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A BACTERIAL PHENYLALANINE AMINOTRANSFERASE LACKING PYRIDOXAL 5′-PHOSPHATE AS COFACTOR

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Summary

A bacterium has been isolated from soil which metabolises phenylalanine initially through the action of a phenylalanine aminotransferase. This enzyme has been purified by conventional techniques and affinity chromatography and shown to be unusual among aminotransferases in not containing pyridoxal 5′-phosphate as cofactor.

Introduction

Although the metabolism of phenylalanine has been investigated extensively in mammalian systems, due to its importance in certain hereditary diseases such as phenylketonuria, in bacteria little is known of its catabolism. In mammalian systems there also appears to be some controversy whether phenylalanine aminotransferase activity is due to a specific enzyme or to a multifunctional transaminase [1,2].

In bacteria, a phenylalanine hydroxylase [3] has been described with properties similar to the mammalian enzyme. Of the two bacterial phenylalanine aminotransferases that have been reported, the one from *Achromobacter eurydice* is a catabolic enzyme [4] and the other from *Escherichia coli* plays a biosynthetic role [5,6]. Both enzymes require pyridoxal 5′-phosphate as cofactor. This paper describes the selection and growth characteristics of a soil bacterium with phenylalanine aminotransferase activity and the purification and some properties of this enzyme. The enzyme is unusual among transaminases in that it appears to contain no pyridoxal 5′-phosphate and does not require this cofactor for full activity. This work was conducted as part of a wider programme of investigation of microbial enzymes of potential use in cancer therapy.

Materials and Methods

Organism. The organism used was an aerobic gram-negative motile rod isolated from soil. It gave a weak fermentative result in the oxidation/fermentation test of Hugh and Leifson [7] and failed to identify with a particular taxon after further testing (21 tests). An analysis using a computer programme based on that developed by Lapage et al. [8] showed it to have the greatest affinity to the genus *Aeromonas*. When analysed as an oxidative organism it identified as *Pseudomonas fluorescens*.

Media. Nutrient broth (Medium A) No. 2 (Oxoid Ltd., London) and nutrient broth plus 1.2% Oxoid No. 3 agar (Medium B) were used as complete media. A chemically defined minimal medium (Medium C) contained the following constituents per l: K_2HPO_4, 1.5 g; KH_2PO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; trace elements [9], 5 ml; L-phenylalanine, 2 g. A second minimal medium (Medium D) contained the following constituents per l: NaH_2PO_4, 1.5 g; KCl, 0.75 g; $Na_2SO_4 \cdot 10H_2O$, 0.644 g; sodium citrate, 0.41 g; $MgCl_2$, 0.119 g; $CaCl_2$, 0.002 g; $NaMoO_4$, 0.02 mg; trace elements [9], 5 ml L-phenylalanine, 5 g.

Growth. The bacteria were grown in 20-ml batch cultures in tubes rotating on angled platters at 28°C or in 1-l cultures aerated with sterile air at 26°C. Cultures were grown for at least 40 h unless specified.

Bacteria were also grown in continuous culture in a New Brunswick C-30 Fermenter at pH 7.0 stirred at 600 rev./min at 30°C and aerated with 940 ml/min sterile air.

For large-scale enzyme purification, bacteria were grown in 400-l stainless steel culture vessels stirred at 250 rev./min at 30°C, from 15-l seed cultures. Cultures were aerated with 300 l/min sterile air and the pH was maintained at pH 7.5 by the automatic addition of 20% H_3PO_4. On completion of growth, indicated by measurements of turbidity, the culture was cooled by circulating cold water (5°C) through the jacket of the culture vessel and the cells were harvested by passing the culture at 100 l/h through a continuous centrifuge (Laval Type 1700, Alfa-Laval Co. Ltd.).

Enzyme assay. Phenylalanine aminotransferase was assayed by the method of Scandura and Canella [10] modified for use with a Pye Unicam AC60 Chemical Processing Unit. The standard reaction mixture contained 8.33 mM L-phenylalanine, 10 mM α-ketoglutarate in 0.1 M Tris · HCl (pH 8.5) and up to 0.1 unit of phenylalanine aminotransferase. 20 μl enzyme was added and, after 5 min at 37°C, the reaction was stopped by the addition of 10 M NaOH and the absorbance read at 318 nm. The extinction coefficient (E_M^{318nm}) of phenylpyruvate under these conditions is 15 700 cm^{-1}.

Routinely, the reagent blank against which activity was measured, was the complete reaction mixture minus enzyme, but when assaying crude bacterial extracts the blank also contained enzyme, added after the alkali.

A unit of phenylalanine aminotransferase was defined as the amount of enzyme which, under the conditions described above, produced 1 μmol/min phenylpyruvate. The K_m of the enzyme was determined by carrying out a series of reactions at various substrate concentrations and deriving the constant from Lineweaver-Burk plots.

Protein assay. Protein was assayed by the method of Lowry et al. as

modified for the Technicon Autoanalyser [11] with bovine serum albumin as standard.

Affinity chromatography media. CNBr-activated Sepharose 4B and epoxy-activated Sepharose 6B were purchased from Pharmacia (Great Britain) Ltd., 3,3'-diaminodipropylamine and *p*-aminobenzoyl azide from Eastman Kodak Ltd., *m*-hydroxycinnamic acid from Aldrich Chemical Co. Ltd. and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC) from the Sigma Chemical Co. Affinity chromatography adsorbents were synthesised by standard methods [12,13].

Results

Selection of bacteria

Bacteria isolated from soil were selected for their ability to degrade L-phenylalanine by growth on Medium C. Of these, strain MRE 5119 was found to be the best producer of phenylalanine aminotransferase and was the parent strain of those described below. A soluble extract from this strain, grown in Medium C and extracted by ultrasonic irradiation and centrifugation of cell debris expressed a specific activity of 1.14 units/mg protein and a K_m for L-phenylalanine of $7.2 \cdot 10^{-4}$ M. Growth in this medium was poor, but a 3-fold improvement was obtained in Medium D. Continuous culture in this medium was used to select a fast-growing variant. A steady state was achieved at a dilution rate (volume changes/h) of 0.1, but could not be maintained at higher dilution rates. After 10 days, the population in the culture had stabilised and was found to have improved growth characteristics in batch culture in Medium C; this variant was given the separate identity strain MRE 5388. Strains MRE 5119 and MRE 5388 both exhibited two morphological types when plated on Medium B. Batch cultures of these morphological types bred true and were examined for growth and phenylalanine aminotransferase production in Media C and D. One of these isolates, strain MRE 5388B, was given the new identity of strain MRE 5390 because of its higher specific activity in both media (see Table I) and was used for all subsequent work.

The growth of strain MRE 5390 was followed in a New Brunswick C-30 Fermenter operating in batch mode (28°C, 550 ml/min air, stirring at 400 rev./min, initial pH 7.0) in Medium D. Good growth and enzyme yield were obtained (Fig. 1), but in subsequent experiments growth was variable. The addition of 0.2% (w/v) sodium succinate to the medium resulted in consistently heavier growth in batch culture (4.23 g wet weight bacteria/l) and a faster growth rate (generation time 3 h) in continuous culture. The enzyme yield increased to 400 units/l of batch culture.

For growth in the 400-l vessel, kindly performed by Mr. A.R. Whitaker, Medium D supplemented with 0.3% (w/v) sodium succinate was used, at a constant pH of 7.5 at 30°C. Growth was typically obtained as shown in Table II and, on harvesting after 37 h, resulted in 6.71 kg wet weight of cells containing $1.12 \cdot 10^6$ units phenylalanine aminotransferase (16.8 g wet weight bacteria/l and 2810 units/l phenylalanine aminotransferase, both significant improvements on the yields from small-scale batch cultures). The cells were stored as 100-g lots at −20°C.

TABLE I

GROWTH AND PHENYLALANINE AMINOTRANSFERASE OF MORPHOLOGICAL VARIANTS OF STRAINS MRE 5119 AND MRE 5388

Bacteria were grown at 28°C as 20-ml tube cultures for 42 h. Absorbances were measured at 520 nm in a Bausch and Lomb Spectronic 20 spectrophotometer and enyme and specific activities relate to cell-free extracts of disrupted bacteria harvested from the cultures.

Strain	Growth in Medium C			Growth in Medium D		
	Absorbance	Phenylalanine aminotransferase (units/ml)	Specific activity (units/mg protein)	Absorbance	Phenylalanine aminotransferase (units/ml)	Specific activity (units/mg protein)
MRE 5119A	0.03	0.19	0.73	5.4	1.68	1.18
MRE 5119B	0.2	1.03	1.61	4.5	1.36	1.10
MRE 5388A	0.07	0.023	—	1.3	0.55	0.65
MRE 5388B *	0.17	1.03	2.45	4.0	1.47	1.28

* This morphological variant of Strain MRE 5388 was given the new identity of Strain MRE 5390.

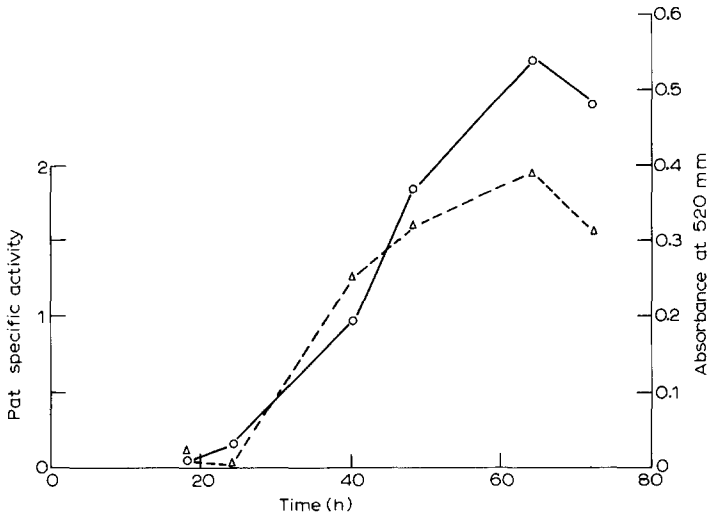


Fig. 1. The growth of strain MRE 5390 in Medium D. Samples were withdrawn for measurement of absorbance and of phenylalanine aminotransferase activity and total protein. Conditions of growth are as described in the text. △, specific activity; ○, absorbance at 520 nm; Pat, phenylalanine aminotransferase.

Purification of phenylalanine aminotransferase

The bacteria from a 400 l culture of strain MRE 5390 contained 724 000 units phenylalanine aminotransferase at the time of harvesting but after storage at −20°C for 1 month the activity dropped to 475 000 units and to 450 000 units after 2 months. The cell paste, containing 450 000 units, was suspended in 50 mM Tris · HCl (pH 7.5) and passed twice through a Manton-Gaulin homogeniser (APV Ltd., Crawley, Sussex) at 8000 lb/inch^2. The material was centrifuged in a Sharples No. 6 continuous-flow centrifuge (Sharples Instruments, Camberley, Surrey) at 40 l/h and the supernatant stored at 4°C. The deposit was resuspended in buffer, passed again through the homogeniser and clarified by centrifugation. The supernatants were pooled, adjusted to

TABLE II

THE GROWTH OF STRAIN MRE 5390 IN A 400 l CULTURE VESSEL

Time (h)	Absorbance *	Viable (count × 10^{-3}/ml)	Phenylalanine (g/l)	Succinate (g/l)	Phenylalanine aminotransferase (units/ml culture)
0	0.13	175.2	5.8	3.0	0
4	0.16	328	6.3	2.83	0.05
8	0.27	726	—	2.68	0.2
12	0.31	1 140	—	2.39	0.43
16	0.44	1 800	—	1.56	0.66
20	0.70	4 120	5.0	0.73	0.94
24	0.84	4 460	—	0.48	1.22
28	1.48	9 700	5.1	0.15	2.78
32	1.72	13 400	0.05	0	3.21
36	1.72	—	0.05	0	3.21
37	1.72	13 900	0.05	0	2.81

* Arbitrary units.

pH 7.5 with NaOH and diluted with water to the same conductivity as the buffer. The solution was concentrated using an Amicon DC-30 with five SM10 hollow-fibre cartridges then diluted with buffer and further concentrated.

The concentrated extract was pumped at 660 ml/h on to a column (14 cm diameter × 24 cm) DEAE-cellulose (DE-52, Whatman Biochemicals Ltd.) which had been equilibrated with 50 mM Tris · HCl (pH 7.5). The column, which absorbed all the enzyme, was washed with two column volumes of buffer and subjected to gradient elution with a linear gradient (LKB Ultrograd) of 0—0.2 M NaCl in 50 mM Tris · HCl (pH 7.5). Fractions with a specific activity greater than 2 units phenylalanine aminotransferase/mg protein were pooled and concentrated using an Amicon DC-2 equipped with an HIDP10 hollow-fibre cartridge.

The product was fractionated in three batches on a Sepharose 6B column (10 cm diameter) equilibrated against 0.1 M Tris · HCl (pH 7.5). Fractions with specific activities greater than 6 units phenylalanine aminotransferase/mg protein were pooled and concentrated.

The fraction from the Sepharose 6B column was put on to a column (5 cm diameter) of DEAE-cellulose (DE-52) equilibrated against 0.1 M Tris · HCl (pH 7.5). The column was then washed with two column volumes of buffer before linear gradient elution at 0—0.15 M NaCl in buffer.

Fractions from the second DEAE-cellulose column, with specific activities greater than 20 units phenylalanine aminotransferase/mg protein, were pooled and concentrated in a stirred ultrafiltration cell with an Amicon PM-30 membrane. The concentrate was passed through a column (3.2 cm diameter) of Sephadex G-150 equilibrated with 0.1 M Tris · HCl buffer (pH 7.5). Fractions with specific activities greater than 60 units phenylalanine aminotransferase/mg protein were pooled and sterilised by Millipore filtration. The purification is summarised in Table III.

Following sterilisation, the product was stored either at 4°C or as a freeze-dried powder at −20°C. Considerable losses of activity were experienced on freezing the enzyme to −20°C, then thawing before use.

TABLE III

THE PURIFICATION OF PHENYLALANINE AMINOTRANSFERASE FROM STRAIN MRE 5390

This purification was carried out on 5 kg wet weight of cells and gave an overall purification of ×105.

Stage	Volume (l).	Enzyme (units × 10^{-3})	Protein (g)	Specific activity (units/mg protein)	Recovery (%)
Whole suspension	25	451	705	0.64	100
1st supernatant	18	275	331	0.83	78.6
2nd supernatant	12	78.1	117	0.67	78.6
DC30 concentrate	17	362	441	0.82	80.3
DEAE-cellulose elutate I	0.62	205	66.6	3.08	45.4
Sepharose 6B eluate	0.88	124	14.3	8.68	27.5
DEAE-cellulose eluate II	0.465	71.5	2.5	29.1	15.9
Sephadex G-150 eluate	0.179	69.4	1.04	67	15.4

Substrate specificity of phenylalanine aminotransferase

The substrate specificity of phenylalanine aminotransferase was determined at various stages in the purification. For this determination the reaction mixture contained 1 mM each of all the naturally occurring amino acids (except tryptophan), 12 mM α-ketoglutarate and 1 unit phenylalanine aminotransferase in 0.1 M Tris · HCl (pH 8.5). After 30 min at 37°C, the reaction was terminated by the addition of one volume of 10% sulphosalicylic acid/0.5 mM norleucine. Blank values were obtained by adding the enzyme after the sulphosalicylic acid. Residual amino acids were determined [14] and compared with those present in the blank tests. The results (Table IV) show that, as the purity of the preparation increased, the specificity became restricted to phenylalanine and tyrosine. A separate experiment using tryptophan indicated that it was also transaminated by phenylalanine aminotransferase at 80% of the rate of phenylalanine.

Homogeneity of phenylalanine aminotransferase preparations

The fraction from the Sephadex G-150 column with the highest specific activity (90 units/mg protein) was subjected to gel isoelectric focusing [15] using pH 4—6 Ampholines. The gel was cut in half along the direction of migration; one half was stained for protein with Coomassie Blue and the other for phenylalanine aminotransferase activity. The method was based on that for the visualisation of glutaminase [10]. The protein stain revealed at least eight bands and the enzyme activity was associated with three adjacent bands with a pI of about 4.4. Integration of the area under the active bands indicated that the enzyme accounted for half of the total protein suggesting that the pure enzyme may have a specific activity approx. 200 units/mg protein.

Affinity chromatography of phenylalanine aminotransferase

Since the purification of phenylalanine aminotransferase by more conventional means resulted in a product only 50% pure, affinity chromatography was investigated. A variety of compounds were investigated as potential inhibitors of phenylalanine aminotransferase (Table V), and of these *m*-hydroxycinnamate was examined as a potential ligand. Adsorbents 1 and 2 (Table VI) were synthesised by published methods [12].

The enzyme was not bound to adsorbent 1 under a variety of conditions, with or without α-ketoglutarate (the co-substrate of the enzyme). Adsorbent 2 did not bind the enzyme at pH 6.0, but, in 50 mM Tris · HCl (pH 7.5), was

TABLE IV

SUBSTRATE SPECIFICITY OF PHENYLALANINE AMINOTRANSFERASE

Stage	Relative rate of degradation of amino acids					
	Phe	Tyr	Met	Ile	Leu	His
DEAE-cellulose Eluate I	100	81	42	42	36	36
Sepharose GB eluate	100	82	35	56	47	24
DEAE-cellulose eluate II	100	84	33	0	0	30
Sephadex G-150 eluate	100	82	0	0	0	0

TABLE V

POTENTIAL INHIBITORS OF PHENYLALANINE AMINOTRANSFERASE

Inhibitors were added to standard reaction mixtures and reagent blanks at a concentration of 9.5 mM with the exception of tyramine and *m*-hydroxycinnamate, because of their low solubility, could only be added at 0.5 mM. Glycine, alanine, glutamine, pyruvate, D-phenylalanine, *p*-amino-DL-phenylalanine and tyramine did not inhibit.

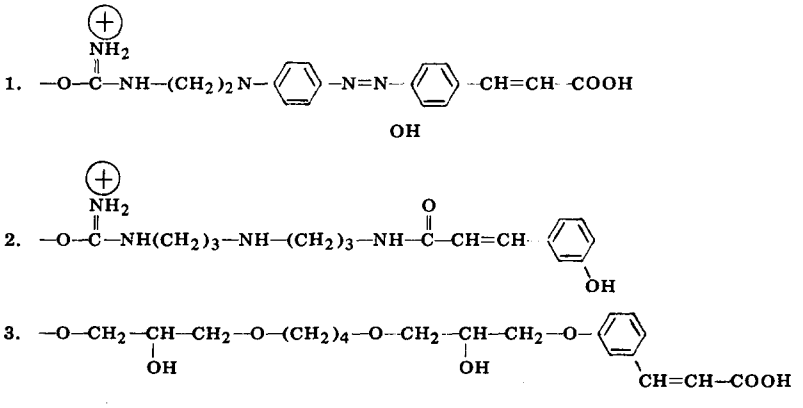
Compound	Inhibition (%)
m-Hydroxycinnamate	15
p-Aminobenzoate	15
DL-*p*-Fluorophenylalanine	21
Phenylacetamide	22
Phthalate	24
Benzoate	35
Thienylalanine	38
Phenylacetate	45
Hydrocinnamate	60

found to bind 300 units phenylalanine aminotransferase/ml gel from a preparation containing 19.25 units/mg protein. Raising the ionic strength of the starting buffer from 50 mM to 0.1 M markedly reduced the capacity of the adsorbent, while at pH 8.5 no binding occurred. a pH of about 9 was necessary to elute the enzyme from the adsorbent. When the enzyme (specific activity 77 units/mg protein) was bound to adsorbent 2 and eluted with 50 mM bicarbonate (pH 9.3), 85% of the enzyme was recovered with a specific activity of 165 units/mg protein; the maximum specific activity observed in column fractions was 199 units/mg protein.

Since the enzyme could not be eluted from adsorbent 2 with 10 mM L-phenylalanine, it is likely that the interaction is not one of true biospecific

TABLE VI

AFFINITY CHROMATOGRAPHY ADSORBENTS FOR PHENYLALANINE AMINOTRANSFERASE PURIFICATION



Adsorbents 1 and 2 were synthesised from CNBr-activated Sepharose 4B while adsorbent 3 was synthesised from epoxy-activated Sepharose 6B.

recognition. In an attempt to elucidate the nature of the interaction, adsorbent 3 was synthesised; this adsorbent does not contain the charged isourea group present in adsorbents prepared from CNBr-activated Sepharose [17]. In 50 mM Tris · HCl (pH 7.5), adsorbent 3 showed only 10% of the capacity for phenylalanine aminotransferase exhibited by adsorbent 2.

The nature of the reaction catalysed by phenylalanine aminotransferase

One unit of phenylalanine aminotransferase was allowed to react in the standard reaction mixture for 30 min when the reaction was stopped by the addition of one volume of 10% sulphosalicylic acid. Amino acid analysis of the mixture revealed a mol to mol relationship between the disappearance of L-phenylalanine and the appearance of L-glutamate. Thin-layer chromatography of reaction mixtures on Camag DF-B silica gel plates in butanol/acetic acid/water (12 : 3 : 5, v/v) and 95% ethanol/0.88 ammonia (7 ; 3, v/v), following 2 h tank saturation, indicated the presence of four compounds with the same mobilities as L-phenylalanine, L-glutamate, α-ketoglutarate and phenylpyruvate. The compounds were visualised both by viewing under an ultraviolet lamp and spraying with ninhydrin.

A separation of the substrates and products of the reaction was also performed using an LKB Tachophor with 10 mM HCl adjusted to pH 9.4 with amediol as leading ion and 20 mM β-alanine adjusted to pH 10 with solid $Ba(OH)_2$ as terminating ion. Runs were carried out at 100 μA and the voltage increased from 500 to 6000 V in the course of each run. The results showed the appearance of only two compounds following phenylalanine aminotransferase action corresponding to L-glutamate and phenylpyruvate.

When reactions were carried out under N_2 in Thunberg tubes the normal rate of reaction was observed and the molar ratio of L-phenylalanine consumed to L-glutamate produced was maintained. The reaction was also carried out in the presence of added NH_4^+ at concentrations of up to 0.5 M, with again no change in the ratio of 1 mol L-phenylalanine consumed to 1 mol L-glutamate produced.

The presence of a cofactor in phenylalanine aminotransferase

At no stage in the purification of phenylalanine aminotransferase was the addition of pyridoxal 5'-phosphate found to stimulate the activity of the enzyme. A wide range of methods [18—22] were employed in an attempt to resolve phenylalanine aminotransferase into apoenzyme and cofactor but in no instance did the procedure result in phenylalanine aminotransferase requiring the addition of exogenous pyridoxal 5'-phosphate for it to exhibit full activity.

Preparations of the enzyme were colourless at pH values between 6 and 10 and in 0.1 M Tris · HCl (pH 8.5) the spectrum was that of a simple protein with none of the absorbtion bands characteristic of pyridoxal 5'-phosphate-containing enzymes [1].

The ORD spectrum of phenylalanine aminotransferase was recorded using a Fica Spectropol 1b spectropolarimeter between 200 and 600 nm using a solution of 420 units phenylalanine aminotransferase/ml in 0.1 M Tris · HCl (pH 8.5) in 2-mm silica cells. A smooth curve was obtained, with none of the perturbations normally associated with pyridoxal 5'-phosphate-containing enzymes [23] in the region 350—450 nm.

Fluorescence measurements were carried out using a Baird Atomic fluorimeter with an excitation wavelength of 320 nm. Using maximum sensitivity and full gain, the emission spectra (320—500 nm) of the solvent, 0.1 M Tris · HCl (pH 8.5), the native enzyme at 100 units/ml in buffer, the enzyme solution plus 8 M urea and the enzyme solution plus 8 M urea plus 4 mM KCN were recorded. No change in the fluorescence spectrum on addition of CN^- to the urea-treated enzyme was observed as described for a pyridoxal 5'-phosphate-containing enzyme [24].

The absence or presence of pyridoxal 5'-phosphate in phenylalanine aminotransferase was further checked by a microbiological assay for pyridoxal 5'-phosphate using *Saccharomyces carlsbergensis* ATCC 9080 as test organism [25]. 3 mg phenylalanine aminotransferase (purified by affinity chromatography) were acid hydrolysed for 24 h, taken to dryness and redissolved in 1 ml water. The assay indicated the presence of less than 5% of the amount of pyridoxal 5'-phosphate predicted, on the basis of the normal ratio of 2 mol pyridoxal 5'-phosphate per mol of protein.

Treatment of phenylalanine aminotransferase with 10 mM phenylhydrazinium hydrochloride (pH 5.5) resulted in the appearance of a new peak in the spectrum of the enzyme with an absorption maximum of about 325 nm (Fig. 2), the absorption maximum of pyruvoylphenylalanine phenylhydrazone [26]. This change in spectrum is unlikely to be due to the formation of the phenylhydrazone of pyridoxal 5'-phosphate since it has an absorption maximum of 410 nm [27] and those of phenylpyruvate, oxaloacetate and α-ketoglutarate have absorption maxima of 340, 385 and 380 nm, respectively.

General properties

The molecular weight of phenylalanine aminotransferase was determined

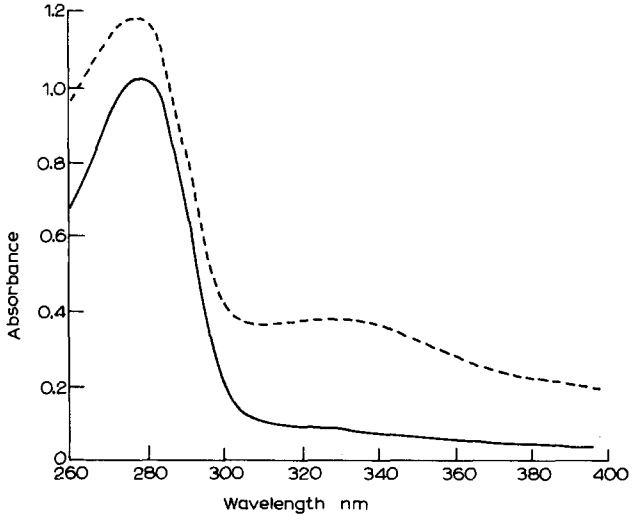


Fig. 2. The ultraviolet absorption spectrum of native phenylalanine aminotransferase in 0.1 M Tris · HCl (pH 8.5) (———) and phenylalanine aminotransferase after treatment at pH 5.5 with 10 mM phenylhydrazinium hydrochloride (- - - - - -).

using a Sephadex G-150 gel filtration column (3.2 cm diameter × 85 cm), calibrated with catalase, L-asparaginase, bovine serum albumin and chymotrypsinogen. The enzyme showed an apparent molecular weight approx. 90 000. This value corresponds to that calculated from the observed sedimentation coefficient, $s_{20,w}$, of 4.9 S.

The kinetics of phenylalanine aminotransferase action do not conform to Michaelis-Menten theory. Lineweaver-Burk plots were not linear, although, at substrate concentrations higher than 0.5 mM, linear plots are obtained (Fig. 3). These give K_m values of 0.74 mM for L-phenylalanine, 0.63 mM for α-ketoglutarate and 0.13 mM for oxaloacetate. At low substrate concentrations, the rate of reaction is greater than that predicted by these values. The non-linear kinetics could be explained either by the enzyme having two active sites of different K_m or by the three isoenzymes having different K_m values. This question could only be resolved if one could separate the isoenzymes and determine their individual kinetics.

The pH vs. activity curve of phenylalanine aminotransferase (Fig. 4) shows variations due to the buffer used, but the best-fit line indicates a pH optimum at pH 8.1. The enzyme is remarkably active at alkaline pH values but, below pH 6.5, the activity falls away rapidly.

In addition to the compounds listed in Table V, a number of carbonyl group reagents were found to inhibit the enzyme (Table VII). The failure of phenylhydrazine to cause complete inhibition of the enzyme is consistent with the results obtained with D-proline reductase [28] where $5 \cdot 10^{-2}$ M phenylhydrazine caused less than 90% inhibition. This may be due to either inhomogeneity of the enzyme molecules or the reaction at one active site rendering a second site inaccessible to the reagent. Protection from inhibition by borohydride could be effected by pretreating the enzyme with either L-phenylalanine or α-ketoglutarate. The enzyme was also found to be product inhibited by phenylpyruvate; at 16.7 mM, 94% inhibition was observed.

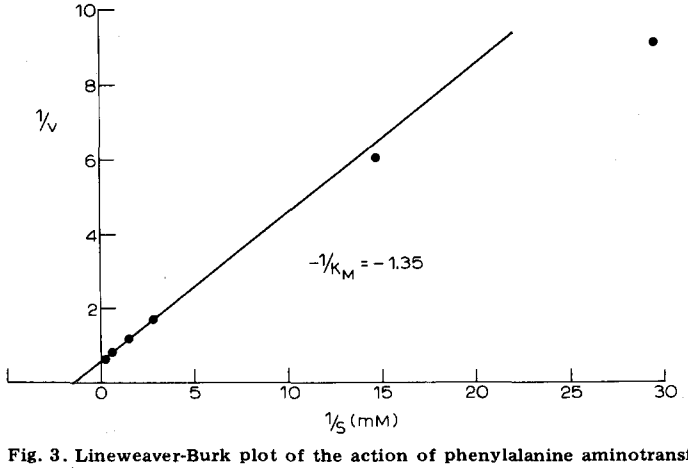


Fig. 3. Lineweaver-Burk plot of the action of phenylalanine aminotransferase on phenylalanine at a constant α-ketoglutarate concentration of 11.1 mM. Assays were carried out at pH 8.5 and 37°C.

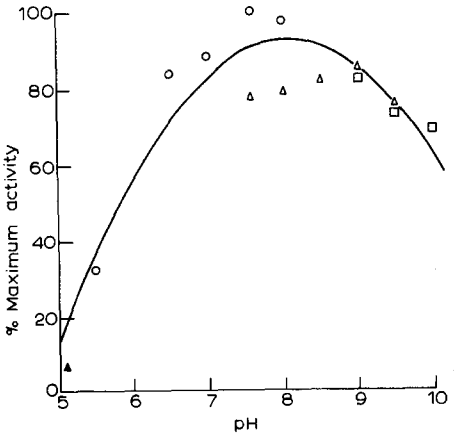


Fig. 4. The pH vs. activity curve of phenylalanine aminotransferase from strain MRE 5390. The enzyme was assayed under standard conditions using 0.1 M acetate buffer (△); 0.1 M phosphate buffers (○); 0.1 M Tris · HCl buffers (△) and 50 mM borate buffers (□). The curve was drawn from a least squares fit quadratic regression analysis of the data.

TABLE VII

THE INHIBITION OF PHENYLALANINE AMINOTRANSFERASE BY CARBONYL GROUP REAGENTS

Enzyme at 30 units/ml reacted with inhibitors in 0.1 M Tris · HCl (pH 8.5) for 30 min at room temperature. 20-μl samples were then taken for assay.

Inhibitor	Concentration (M)	Inhibition (%)
Sodium borohydride	$2 \cdot 10^{-2}$	100
Hydroxylamine hydrochloride	$5 \cdot 10^{-2}$	67
	$5 \cdot 10^{-3}$	49
	$5 \cdot 10^{-4}$	24
Phenylhydrazine hydrochloride	$5 \cdot 10^{-2}$	69
	$5 \cdot 10^{-3}$	81
	$5 \cdot 10^{-4}$	78
	$5 \cdot 10^{-5}$	60

Discussion

The results suggest that phenylalanine aminotransferase from strain MRE 5390 is a true transaminase but lacks pyridoxal 5'-phosphate as cofactor. The evidence that pyridoxal 5'-phosphate is not a cofactor for this enzyme is based on methods whose sensitivity, particularly the microbiological assay, is such that despite the very small amounts of pyridoxal 5'-phosphate involved would have revealed its presence had this enzyme contained the amount normally found in pyridoxal 5'-phosphate-containing enzymes.

There are, however, among other groups of enzymes which are normally expected to contain pyridoxal 5'-phosphate as cofactor, examples which contain none. Among the decarboxylases, which normally contain pyridoxal 5'-phosphate as cofactor, the histidine decarboxylase from *Lactobacillus* 30a lacks pyridoxal 5'-phosphate [26] and among the γ-aminobutyrate-α-ketoglutarate

aminotransferases the enzyme from *Ps. fluorescens* probably lacks pyridoxal 5'-phosphate [29] while the enzymes of identical function from *Pseudomonas aeruginosa* [30] and human brain [31] both show a requirement for it. The phenylalanine aminotransferase reported here is similar in many respects (Table VIII) to the enzymes from *A. eurydice* [4] and *E. coli* [6] although both these enzymes contain pyridoxal 5'-phosphate. The nature of the cofactor, if any, in the enzyme from strain MRE 5390 is uncertain. The only information as to its identity is the change in absorption spectrum on treatment of the enzyme with phenylhydrazine. Although the spectral change is almost identical with that observed for the pyruvate-containing histidine decarboxylase from *Lactobacillus* 30a the spectrum of the keto acid phenylhydrazone is altered by the nature of the molecule to which it is linked [26]. Without isolation of the phenylhydrazone fragment and chemical identification of the carbonyl group containing moiety, no positive identification of the cofactor can be given although it seems likely that it will be one of the family of prosthetic groups described by Snell [32] since the susceptibility of the enzyme to inhibition by borohydride and phenylhydrazine certainly indicates the presence of an essential carbonyl group.

The enzyme does appear to catalyse a true transamination reaction. The mechanism of L-phenylalanine utilisation by the strain is unlikely to be through an oxygenase mechanism since phenylalanine aminotransferase is active under anaerobic conditions and the reaction mechanism is unlikely to be a two-enzyme ammonia transfer system since, even in the presence of added NH_4^+ (up to 0.5 M), the mol to mol relationship between the utilisation of L-phenylalanine and the production of L-glutamate was maintained. Thus it seems probable that the enzyme described is a true transaminase with a catabolic function similar to the aromatic amino acid transaminase of *A. eurydice* [4].

The behaviour of phenylalanine aminotransferase on the affinity chromatography sorbents described appears to depend both on the nature of the spacer arm and the manner of attachment to the ligand. The failure of phenylalanine aminotransferase to bind to adsorbent 1 (Table VI) may be due to the bulky nature of the grouping caused by the azo linkage. Alternatively, binding may be dependent on the arm terminating in an aromatic ring as in adsorbent 2. The interaction does, however, show a degree of specific recognition of the ligand

TABLE VIII

A COMPARISON OF THE PROPERTIES OF AROMATIC AMINO ACID TRANSAMINASES OF BACTERIAL ORIGIN

Properties	Source		
	MRE 5390	*E. coli* *	*A. eurydice* **
Molecular weight	90 000	88 000	—
p*I*	pH 4.4	pH 4.6	pH 6
Rate of substrate utilisation	Phe > Tyr = Trp	Tyr > Trp > Phe	Phe > Tyr > Trp > Asp
Enzyme function	catabolic	anabolic	catabolic
Presence of pyridoxal 5'-phosphate	no	yes	yes

* Data taken from ref. 6.
** Data taken from ref. 4.

since if L-phenylalanine is substituted for *m*-hydroxycinnamate in adsorbent 2, no binding occurs, but if no charged isourea group is present as in adsorbent 3 and an aromatic ring is not the terminal group of the arm some binding still occurs. These results indicate that the interaction of phenylalanine aminotransferase with the affinity adsorbents described is complicated and is not a simple biological recognition of the ligand.

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