point. The trapping efficiency of the coupled enzyme system was monitored by including a trace amount of D-[U-^{14}C]alanine in some incubations and measuring the partitioning of ^{14}C between N-acetyl-D-alanine and unreacted alanine.

Sodium borohydride reductions of holo-tyrosine phenol-lyase and complexes with D- or L-alanine

In a typical experiment 70 mg of apo-tyrosine phenol-lyase in 10 mM potassium phosphate buffer, pH 8.0, containing 5 mM mercaptoethanol was converted to the holo [4′-^{3}H]PLP form by incubating with a 20−28-fold excess of [4′-^{3}H]PLP (spec. radioact. = 5.8 μCi/μmol or 11 μCi/μmol) for 15 min at 30°C [24]. Excess PLP was removed by chromatography on a Sephadex G-25 (medium) column (2 × 25 cm) equilibrated with 10 mM potassium phosphate buffer, pH 8.0, containing 5 mM mercaptoethanol.

A solution containing 10−19 mg of reconstituted [4′-^{3}H]PLP tyrosine phenol-lyase was incubated 15 min at 22°C either in 10 mM potassium phosphate, pH 8.0, with 67 mM D- or L-alanine to generate PLP-substrate complexes or in the absence of substrate for holo enzyme reduction experiments. Sodium borohydride was then added to a final concentration of 50 mM (12−17 mg) and the solution was allowed to stand at room temperature for 1.5 h. The reaction was terminated by the addition of conc. HCl. Pre-

cipitated protein was removed by centrifugation and washed twice with 4 ml H_2O.

Supernatant fractions from reductions in the presence of alanine containing phosphopyridoxylalanine were combined, lyophilized, dissolved in 6 ml of 6 N HCl and dephosphorylated by refluxing under N_2 for 4 h. The reaction mixture was evaporated to dryness under vacuum, the residue dissolved in 2 ml H_2O containing 17—22 mg unlabeled pyridoxylalanine, neutralized and applied to a column of Dowex AG 50 × 8, H^+, 200—400 mesh (1.8 × 15 cm). After washing the column with 200 ml H_2O and 200 ml 1 N HCl, pyridoxylalanine was eluted with an 800 ml gradient of 1 to 5 N HCl. Fractions absorbing at 294 nm were combined and evaporated to dryness. Final purification was accomplished by chromatography in system C and/or D.

Precipitated protein from the reduction of holoenzyme in the absence of substrate was refluxed with 6 ml 6 N HCl for 6 h under N_2 and evaporated to dryness *in vacuo*. The residue was dissolved in 2 ml H_2O containing 11 mg ε-pyridoxyl-L-lysine, chromatographed on a Dowex AG 50 column as described for pyridoxylalanine and finally purified by paper chromatography in system C and then system E.

Pyridoxylalanine (6—8 mg) was degraded to pyridoxamine as described in [19], passed over a Dowex AG 50 × 8 H^+ column (1.5 × 18 cm), which was washed with 200 ml 1 N HCl and then eluted with 800 ml of a linear gradient of 2.5 to 5 N HCl. Fractions absorbing at 294 nm were combined and evaporated to dryness *in vacuo*. Final purification of pyridoxamine was accomplished by paper chromatography in system C.

ε-Pyridoxyllysine samples were degraded to pyridoxamine as described in [19] and passed over a cation exchange column (Amberlite CG 50, H^+ form, 100—200 mesh 1.5 × 18 cm). The column was washed with 200 ml water and then eluted with 700 ml of a linear gradient of water to 0.1 N HCl. Fractions absorbing at 293 nm were combined and evaporated to dryness *in vacuo*. Final purification of pyridoxamine was accomplished by paper chromatography in systems C and D.

Stereochemical analysis of the pyridoxamine was carried out by incubating the samples with apo-aspartate aminotransferase, α-ketoglutarate and glutamate for exchange of the *pro*-4′*S* hydrogen of PM with solvent as described [16] or, in some cases, in the absence of glutamate for conversion to py-
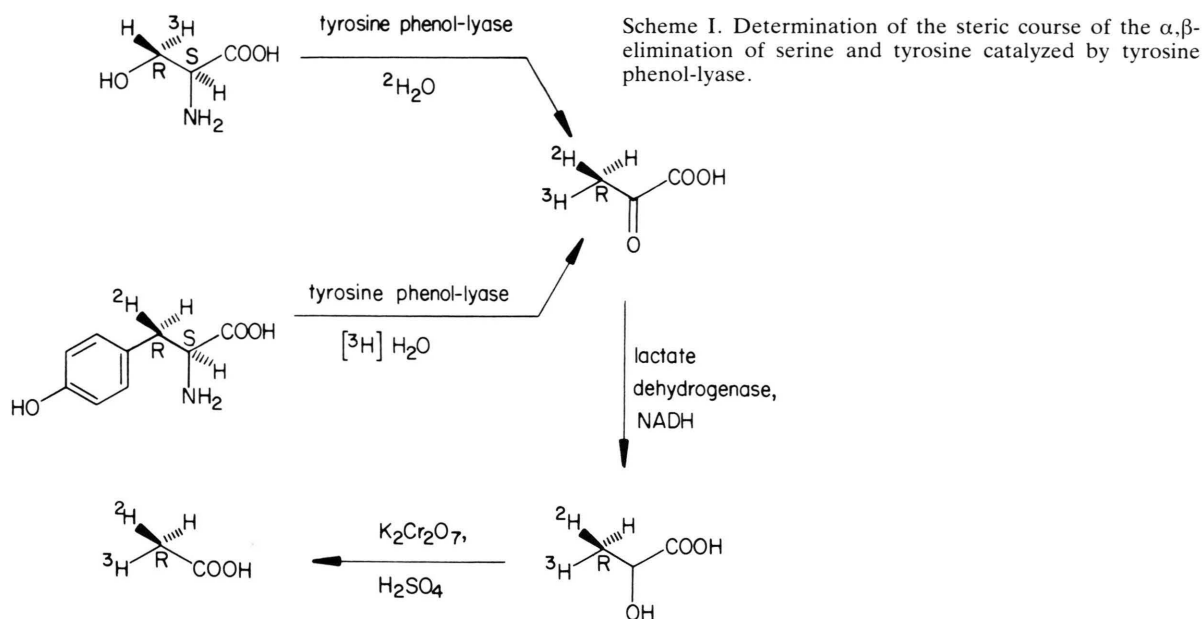
ridoxal with elimination of the *pro*-4′*S* hydrogen from PM [19].

Results

Stereochemical course of α,β-elimination reactions

The α,β-elimination reaction of serine catalyzed by tyrosine phenol-lyase produces pyruvate and ammonia; in this conversion the hydroxyl group of serine is replaced by an atom of hydrogen. When this reaction is carried out in ^2H_2O with serine stereospecifically tritiated at C-3 as substrate, the incorporation of deuterium from solvent will give a pyruvate methyl group which is chiral because it contains all three isotopes of hydrogen. If the addition of the third hydrogen is stereospecific, one enantiomer of such a chiral methyl group will result, and its configuration will reveal the overall steric course of the reaction (Scheme I). In the experimental setup the formation of pyruvate was tightly coupled to its further conversion into lactate with lactate dehydrogenase, generating a stable product which can be oxidized to acetic acid for configurational analysis of the methyl group by established methods [21—23]. In the chiral methyl group analysis, acetate is enzymatically converted to malate by the action of acetate kinase, phosphotransacetylase and malate synthase. A kinetic isotope effect in the malate synthase reaction results in the formation from chiral acetate of malate with an uneven tritium distribution at C-3. Upon incubation with fumarase, which equilibrates the *pro*-3-*R* hydrogen of malate with solvent protons, malate derived from *R* acetate retains more than half of the original tritium. Conversely, malate derived from *S* acetate loses more than half of its tritium in the fumarase reaction. The *F* value refers to the percentage of tritium retained carbon-bound in incubations with fumarase and is a measure of both the configuration and chiral purity of the methyl group. Calibrations of the chirality assay with optically pure acetate samples have given *F* values of 21 ± 2 for *S* acetate and 79 ± 2 for *R* acetate (*cf.* [23]).

The results listed in Table I demonstrate that the acetate samples produced from the first pair of incubations with serine (experiments 1 and 2) are chiral, indicating that protonation at C-3 occurs stereospecifically. The reaction proceeds with retention of configuration at C-3 since (3*R*)-[3-^3H]serine gave *R*-acetate and the 3*S* isomer gave *S*-acetate. The diminished chiral purity of the acetate samples derived

Scheme I. Determination of the steric course of the α,β-elimination of serine and tyrosine catalyzed by tyrosine phenol-lyase.

Table I. Stereochemistry of the α,β-elimination of serine and tyrosine catalyzed by tyrosine phenol-lyase.

$^3H/^{14}C$ of	$(2S,3R)$-[U-^{14}C, 3-^{3}H]serine in ^{2}H$_2$O	$(2S,3S)$-[U-^{14}C, 3-^{3}H]serine in ^{2}H$_2$O	$(2S,3R)$-[U-^{14}C, 3-^{3}H]serine (reisolated from Expt. 1) ^{2}H$_2$O	$(2S,3S)$-[U-^{14}C, 3-^{3}H]serine (reisolated from Expt. 2) in ^{2}H$_2$O	$(2S,3R)$-[3-^{2}H] tyrosine in ^{3}H$_2$O	$(2S,3S),(2R,3R)$- [2,3-^{2}H$_2$]tyrosine in ^{3}H$_2$O
	(Expt. 1)	(Expt. 2)	(Expt. 3)	(Expt. 4)	(Expt. 5)	(Expt. 6)
Serine	3.11	2.38	n.d.[a]	n.d.	–	–
Lactate	2.82	2.41	n.d.	n.d.	–	–
Acetate	4.10	3.91	3.80	2.72	8.56[b]	8.43[b]
Malate	3.01	2.84	2.54	1.79	5.39	4.61
Fumarate	2.18	1.10	1.44	0.89	3.32	2.24
F value[c]	72.4	38.7	56.7	49.7	61.6	48.6
(ee)	(77%)	(39%)	(23%)	(1%)	(40%)	(5%)

[a] n.d. = not determined.
[b] [^{14}C]Acetate added to tritiated samples for analysis.
[c] *F* value = % tritium retention in fumarase reaction; ee = enantiomeric excess, $\dfrac{|50\text{-}F|}{29} \times 100[\%]$.

from the second incubations (experiments 3 and 4), using serine which had been reisolated from the first, demonstrates that serine is extensively racemized at C-3 during incubation with tyrosine phenol-lyase.

The α,β-elimination of tyrosine, in which the phenol moiety is replaced by hydrogen, also proceeds with retention of configuration C-3 (Table I, experiment 5). The methyl group obtained by degradation

of lactate produced from racemic $(2S,3S)(2R,3R)$-[2,3-^{2}H$_2$]tyrosine in ^{3}H$_2$O was nearly racemic (Table I, experiment 6). This suggests that the α,β-elimination reaction of both D- and L-tyrosine occurs with retention of configuration at C-β, producing upon completion of the reaction equal amounts of *S* methyl groups (from $2S,3S$-tyrosine) and *R* methyl groups (from the $2R,3R$ isomer).

Table II. Transfer of hydrogen from the α-carbon of tyrosine to C-4 of phenol.

Substrate	Solvent	% H / % D at C-4 of Phenol[a]	% Transfer
L-[2-^{2}H]tyrosine	H_2O	75.6/24.4	24.4
L-[2,3-^{2}H$_1$]tyrosine	H_2O	84.2/15.8	15.8
L-tyrosine	^2H_2O	0/100	not detectable
L-[2-^{2}H]tyrosine	^2H_2O	0/100	–

[a] Obtained by integration of ^{1}H NMR spectra.

Transfer of α-^{2}H to C-4 of phenol

The fate of the α-hydrogen in the α,β-elimination reaction of tyrosine was determined in incubations of L-[2-^{2}H]tyrosine with tyrosine phenol-lyase. Phenol produced in this reaction was isolated and analyzed by ^{1}H NMR spectroscopy for its deuterium content at C-4. Incubations were carried out with and without trapping of the second product, pyruvate, by lactate dehydrogenase to minimize back reaction. The results of a number of incubations demonstrate deuterium transfer with greatly varying efficiencies, depending on reaction conditions and on other, less controllable variables like, apparently, age of the enzyme [25]. The highest transfer of deuterium from the α-position of the tyrosine side chain to C-4 of the leaving group observed was 24% (Table II). No internal hydrogen transfer was detected in incubations containing unlabeled tyrosine in ^2H_2O; the phenol generated under these conditions was completely deuterated at C-4. In addition, attempts to demonstrate hydrogen transfer in the reverse reaction, *i.e.*, in the synthesis of tyrosine from [4-^{2}H]phenol in H_2O or unlabeled phenol in ^2H_2O revealed no detectable transfer (data not shown).

Internal return of H-α in the racemization of alanine

Tyrosine phenol-lyase catalyzes the racemization of D- and L-alanine at 2.5% of the rate of L-tyrosine degradation [2]. This process can in principle be catalyzed by a single base which both abstracts the α-proton from one enantiomer and protonates the resulting carbanion on the opposite face, or it may involve two base groups on the enzyme positioned on opposite sides of the bound substrate. In the first case it may be possible to observe internal return of the α-hydrogen in the conversion of one enantiomer to the other, providing that the rate of exchange of the proton on the base with solvent is slower than transfer to product. Internal return of the α-hydrogen in the conversion of L- to D-alanine was probed by trapping product with an excess of D-amino acid N-acetyltransferase (Scheme II).

The ratio of racemase to acetylase activity was about 1:10, and trapping efficiencies ranged from 79 to 97% [10, 25]. The conversion of L-alanine carrying deuterium in the α-position gave N-acetyl-D-alanine containing 3% deuterium (Table III). The L-alanine sample was 82% enriched in deuterium; therefore the results indicate that about 3.5% (3.07/0.82) of the deuterium is returned to product in the racemization of L-alanine. The complementary experiment, in which unlabeled L-alanine was converted to the D-isomer in ^2H_2O, is complicated by the fact that the enzyme can also catalyze hydrogen exchange at C-β. In this case, the presence of 6% of the undeuterated species again indicates some internal return of the α-hydrogen. The results of the mass spectral and ^{1}H NMR analysis of the N-acetyl-D-alanine obtained in this incubation show that the monodeuterated, dideuterated, and trideuterated species consist predominantly of α-^{2}H-β-^{1}H$_3$, α-^{2}H-β-^{1}H$_2$^{2}H and α-^{2}H-β-^{1}H^{2}H$_2$
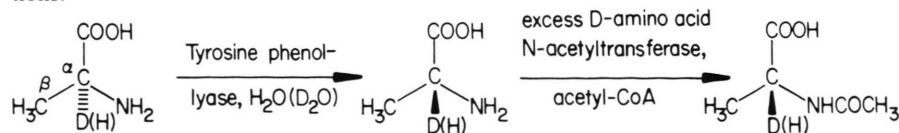
Scheme II. Internal return of α-hydrogen in the conversion of L- into D-alanine under nearly single-turnover conditions.

Table III. Internal return of α-hydrogen in the tyrosine phenol-lyase catalyzed racemization of alanine.

Substrate	Mass spectral data for N-acetyl-D-alanine		Distribution of ^{2}H in N-acetyl-D-alanine	
	m/z	relative intensity	species present[a]	abundance
L-[α-^{2}H]alanine (82% ^{2}H) in H$_2$O	132(MH$^+$)	100	^{2}H$_0 = $ α-^{1}H, β-^{1}H$_3$	97.0 ± 0.2
	133(MH$^+$ + 1)	8.75 ± 0.02	^{2}H$_1 = $ α-^{2}H, β-^{1}H$_3$	3.1 ± 0.2
	134(MH$^+$ + 2)	0.92 ± 0.02		
L-alanine in ^{2}H$_2$O (98% ^{2}H)	132(MH$^+$)	19.1 ± 1.9	^{2}H$_0 = $ α-^{1}H, β-^{1}H$_3$	6.0 ± 0.8
	133(MH$^+$ + 1)	100	^{2}H$_1 = $ α-^{2}H, β-^{1}H$_3$ + α-^{1}H, β-^{1}H$_2$^{2}H	31.2 ± 0.7
	134(MH$^+$ + 2)	76.7 ± 1.7	^{2}H$_2 = $ α-^{2}H, β-^{1}H$_2$^{2}H + α-^{1}H, β-^{1}H^{2}H$_2$	22.3 ± 0.4
	135(MH$^+$ + 3)	71.7 ± 0.03	^{2}H$_3 = $ α-^{2}H, β-^{1}H^{2}H$_2$ + α-^{1}H, β-^{2}H$_3$	21.3 ± 0.5
	136(MH$^+$ + 4)	64.8 ± 0.7	^{2}H$_4 = $ α-^{2}H, β-^{2}H$_3$	19.2 ± 0.1
	137(MH$^+$ + 5)	5.1 ± 0.05		

[a] ^{2}H$_0$ = undeuterated species, ^{2}H$_1$ = monodeuterated species, etc.

species, respectively, *i.e.*, most of the N-acetyl-D-alanine molecules with deuterium in the β position also contain deuterium in the α position. This suggests that the exchange of β protons requires the prior removal of the α proton in the same molecule [26, 27].
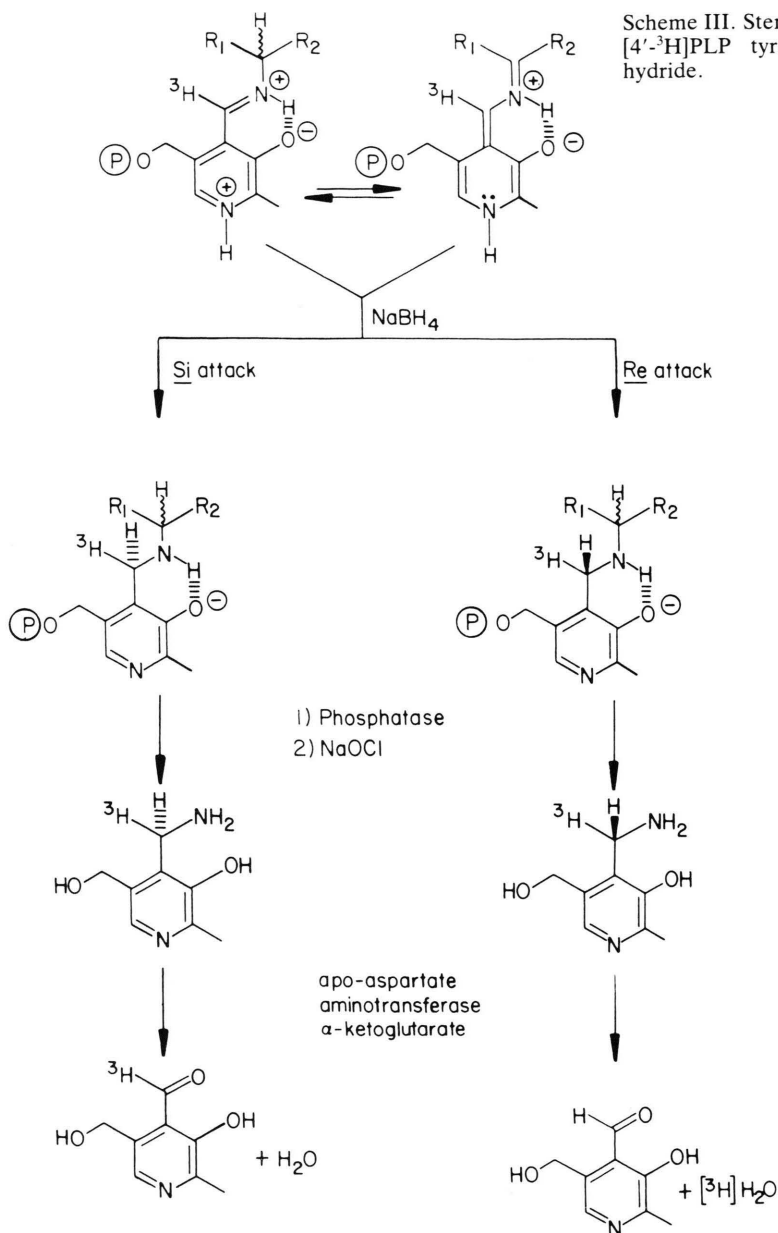
Stereochemistry of NaBH$_4$ reduction of enzyme-bound Schiff's bases

Trapping of Schiff's bases of enzyme-bound PLP by reduction with sodium borohydride has been used extensively in stereochemical studies of PLP enzymes [28]. Configurational analysis of the product of the reduction of unlabeled Schiff's base with [^{3}H]NaBH$_4$ (*cf.* [28]) or of [4'-^{3}H]PLP Schiff's base with unlabeled NaBH$_4$ [19] reveals the Schiff's base geometry and the relative accessibility of the two faces of native enzyme or enzyme-substrate imines to external reagents. The methodology employed here involved the conversion of apo-tyrosine phenol-lyase into the [^{3}H]PLP form by reconstitution with C-4' tritium-labeled cofactor. Sodium borohydride reduction was then carried out on the reconstituted holoenzyme in the presence of saturating amounts of D- or L-alanine, as well as in their absence. The products of these reductions, phosphopyridoxyllysine for native enzyme and phosphopyridoxylalanine from reductions in the presence of substrate, were isolated and degraded to pyridoxamine by standard procedures [19]. Analysis of the tritium distribution at C-4' of PM was accomplished by incubation with apo-aspartate aminotransferase which catalyzes the stereospecific exchange of the *pro-S* hydrogen at that position [16]. In this sequence, ^{3}H is released from labeled pyridoxamine when borohydride reduction

Table IV. Stereochemical analysis of pyridoxamine from sodium borohydride reductions of tyrosine phenol-lyase and its complexes with D- and L-alanine.

Substrate	Specific radioactivity of PM (dpm/μmol)	Specific radioactivity of PM or pyridoxal after apo-aspartate aminotransferase reaction (dpm/μmol)	% Tritium released	Predominant facial selectivity of attack at C-4'
D-Alanine-enzyme	6.3 × 10^3	4.6 × 10^3	27	*Si*
L-Alanine-enzyme	1.5 × 10^4	1.0 × 10^4	31	*Si*
Racemic [4'-^{3}H]PM (control)	3.9 × 10^3	1.8 × 10^3	55	−
Holoenzyme	6.9 × 10^3	2.7 × 10^3	61	*Re*
Racemic [4'-^{3}H]PM (control)	2.9 × 10^3	1.5 × 10^3	51	−

Scheme III. Stereochemical possibilities in the reduction of [4'-³H]PLP tyrosine phenol-lyase with sodium borohydride.

occurs mainly on the *Re* face with respect to C-4', and is retained in pyridoxamine for samples derived from *Si* face reduction (Scheme III). The results of the stereochemical analysis of PM samples from reductive trappings of the PLP-substrate imines of both D- and L-alanine demonstrate predominant attack on the *Si* face, since tritium is retained in both of these samples (Table IV). Importantly, the reduction of the D- and of the L-alanine complex show virtually

the same degree and direction of facial selectivity. Reduction of the native enzyme occurs preferentially on the *Re* face, albeit marginally so.

Discussion

Pyridoxal phosphate-requiring enzymes that catalyze α,β-elimination and nucleophilic β-replace-

ment reactions of amino acids exhibit several common mechanistic features [3, 4]. The cofactor is bound to native enzyme through a Schiff's base linkage with the ε-amino group of a lysine residue. Transimination with the amino group of the substrate yields a new PLP-substrate imine. Abstraction of a proton from C-α of the substrate-PLP imine by a basic group on the enzyme, aided by conjugation through the planar π system to the pyridine ring, gives a resonance-stabilized carbanionic intermediate. This facilitates elimination of an anionic substituent from C-β to produce an aminoacrylate Schiff's base (Scheme IV). Hydrolysis of this intermediate ultimately gives pyruvate and ammonia in α,β-elimination reactions, whereas nucleophilic attack at C-β produces a new amino acid in β-replacement reactions.

As originally suggested by Dunathan [28], orientation of the substrate such that the bonds to be broken are aligned perpendicular to the extended π system of the PLP-substrate imine should favor bond cleavage. In this conformation maximal orbital overlap between the sigma bond, either at C-α or C-β, and

the π system is achieved, resulting in substantial rate enhancement for cleavage of the sigma bond.

Stereochemical probes can provide information about the orientation of substrate and cofactor in the active site and can be used to detect conformational changes that occur during the catalytic process. In this study we have demonstrated that the α,β-elimination reactions of L-serine and L-tyrosine involve stereospecific protonation at C-β. Hence the protonation of the aminoacrylate intermediate must occur on the enzyme and its hydrolysis is apparently enzyme mediated. The steric course of the reaction, net retention of configuration in the replacement of the OH or *p*-hydroxyphenyl group by hydrogen, indicates that the proton is delivered on the same face from which the leaving group departed. This conforms to the findings for all other PLP-catalyzed α,β-elimination reactions studied, *i.e.*, those catalyzed by tryptophanase, D-serine dehydratase, S-alkylcysteine lyase, tryptophan synthase β₂ protein [see 3, 4] and D- and L-threonine dehydratase [29]. Samples of stereospecifically β-tritiated serine reisolated from incubations with tyrosine phenol-lyase were exten-

Scheme IV. Stereochemical mechanism of the α,β-elimination reactions catalyzed by tyrosine phenol-lyase.
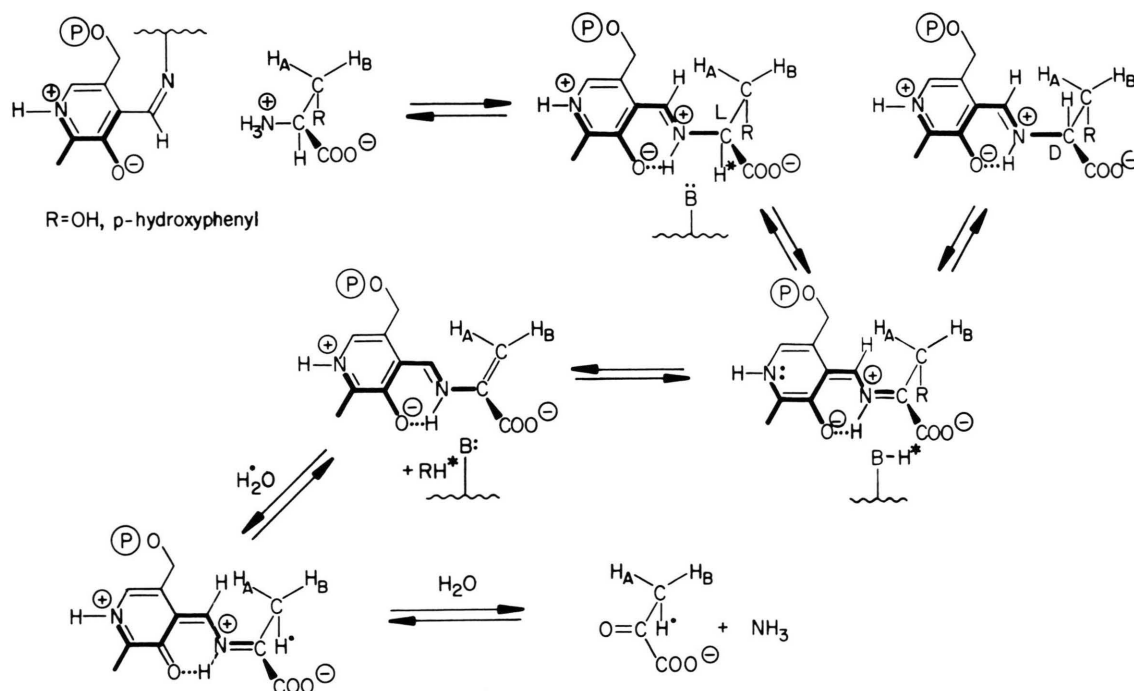
sively racemized at C-β, indicating that the reaction sequence at least up to the β-protonation of the aminoacrylate Schiff's base is reversible. It is known that the enzyme can synthesize L-tyrosine from phenol, pyruvate and ammonia [1, 30], but synthesis of L-serine from pyruvate and ammonia has not been reported.

The formation of nearly racemic methyl groups in the deamination of racemic (2S,3S),(2R,3R)-[2,3-^{2}H$_2$]tyrosine in [^{3}H]H$_2$O confirms that both L- and D-tyrosine can be converted to pyruvate [1]. The L isomer is known to react about 4 times faster than the D [1], and the small residual *S* chirality of the resulting methyl group (Table I, Expt. 6), expected from the (2S,3S) isomer, is in accord with that. It follows that the (2R,3R) isomer must have produced *R* methyl groups, and hence the reaction at C-β proceeds with retention of configuration irrespective of the configuration at C-α. The most plausible conformation of the enzyme-bound PLP- D-tyrosine Schiff's base is one in which the carboxyl group and the phenyl ring occupy approximately the same positions, and therefore binding sites, as in the complex with L-tyrosine, but H-α is displayed on the opposite face. If one assumes that H-α and the *p*-hydroxyphenyl group are *syn* oriented in the L-tyrosine complex (see below), the conformation of the PLP-

D-tyrosine complex must place these two groups in an *anti* relationship. Abstraction of H-α from the two Schiff's bases on opposite faces then produces the same resonance-stabilized carbanion (Scheme IV).

The demonstration of transfer of deuterium from C-α of L-tyrosine to C-4 of the phenol generated in the·α,β,-elimination reaction in H$_2$O agrees with observations reported by Faleev *et al.* [31], although quantitatively, our data differ substantially from theirs. This may, however, be due to differences in the enzyme preparation and experimental conditions and is not too surprising in view of the considerable variability in the results we have experienced. Internal transfer of the α-hydrogen implies that abstraction of the α-proton and protonation at C-4 of the phenyl ring are catalyzed by the same base, suggesting a *syn* orientation of H-α and the phenyl substituent. However, this latter conclusion may not be very meaningful in view of the demonstration of a single base mechanism for the racemization of alanine (see below), which indicates considerable mobility in the active site. The results parallel those obtained with tryptophanase where intramolecular transfer of hydrogen from C-α of tryptophan has been demonstrated to C-3 of the indole generated [32]. Subsequent work by Miles and coworkers [33, 34] with 2,3-dihydrotryptophan as inhibitor has pro-

Scheme V. Mechanism of the carbon-carbon bond cleavage in the α,β-elimination reaction of tyrosine.
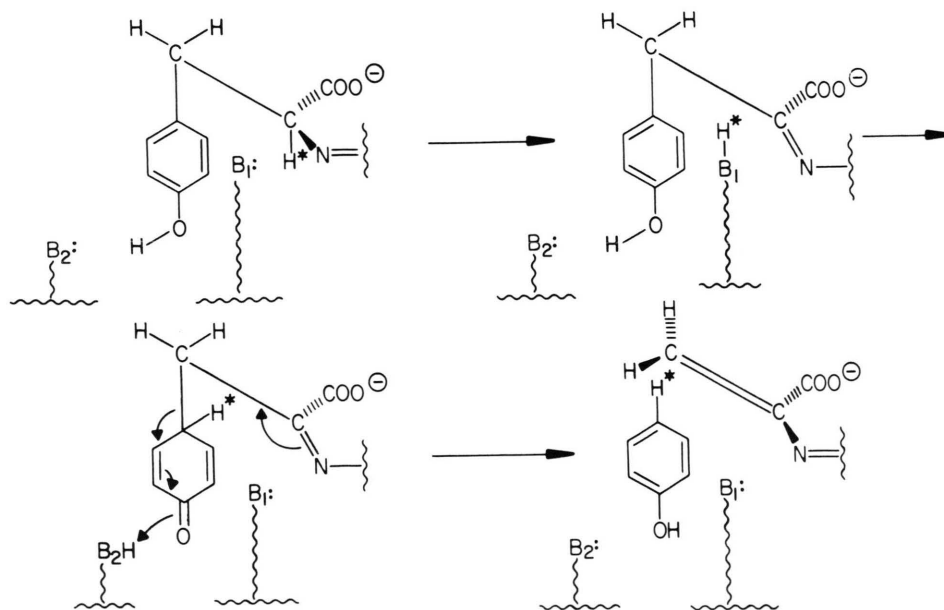
vided strong evidence for the involvement of an indolenine intermediate both in the tryptophanase and the related tryptophan synthase reaction. Protonation at C-3 of the indole moiety and concommitant deprotonation/protonation at N-1 transforms the indole moiety into a better leaving group. Analogously, protonation of the PLP-tyrosine derived α-carbanion para to the phenolic OH group and removal of the OH proton transforms the *p*-hydroxyphenyl moiety, a poor leaving group, into the more reactive cyclohexadienonyl moiety. Elimination of phenol then occurs by a dienone-phenol rearrangement (Scheme V) [31].

In most of the reactions it catalyzes, tyrosine phenol-lyase follows the general principle that PLP enzyme reactions take place, with few exceptions (*cf.* [4, 35]), on only one face of the planar Schiff's base [3–6]. However, the enzyme is unique in its ability to handle both D- and L-amino acids and to interconvert them, a feature it shares with amino acid racemases. Such reactions of necessity require that the enzyme must operate on both faces of the PLP-substrate complex. Proton abstraction and addition on opposite faces of the substrate can occur by a two-base mechanism, as in proline racemase [36], or be mediated by a single base. The demonstration of internal return of α-hydrogen in the conversion of L- to D-alanine suggests that tyrosine phenol-lyase operates by a single-base mechanism. The similar magnitude of transfer of ^{2}H in H_2O and ^{1}H in ^2H_2O points to a monoprotic base [37], consistent with other evidence implicating a histidine in the catalytic process [38], probably the abstraction of H-α [31, 38].

A single base mechanism for deprotonation/protonation on opposite faces of the coenzyme-substrate complex requires that base and substrate must move relative to each other during the catalytic process. Circular dichroism studies [39] and particularly sodium borohydride reduction experiments [19, 40, 41] have demonstrated for several PLP enzymes a cofactor reorientation upon substrate binding, and this observation has been confirmed in the X-ray work on aspartate aminotransferase [42]. In the native enzyme, reduction of the PLP-lysine Schiff's base occurs preferentially on the *Re* face relative to C-4', whereas in the PLP-substrate or -inhibitor complexes the *Si* face is more accessible to the external reagent. In view of this mobility of the cofactor it is attractive to postulate, as did Henderson and Johnson [43] in their "swinging door" mechanism, that in

PLP-dependent racemases the base handling H-α is relatively fixed and the PLP-substrate complex pivots to expose different faces to the base. If this is true, one might expect that different faces of the cofactor are exposed to an external reagent when the enzyme is charged with a D- and with an L-amino acid. Our attempt to demonstrate this by $NaBH_4$ reduction of complexes of the [^{3}H]PLP-enzyme with D- and L-alanine, however, showed no significant difference in the facial preference of attack by the reducing agent. This may, of course, simply mean that both the D- and L-alanine complex are rapidly deprotonated to the resonance-stabilized α-carbanion, resulting in reduction of the same species in both cases. However, it is also quite possible that the concept of a fixed base and mobile substrate is overly simplistic and that both show some mobility in the process.

The fact that both enzyme-substrate complexes are reduced preferentially from the *Si* face, whereas reduction of the holoenzyme shows preference for *Re* attack conforms to the observations with other PLP enzymes [19, 40, 41]. However, the degrees of facial preference in both cases are much less pronounced than with other PLP enzymes, suggesting a much less tightly constraining active site for tyrosine phenol-lyase. This relative "looseness" of the active site may be the reason for the remarkably broad substrate and reaction specificity of this enzyme. In this context it should be noted that Faleev *et al.* [44] have questioned whether the L- and D-serine dehydratase and alanine racemase activities are truly properties of tyrosine phenol-lyase. By induction experiments with different substrates they showed that *E. intermedia* can produce L- and D-serine dehydratase and alanine racemase, and they pointed out that contamination with 0.05%, 0.1% and 0.003%, respectively, of these enzymes would account for the observed activities of tyrosine phenol-lyase. While their point is well taken, considerable circumstantial evidence supports the notion that tyrosine phenol-lyase itself does possess racemase activity, namely (i) D- and L-alanine binding cause large absorption changes at 500 nm [45], much larger than can be accounted for by 0.003% of a contaminating enzyme, suggesting that both can be deprotonated at C-α, (ii) the enzyme can deaminate both D- and L-tyrosine at rates not vastly different, demonstrating its competence to react both enantiomers of amino acids, (iii) unlike tyrosine phenol-lyase, alanine racemase from *E. coli* shows no internal return of α-hydrogen in the con-

version of L- to D-alanine [10], and (iv) whereas bacterial alanine racemases tend to have a very narrow substrate specificity [46−48], tyrosine phenollyase racemizes other amino acids as well, for example, phenylalanine faster than alanine [1].

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