Use of Nitrogen-15 and Deuterium Isotope Effects To Determine the Chemical Mechanism of Phenylalanine Ammonia-lyase†

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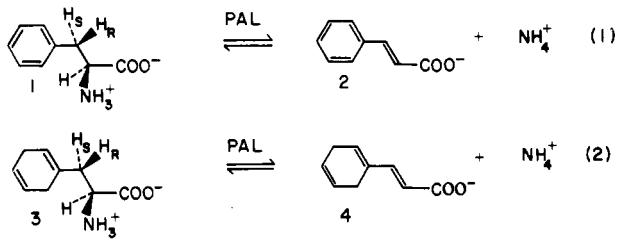
ABSTRACT: Phenylalanine ammonia-lyase has been shown to catalyze the elimination of ammonia from the slow alternate substrate 3-(1,4-cyclohexadienyl)alanine by an E1cb mechanism with a carbanion intermediate. This conclusion resulted from comparison of ^{15}N isotope effects with deuterated (0.9921) and unlabeled substrates (1.0047), and a deuterium isotope effect of 2.0 from dideuteration at C-3, with the equations for concerted, carbanion, and carbonium ion mechanisms. The ^{15}N equilibrium isotope effect on the addition of the substrate to the dehydroalanine prosthetic group on the enzyme is 0.979, while the kinetic ^{15}N isotope effect on the reverse of this step is 1.03–1.04 and the intrinsic deuterium isotope effect on proton removal is in the range 4–6. Isotope effects with phenylalanine itself are small (^{15}N ones of 1.0021 and 1.0010 when unlabeled or 3-dideuterated and a deuterium isotope effect of 1.15) but are consistent with the same mechanism with drastically increased commitments, including a sizable external one (i.e., phenylalanine is sticky). pH profiles show that the amino group of the substrate must be unprotonated to react but that a group on the enzyme with a pK of 9 must be protonated, possibly to catalyze addition of the substrate to dehydroalanine. Incorrectly protonated enzyme–substrate complexes do not form. Equilibrium ^{15}N isotope effects are 1.016 for the deprotonation of phenylalanine or its cyclohexadienyl analogue, 1.0192 for deprotonation of NH_4^+, 1.0163 for the conversion of the monoanion of phenylalanine to NH_3, and 1.0138 for the conversion of the monoanion of aspartate to NH_4^+. The value of 1.0246 for the deprotonation of ND_4^+ in D_2O shows that replacing an N–H with an N–D bond raises the ^{15}N fractionation factor by 0.5%.

We have recently reported that by determining a heavy atom isotope effect with a deuterated and nondeuterated substrate it was possible to determine whether the two isotope effects were on the same step, and if they were not, do deduce the order of the steps, and thus the nature of the intermediate in the reaction (Hermes et al., 1982). This method has shown that the oxidative decarboxylations catalyzed by malic enzyme (Hermes et al., 1982), isocitrate dehydrogenase (Grissom & Cleland, 1983), and 6-phosphogluconate dehydrogenase (Rendina et al., 1984) are stepwise with dehydrogenation preceding decarboxylation but that prephenate dehydrogenase catalyzes a concerted decarboxylation of prephenate (Hermes et al., 1984). We have now applied this method to phenylalanine ammonia-lyase (EC 4.1.3.5) (PAL),[1] which catalyzes the overall elimination of $-NH_3^+$ and the *pro-3S* hydrogen of L-phenylalanine (**1**) to yield *trans*-cinnamate (**2**), in order to



determine whether the chemical reaction was concerted or involved a carbanion or carbonium ion intermediate.

The enzyme used in this investigation was from the yeastlike fungus *Rodotorula glutinus*. This protein is similar in physical characteristics to enzymes from maize and potatoes and consists of a tetramer of identical subunits of overall molecular weight of 4 × 83 000 (Havir & Hanson, 1975). The higher plant enzymes show negative cooperativity (Conway & Koshland, 1968) while PAL from microorganisms such as *R. glutinus* displays Michaelis–Menten kinetics (Hodgins, 1971). Enzymes from all sources appear to contain the dehydroalanine (2-aminoacrylate; see Figure 1) prosthetic group as part of the polypeptide chain (Havir & Hanson, 1975). This electrophilic center has been presumed to be essential in activating the amino group to form a leaving group better than $-NH_3^+$. Deamination of the prosthetic group occurs after the release of cinnamate, so that the kinetic mechanism is an ordered Uni-Bi one (Havir & Hanson, 1975). In addition, exchange of the α-hydrogen of phenylalanine does not occur from an enzymic intermediate prior to release of cinnamate (Hanson & Havir, 1972). Presumably cinnamate is releaased into solution before the catalytic base is uncovered and free to exchange with the solvent.

In the present work we have measured ^{15}N isotope effects (by following changes in the natural abundance of ^{15}N in the ammonia formed) with both phenylalanine and its dihydro analogue which is a slow substrate. We have used both 3-dideuterated and unlabeled substrates and have also determined the deuterium isotope effects by comparison of rates with labeled and unlabeled substrates. We have determined the ^{15}N equilibrium isotope effects for the overall reaction, for the reaction in which aspartate is converted to fumarate and NH_4^+, and for the deprotonation of NH_4^+ to NH_3. We

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[1] Abbreviations: dihydrophenylalanine, 3-(1,4-cyclohexadienyl)alanine; PAL, phenylalanine ammonia-lyase; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonate.

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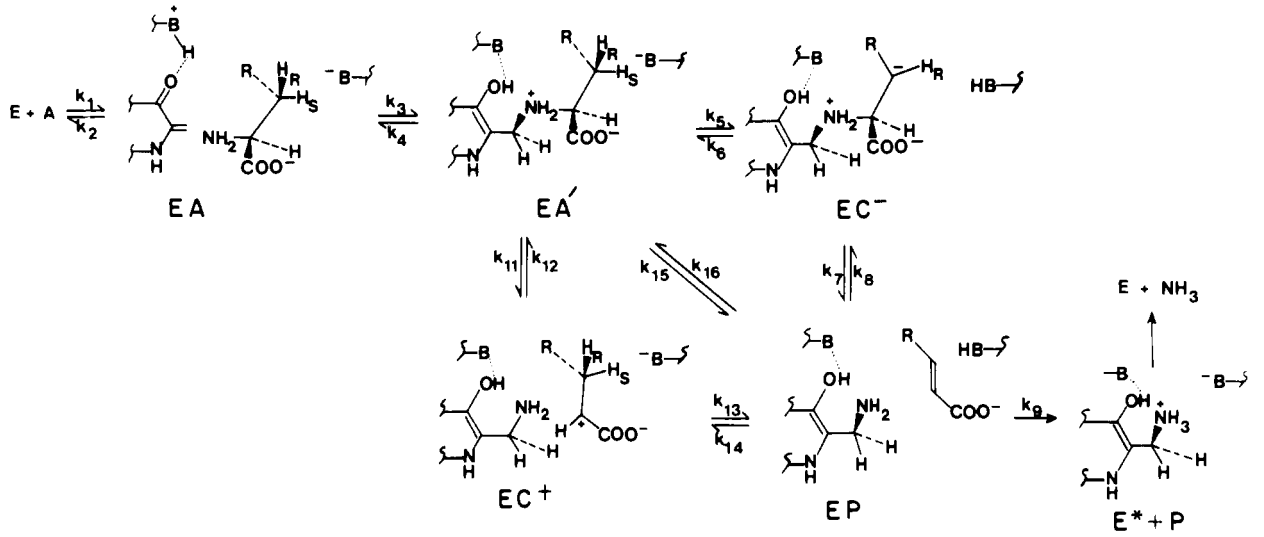


FIGURE 1: Possible chemical mechanisms for the reaction catalyzed by phenylalanine ammonia-lyase.

conclude that the elimination of ammonia from phenylalanine involves a carbanion intermediate.

Materials and Methods

Materials. Phenylalanine ammonia-lyase from *Rhodotorula glutinus*, aspartase from *Hafnia alvei*, and bovine glutamate dehydrogenase were from Sigma. L-, DL-, or D-phenylalanine, sodium phenylpyruvate, *trans*-cinnamic acid, and sodium cyanoborohydride were from Aldrich. D_2O (99.8% D) used for synthesis of deuterated compounds was from Bio-Rad. The reduction catalyst palladium on barium sulfate (5%) and methyl alcohol-*d* were from Sigma. Assay of ammonia was with the Nessler's-based Sigma ammonia color reagent. G-10 gel filtration and AG 50W-X8 cation-exchange resins were from Pharmacia and Bio-Rad, respectively.

Phenylalanine-3,3-d_2. A 1.0-g (4.9 mmol) sample of sodium phenylpyruvate monohydrate (exchanged in D_2O) was suspended in 20 mL of D_2O, and the pH was raised to 11 with NaOD. Upon formation of the enol, the phenylpyruvate dissolved completely to give a faintly yellow solution. After the sample was stirred for 5 min at room temperature, the pH was lowered to 6 with DCl, and the solution was lyophilized to give sodium phenylpyruvate-*3,3-d_2*. Proton NMR at 270 MHz showed the material to be over 98% deuterated (no detectable signal at δ 4.08). For reductive amination (Borch et al., 1971), a solution of 1.01 g of deuterated phenylpyruvate (4.9 mmol), 2.45 g (25 mmol) of ammonium bromide (exchanged in D_2O), and 0.615 g (9.8 mmol) of $NaBH_3CN$ in 100 mL of methanol-*d* was stirred for 48 h at 25 °C (when the reaction was run in unlabeled methanol, the product was only 80% deuterated at C-3). After the solution was evaporated in vacuo, the residue was dissolved in 20 mL of water and placed on a column of AG 50W-X8-H^+ (300 mequiv capacity). The column was washed with 500 mL of water and the phenylalanine eluted with 300 mL of 1 N ammonia. Rotary evaporation left 430 mg (51%) of phenylalanine-*3,3-d_2*: proton NMR (D_2O, pD 1.5) δ 4.22 (s, 1 H, H-2), 7.33 (m, 5 H, aromatic). The residual signals at δ 3.13 and 3.30 for the CH_2 protons indicated that the extent of deuteration was 90%.

Dihydrophenylalanine [*3-(1,4-Cyclohexadienyl)*-DL-*alanine*] (**3**). This compound was prepared by the method of Snow et al. (1968). In a 1-L three-necked flask fitted with a gas inlet tube and a N_2 bubbler (static), 2.5 g (15.2 mmol) of DL-phenylalanine was placed in 85 mL of dry methanol (stored over Linde 4 Å molecular sieves). The flask was immersed in a dry ice/acetonitrile bath, and 0.6 L of liquid ammonia was condensed. The temperature was maintained near −40 °C while 12.0 g (522 mmol) of sodium was added to the stirred solution in small portions (~0.5 g) over a period of 1.5 h. The blue color was allowed to fade before each successive addition of sodium. When addition of sodium was complete, 29.2 g (541 mmol) of ammonium chloride was added carefully in portions, and the ammonia was allowed to evaporate overnight. Methanol and residual ammonia were removed by evacuating the flask with a vacuum pump for 1 h. The dried reaction residue was suspended in 30 mL of cold water, adjusted to pH 7 with 6 N HCl, and filtered. The thick solid was resuspended in 30 mL of water and filtered. This procedure was repeated 5 times to remove all inorganic salts. The material was then washed on a fritted glass filter several times, first with ethanol and then with diethyl ether. The product was recrystallized from 50% ethanol (25 mL/g) to yield 884 mg of **3** (35%). The reaction was also scaled up to 10 g of DL-phenylalanine, 340 mL of methanol, 3 L of liquid ammonia, 48 g of sodium, and 117 g of NH_4Cl to give a similar yield: proton NMR (D_2O, pD 6.0) δ 2.4–2.6 (m, 2 H, CH_2 of side chain), 2.65 (broad, 4 H, $CH_2CH{=}CH$ on C-3 and C-6 of ring), 3.71 (dd, 1 H, $CHNH_3^+$), 4.72 (s, exchangeable H), 5.64 (s, CH=C on C-2 of ring), 5.74 (s, 2 H, CH=CH on C-4 and C-5 of ring); no detectable phenylalanine.

Dihydrophenylalanine-3,3-d_2 (**3**-d_2). Synthesis was identical with that for dihydrophenylalanine, except that DL-phenylalanine-*3,3-d_2* was used: proton NMR (D_2O, pD 6.0) δ 2.65 (broad, 4 H, $CH_2CH{=}CH$ on C-3 and C-6 of ring), 3.65 (s, 1 H, $CHNH_3^+$), 4.72 (s, exchangeable H), 5.63 (s, CH=C on C-2 of ring), 5.75 (s, 2 H, CH=CH on C-4 and C-5 of ring); deuteration greater than 88%; no detectable phenylalanine.

DL-*β-Cyclohexylalanine.* A total of 1.0 g (6.0 mmol) of **3** was dissolved in 10 mL of 70% acetic aciid containing 500 mg of prereduced palladium-on-barium sulfate catalyst. Hydrogenation was allowed to proceed overnight on a Parr hydrogenation apparatus. Hydrogen uptake was appropriate for 2 molar equivalents. The catalyst was removed by filtration through a Celite pad, and the filtrate was concentrated to dryness. The residue was taken up in a minimum volume of boiling water (~30 mL) and allowed to crystallize. Yield was 0.49 g (48%) of white crystals: proton NMR (D_2O, pD 6.0)

δ 0.9–2.05 (m, 13 H), 3.75 (m, 1 H, C*H*NH_3^+), 4.72 (s, exchangeable H).

Nomenclature. The nomenclature used is that of Northrop (1977), in which isotope effects on kinetic or thermodynamic parameters are defined by leading superscripts. Thus, D and 15 refer to deuterium or ^{15}N isotope effects. For example, $^{15}(V/K_{Phe\text{-}3,3\text{-}d_2})$ is the ^{15}N isotope effect on V/K (value with ^{14}N/value with ^{15}N) with phenylalanine-*3,3,*-d_2 as substrate. For a further discussion of nomenclature, see Cook & Cleland (1981).

Initial Velocity Studies. Initial velocity studies were performed at 25 °C by monitoring absorbance changes at either 290 [*trans*-cinnamate (**2**)] or 267 nm [*trans*-(1,4-cyclohexadienyl)acrylic acid (**4**)]. The extinction coefficients at these wavelengths for **2** and **4** are 10000 and 15000 M^{-1}, respectively. When the inhibition of PAL by cinnamate was determined, a coupled assay for NH_3 using NADPH and glutamate dehydrogenase was used. In this case, measurement was at 340 nm, and the extinction coefficient of 6220 M^{-1} of NADPH was used. A comparison of the two assay procedures gave V_{max} and K_{Phe} values which agreed within 10% at pH 8.38.

Deuterium isotope effects were obtained by comparing the initial velocities with deuterated and unlabeled substrates. V/K isotope effects determined by comparing the slopes of reciprocal plots are not sensitive to the presence of inhibitors in the deuterated or unlabeled substrates but are only as precise as the relative concentrations of the substrates are known (see methods used for calibration below). The V isotope effects do not depend on substrate calibration but are sensitive to the presence of impurities in the substrates. The proteo and deuterio substrates were in all cases synthesized in identical fashion as racemic mixtures. Note, however, that unless the K_m values for the proteo and deuterio substrates are the same or the inactive isomer is a very poor inhibitor, the V isotope effect obtained from the racemic mixtures may be in error (Grimshaw & Cleland, 1980).

Determination of Substrate Concentrations. L-Phenylalanine concentrations were determined enzymatically by using PAL in 50 mM sodium borate, pH 8.8. The K_{eq} for the production of cinnamate is 4.7 M (Havir & Hanson, 1968). The concentrations of L-dihydrophenylalanine stock solutions were determined by measurement of the absorbance at 225 nm (ϵ = 302 M^{-1}; Snow et al., 1968) and by integration of resonances in the proton NMR of a sample which had been diluted into a standard solution of sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), lyophilized, and redissolved in D_2O (100.0 atom % D). Since the same standard DSS solution was used for both the labeled and unlabeled substrates, their relative concentrations were accurately known. Both the UV determination and the proton NMR method of calibration gave the same concentration (within 5%), as did measurement based on weight. An additional check of the purity of the solid was by Kjeldahl analysis for total nitrogen. When dissolved in solution, neither dihydrophenylalanine or its deuterated analogue showed any free ammonia by Nessler's assay. Kjeldahl digestion followed by steam distillation gave an amount of ammonia consistent with the UV, NMR, and weight determinations.

^{15}N Kinetic Isotope Effects. The $^{15}(V/K)$ isotope effects were determined by isotope ratio mass spectral analysis of the ammonia from complete or low conversion (10–20% reaction) samples by using the natural abundance of ^{15}N as the label. The complete conversion samples give the mass ratio in the original substrate, while the low conversion samples reflect the isotopic discrimination. The details of ammonia isolation and conversion to N_2 for analysis are given below.

^{15}N Equilibrium Isotope Effects. To determine the ^{15}N equilibrium isotope effect for the aspartate/ammonium ion equilibrium, the following were incubated in 100 mL with 50 units of aspartase: 100 mM Hepes, pH 7.9, 60 mM $MgCl_2$, 313 mM fumarate, and 200 mM NH_4Cl. After 10 times the time required to reach thermodynamic equilbrium, the pH was lowered to 1.5 with 3 drops of concentrated H_2SO_4 to denaturate the enzyme, and the ammonia was isolated by steam distillation and analyzed by isotope ratio mass spectrometry. The $^{15}N/^{14}N$ ratio of this sample was compared to that of the initial NH_4Cl to calculate the $^{15}K_{eq}$ isotope effect. These results are shown in Table IV.

An analogous experiment was performed to determine the ammonia/phenylalanine ^{15}N equilibrium isotope effect with 8 M ammonia at pH 10, 8.5 mM *trans*-cinnamate, and 10 units of PAL. Phenylalanine was isolated by chromatography on AG 50W-H^+, and the mass ratio of its nitrogen (after Kjehldahl digestion and isolation of the ammonia) was compared with that of the initial ammonia. These results are also in Table IV.

To determine the ammonium ion/ammonia ^{15}N equilibrium isotope effect, we employed a Millipore filter that only allows diffusion of unprotonated ammonia. To one flask of an apparatus consisting of two sealed and magnetically stirred 50-mL Erlenmeyer flasks separated by a Millipore FALP (04700) membrane filter was added 50 mL of 50 mM NH_4Cl in 50 mM borate, pH 8.4. The other flask contained water or 250 mM NaCl. Chemical equilibration was observed in ~3 h, as monitored by Nessler's assays. After 23 h, the water side was removed and acidified with 3 drops of concentrated H_2SO_4, and the ammonia was isolated by alkaline steam distillation. Comparison of the mass ratio of this material with that of the starting NH_4Cl gave the results in Table IV. These experiments were also carried out by adding NaOH or NaOD to 120 mM NH_4Cl or ND_4Cl in H_2O or D_2O and allowing diffusion into 70 mM NaCl in H_2O or D_2O.

Assay Conditions for Nitrogen Isotope Effect Studies. All solutions were checked for ammonia by using either the glutamate dehydrogenase or Nessler's assay. Fifteen milliliters of 40 mM (active isomer concentration) phenylalanine, dihydrophenylalanine, or their 3-dideuterated counterparts in 50 mM sodium borate, pH 8.4–9.6, was incubated with 1 unit of PAL (desalted on a 1.6 × 30 cm G-10 column and free of ammonia by Nessler's assay) in tightly stoppered vessels for sufficient time to give ~20% reaction. The progress of the reaction was followed by removing 10-μL aliquots for assay of both *trans*-cinnamate (at 290 nm) or *trans*-3-(1,4-cyclohexadienyl)acrylate (at 267 nm) and ammonia (by Nessler's assay). The reactions were quenched with 3 drops of concentrated H_2SO_4 (pH to 1) and the protein and cinnamic acid precipitates removed by filtration.

The ammonia from the quenched reactions was isolated by steam distillation (Bremner, 1965). The assay solution was first diluted with water to 30 mL and placed in the distilling flask. Through a septum was introduced 5 mL of 13 M NaOH. The collection flask contained 10 mL of 0.1 N H_2SO_4 into which the distillate tube was immersed. The distillation was continued until 40 mL had been collected. Nessler's assay at this point in control experiments verified that all ammonia was recovered, and none was produced from unreacted substrates. The distillate was then reduced in volume to ~1 mL with a rotary evaporator connected to a vacuum pump. Since the solution is at very low pH, no ammonia is lost during the concentration procedure.

The hypobromite oxidation of NH_3 to N_2 has been shown to be quantitative and thus without ^{15}N fractionation (Bremner, 1965). The reagent is prepared by adding 60 mL of Br_2 to 300 mL of 17 M NaOH and diluting this 1:1 with 12 mM KI; this mixture is filtered through a sintered glass funnel 48 h after preparation and stored at 4 °C in the dark. The ~1-mL sample of ammonia was oxidized with 1.0 mL of hypobromite reagent in a Y-tube (Bremner, 1965).

The $^{15}N/^{14}N$ ratio of the resulting N_2 was measured immediately with a Varian MAT 250 dual inlet isotope ratio mass spectrometer. The reproducibility of this oxidation is typically ±0.002% of the mass ratio. The manipulations involved in the isolation of the NH_3 by steam distillation have been shown not to introduce any isotopic fractionation by comparing the mass ratios of stock NH_4Cl with that carried through the described procedure (variation less than ±0.02%).

Though the K_{eq} for the PAL-catalyzed elimination of ammonia from phenylalanine is favorable enough to permit the enzymatic generation of 100% conversion samples to establish the mass ratio in the original substrate, it is more economical to use Kjeldahl digestion to isolate the ammonia (Burris, 1957). In the case of the slow substrate, dihydrophenylalanine, this is the only practical way to ensure complete conversion to ammonia. To 50 mg (~280 μmol) of phenylalanine or dihydrophenylalanine in a Kjeldahl flask was added 1.5 g of K_2SO_4, 1.5 mL of mercuric sulfate (12 mL of concentrated H_2SO_4 diluted to 100 mL containing 10 g of red mercuric oxide), 3.0 mL of concentrated H_2SO_4, and a few boiling chips. The mixture was heated to boiling on a digestion rack for ~1.5 h (30 min past clearing of the solution). The solution was then cooled to room temperature, 25 mL of water was added, and the solution was again cooled. Zinc dust (0.6 g) was added and the flask allowed to sit for 30 min. After the solution was filtered, the filtrate was steam distilled to recover the ammonia after addition of 15 mL of 13 N NaOH to make the solution basic. Nessler's assay of the recovered ammonia indicated that the yields were quantitative.

Data Analysis. Reciprocal initial velocities were plotted vs. the reciprocal of substrate concentration, and the data were fitted by the least-squares method assuming equal variances for the values of v or $\log y$ by using a digital computer and the programs of Cleland (1979). Individual saturation curves were fitted to eq 3. pH profiles in which the log of the

$$v = VA/(K + A) \tag{3}$$

parameter decreased both above pK_2 with a slope of −1 and below pK_1 with a slope of 1 were fitted to eq 4. The ^{15}N

$$\log y = \log [C/(1 + H/K_1 + K_2/H)] \tag{4}$$

isotope effects as a function of pH were fitted to eq 5. y_L and

$$y = [y_L + y_H(K_1/H)]/(1 + K_1/H) \tag{5}$$

y_H are the pH-independent values of the ^{15}N isotope effect at low and high pH, respectively.

Deuterium isotope effects were obtained by fitting the experimental velocities to eq 6. In eq 3–6, V is the maximum

$$v = VA/[K(1 + F_iE_{V/K}) + A(1 + F_iE_V)] \tag{6}$$

velocity and K the Michaelis constant of phenylalanine or its dihydro analogue. In eq 6, F_i is the fraction of deuterium in the substrate and E_V and $E_{V/K}$ are the isotope effects minus one on V and V/K. In eq 4 and 5, K_1 and K_2 are the acid dissociation constants of either enzymic or substrate groups and H is hydrogen ion concentration.

To determine ^{15}N kinetic isotope effects, eq 7 was used,

$$^{15}(V/K) = \log (1 - f)/\log (1 - fR/R_0) \tag{7}$$

where R is the $^{15}N/^{14}N$ ratio in the product at the fraction of reaction f, and R_0 is the mass ratio in the initial substrate. ^{15}N equilibrium isotope effects were calculated from eq 8

$$^{15}K_{eq} = y_2/y_1 + x_1(y_2/y_1 - 1) \tag{8}$$

where x_1 is the [product]/[reactant] ratio at equilibrium, y_1 is the $^{15}N/^{14}N$ ratio of the product at equilibrium, and y_2 is the mass ratio of the initial reactant.

THEORY

Hermes et al. (1982) have shown that by measuring a heavy atom isotope effect with both a deuterated and an unlabeled substrate one can tell which isotope-sensitive step comes first in the mechanism or whether both isotope effects are on the same step. The application of this method to PAL is somewhat more complicated, since there exist two separate ^{15}N-sensitive steps in each possible mechanism. We thus give below the equations for each of the three possible mechanisms for the elimination reaction (see Figure 1 for the structures of the intermediates in each case).

Carbanion Intermediate (E1cb Mechanism). The relevant equations in this case are

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA' \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EC^- \underset{k_8}{\overset{k_7}{\rightleftharpoons}} EP \overset{k_9}{\rightharpoonup} E^* + P \tag{9}$$

$$^{D}(V/K) = \frac{^{D}k_5 + a(1 + b) + c}{1 + a(1 + b) + c} \tag{10}$$

$$^{15}(V/K)_H = \frac{^{15}K_{eq3} + a(^{15}k_3 + b) + c(^{15}K_{eq3}{}^{15}k_7)}{1 + a(1 + b) + c} \tag{11}$$

$$^{15}(V/K)_D = \frac{^{15}K_{eq3} + a(^{15}k_3 + b)/^{D}k_5 + c(^{15}K_{eq3}{}^{15}k_7)/^{D}k_5}{1 + a(1 + b)/^{D}k_5 + c/^{D}k_5} \tag{12}$$

where a is k_5/k_4, b is k_3/k_2, and c is k_6/k_7 (we will assume that $k_9 \gg k_8$ with the slow substrate dihydrophenylalanine). The $EA' \rightarrow EC^-$ step is deuterium but not ^{15}N sensitive, while both the $EA \rightarrow EA'$ and $EC^- \rightarrow EP$ steps are ^{15}N sensitive. We will assume that $k_2 \gg k_3$ for the slow substrate dihydrophenylalanine and that $^{15}k_4 = {}^{15}k_7$ so that $^{15}k_3 = {}^{15}K_{eq3}{}^{15}k_7$. We will also assume that $^{D}K_{eq}$ is unity for the proton transfer step (this will be a good assumption as long as the base to which the proton is transferred is a carboxyl or imidazole group). By using the experimental value of $^{D}(V/K)$ and assuming a value of $^{15}K_{eq3}$, these equations can be solved for the expected $^{15}(V/K)_H$ and $^{15}(V/K)_D$ values as a function of $^{D}k_5$ and $^{15}k_7$:

$$a + c = [^{D}k_5 - {}^{D}(V/K)]/[^{D}(V/K) - 1] \tag{13}$$

$$^{15}(V/K)_H = {}^{15}K_{eq3}[1 + (a + c)^{15}k_7]/(1 + a + c) \tag{14}$$

$$^{15}(V/K)_D = {}^{15}K_{eq3}[1 + (a + c)^{15}k_7/^{D}k_5]/[1 + (a + c)/^{D}k_5] \tag{15}$$

Since $^{15}K_{eq3}$ is a factor of both eq 14 and 15, the ratio of $^{15}(V/K)_H/{}^{15}(V/K)_D$ is not sensitive to this value. One can thus pick values of $^{D}k_5$ and $^{15}k_7$ that give the correct ratio of observed ^{15}N isotope effects, and adjust $^{15}K_{eq3}$ so that the absolute values are matched.

Table I: Comparison of PAL Substrate and Inhibitor Characteristics[a]

compound[b]	K_m (μM)	relative V_{max}	relative V/K	pK of amino group
phenylalanine	216 ± 9	100	100	9.13 ± 0.02
dihydrophenylalanine	122 ± 4	7	12	9.27 ± 0.01
β-cyclohexylalanine	1800[c]			9.93 ± 0.04

[a] At 25 °C 50 mM borate, pH 8.9 (the pH optimum). [b] Racemic mixtures. [c] Slope inhibition constant.

Carbonium Ion Intermediate (E1 Mechanism). The equations in this case are

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA' \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} EC^+ \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} EP \xrightarrow{k_9} E^* + P \quad (16)$$

$$^{D}(V/K) = \frac{^{D}k_{13} + [1 + a(1 + b)]/c}{1 + [1 + a(1 + b)]/c} \quad (17)$$

$$^{15}(V/K)_H = \frac{^{15}K_{eq3}{}^{15}k_{11} + a(^{15}k_3 + b) + c(^{15}K_{eq3}{}^{15}K_{eq11})}{1 + a(1 + b) + c} \quad (18)$$

$$^{15}(V/K)_D = \frac{^{15}K_{eq3}{}^{15}k_{11} + a(^{15}k_3 + b) + c(^{15}K_{eq3}{}^{15}K_{eq11})^{D}k_{13}}{1 + a(1 + b) + c^{D}k_{13}} \quad (19)$$

where a is k_{11}/k_4, b is k_3/k_2, and c is k_{12}/k_{13} (we assume, as above, that $k_9 \gg k_{14}$). The EA → EA′ and the EA′ → EC$^+$ steps are ^{15}N sensitive, while the EC$^+$ → EP step is deuterium sensitive. Making the assumptions that we did above that $k_2 \gg k_3$ and $^{15}k_4 = {}^{15}k_{11}$ gives

$$(1 + a)/c = [^{D}k - {}^{D}(V/K)]/[^{D}(V/K) - 1] \quad (20)$$

$$^{15}(V/K)_H = {}^{15}k_3[1/{}^{15}k_{12} + (1 + a)/c]/[1 + (1 + a)/c] \quad (21)$$

$$^{15}(V/K)_D = {}^{15}k_3[^{D}k_{13}/{}^{15}k_{12} + (1 + a)/c]/[^{D}k_{13} + (1 + a)/c] \quad (22)$$

Since $^{15}k_3$ is a factor of both eq 21 and 22, the ratio of $^{15}(V/K)_H$ and $^{15}(V/K)_D$ is sensitive only to the assumed values of $^{D}k_{13}$ and $^{15}k_{12}$, and one can pick values of these constants that give the correct ratio of observed ^{15}N isotope effects, and adjust $^{15}k_3$ to match the absolute values. It should be noted, however, that since the calculated $^{15}K_{eq}$ value for the reaction to give the amino enzyme E* is normal,[2] the ^{15}N isotope effects with both deuterated and unlabeled substrates will always be normal, although the one with the deuterated substrate will be closer to unity.

Concerted Reaction (E2 Mechanism). The equations in this case are

$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EA' \underset{k_{16}}{\overset{k_{15}}{\rightleftharpoons}} EP \xrightarrow{k_9} E^* + P \quad (23)$$

$$^{D}(V/K) = \frac{^{D}k_{15} + a(1 + b)}{1 + a(1 + b)} \quad (24)$$

$$^{15}(V/K)_H = \frac{^{15}K_{eq3}{}^{15}k_{15} + a(^{15}k_3 + b)}{1 + a(1 + b)} \quad (25)$$

$$^{15}(V/K)_D = \frac{^{15}K_{eq3}{}^{15}k_{15} + a(^{15}k_3 + b)/{}^{D}k_{15}}{1 + a(1 + b)/{}^{D}k_{15}} \quad (26)$$

[2] The value should be about 1.004, since in the substrate the nitrogen is attached to a carbon bonded to two carbons and a hydrogen, while in E* it is attached to a carbon bonded to one carbon and two hydrogens. See footnote *r* of the table in Cleland (1980).

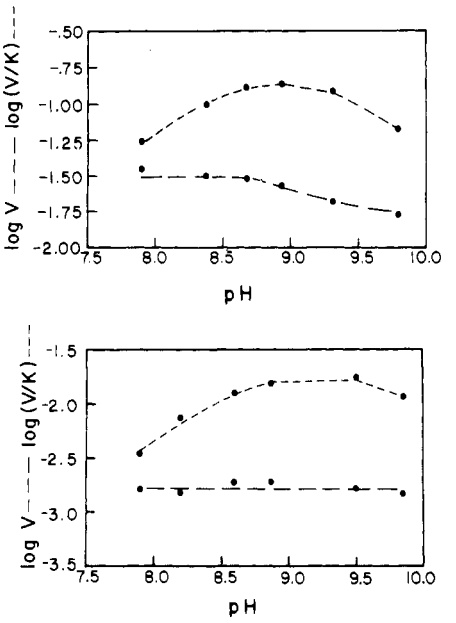


FIGURE 2: pH profiles for phenylalanine (top) and dihydrophenylalanine (bottom) as substrates for phenylalanine ammonia-lyase. Data at each pH value were fitted to eq 3.

where a is k_5/k_4 and b is k_3/k_2 (we assume, as above, that $k_9 \gg k_{16}$). The interconversion of EA′ and EP is the deuterium-sensitive step, while the EA → EA′ and EA′ → EP steps are ^{15}N sensitive. If one assumes that $k_2 \gg k_3$ for the slow substrate dihydrophenylalanine and $^{15}k_4 = {}^{15}k_{15}$ so that $^{15}K_{eq3}{}^{15}k_{15} = {}^{15}k_3$, then

$$a = [^{D}k_{15} - {}^{D}(V/K)]/[^{D}(V/K) - 1] \quad (27)$$

$$^{15}(V/K)_H = {}^{15}(V/K)_D = {}^{15}k_3 \quad (28)$$

If $^{15}k_3$ is less than $^{15}K_{eq3}{}^{15}k_{15}$ (×0.99, say), then $^{15}(V/K)_D$ will be *larger* than $^{15}(V/K)_H$, regardless of the values of $^{D}k_{15}$ and $^{15}k_3$ chosen, and both ^{15}N isotope effects will be larger than $^{15}k_3$.

Results and Discussion

Comparison of Substrate and Inhibitor Characteristics. A comparison of the kinetic properties of phenylalanine and 3-(1,4-cyclohexadienyl)alanine (dihydrophenylalanine) as substrates for PAL are in Table I, along with the pK's of the α-amino groups of these compounds and of the inhibitor β-cyclohexylalanine determined by titration.

pH Profiles. The V/K profiles for both phenylalanine and dihydrophenylalanine are in Figure 2. Both of these substrates display decreases in V/K at high and low pH. Fitting the data to eq 4 gives pK's of 8.40 ± 0.04 and 9.43 ± 0.04 for phenylalanine and 8.87 ± 0.12 and 9.53 ± 0.16 for dihydrophenylalanine. This is the typical behavior when one group must be protonated and the other unprotonated for activity. In V/K profiles substrate pK's are seen if protonation state of the substrate influences either binding or catalysis. For PAL one would expect the protonation state of the substrate amino group to be critical for binding and formation of the activated amine (EA′ of Figure 1), and therefore, one of the pK's for each V/K profile likely corresponds to the acid dissociation constant for the α-amino group. Since the two pK's seen in each profile are only slightly different, one cannot a priori tell which belongs to the substrate amino group (Cleland, 1977). It is quite possible for reverse protonation to be required (that is, for the group with the lower pK to have to be protonated, and the one with the higher pK to have to be ionized). The

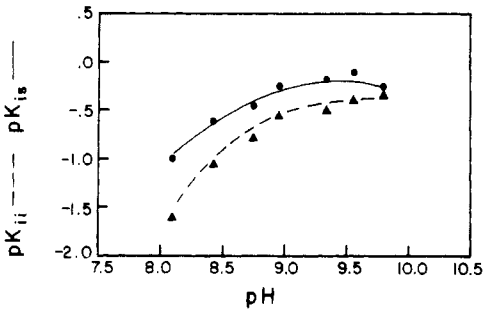


FIGURE 3: pH variation of the inhibition constants for β-cyclohexylalanine with phenylalanine as variable substrate. K_{is} and K_{ii} are slope and intercept inhibition constants, respectively.

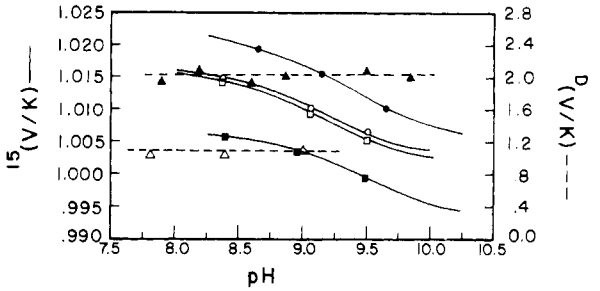


FIGURE 4: pH variation of kinetic isotope effects with phenylalanine (open symbols) or dihydrophenylalanine (solid symbols). ^{15}N isotope effects with unlabeled (○, ●) or 3-dideuterated (□, ■) substrates. Deuterium isotope effects with dideuterated substrates (△, ▲).

pK's of phenylalanine and dihydrophenylalanine are 9.13 ± 0.02 and 9.27 ± 0.01, respectively. These are closest to the higher pK's seen in the V/K profiles, and thus, a reverse protonation situation likely exists, with the actual substrate being the unprotonated amino acid with the free amino group available for nucleophilic addition to the dehydroalanine residue (see Figure 1). The lower pK's in these profiles may correspond to the group on the enzyme that protonates the amide oxygen upon formation of EA′. When pK's are close together, a fit to eq 4 determines their average accurately, but the individual values with more uncertainty. The actual lower pK's for the V/K profiles of both substrates are probably identical and ~9. The identity of the group is unknown, but the pK and postulated role in catalysis are consistent with a lysine.

Inhibition by the saturated analogue of phenylalanine, β-cyclohexylalanine, was noncompetitive, and the pH profiles for the inhibition constants are in Figure 3. The pK_{is} profile is bell shaped, with K_{is} increasing below pK's of 9.12 ± 0.05 and 9.72 ± 0.05. The pK of β-cyclohexylalanine is 9.93 ± 0.04, and the presence of the pK at 9.12 in the pK_{is} profile suggests that the enzymic group implicated above in catalysis is involved in inhibition and that the inhibitor forms a covalent complex analogues to EA′. Evidence also exists for reverse protonation in the irreversible inhibition by nitromethane which involves attack of the nitromethane anion (pK = 10.3) on the β-carbon of dehydroalanine. Havir & Hanson (1975) found that the rate of inactivation of PAL by nitromethane decreased below pH 8.7 and above pH 10.3. The largest concentrations of nitromethane anion and protonated enzymic base are found in the pH range between the respective pK's, so that inactivation rates are highest in this pH range. The pK for the base expected to facilitate removal of the *pro-S* proton is presumably below the pH range tested (i.e., below pH 7.5). In the analogous fumarase reaction, this group is a carboxyl with a pK of 5.8 (Blanchard & Cleland, 1980).

The V_{max} values for phenylalanine and dihydrophenylalanine are relatively pH independent, with that for phenylalanine showing a slight decrease at high pH (possibly as the result of a slower release of ammonia from the enzyme when the amino group of E* is not protonated). This behavior is diagnostic for formation of an enzyme–substrate complex only when both the substrate and enzyme are in the correct protonation states for catalysis, so that no incorrectly protonated complexes are possible (Cleland, 1977).

In contrast to the behavior of β-cyclohexylalanine, inhibition by cinnamate was competitive vs. phenylalanine with an inhibition constant of 24 ± 3 μM that was pH independent from pH 8.3 to 9.3. D-Phenylalanine is not a substrate for PAL but was a competitive inhibitor. At pH values of 8.68, 9.21, and 9.68 the inhibition constants were 0.93 ± 0.05, 0.77 ± 0.04, and 1.8 ± 0.2 mM, respectively.

Deuterium Isotope Effects. The concentrations of DL-phenylalanine and DL-phenylalanine-*3,3-d*$_2$ were varied from 0.2 to 5 times the K_m (~300 μM) at various pH's, and the isotope effects on V and V/K were very small (~1.15 on V/K) and apparently pH independent. These isotope effects are for dideuterated substrates and thus represent the product of the primary (*pro-S* hydrogen) and α-secondary (*pro-R* hydrogen) isotope effects. This causes no problem with interpretation, however, even if the secondary isotope effect is largely on a step different from proton transfer, since the primary isotope effect will be much larger than the secondary one.

The small magnitude of the deuterium isotope effect with phenylalanine shows that the commitments are high for the proton removal step, and led us to search for an alternate substrate which would possess a less acidic C-3 proton so that proton transfer would be slower. The partially ring-saturated analogue, dihydrophenylalanine (**3**), was ideal, since the electron-withdrawing properties of the ring were diminished, but its K_m was only slightly different, and V was only 7% that of phenylalanine. Hanson et al. (1979) suggest that the lower rates of reaction with dihydrophenylalanine result from electronic rather than steric or hydrophobic differences from phenylalanine. The deuterium isotope effect for dideuterated substrate was the same on V and V/K and pH independent. The value of 2.0 shows that proton removal in indeed more rate limiting than with phenylalanine, and the equal size of the isotope effects on V and V/K implies that hydrolysis of the amino–enzyme intermediate (E* in Figure 1) is much faster than the β-elimination reaction. The pH-independent nature of the V/K isotope effects again supports the idea that only the correctly protonated state of enzyme and substrate can combine to form an enzyme–substrate complex. External commitments can be eliminated and V/K isotope effects increased by changing pH if an enzyme–substrate complex can form in an incorrect protonation state (Cleland, 1977). Since intrinsic primary deuterium isotope effects on elimination reactions are usually in the 5–7 range, the $^{D}(V/K)$ value of 2.0 indicates that steps other than proton removal are also partly rate limiting, but this isotope effect is of sufficient size to make practical the measurement of ^{15}N isotope effects with deuterated and unlabeled substrates as a test of mechanism.

^{15}N Kinetic Isotope Effects. The $^{15}(V/K)$ isotope effects for phenylalanine, phenylalanine-d_2, dihydrophenylalanine, and dihydrophenylalanine-d_2 were determined from the mass ratios in the initial substrate an in ammonia after partial reaction by the use of eq 7 as shown in Table II. Figure 4 shows that the $^{15}(V/K)$ isotope effects with all four compounds decrease at higher pH, with the pK for the change apparently being that of the α-amino group of the substrate. Fitting the three points of each curve to eq 5 with various assumed pK values

Table II: ^{15}N Kinetic Isotope Effects for PAL

substrate	pH	$^{15}(V/K)^a$
phenylalanine	8.40	1.0146 ± 0.0005 (4)
	9.05	1.0106 ± 0.0006 (4)
	9.50	1.0066 ± 0.0003 (3)
phenylalanine-d_2	8.38	1.0141 ± 0.0006 (3)
	9.05	1.0097 ± 0.0005 (3)
	9.50	1.0052 ± 0.0003 (3)
dihydrophenylalanine	8.55	1.0195 ± 0.0002 (3)
	9.10	1.0162 ± 0.0005 (3)
	9.60	1.0102 ± 0.0005 (3)
dihydrophenylalanine-d_2	8.40	1.00542 ± 0.00002 (2)
	8.97	1.0033 (1)
	9.50	0.9993 ± 0.0002 (2)

[a] Number of determinations in parentheses.

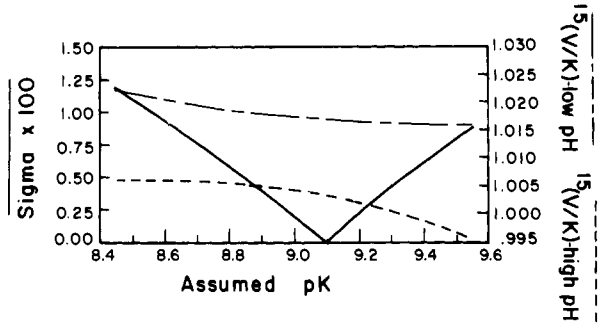
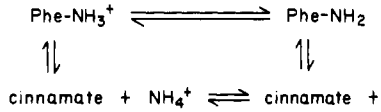


FIGURE 5: Plots of σ (square root of the sum of the squares of the residuals) and low and high pH plateau values of $^{15}(V/K)$ from fits of observed ^{15}N isotope effects to eq 5 as a function of assumed pK for phenylalanine. A similar plot was seen for dihydrophenylalanine, except that the minimum for σ was at pH 9.3. Similar plots were seen with dideuterated substrates.

gave the low and high pH plateau values and the σ values (square root of the sum of the squares of the residuals) shown for phenylalanine in Figure 5 (similar curves were seen with dihydrophenylalanine). The best fit (lowest σ value) is in both cases precisely equal to the pK of the amino acid (9.1 for phenylalanine and 9.3 for dihydrophenylalanine), showing that the change in the ^{15}N isotope effects is due to the change in ionization of the substrate. The pH dependence of the kinetic parameters for the substrates and the inhibition constant for β-cyclohexylalanine suggested that the unprotonated free amino form of the substrate was the species actually bound by the enzyme. At pH values below the pK of the amino group the substrate will be mainly protonated and the kinetic ^{15}N isotope effect will be multiplied by the ^{15}N equilibrium isotope effect on proton dissociation. This effect is normal (greater than unity), since the nitrogen is more stiffly bonded in protonated $-NH_3^+$ than in $-NH_2$. The high and low pH plateau values for the observed ^{15}N isotope effects obtained from fitting the experimental data to eq 5 are in Table III.

^{15}N Equilibrium Isotope Effects. The average ratio of the low and high pH plateau values in Table III is 1.016 ± 0.002. To show that this value is consistent with the ^{15}N equilibrium isotope effect for the deprotonation of phenylalanine, the equilibrium isotope effects in Table IV were measured. Our aim was to determine indirectly the equilibrium isotope effect for the deprotonation of phenylalanine by constructing three sides of the thermodynamic box:



The $NH_4^+ \rightleftharpoons NH_3$ (1.0192) and Phe–$NH_2 \rightleftharpoons NH_3$ (1.0163) ^{15}N equilibrium isotope effects[3] were readily determined, but

Table III: Low and High pH Plateau Values for ^{15}N Isotope Effects[a]

substrate	assumed pK	$^{15}(V/K)_{low\ pH}$	$^{15}(V/K)_{high\ pH}$	difference
phenylalanine	9.1	1.0170	1.0021	0.0149
phenylalanine-d_2	9.1	1.0167	1.0010	0.0157
dihydrophenylalanine	9.3	1.0228	1.0047	0.0181
dihydrophenylalanine-d_2	9.3	1.0065	0.9921	0.0144

[a] The standard deviation on all fitted values was ± 0.0001 or less.

Table IV: ^{15}N Equilibrium Isotope Effects

reaction	pH	[product]/[reactant] at equilibrium	$^{15}K_{eq}{}^i$
aspartate$^- \rightleftharpoons$ fumarate + NH_4^+	7.9	0.21[a]	1.0138 ± 0.0001[b] (2)
NH_3 + cinnamate $\rightleftharpoons$ phenylalanine$^-$	10.0	0.003[c]	0.9840 ± 0.0001[d] (2)
$NH_4^+ \rightleftharpoons NH_3$		0.16[e]	1.0210 ± 0.0006 (2)
		0.21[f]	1.0190 ± 0.0001 (4)
		0.32[g]	1.0204 ± 0.0003 (3)
$ND_4^+ \rightleftharpoons ND_3$		0.77[h]	1.0246 ± 0.0001 (3)

[a] Equilibrium was established in a sealed vessel containing, in 100 mL, 100 mM Hepes, pH 7.9, 60 mM $MgCl_2$, 313 mM fumarate, 200 mM NH_4Cl, and 50 units of aspartase. [b] Experimental value of 1.0146 corrected for the proportions of aspartate and ammonium ion not protonated at pH 7.9 by using the ^{15}N equilibrium isotope effects on deprotonation. [c] Equilibrium was established at pH 10.0 in a sealed vessel containing, in 15 mL, 8 M NH_3, 8.5 mM *trans*-cinnamate, and 10 units of PAL. [d] Experimental value of 0.9847 corrected for the proportions of ammonia and phenylalanine that are protonated at pH 10 by using the ^{15}N equilibrium isotope effects on protonation. [e] Equilibrium was established by allowing diffusion on NH_3 through a ammonia-permeable membrane from 50 mL of 50 mM NH_4Cl in 50 mM borate, pH 8.4, into 50 mL of water. The [product]/[reactant] value is [total ammonia in both compartments]/[ammonium ion in the starting compartment]. Nessler's reagent was used to assay each compartment after equilibrium was reached. [f] As in footnote *d*, except diffusion into 250 mM NaCl (ionic strength equal in both compartments). [g] NaOH added to 120 mM NH_4Cl; diffusion into 70 mM NaCl. [h] Same as in footnote *g*, except in D_2O. [i] Number of determinations in parentheses.

the high ionic strength that results from the ~8 M NH_4Cl required to established the Phe–$NH_3^+ \rightleftharpoons NH_4^+$ equilibrium denatured PAL. The aspartase-catalyzed equilibrium Asp–$NH_3^+ \rightleftharpoons NH_4^+$ ($^{15}K_{eq}$ = 1.0138) can be readily established at a pH below the pK's of aspartate and ammonia, and this ^{15}N equilibrium isotope effect is not likely to be much different from that of Phe–$NH_3^+ \rightleftharpoons NH_4^+$. Using the experimental values in Table V gives a calculated equilibrium isotope effect for Phe–$NH_3^+ \rightleftharpoons$ Phe–NH_2 of 1.0167, which is in excellent agreement with the average ratio of low and high pH plateau values of 1.016 ± 0.002 in Table III. The pH variation of the ^{15}N kinetic isotope effects with both phenylalanine and dihydrophenylalanine is thus wholly attributable to the equilibrium isotope effect on deprotonation of the amino acid. Note from these values that the stiffening effect of protonation

[3] An unpublished value for the equilibrium isotope effect for deprotonation of ammonium ion of 1.039 was quoted by O'Leary (1978) and has been included in lists of fractionation factors [for example, in Cleland (1980)]. This value was measured by sampling the first portion of ammonia diffusing through a membrane into an acid trap, and is the product of the equilibrium isotope effect on deprotonation of NH_4^+, and the kinetic isotope effect on diffusion of ammonia through the membrane (which, if the entity diffusing is NH_3, might be expected to be as large as 1.029). Early less precise values for the equilibrium isotope effect on deprotonation were 1.024 (Thode et al., 1945) and 1.021 (Urey & Aten, 1936).

if 0.25% less when the nitrogen is already bonded to carbon than in NH_3, and conversely, the stiffening effect of replacing H with C is 0.25% less when the nitrogen has a positive charge than when it is neutral.

Another apparent ^{15}N equilibrium isotope effect was observed during the synthesis of phenylalanine-d_2 when this was carried out in methanol-d with ND_4Br. The product was about 1% depleted in ^{15}N relative to the NH_4Br, while no discrimination was observed when NH_4Br and unlabeled methanol were used. During this synthesis the protonated imine of phenylpyruvate is reduced by cyanoborohydride, and since ammonium bromide was present at 5 times the level of phenylpyruvate, the isotope ratio in the product presumably represents the fractionation factor of protonated imine relative to ammonium ion. This must be near unity in methanol but ~0.99 with $=ND_2^+$ and ND_4^+. This change is reasonable, since bonds to deuterium are stiffer than ones to hydrogen, and four bonds are stiffened in ND_4^+, while only two are affected in $=ND_2^+$. To confirm that this effect exists, we measured the $ND_4^+ \rightleftharpoons ND_3$ ^{15}N equilibrium isotope effect in D_2O, and as shown in Table IV, it is about 0.5% higher than the value for $NH_4^+ \rightleftharpoons NH_3$. Since four bonds are stiffened in ND_4^+ and only three in ND_3, an effect half of what is seen with the imine is expected, and the size of this deuterium isotope effect on ^{15}N fractionation factors can be taken as established.

Mechanism of the Elimination Catalyzed by PAL. The high pH $^{15}(V/K)$ values in Table III for dihydrophenylalanine are consistent only with the predictions for an E1cb mechanism with a carbanion intermediate. Since the value with deuterated dihydrophenylalanine is clearly inverse and lower than the value with unlabeled substrate, both the concerted and carbonium ion mechanisms are ruled out (see Theory). The ratio of the $^{15}(V/K)_H$ and $^{15}(V/K)_D$ values at high pH is 1.0127, and in order to match the actual observed values, $^{15}K_{eq3}$ has to be 0.979. Choosing 0.978 or 0.980 gives calculated values significantly lower or higher, respectively, than the observed one. This is a very reasonable value for the ^{15}N equilibrium isotope effect on the addition of the amino group of the substrate to dehydroalanine to give the protonated secondary amino shown as EA′ in Figure 1 and supports the postulated chemistry of the reaction. Note that the effect of adding a carbon to the nitrogen of the substrate (2.1%) is somewhat greater than that of adding just a hydrogen (1.6–1.8%).

The exact values of $^Dk_5$ and $^{15}k_7$ in mechanism 9 are not specified by the solution of eq 13–15, but representative values calculated with $^{15}K_{eq3} = 0.979$ that match the observed ^{15}N isotope effects are 5.6 and 1.033, 5.2 and 1.034, and 4.6 and 1.036, all of which are reasonable for the postulated chemistry. The value of $^{15}k_7$ appears to be well above the ^{15}N equilibrium isotope effect for this step (estimated to be 1.004/0.979 = 1.025), so that $^{15}k_8$ (and also $^{15}k_3$) will be normal (~1.01) as one might expect for a primary isotope effect involving heavy atom motion in the reaction coordinate, even though the equilibrium isotope effect is strongly inverse.

The isotope effects with phenylalanine itself are small, but if $^Dk_5$ has a value of 5.2, the sum of the commitments $[k_5/k_4(1 + k_3/k_2) + k_6/k_7]$ for the proton transfer is ~27, compared to a value of 3.2 for dihydrophenylalanine. The distinction between k_5/k_4, k_3/k_2, and k_6/k_7 cannot be determined, except that to match the observed ^{15}N isotope effects, at least part of the forward commitment (>3) must be contributed by k_3/k_2, so that phenylalanine has an external commitment and is sticky. The slight decrease in the $^{15}(V/K)$ value with deuterated phenylalanine, but with the value remaining slightly normal, is consistent with eq 14 and 15 and a carbanion mechanism but is not sufficient to allow mechanistic distinction. Since proton removal is easier with phenylalanine than with the dihydro analogue (that is, the carbanion is more stable), it is likely that the carbanion mechanism applies equally to both substrates.

The fascinating question is how does the enzyme stabilize the carbanion intermediate? When carbanions are formed α to a carboxyl group, the electrons appear to be delocalized into the carboxyl group to give an *aci*-carboxylate structure, since the corresponding nitronate analogues are very tight inhibitors [fumarase and aspartase (Porter & Bright, 1980); aconitase (Schloss et al., 1980); isocitrate lyase (Schloss & Cleland, 1982); enolase (Anderson et al., 1984)]. With phenylalanine ammonia-lyase, however, the electrons would have to be delocalized into the ring of phenylalanine or into the single double bond that is properly placed in dihydrophenylalanine. The lack of such a double bond is apparently what keeps β-cyclohexylalanine from being a substrate, although it appears to bind in the same way as the substrates and may form the secondary amino structure EA′ in Figure 1. Possibly a positively charged group is suitably placed to stabilize any negative charge which is delocalized into the ring. For now the facility of enzymes in forming carbanions remains a challenging puzzle.

The present study was undertaken because we suspected that PAL might catalyze a reaction that was concerted because of the apparent difficulty of delocalizing the electrons in a carbanion intermediate. The results show clearly that our suspicions were unfounded and illustrate the power of multiple isotope effect methodology to make clear distinctions between possible mechanisms. They also emphasize the point that the natural substrate for an enzyme is often unsuitable for analysis of mechanism because of high commitments, while a slowly reacting alternate substrate will reveal the secrets of the enzymatic mechanism.

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Circular Dichroic Evidence for an Ordered Sequence of Ligand/Binding Site Interactions in the Catalytic Reaction of the cAMP-Dependent Protein Kinase†

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ABSTRACT: A limiting requirement for substrate specificity of the cAMP-dependent protein kinase is the presence of one or two basic residues located to the N-terminal side of the target substrate serine. Furthermore, circular dichroic (CD) studies have shown that binding of protein substrate involves a series of at least two independent conformational changes in the enzyme, each of which is initiated by a recognition signal on the substrate protein. The present study attempts to elucidate further the complete sequence of enzyme/ligand interactions by using the synthetic substrate peptide Kemptide and analogues differing from it at crucial points in the sequence: the Ala-peptide, where alanine is substituted for the target serine, and D-Ser-Kemptide, where the target serine is in the D rather than the L configuration. Examination of the effects of binding of these substrates on the intrinsic UV CD of the enzyme and the induced CD in the presence of Blue Dextran has revealed a third step in the substrate/enzyme binding interaction. Although sections of the conformational change at the active site are dependent on the basic subsite and the serine hydroxyl group on the peptide, respectively, the complete conformational change requires that the substrate be bound in random coil conformation. Where this does not occur, the kinetics show that the peptide will not act either as substrate or as inhibitor of the enzyme. Further, the interaction between the serine hydroxyl group and an enzyme tyrosine residue, previously observed, appears to be dependent on the correct orientation as well as the mere presence of the target –OH group. Taken together, the data allow one to construct an ordered sequence of conformational changes taking place upon substrate binding leading to the final form of the active site.

The cAMP-dependent protein kinase is the primary, if not sole, effector of hormonally induced actions that are mediated by increases in cellular levels of cAMP. A broad range of proteins has been shown to be phosphorylated both in the cell in response to a cAMP signal and in vitro by the cAMP-dependent protein kinase. Despite this extreme diversity of substrates, a certain degree of specificity must pertain to the kinase in its role as mediator of hormonal control. It is, therefore, to be expected that there exist certain unique features of a protein substrate's structure that are recognized by the kinase and dictate substrate specificity.

Initial investigations of the sequence at the site in phosphorylated proteins led to the use in particular of the heptapeptide Kemptide, whose sequence (Leu-Arg-Arg-Ala-Ser-Leu-Gly) is that of the phosphorylation site of porcine hepatic pyruvate kinase, and of peptide analogues of this sequence. Such studies showed that a limiting requirement in dictating protein kinase substrate specificity is the presence of one or two basic residues optimally located two and three residues away from the target seryl residue on its N-terminal side (Daile et al., 1975; Zetterquist et al., 1976; Kemp et al., 1977; Feramisco et al., 1979; Meggio et al., 1981).

More recent information on the interactions occurring between the protein kinase and its peptide substrates has come from several approaches. Granot, Mildvan, and co-workers

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