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L-Phenylalanine Ammonia-Lyase (Maize)

EVIDENCE FOR A COMMON CATALYTIC SITE FOR L-PHENYLALANINE AND L-TYROSINE[1]

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ABSTRACT

L-Phenylalanine ammonia-lyase (E.C. 4.3.1.5) from maize is active with L-tyrosine and L-phenylalanine and exhibits atypical Michaelis-Menten kinetics with both substrates. With phenylalanine as a substrate, the pH optimum is 8.7 and with tyrosine, 7.7. The estimated Km at high substrate concentrations is 0.27 mM for phenylalanine and 0.029 mM for tyrosine. However, the V_{max} with phenylalanine is eight times higher than the V_{max} with tyrosine when both are measured at pH 8.7, and 7 times higher when both are measured at their pH optima. The following evidence leads us to the conclusion that there is a common catalytic site for both substrates: (*a*) It is impossible to appreciably alter the ratio of the two activities during purification and isoelectric focusing. (*b*) The ratio of the products formed in mixed substrate experiments is in good agreement with the ratio predicted from the estimated Km values. (*c*) $NaBH_4$ reduces both activities to the same degree and L-phenylalanine, L-tyrosine, cinnamate, and *p*-coumarate protect both activities against $NaBH_4$ reduction to the same degree. In contrast, the enzyme isolated from potato, which does not act on L-tyrosine, is not protected against reduction by either L-tyrosine or *p*-coumarate. However, both enzymes appear to have a dehydroalanine-containing prosthetic group.

The enzyme L-phenylalanine ammonia-lyase (EC 4.1.3.5), which converts L-phenylalanine to *trans*-cinnamate and ammonia has been isolated and purified to varying degrees from a number of sources (6, 10, 13, 16, 19, 20, 23, 26). Many of these preparations have the ability to catalyze the elimination of ammonia from a variety of *o*-, *m*-, and *p*-substituted phenylalanines including L-tyrosine. A question which immediately arises is whether one enzyme with a broad specificity is responsible for all of the observed activities or, alternatively, several enzymes function in these reactions. It has been suggested that separate enzymes specific for L-tyrosine and L-phenylalanine are present in some plant tissues (22). In other cases, however, the experimental results would favor the view that one enzyme is capable of catalyzing the two reactions (23). In surveys made with a wide variety of organisms there are no examples in which an extract has activity with tyrosine but not with phenylalanine, and indeed, no cases in which higher activity is observed with tyrosine than with phenylalanine (1, 2, 21, 28, 29). Attempts to purify a specific tyrosine ammonia-lyase have resulted in preparations which are three to four times more active with phenylalanine than tyrosine when assayed at saturating substrate concentrations (15, 22).

Resolving the question of enzyme specificity or lack of it when working with a nonhomogeneous system is a very difficult one. Factors such as relative V_{max} and Km, as well as pH optima, influence the results of certain types of inhibitor and mixed substrate experiments and these factors must be taken into account when the results are interpreted. In this paper we characterize the L-tyrosine ammonia-lyase activity of maize and present the several lines of evidence which lead us to believe that both L-tyrosine and L-phenylalanine react at the same active site. The question of specificity must be answered before we can understand the *in vivo* function and control of this enzyme.

MATERIALS AND METHODS

Enzyme Sources. L-Phenylalanine ammonia-lyase was purified through the Agarose step from light exposed potato tuber slices (12). The enzyme from maize was purified by a revised procedure (24) which will be published later (Reid *et al.*, in preparation). Protein was determined by the Lowry method (18).

Spectrophotometric Assay. Conditions of assay with L-phenylalanine as substrate and definition of a unit of activity were as previously described (10) except that a Gilford 2400 recording spectrophotometer was used. The specific activity of the maize preparations used in these experiments was 75 milliunits/mg protein or higher. In assaying for activity with L-tyrosine, 1.67 mM L-tyrosine replaced the L-phenylalanine. The reaction, followed at 290 nm, was proportional to the amount of protein added (equivalent to 5–35 milliunits of activity, measured with L-phenylalanine as substrate,) and the rate was linear for at least 20 min. At pH 8.7 (sodium-borate buffer), 1 μmole of *p*-coumarate in 3.0 ml has an absorbancy of 5.8. The *p*-coumarate, which was used as a standard, was recrystallized from hot water after addition of activated charcoal. The product formed from L-tyrosine was identified as *p*-coumarate by its absorption spectrum and by cochromatography with authentic *p*-coumarate in several solvent systems.

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RESULTS

KINETIC STUDIES

pH Activity Relationship. The effect of pH on initial velocity was determined with both L-phenylalanine and L-tyrosine. The results are shown in Figure 1. With L-phenylalanine as substrate the pH optimum is 8.7, similar to the value obtained with the enzyme from potato (10). With L-tyrosine, the pH optimum is shifted to pH 7.7. This spectrophotometric assay with tyrosine is subject to the slight variation in absorbancy which *p*-coumarate exhibits in different buffers. The curve in Figure 1 has not been corrected for this variation. The true initial velocities at pH 5.8 to 7.2 should be approximately 5 to 10% higher than shown; for pH 9.1 to 10, 5 to 10% lower. The extinction coefficient of cinnamate does not show this variation. The substrate concentration was increased at several points in order to make certain the enzyme was saturated at each pH. The curves thus represent the changes in V_{max} with pH.

Initial Velocity and Substrate Concentration. In earlier studies of the kinetic properties of the enzyme from maize, it was difficult to obtain reproducible results (19). The revised purification method has resulted in a stable preparation whose properties do not change on storage. The results of measurement of initial velocity as a function of either L-phenylalanine or L-tyrosine concentration are shown in Figures 2 and 3. The data has been plotted in the form *v versus* v/S in order to detect more easily departures from normal Michaelis-Menten kinetics (5). A straight line relationship is not observed, indicating that the enzyme does not follow normal Michaelis-Menten kinetics. The shape of the curves is very similar to that reported earlier (10, 19); that is, as substrate concentration increases the apparent K*m* (estimated as the slope of tangents to the curve) also increases. The apparent K*m* for phenylalanine estimated near saturation is approximately 9-fold higher than for tyrosine (0.27 mM *versus* 0.029 mM), but very near that for the potato enzyme (0.26 mM).

Relative Activity, V_{max}. The V_{max} with L-phenylalanine as substrate, measured at pH 8.7, is eight times higher than the V_{max} with L-tyrosine. If the V_{max} with L-phenylalanine, pH 8.7, is compared to the V_{max} for L-tyrosine, pH 7.7, the ratio drops

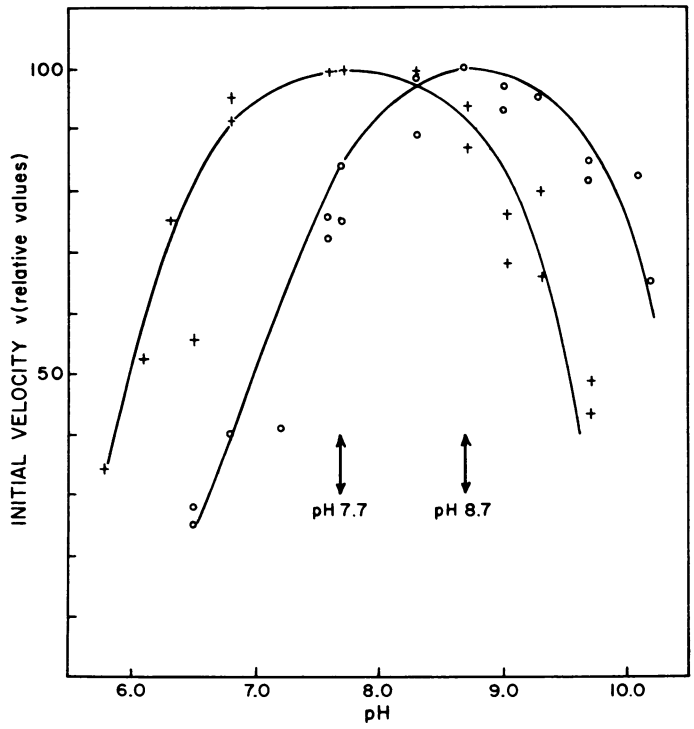

FIG. 1. Variation in initial velocity as a function of pH. The spectrophotometric assay contained 15 milliunits of enzyme; the substrates were at saturation, 6.67 mM for L-phenylalanine (○) and 1.67 mM for L-tyrosine (+). The following buffers (33 mM final concentration) were used: phosphate, pH 5.8 to 7.6; tris, pH 8.3; borate, pH 8.7 to 9.6; glycine, pH 9.6 to 10.2. Values of *v* are expressed relative to the maximum velocity for each substrate.

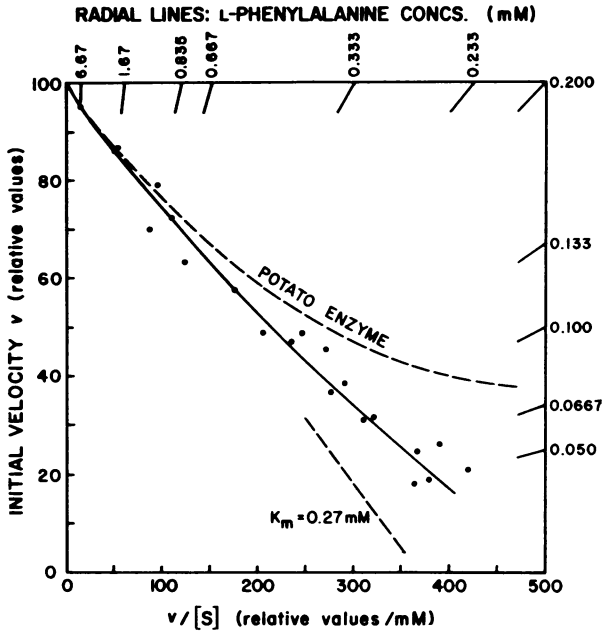

FIG. 2. Initial velocity of cinnamate formation as a function of L-phenylalanine concentration. Temperature 29 to 30 C, pH 8.7, buffer 33 mM sodium borate, 10 milliunits enzyme per assay. Values of *v* are expressed relative to *v* for the standard assay which is set at 96. This adjustment makes $V_{satn} = 100$.

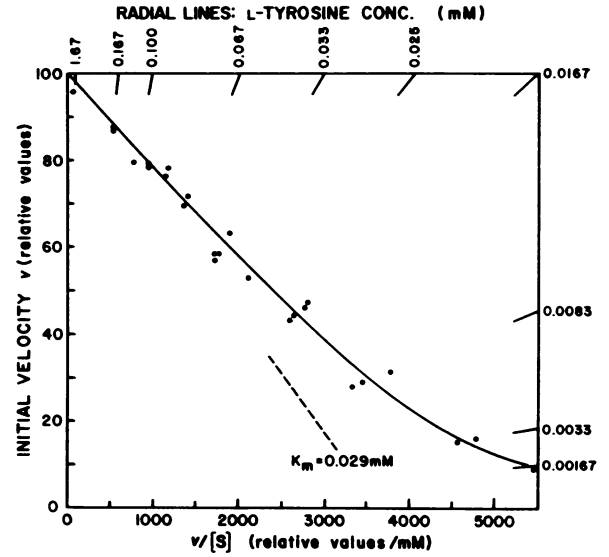

FIG. 3. Initial velocity of *p*-coumarate formation as a function of L-tyrosine concentration. Temperature 29 to 30 C, pH 8.7, buffer 33 mM sodium borate, 20 milliunits enzyme per assay. Values of *v* are expressed relative to *v* for the standard assay which is set at 96. This adjustment makes $V_{satn} = 100$.

Table I. *Mixed Substrate Experiments*

There were three assay mixtures for each experiment: one contained L-phenylalanine, the second L-tyrosine, and the third, equimolar concentrations of each. In addition to unlabeled substrate (concentrations specified below), each reaction mixture contained 0.6 × 10^6 cpm of L-phenylalanine *U-t*, L-tyrosine *U*-^{14}C or both, 40 mM sodium borate buffer, pH 8.7, and 15 milliunits of enzyme. Total volumes were 0.2 to 0.3 ml. Aliquots (50 μl) were withdrawn at various time intervals, placed on ChromAR sheet (Mallinkrodt, St. Louis) and dried immediately in a stream of warm air. Separation was carried out in benzene-acetic acid-water (2:2:1, upper phase; see Fig. 5). After removal of solvent, the positions of marker cinnamate and *p*-coumarate were determined under ultraviolet light (Mineralight, Ultra-Violet Products, Inc., South Pasadena, Calif.). Strips of the chromatogram were cut out and placed in vials containing scintillation fluid for counting. When known amounts of labeled cinnamate and *p*-coumarate were chromatographed in this system, the recovery of radioactivity was 80 to 90%. The relative velocities, v_{Phe} and v_{Tyr}, are the velocities observed when L-phenylalanine and L-tyrosine are mixed. They are expressed as a percentage of the velocity observed when each substrate is present alone. For each set of values, the ratio K_{Phe}/K_{Tyr} was calculated from equation 2 (see text). The average values and their standard errors are given in the last line.

Substrate Concn	Time	Relative Velocity		K_{Phe}/K_{Tyr}
		v_{Phe}	v_{Tyr}	
mM	*min*			
4.5	10	10.1	95.5	9.5
	30	13.8	82.5	6.0
	60	14.4	85.3	5.9
5.0	60	12.1	86.7	7.2
5.9	5	15.7	98.8	6.3
	10	13.0	99.2	7.6
	15	12.0	82.0	6.8
Average values ± standard error		13.0 ± 1.8	90.0 ± 7.6	7.1 ± 1.3

to 7. These relative activities are constant throughout the purification procedure.

Mixed Substrate Experiments. When L-phenylalanine (6.67 mM) and L-tyrosine (1.67 mM) were mixed in the standard spectrophotometric assay, the change in absorbancy per minute was considerably less than the sum of the changes for the two substrates determined independently. The spectrophotometric assay does not discriminate between cinnamate and *p*-coumarate formation, so a radioactive assay was devised. The results of seven mixed substrate experiments using this assay technique are shown in Table I and Figure 4. In these experiments, the time of reaction ranged from 10 to 60 min and the concentration of the substrate (equimolar) varied from 4.5 mM to 5.9 mM. Since there was no obvious dependence of the data on the conditions shown, all experimental results were averaged, and the standard error of the samples was determined. The saturation reaction velocities (in cpm) for each substrate alone, V_{Phe} and V_{Tyr}, were set at 100. The rate of formation of *p*-coumarate in the presence of L-phenylalanine, v_{Tyr}, was 90.0 ± 7.6% of V_{Tyr}. Similarly, the rate of formation of cinnamate in the presence of L-tyrosine, v_{Phe}, was 13.0 ± 1.8% of V_{Phe}.

Dixon and Webb (4) have derived the following expression to predict the behavior of a system in which a nonspecific enzyme is mixed with two of its substrates:

$$\frac{v_a}{v_b} = \frac{V_a K_b}{V_b K_a} \quad (1)$$

Briefly, they have shown that the reaction velocity for each substrate in a mixture (v_a and v_b) is proportional to the velocity observed when each substrate is present alone (V_a and V_b) and its K*m* (K_a and K_b). This expression can be rearranged to give:

$$\frac{v_a/V_a}{v_b/V_b} = \frac{K_b}{K_a} \quad (2)$$

in which the quantities v_a/V_a and v_b/V_b are equivalent to the relative velocities expressed in Table I. The ratio of the K*m* values, K_{Phe}/K_{Tyr}, was calculated by substituting in equation 2. The average value for the ratio and its standard error is 7.1 ± 1.3. This value is not far from the ratio of approximately 9 determined by estimating apparent K*m* values at saturating substrate concentrations from the curves in Figures 2 and 3.

INHIBITOR STUDIES

Inhibition by Sulfhydryl Reagents. Maize enzyme (10 milliunits) was incubated for 15 min at 4 C with each of the following compounds at the specified concentration: *N*-ethyl maleimide (1 mM), iodoacetic acid (5 mM), *p*-chloromercuribenzoate (5 mM). Aliquots of the incubation mixtures were assayed for both L-tyrosine and L-phenylalanine ammonia-lyase activity under standard assay conditions. The greatest amount of inhibition observed under these conditions was 5%. The enzyme from maize is therefore similar to the one from potato in that it is not possible to demonstrate a requirement for a sulfhydryl group for activity.

Inhibition by Substrate Enantiomers and by Reaction Products. Previous work has established that D-phenylalanine com-

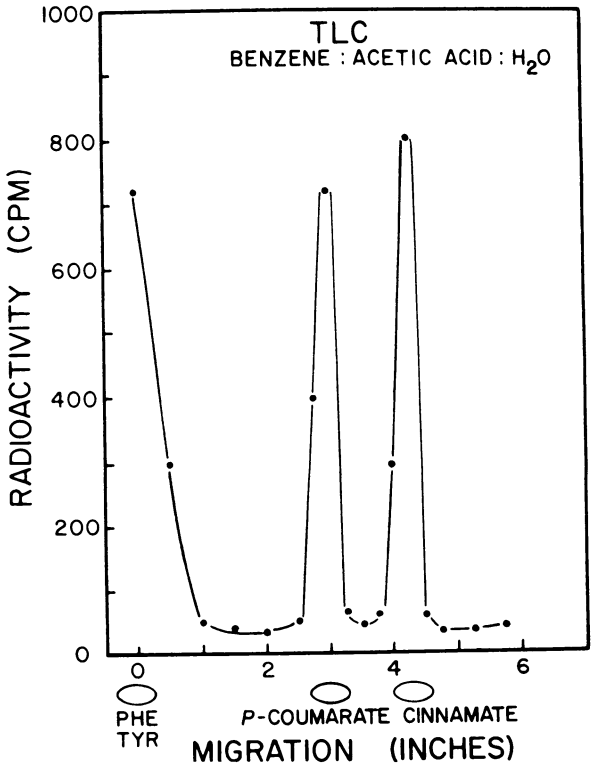

FIG. 4. Separation of cinnamate and *p*-coumarate by thin layer chromatography. The components of the system and conditions of separation are shown in Table I.

Table II. *Protection of L-Phenylalanine Ammonia-Lyase Activities of Maize and Potato against Sodium Borohydride Inactivation*

Aliquots of each enzyme preparation (15 milliunits) were incubated at 4 C with the compounds given in the table below for 5 min before $NaBH_4$ was added (30 μM, final concentration). After 5 min the incubation mixture was pipetted into a cuvet, and enzymatic activity was determined under the standard assay conditions.

Additions	Amount	Relative Activity[1]	
		Potato	Maize
	mM concn	%	
None		4	16
L-Phenylalanine	30	95	68
L-Tyrosine	5	<1	81
Cinnamate	3.0	93	84
p-Coumarate	3.0	10	89
L-Alanine	30	<1	10

[1] The activity of enzyme to which water was added in place of $NaBH_4$ was taken as 100.

petitively inhibits L-phenylalanine ammonia-lyase from potato and maize (11, 19). Cinnamate, when tested with the enzyme from potato, exhibited a strong inhibition which could not be classified as either competitive or noncompetitive. The effect of D-tyrosine, D-phenylalanine, cinnamate, and *p*-coumarate on both phenylalanine and tyrosine ammonia-lyase activities of maize and on the phenylalanine ammonia-lyase activity of potato was tested. The enzyme from potato which does not act on L-tyrosine was not inhibited by D-tyrosine or *p*-coumarate. In contrast, all the above mentioned compounds were inhibitors of both activities of the maize preparations. Preliminary results indicate that the inhibitor constants for these compounds is approximately the same when tested with either L-phenylalanine or L-tyrosine as substrate. The difficulties encountered in interpreting inhibition data are presented in the discussion.

Borohydride Inactivation. Sodium borohydride inactivates L-phenylalanine ammonia-lyase isolated from several sources (11, 13). This inactivation can be prevented by the prior addition of L-phenylalanine or cinnamate to the enzyme, leading to the conclusion that the reduction of a prosthetic group at the active site is the mode of inactivation (9, 11).

The enzyme from maize is inactivated by $NaBH_4$ at concentrations (6–30 μM) which cause approximately equal inactivation of the potato enzyme (Table II and Fig. 5). In Table II, the effectiveness of L-tyrosine, L-phenylalanine, cinnamate, and *p*-coumarate in protecting the phenylalanine ammonia-lyase activities of potato and maize are compared. Cinnamate and L-phenylalanine are effective for both enzymes, but L-tyrosine and *p*-coumarate, while protecting the enzyme from maize, are ineffective as protectors for the potato enzyme. An amino acid such as L-alanine which is neither a substrate nor an inhibitor does not protect either enzyme. It is apparent that L-tyrosine and *p*-coumarate bind to the same site as L-phenylalanine and cinnamate in the maize enzyme, protecting the enzyme against inactivation by borohydride.

The correlation between loss of phenylalanine activity and tyrosine activity is shown in Figure 5. After each addition of protecting compound and/or borohydride, one aliquot was assayed with phenylalanine and another with tyrosine. It can be seen that it is impossible to change appreciably the ratio of activities, *i.e.*, the loss of activity with one substrate is parallel to the loss of activity with the other. The scatter about the theoretical line is within the limits of the experimental error in sampling and assay.

Evidence for the Existence of Dehydroalanine at the Active Site. In the preceding section, the evidence was presented that borohydride causes inactivation by reducing a prosthetic group at the active site. It should be possible to identify this group in the following way: use tritiated $NaBH_4$ ($NaBH_4$-t_4) for reduction in order to label it, then hydrolyze the tritiated protein, and characterize the product containing the tritium.

The techniques used to reduce enzyme with $NaBH_4$-t_4, hydrolyze it and identify the major tritiated product, have been described fully elsewhere (9). Only an outline of the procedure and the results will be given here. Enzyme (425 milliunits, 17 mg of protein) was reduced with the minimal amount of $NaBH_4$-t_4 necessary to cause inactivation. Prereduction of the enzyme with $NaBH_4$ in the presence of cinnamate was not attempted. Immediately after reduction the enzyme was passed through a column of Sephadex G-25 medium (2.0 × 18 cm) equilibrated with 10 mM phosphate buffer, pH 6.8. The fractions containing tritiated protein were combined (5.5 mg). The incorporation was 2.4×10^5 cpm per mg protein.

After hydrolysis of the tritiated protein with 6 N HCl and removal of the acid by sublimation, approximately 60% of the radioactivity was recovered in the sublimate and 40% remained in the residue; similar results have been obtained with the enzyme from potato. The chromatography and electrophoresis of the hydrolyzed sample obtained from the maize en-

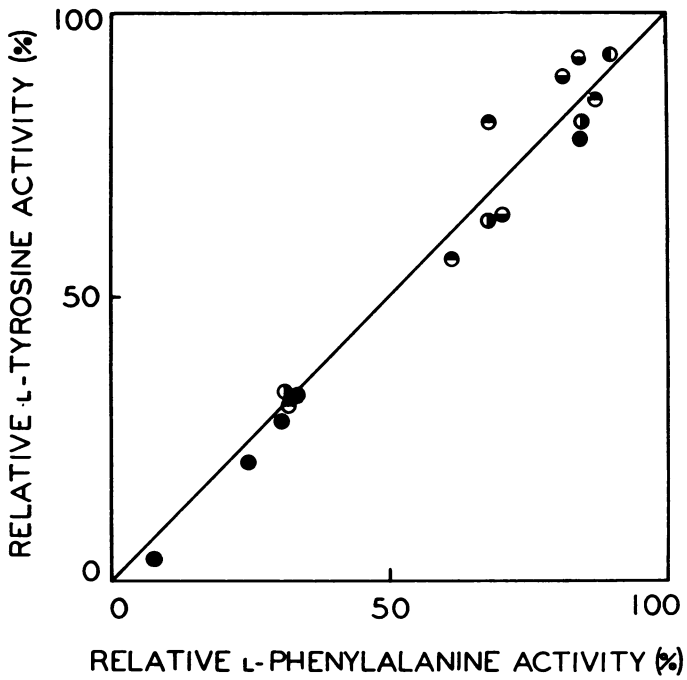

FIG. 5. Correlation of loss of L-phenylalanine ammonia-lyase activity with loss of L-tyrosine ammonia-lyase activity upon $NaBH_4$ inactivation. Incubation mixtures containing maize enzyme (25 milliunits) were set up as described in Table II. After the incubation with $NaBH_4$, one aliquot was withdrawn and assayed with phenylalanine as the substrate, and another aliquot was assayed with tyrosine. The relative activity for each substrate was calculated as the percentage of the activity determined with neither protecting substance nor $NaBH_4$ added. In experiments with $NaBH_4$ alone (●), the concentrations used were 6 to 30 μM. For all other experiments, the $NaBH_4$ concentration was kept at 30 μM and the concentration of the protecting compound varied as follows: L-phenylalanine (◓), 10 to 30 mM; L-tyrosine (◒), 1 to 3 mM; cinnamate (◑), 0.5 to 2.0 mM; *p*-coumarate (◐), 0.5 to 3.0 mM.

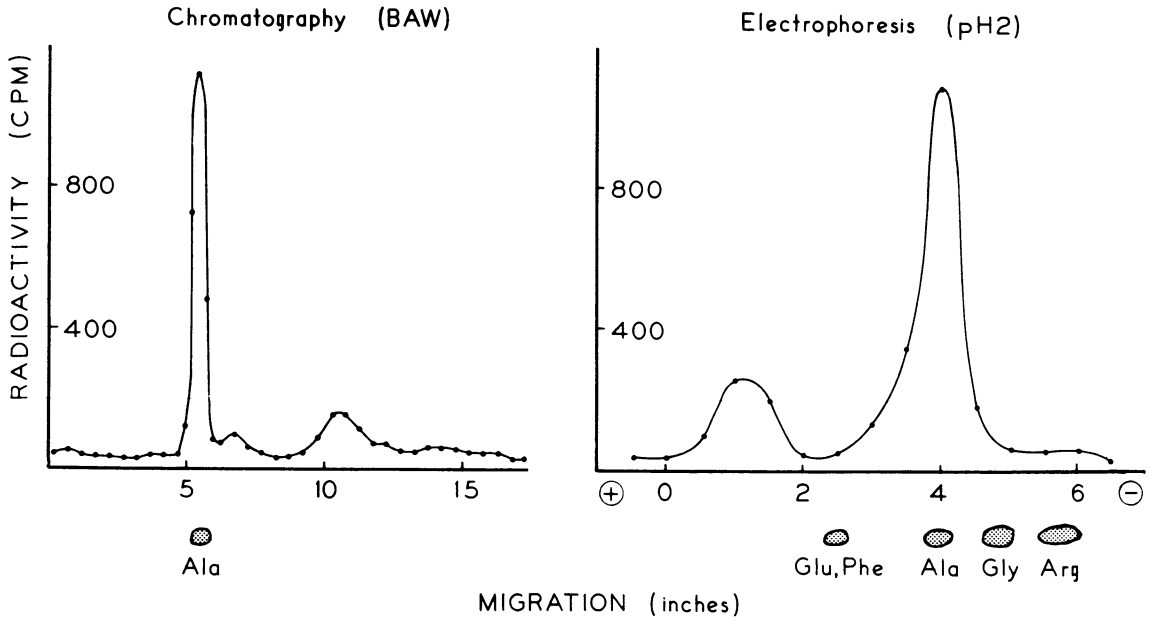

FIG. 6. Identification of alanine-*t* as the major radioactive product after hydrolysis of maize enzyme inactivated with $NaBH_4$-t_4. The solvent for descending paper chromatography (18 hr) was 1-butanol-acetic acid-water (4:1:5, v/v, upper phase). The buffer for the electrophoresis was acetic acid-formic acid (240 ml + 60 ml, diluted to 2 liters). Conditions of electrophoresis were 20 min at 60 v/cm in a Savant (Hicksville, N.Y.) 45-cm flat plate system at 12 C. After separation of the hydrolysate and location of the positions of the amino acid markers with ninhydrin, strips were cut from the papers, placed in vials containing scintillation fluid, and counted (Nuclear Chicago).

zyme is shown in Figure 6. The results of chromatography and electrophoresis of the tritiated product with authentic alanine in these systems makes it apparent that, as with the potato enzyme, alanine-*t* is the major product of reduction with $NaBH_4$-t_4. Alanine-*t* has also been recovered from hydrolysates of histidine ammonia-lyase which was reduced with $NaBH_4$-t_4 (27). The peak in the region of alanine accounts for approximately 60% of the radioactivity; there appear to be at least two other unidentified compounds present. These minor products may arise either from contaminating proteins or side reactions which occur. In experiments with the potato enzyme in which the prereduction step was omitted, the alanine accounted for 66 to 80% of the radioactivity in the residue. If enough maize enzyme had been available to do the prereduction step, it is likely that the results would be similar to those obtained with the potato enzyme which was prereduced, *i.e.*, over 90% of the residue counts were in alanine-*t*.

A large batch of hydrolyzed tritiated protein was separated by 1-butanol-acetic acid-H_2O (4:1:5, v/v, upper phase) descending paper chromatography. The radioactivity in the alanine region was eluted from the paper. The purified alanine proved to be a mixture of 49% L-enantiomer and 39% D. There was a 12% residue of unreacted material which may represent a contaminant in the alanine or may reflect the difficulty of doing the sequential oxidations with D- and L-amino acid oxidases (9). On acid permanganate oxidation of the alanine-*t*, about 60% of the tritium was found to be attached to the β-carbon. A number of samples of alanine from the potato enzyme were examined for tritium distribution. More than 85% of the label was in the β-position for most of these samples, but several samples obtained from stored tritiated enzyme had much lower values for tritium in the β-position (9).

DISCUSSION

In order to investigate the physical and catalytic properties of an enzyme it is essential to have an easily available preparation with stable and reproducible properties. The new purification procedure for phenylalanine ammonia-lyase from maize has given us such a preparation and we have been able to extend the observations made earlier (19).

Earlier work showed that the enzymes from maize and potato are alike in a number of ways. (*a*) These enzymes from higher plants are approximately the same molecular weight (about 330,000) and are appreciably larger than the phenylalanine ammonia-lyase from *Streptomyces verticillatus* which has a molecular weight of about 226,000 (6, 10, 19). (*b*) Both the potato and the maize enzyme are inactivated by $NaBH_4$ and protected against inactivation by compounds which bind to the active site. Hodgins (13) has reported a similar finding for the enzyme from *Rhodotorula glutinis*. Furthermore, experiments in which $NaBH_4$-t_4 was used to label the active site revealed that the enzymes from potato and maize contained the same prosthetic group which has been postulated to contain dehydroalanine (8). The mechanism of action of the enzyme has been fully explored elsewhere (9). (*c*) Both exhibit departure from typical Michaelis-Menten kinetics, *i.e.*, the apparent K*m* varies with substrate concentration. Because of the departure from linearity which is observed, the apparent K*m* must be determined as the slope of a tangent to the curve. For comparison purposes, apparent K*m* values at high substrate concentration are used. For the potato enzyme, the value is 0.26 mM (11) and for the maize enzyme, 0.27 mM (Fig. 2). The value determined earlier for the maize enzyme was much higher, 0.70 mM, and varied considerably (19). Our earlier suggestion that this variation was due to modification of the enzyme after isolation from the tissue is most likely true, since in the revised method for purification, contaminating substances are removed much more quickly. While the apparent K*m* values at high substrate concentration are the same for the potato and maize enzymes, the *v* versus *v*/S plot for the potato enzyme exhibits much more pronounced departure from linearity.

The kinetic data for the potato enzyme appears to meet the criteria for negative cooperativity as defined by Levitski and

Koshland (17), *i.e.*, the binding of the first ligand (L-phenylalanine) makes it more difficult for the second and subsequent ligands to be bound. The effect on the successive binding constants of substrate to enzyme can be as large as a factor of 10^2 (3). Negative cooperativity has the definite advantage of maintaining enzyme activity even at very low substrate concentrations. This is of great importance if the enzyme participates in a major pathway. Whereas the potato enzyme exhibits pronounced negative cooperativity, the effect is somewhat less for the maize enzyme, as is evident from the smaller departure from linearity shown in Figure 2. The degree of difference in the kinetic behavior may be an important reflection of differences in the *in vivo* control of enzyme function in two plant tissues.

One important difference between the two enzymes is in their substrate specificity. The enzyme from potato does not deaminate L-tyrosine to any appreciable extent at any stage of purification. The maize enzyme preparations, on the other hand, exhibit activity toward both L-phenylalanine and L-tyrosine at all stages of purification. With tyrosine as substrate (Fig. 3), the shape of the curve is similar to the one for phenylalanine, but the apparent K*m* at high substrate concentration is lower by an order of magnitude (0.029 mM).

We believe that in maize the deamination of both phenylalanine and tyrosine are catalyzed at the same active site for the following reasons:

1. The two activities are not separable by the physical means which have been employed. Not only is the ratio constant during purification (24), but the two activities migrate as a single peak in zone electrophoresis in a sucrose density gradient at pH 6.4 and 7.8. During a number of determinations of the isoelectric point under varying conditions of migration, the value varied from pH 4.95 to 5.15 but the two activity peaks always coincided (unpublished results).

2. Cinnamate, *p*-coumarate, D-tyrosine, and D-phenylalanine inhibit both activities to approximately the same extent. In contrast, the potato enzyme, which shows no activity with tyrosine, is not inhibited by D-tyrosine or *p*-coumarate.

3. In inactivation studies with $NaBH_4$, it has not been possible to change the ratio of activities appreciably (Fig. 5). The addition of phenylalanine and cinnamate has been shown to protect the active site of the potato enzyme against $NaBH_4$ reduction. When the enzyme from maize is mixed with any of the following before the addition of borohydride, both activities are protected to an equal extent: L-phenylalanine, L-tyrosine, *p*-coumarate, cinnamate. Again, there is a strong contrast with the potato enzyme in which L-tyrosine and *p*-coumarate are completely ineffective in protecting against $NaBH_4$ inactivation.

The variation in pH optima with substrate reported here (Fig. 1) has been observed by others and used as proof of the existence of separate enzymes (22, 23). This is not a valid argument. The position of a pH optimum at saturating substrate concentration is determined by the ionization state of the groups of the enzyme-substrate complex. It is therefore not surprising that the pH optimum varies with substrate. There are a number of well documented cases in which enzymes as varied in function as α-chymotrypsin, glutamic acid dehydrogenase, and lysosomal α-glucosidase, each of which catalyzes several reactions, have widely separated pH optima for each reaction (7, 14, 25).

A second argument used in favor of the two enzyme theory is based on the observation that when enzyme is incubated with a mixture of phenylalanine and tyrosine, the amount of cinnamate produced is reduced to a large extent, but the *p*-coumarate production is affected only slightly (see Table I). The explanation usually given is that tyrosine strongly inhibits the enzyme for phenylalanine but phenylalanine only slightly inhibits the enzyme for tyrosine. It is equally valid to assume that the two amino acids are competing for the same active site and that the observed rates are a reflection of the relative K*m* values for the individual amino acids. When a quantitative experiment is done with both substrates at saturation, the ratio of the K*m* values (phenylalanine/tyrosine) predicted by theory (4) is 7.1 ± 1.3; considering the certain amount of error inherent in the radioactive assays this value is in good agreement with the ratio of approximately 9 estimated from tangents to the curves shown in Figures 2 and 3. Another type of approach would be to determine the K_I for tyrosine when phenylalanine is the substrate and vice versa. If both substrates are acting at the same site as we believe, then the values for K_I and apparent K*m* will be quite similar. Unfortunately there are two difficulties in this approach: (*a*) the radioactive assay must be used, and the values at low substrate concentrations (*i.e.*, low activity) which are critical in such a determination will have a large error; (*b*) the complex nature of the kinetics makes it difficult to determine meaningful K_I values. This approach will be valuable for inhibitors which "straighten out" the kinetics as cinnamate and D-phenylalanine do for the enzyme from potato (11). The results on the effect of D-phenylalanine, D-tyrosine, cinnamate and *p*-coumarate on the maize enzyme are only preliminary. It is clear, however, that all the compounds mentioned inhibit both activities of the maize enzyme.

In summary, the available evidence indicates that maize phenylalanine ammonia-lyase has one type of catalytic site which can act on tyrosine and phenylalanine. The enzyme from potato tubers, on the other hand, is restricted to phenylalanine as a substrate. The question of why the two enzymes differ in their catalytic properties will be a much more difficult one to answer.

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