THE PRESENCE OF A CARBONYL GROUP AT THE ACTIVE SITE OF L-PHENYLALANINE AMMONIA-LYASE*

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L-phenylalanine ammonia-lyase (E.C.4.1.3.5.), the enzyme catalyzing the deamination of L-phenylalanine to cinnamic acid and ammonia, has been purified to near homogeneity (as judged by disc gel electrophoresis). The enzyme possesses a non-pyridoxal carbonyl group at its active site which participates in the catalytic reaction. When the enzyme is reduced with Na B $^{3}H_{4}$ radioactivity is non-exchangeably incorporated, indicating the formation of a carbon-hydrogen bond. The recently proposed (Havir and Hanson, 1968) role of an enzyme carbonyl group in the enzymatic deamination of L-phenylalanine by potato L-phenylalanine ammonia-lyase is thus substantiated.

MATERIALS AND METHODS

L-phenylalanine ammonia-lyase is obtained from sonic extracts of Rhodotorula glutinis (A.T.C.C. 15385) (Ogata, et. al., 1966) and purified by protamine sulfate treatment, ammonium sulfate fractionation and DEAE cellulose and Sephadex G-200 chromatography (Hodgins, 1968).

Enzyme assays were carried out under the following conditions: 8.33×10^{-4} M L-phenylalanine, 1×10^{-1} M Tris-HCl, pH 8.5, and enzyme in a volume of 3.0 ml were incubated at 30 degrees C. The appearance of cinnamic acid is measured by the increase in optical density at 290 mμ (Zucker, 1965). An enzyme unit is defined as that amount of protein catalyzing the appearance of 1μm of cinnamic acid per minute under these conditions.

Polyacrylamide gel electrophoresis at pH 8.3 was carried out as described by Ornstein (1964). The protein bands in the gel were stained with solutions of amido Black B. In studies following the enzyme activity, a gel was sectioned into 5 mm pieces and eluted with 1 ml 0.05 M Tris, pH 8.5.

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Protein determinations were done by the modified Lowry method of Zak and Cohen (1961) and comparison with bovine serum albumin standards.

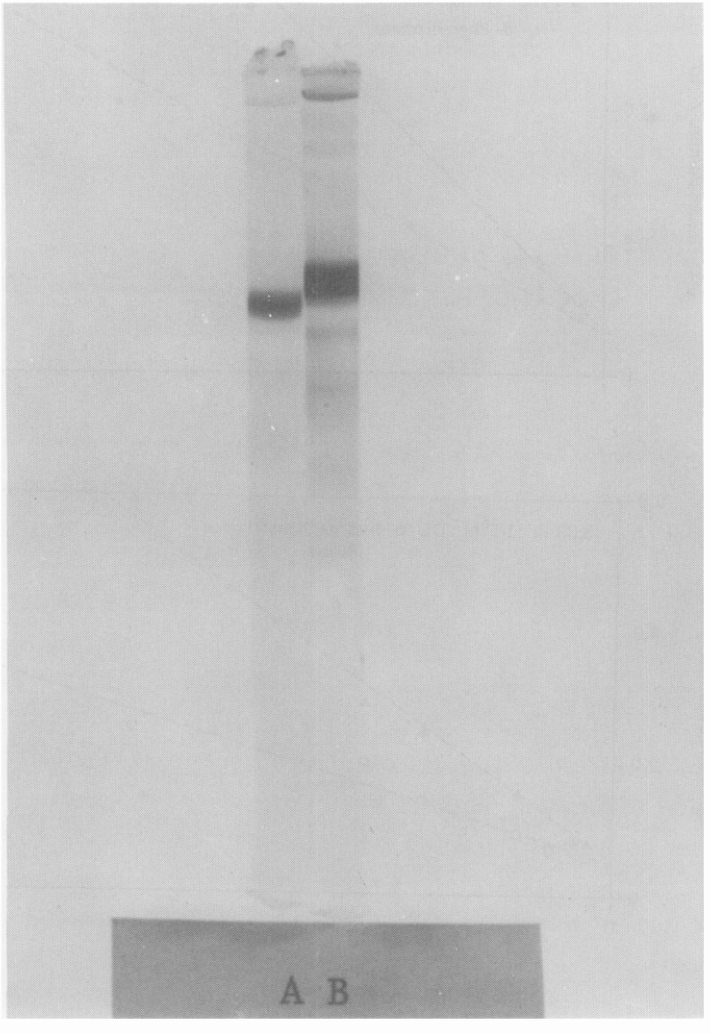

FIGURE 1 - ACRYLAMIDE DISC GEL ELECTROPHORESIS OF L-PHENYLALANINE AMMONIA-LYASE
Gel (A) contains 13.7 μg and gel (B) 54.2 μg of enzyme (0.205 units/mg). The gels were allowed to run at 3.4 mA per tube until the bromphenol blue marke had reached the bottom.

RESULTS

L-phenylalanine ammonia-lyase, one hundred-fold purified is shown in Figure 1 to be a largely homogeneous band on acrylamide gel disc electrophoresis.

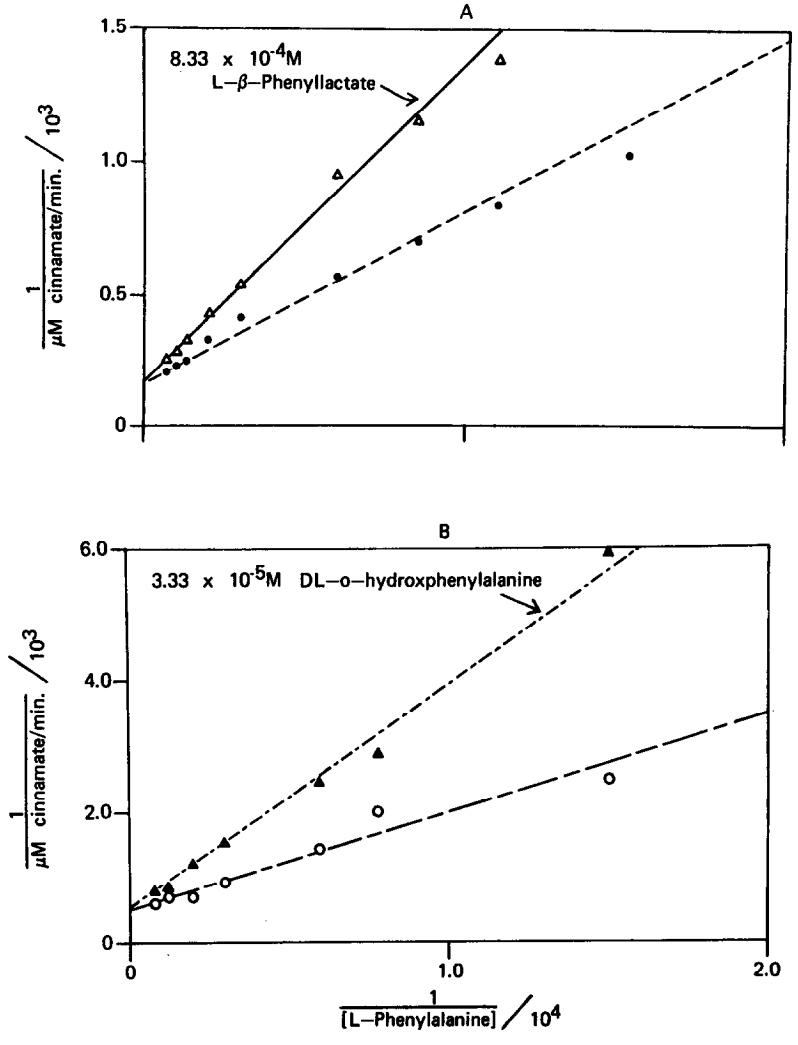

FIGURE 2 - LINEAWEAVER-BURK PLOTS OF L-PHENYLALANINE AMMONIA-LYASE INHIBITION BY SUBSTRATE ANALOGS. The concentration of L-phenylalanine is expressed in moles/liter. The cinnamic acid was measured by the change in O.D. at 290 mμ.

A. The incubation mixtures consisted of L-β-phenyllactate, where indicated, L-phenylalanine, 1 x 10^{-1}M Tris-HCl, pH 8.5 and 2.4 x 10^{-2}mgs enzyme (0.506 units/mg) in a volume of 3.0 ml at 30 degrees C.

B. The incubation mixtures consisted of DL-o-hydroxyphenylalanine, where indicated, L-phenylalanine, 1 x 10^{-1}M Tris-HCl, pH 8.5 and 2.41 x 10^{-2}mgs enzyme (0.167 units/mg) in a volume of 3.0 ml at 30 degrees C.

Elution of a duplicate (of 'B' on Fig. 1) has shown that the enzyme activity is associated with the dense protein band. The purest preparations of enzyme vary in activity from 0.2 to 0.8 enzyme units per mg.

The effects of two substrate analogs, L-β-phenyllactic acid and DL-o-hydroxyphenylalanine, on the kinetics of the lyase reaction are shown in Figure 2. Both compounds, inactive as substrates, appear to competitively inhibit the deamination of phenylalanine. The K_i values, based on the change in slope of the inhibited reaction are 2.68×10^{-5} M for DL-o-hydroxyphenylalanine and 1.02×10^{-3} M for L-β-phenyllactic acid.

TABLE I

INHIBITION BY CARBONYL REAGENTS

Inhibitor	Concentration	% Activity
$NaBH_4$	2.5×10^{-4}M	5.8
	1.25×10^{-4}M	35.0
	8.25×10^{-5}M	67.5
	2.5×10^{-5}M	80.4
	0	100.0
NaCN	1.25×10^{-2}M	1.2
	6.125×10^{-3}M	20.0
	2.5×10^{-3}M	70.8
	1.25×10^{-3}M	92.9
	0	100.0
$NaHSO_3$	2.5×10^{-2}M	3.1
	1.25×10^{-2}M	27.1
	6.125×10^{-3}M	61.0
	2.5×10^{-3}M	85.0
	0	100.0

The $NaBH_4$ inactivations were carried on as follows: 8.98×10^{-2} mgs enzyme (0.486 units/mg), inhibitor as indicated, and 2×10^{-1}M Tris-HCl pH 8.5 in a volume of 0.04 ml were incubated for 10 minutes at 30 degrees C. The mixtures were diluted fifty-fold with 0.05 M Tris-HCl, pH 8.5 and assayed for residual enzyme activity using the standard system.

The conditions for $NaHSO_3$ and NaCN inactivations were as follows: 8.3×10^{-2} mgs enzyme (0.643 units/mg) 3×10^{-1}M Tris-HCl, pH 8.5 and bisulfite or cyanide in a volume of 0.04 ml were incubated for 10 minutes at 30 degrees C. Dilution and assays were performed as above.

The enzyme is inactivated (and not reactivated by dilution and enzyme assay) by the carbonyl reagents sodium borohydride, sodium cyanide and sodium bisulfite. Table I shows the effect of various concentrations of the reagents on enzyme

TABLE II

THE PROTECTIVE EFFECTS OF SUBSTRATE ANALOGS AGAINST INHIBITION BY CARBONYL REAGENTS

Concentration of L-β-Phenyllactic Acid	% Activity $NaBH_4$	NaCN	$NaHSO_3$
0	4.45	0	4.08
5.0×10^{-4}M	20.9	0	----
1.25×10^{-3}M	57.7	0	9.18
2.5×10^{-3}M	74.1	8.5	30.0
5.0×10^{-3}M	90.0	11.8	43.9
1.25×10^{-2}M	100.0	48.0	79.5
2.5×10^{-2}M	102.1	63.2	90.0
5.0×10^{-2}M	101.8	----	95.0
DL-o-Hydroxyphenylalanine			
0	4.78	0	0
1.25×10^{-5}M	----	2.96	3.3
2.5×10^{-5}M	----	7.39	22.4
5.0×10^{-5}M	48.3	19.9	55.0
1.25×10^{-4}M	74.7	23.6	75.0
2.50×10^{-4}M	88.5	41.4	96.4
*5.0×10^{-4}M	----	67.4	102.1
*1.25×10^{-3}M	99.9	77.8	100.3
*2.5×10^{-3}M	101.0	101.8	99.8
*5.0×10^{-3}M	100.2	----	----

The $NaBH_4$ inactivations were carried out as follows: 8.98×10^{-2}mgs enzyme (0.486 units/mg), 2.5×10^{-4}M $NaBH_4$, 0.2M Tris-HCl, pH 8.5 and protective agent in a volume of 0.04 ml were incubated for 10 minutes at 30 degrees C. Dilution and enzyme assays were the same as described for Table I.

The NaCN inactivations were carried out as follows: 8.25×10^{-2}mgs enzyme (0.297 units/mg) 1.25×10^{-2}M NaCN, 3.0×10^{-1}M Tris-HCl, pH 8.5 and protective agent in a volume of 0.04 ml were incubated for 10 minutes at 30 degrees C. Dilution and enzyme assays are as previously described.

The conditions for the $NaHSO_3$ inactivation were the same as those described for the NaCN experiment, except that 2.5×10^{-2}M $NaHSO_3$ replaced NaCN.

* The relative enzyme activity of these incubations was corrected for the inhibitory effects of DL-o-hydroxyphenylalanine in the assay mixtures.

activity. This inactivation of enzyme by carbonyl reagents is affected by the presence of the competitive inhibitors mentioned earlier. Table II shows the protective effect of these substrate analogs against the carbonyl reagents. As would be expected from the kinetic data, DL-o-hydroxyphenylalanine is more efficient than L-β-phenyllactic acid as a protective agent. These results are consistent with the existence of a carbonyl group at the active site of yeast L-phenylalanine ammonia-lyase.

Further evidence as to the nature of the borohydride sensitive group at the active site of the enzyme was obtained by treatment with Na B $^{3}H_{4}$. The following experiment was performed:

2 ml (22.7 mgs) of the enzyme (0.405 units/mg) was incubated in 7.2×10^{-3} M DL-o-hydroxyphenylalanine and 5×10^{-4} M $NaBH_4$ for 15 minutes at 30 degrees C. The resultant mixture was then extensively dialyzed against 5×10^{-2} M Tris-HCl, pH 8.5 and found to exhibit no loss of enzymatic activity. This dialyzed preparation was then incubated (15 min., 30 degrees C.) with 6×10^{-4} M Na B $^{3}H_{4}$ (194 mc/mM) and then dialyzed exhaustively against 1×10^{-2} M Tris-HCl, pH 8.5. The enzyme had lost 97.6% of its enzymatic activity and had incorporated 3.175×10^{6} DPM/mg protein. This corresponds (assuming no isotope effect in the reduction) to 3.46×10^{4} mg protein/m atom H.

The presence of pyridoxal phosphate in yeast L-phenylalanine ammonia-lyase is unlikely because: (1) the spectrum of highly purified enzyme (0.5 units/mg) at a concentration of 10 mg/ml reveals no significant peaks of ultraviolet absorption above that at 280 mμ; (2) at no time could the activity of the enzyme be enhanced by the addition of pyridoxal phosphate; and (3) dialysis of the enzyme against cysteine (Shaltiel et. al., 1966) produced no loss of catalytic activity.

L-phenylalanine ammonia-lyase from yeast resembles the enzyme from barley (Koukol and Conn, 1961) rather than that from potato (Havir and Hanson, 1968) in its sensitivity to the sulfhydryl agent paramercuribenzoate (PMB). A mixture of 8.98×10^{-2} mgs of enzyme (0.486 units/mg), 2.5×10^{-5} M PMB and 1.0×10^{-1} M Tris-HCl, pH 8.5, in a volume of 0.04 ml was incubated for 10 minutes at 30 degrees C. After a fifty-fold dilution and enzyme assay it was apparent that 100% of all catalytic activity was lost. When 1×10^{-4} M dithiothreitol was added to a duplicate inactivation mixture (after 10 minutes, 30 degrees C.) 68% of the original enzyme activity was recovered within an additional ten minutes of incubation time.

DISCUSSION

The incorporation of non-exchangeable tritium into L-phenylalanine ammonia-lyase after treatment with $NaB^{3}H_{4}$ indicates the formation of a carbon

tritium bond. The treatment of enzyme with $NaBH_4$ and DL-o-hydroxyphenylalanine prior to NaB^3H_4 reduction presumably precludes the incorporation of tritium at any place but the active site of the enzyme. The non-exchangeable character of the incorporated label rules out the reduction of a disulfide bond (Light and Sinha, 1967) as the cause of inactivation and indicates the reduction of an aldehydic or ketonic enzyme group.

These results are consistent with and further the evidence for the presence of a non-pyridoxal phosphate carbonyl group at the active site of L-phenylalanine ammonia-lyase. Previously, the incorporation of radioactive cinnamic acid into L-phenylalanine during the course of the largely irreversible deamination reaction (Young and Neish, 1966; Havir and Hanson, 1968) has been taken as evidence for the formation of an enzyme-ammonia intermediate whose breakdown is rate-limiting. By analogy with L-histidine ammonia-lyase and its similar behavior in exchange reactions (Peterkovsky, 1962) and the later discovery (Smith, et. al., 1967) that this enzyme possesses an essential carbonyl group it would be expected that L-phenylalanine ammonia-lyase should also have an essential carbonyl group.

Havir and Hanson (1968) have recently presented evidence for the sensitivity of potato L-phenylalanine ammonia-lyase to carbonyl reagents and have postulated a mechanism for the lyase reaction. This mechanism, very similar to that proposed for L-histidine ammonia-lyase (Smith et. al., 1967), involves the initial formation of a carbinolamine between the amino group of substrate and a carbonyl group at the active site of the enzyme. Following a Hofmann type elimination of the carbinolamine moiety from the substrate there results an ammonia-enzyme (accounting for the exchange data) and cinnamic acid. The breakdown of the enzyme-ammonia intermediate to native enzyme and ammonia is the final step in their postulated reaction sequence. The incorporation of non-exchangeable tritium into L-phenylalanine ammonia-lyase validates the existence of a carbonyl group at its active site and substantiates the postulated mechanism, which seems to be similar for the potato and yeast enzymes.

The sensitivity of this enzyme to PMB inactivation would seem to indicate the presence of a catalytically necessary sulfhydryl group. This group may serve to facilitate the abstraction of a proton from the β-carbon of substrate during the elimination reaction.

Further work is currently underway in this laboratory to identify the reducible carbonyl group in question.

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