BBA 66103

PURIFICATION AND PARTIAL CHARACTERIZATION OF THE BRANCHED CHAIN AMINO ACID TRANSAMINASE OF *PSEUDOMONAS AERUGINOSA*

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(Received January 13th, 1970)

SUMMARY

The enzyme responsible for catalyzing transfer of amino groups from branched chain amino acids to 2-oxoglutarate in *Pseudomonas aeruginosa* has been purified 370-fold and partially characterized. The purification methods included a two-step heat treatment, removal of nucleic acids with protamine sulfate and chromatography on Sephadex G-200, DEAE-cellulose and cellulose phosphate. Disc electrophoresis with acrylamide gel was used as a check of enzyme purity.

The molecular weight of the enzyme was estimated to be 130 000 g/mole by gel filtration on calibrated G-200 columns. The pyridoxal content was 2 moles/mole of protein.

The enzyme catalyzed amino transfer from L-isomers of isoleucine, leucine, methionine, norvaline, phenylalanine and valine to 2-oxoglutarate. The pH optima for enzyme activity were found to be 7.4 for isoleucine, 8.1 for norvaline, and 8.4 for valine. The enzyme had two pH optima for enzyme activity, at 6.8 and 8.1 with leucine.

Michaelis constants for seven of the fourteen potential substrates ranged from $2.6 \cdot 10^{-4}$ for 2-oxovalerate to $1.3 \cdot 10^{-2}$ M for L-glutamate.

Under the conditions used for growth, the branched chain amino acid transaminase of *P. aeruginosa* functioned as a biosynthetic enzyme while the counterpart from animals is a catabolic enzyme.

INTRODUCTION

The existence of transaminases effecting the transfer of amino groups from branched chain amino acids to 2-oxoglutarate has been known for some time in both animals and microorganisms[1-6]. The enzyme which catalyzes this reaction is classified by the Enzyme Commission as EC 2.6.1.6, L-leucine:2-oxoglutarate aminotransferase and given the trivial name, leucine aminotransferase. TAYLOR AND JENKINS[6] recently prepared very highly purified branched chain amino acid transaminase from pig heart and established that a single enzyme was indeed responsible for amino transfer

from all three branched chain and certain aliphatic amino acids to 2-oxoglutarate. Several years ago, RUDMAN AND MEISTER[2] partially purified two transaminases from *Escherichia coli*; one which they named transaminase A, catalyzed amino transfer from the aromatic amino acids and aspartate to 2-oxoglutarate, the other was named transaminase B and catalyzed amino transfer from the branched chain amino acids to 2-oxoglutarate. Until recently, very little else had been done with bacterial enzymes. Now M. S. COLEMAN AND F. B. ARMSTRONG, JR. (personal communication) have purified and crystallized the branched chain amino acid transaminase from *Salmonella typhimurium* and we have the corresponding enzyme from *Pseudomonas aeruginosa* highly purified. This paper deals with the procedure used to purify the pseudomonad enzyme, some properties of the purified enzyme and a discussion of the role of this transaminase in the biology of the cell.

MATERIALS AND METHODS

Cultural methods

The strain of *Pseudomonas aeruginosa* used in this study is the same one used in previous work done in this laboratory and was maintained as described in an earlier publication[7]. The organism was grown in large quantities for enzyme purification in a defined medium with glucose as the energy source. The basal medium had the following composition: salts C (ref. 8), 65 ml; $(NH_4)_2SO_4$, 13.0 g; KH_2PO_4, 15.6 g; K_2HPO_4, 94.2 g; amino acid solution, 250 ml; distilled water, 11 450 ml. The amino acid solution was prepared by adding the following amino acids to 250 ml of water and adjusting the pH to 7.5 with 1 M KOH: 6.5 g L-aspartic acid, 3.25 g L-glutamic acid, 1.95 g glycine, 1.95 g DL-alanine. Portions of 50 ml and 850 ml of the basal medium were withdrawn for growth of the inoculum. Glucose was autoclaved separately as a 6% solution; 5 ml of sterile glucose solution was added to the 50-ml portion of basal medium, 100 ml added to the 850-ml portion and 1200 ml added to the large batch of medium.

The smallest container of complete medium was inoculated with a suspension of bacteria washed from a slant of valine medium. The culture was aerated with an aquarium pump and incubated for 8 h at 37°. This culture was then added to the flask with 950 ml of medium which was incubated for another 8.5 h at 37° with aeration. The large portion of complete medium, prewarmed to 37° was placed in a 13-l Bio-Kulture assembly (Fermentation Design, Fogelsville, Pa.) and the inoculum added. The temperature of the fermenter was set at 37°, the agitator speed was adjusted to 400 rev./min, and 3.5 l of air were delivered per min from two aquarium pumps. Bacterial growth was monitored by measuring the turbidity of 1:5 dilutions of samples at 660 mμ with a Bausch and Lomb Spectronic 20. Bacteria were harvested at the peak of growth with a Szent–Gyorgi and Blum continuous flow attachment to the Servall RC-2 centrifuge. Harvested bacteria were stored at −20° until they were used.

Materials

The following chemicals were obtained from Sigma Chemical Co.: bovine pancreatic deoxyribonuclease, Type DN-25, Salmine grade II protamine sulfate, potassium 2-oxoglutarate, sodium 2-oxovalerate, all amino acids used in this study, and pyridoxal 5′-phosphate. Sephadex G-200 was obtained from Pharmacia.

Enzymic methods

Protein was estimated by the method of LOWRY *et al.*[9]. The amount of $(NH_4)_2SO_4$ to be added during enzyme purification was calculated from the following formula which was adapted from the data of BRENNER-HOLZACH AND STAEKELIN[10]:

$$\frac{(1.77) \times (\text{ml of solution}) \times (B - A)}{3.54 - B} = X$$

where B is the fractional saturation to be achieved, A is the initial saturation and X is $(NH_4)_2SO_4$ (g) to be added to achieve saturation B.

The quantitative enzyme assay contained the following ingredients in a volume of 1 ml: potassium phosphate buffer (pH 7.5), 100 μmoles; pyridoxal 5'-phosphate, 50 μg; enzyme protein, 0.02 unit; L-norvaline, 60 μmoles; 2-oxoglutarate, 5 μmoles.

Both the norvaline and 2-oxoglutarate solutions were adjusted to pH 7.5 before being added to the reaction mixture. The reaction was started by adding 0.3 ml of 0.2 M L-norvaline. One enzyme unit was defined as that amount which catalyzed the formation of 1 μmole of glutamate per min; specific activity was defined as units per mg protein. The amount of glutamate produced was proportional to the enzyme concentration up to about 0.1 unit of enzyme.

The mixtures were incubated for 15 min at 37° in a shaking water bath, then 0.5 ml of 1.0 M $HClO_4$ was added to stop the reaction; if necessary, the mixtures were centrifuged 15 min at 12 000 $\times g$ to remove protein. The supernatant fluids were neutralized by adding 0.5 ml of 1.0 M KOH. When the precipitation of potassium perchlorate was complete, 20 μl of each mixture was spotted on chromatographic paper. An internal standard with all the components of the reaction mixture and 5 μmoles of L-glutamate, but no protein, was carried through with each determination. The chromatograms were developed for 30 min with the methanol–water–pyridine solvent of REDFIELD[11]. The chromatograms were dried and dipped in the ninhydrin reagents described by HEILMANN and her co-workers[12]. The chromatograms were dried until faint spots appeared, then were placed in sealed jars over concentrated sulfuric acid and incubated for 30 min to 1 h at 37° to develop the color. The glutamate spots were excised and the color was eluted in 2.5 ml of methanol and 0.1 ml of 2 M acetic acid and the absorbance was read at 510 mμ.

In the assay used to study substrate specificity, the standard assay was used with 1 μg of purified transaminase and 15 μmoles of amino acid were used. Very insoluble amino acids such as tyrosine and tryptophan were made up as 0.05 M suspensions and the reaction was started with the addition of 15 μmoles of potassium 2-oxoglutarate (pH 7.5). The incubation time for this assay was 20 min. The assay system used in the pH study contained 2 μg of enzyme and differed from the standard assay in that 30 μmoles of each amino acid and 2-oxoglutarate were used and the incubation time was 20 min. K_m's were determined first for 2-oxoglutarate by adding 60 μmoles of norvaline and varying amounts of 2-oxoglutarate to reaction mixtures; 1 μg of protein was used in these reactions. The constants for the amino acids were found by using 5 μmoles of 2-oxoglutarate and varying amounts of amino acids. MICHAELIS constants[13] were calculated from the following formula:

$$\frac{[S]}{v} = \frac{[S]}{v_{max}} + \frac{K_m}{v_{max}}$$

When $[S]/v$ is plotted as a function of $[S]$, a straight line is obtained. The slope of the line with the $[S]/v$ axis is $1/v_{max}$ and its intercept is K_m/v_{max}; K_m is obtained by multiplying the value of the intercept by v_{max} (refs. 14, 15).

Physicochemical methods

The molecular weight of the transaminase was estimated by filtration of the protein through calibrated columns of Sephadex G-200[16], 2.5 cm × 35 cm. Standard proteins used were bovine pancreatic trypsin, ovalbumin, bovine serum albumin and human γ-globulin. The transaminase was concentrated with an Amicon ultrafilter with nitrogen as a source of pressure for this determination.

Pyridoxal content of the protein was determined by the method of WADA AND SNELL[17] with the volume of the assay reduced to 1 ml. Samples were first dialyzed exhaustively against 0.02 M ammonium bicarbonate to free them of sucrose used in the purification procedure, lyophilized, weighed and hydrolyzed according to the method of RABINOWITZ AND SNELL[18]. The sample was dried, redissolved in distilled water and assayed for pyridoxal with phenylhydrazine. The standard was crystalline pyridoxal 5'-phosphate.

RESULTS

Purification of branched chain amino acid transaminase

Cell-free extracts of *P. aeruginosa* were made by suspending 150 g of frozen bacteria in 200 ml of 0.05 M potassium phosphate buffer (pH 6.5). Suspension of bacteria was aided by adding 40 mg of pancreatic deoxyribonuclease. The slurry was stirred at room temperature for approx. 20 min and bacteria were disrupted by treatment of the suspension with sonic oscillation using a DF 101 Raytheon oscillator with chilled water at 4° circulating through the jacket. Cellular debris was removed from the extract by centrifugation at 12 000 × g for 15 min using a refrigerated centrifuge kept at 10°.

Transaminases have frequently been purified by heating the enzyme with 2-oxoglutarate and pyridoxal phosphate and this procedure was useful in the purification of the enzyme from *P. aeruginosa*. Sufficient potassium 2-oxoglutarate (0.4 M, pH 6.5) was added to the extract to give a final concentration of 0.005 M and solid pyridoxal 5-phosphate was added to a final concentration of 50 μg/ml. The temperature of the extract was brought to 62° quickly in a 95° water bath, the extract was transferred to a 62° bath kept there for 15 min, and then chilled in an ice-salt bath. Denatured protein was removed by centrifuging the suspension for 1 h at 37 000 × g. The pellet was washed by resuspending it in 75 ml of 0.05 M potassium phosphate buffer (pH 6.5), and centrifuging the suspension for 1 h at 37 000 × g. The two clear extracts were combined, heated to 75° in a 95° bath, transferred to a 75° bath for 5 min and then quickly chilled in an ice-salt bath. Denatured protein was again removed by centrifugation. The increase in specific activity at this stage was approx. 6-fold (Table I).

Nucleic acids were removed by treatment of the extract with an amount of 1% protamine sulfate equal to 0.8 the volume of the extract. The suspension was stirred for 10 min, centrifuged for 45 min at 37 000 × g and the pellet discarded.

Solid $(NH_4)_2SO_4$ was added to the extract to 35% satn., the suspension was stirred for 10 min and insoluble proteins were removed by centrifugation of the

TABLE I

PURIFICATION OF BRANCHED CHAIN AMINO ACID TRANSAMINASE OF *P. aeruginosa*

Fraction	*Protein (mg)*	*Units*	*Specific activity (μmoles/min per mg protein)*
Cell-free extract	18 350	2460	0.13
Heated extract	1 980	1540	0.78
Protamined extract	1 660	1590	0.96
$(NH_4)_2SO_4$ fraction	520	1190	2.3
Pool from Sephadex column	136	1360	10
Pool from DEA-cellulose column	29	1080	37
Pools from cellulose phosphate column			
No. 1	1.2	75	62
No. 2	1.4	105	75
No. 3	4.9	235	48

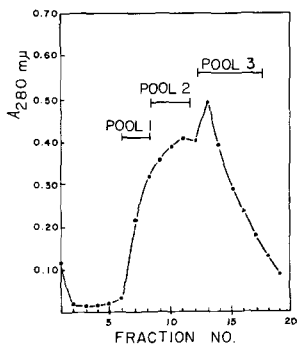
mixture for 30 min at 37 000 × *g*. Enough $(NH_4)_2SO_4$ was then added to bring the solution to 60% satn. and the mixture again stirred for 10 min; insoluble proteins were sedimented by centrifuging the suspension for 30 min at 22 000 × *g*. The pellet was dissolved in 35 ml of 0.05 M potassium phosphate buffer (pH 8.5). There was approximately a 2-fold increase in specific activity as a result of this step.

The protein solution was placed on a 10 cm × 22 cm column of Sephadex G-200 which had been equilibrated with 0.05 M potassium phosphate buffer (pH 8.5). Protein was eluted from the column with the same buffer in 13-ml fractions; the transaminase was eluted between Fractions 60 and 100 and the active fractions were pooled.

The pooled fractions were concentrated by the use of solid sucrose. The enzyme was placed in a dialysis bag which was then folded loosely in a large funnel and enough sucrose was added to cover the bag. The funnel was kept in a refrigerator until the extract was sufficiently concentrated, usually about 6 h.

The concentrated enzyme was placed on a 1.3 cm × 47 cm DEAE-cellulose column which had been equilibrated with 0.05 M potassium phosphate solution (pH 9.2). Protein was eluted with a linear buffer gradient using 500 ml of 0.05 M potassium phosphate and 500 ml of 0.2 M potassium phosphate buffer (pH 7.5). Fractions of 10 ml were collected and the enzyme was eluted between Tubes 60 and 100.

The pooled extract was concentrated by sucrose dialysis to about one-tenth its volume, the pH adjusted to 5.5 with 0.15 M phosphoric acid. The concentrate was diluted 1:5, reconcentrated, diluted 1:3 with distilled water and finally concentrated to about 20 ml. The concentrated enzyme extract was placed on a 1 cm × 45 cm column of cellulose phosphate equilibrated with 0.01 M potassium buffer (pH 5.5). Protein was eluted with a linear gradient obtained with 200 ml of 0.01 M potassium phosphate (pH 5.5) and 200 ml of 0.01 M potassium phosphate (pH 5.5). Fractions were pooled as indicated in Fig. 1 and the homogeneity of the pools was checked by disc gel electrophoresis (Fig. 2). Pools 1 and 2 were nearly homogenous and had the highest specific activity (Fig. 2, Table I), and these pools were used for all critical



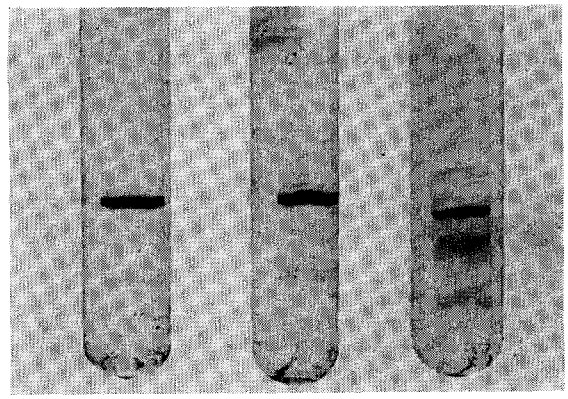


Fig. 1. Elution of protein from the cellulose phosphate column showing the fractions which were pooled. The size of the fractions was 5 ml.

Fig. 2. Disc electrophoresis of the pooled enzyme fractions obtained from the cellulose phosphate column. The pools are from left to right, 1, 2, and 3.

measurements of enzyme activity where purest enzyme preparations were needed. However, the overall yield was low which restricted the kinds of measurements which could be made.

Physicochemical properties

The molecular weight of the transaminase was estimated to be 130 000 by filtration through Sephadex G-200 columns. TAYLOR AND JENKINS[6] reported that pig heart leucine aminotransferase had a molecular weight of 75 000. Two determinations of the pyridoxal content using separate enzyme preparations provided values of 2.05 and 2.40 moles of pyridoxal per mole of enzyme. TAYLOR AND JENKINS were able to separate pyridoxal phosphate from the enzyme by dialysis against phosphate buffer containing leucine or glutamate but this treatment was not successful with the bacterial enzyme. The pyridoxal content of the pseudomonad enzyme (about 1 mole per 65 000 g of protein) is similar to that of the pig heart leucine aminotransferase (1 mole per 75 000 g protein).

Substrate specificity

The specific activity of the enzyme with several L-amino acids donors is shown in Table II. It is clear that the best substrates for the enzyme are the branched chain amino acids, at least under the condition of the standard assay. It is interesting to note, however, that the enzyme was able to use L-phenylalanine, which was true also of the partially purified transaminase B of *E. coli*, but not of the highly purified leucine aminotransferase of pig heart[6].

K_m's

Apparent K_m's were obtained for several substrates for the enzyme by varying the concentration of one substrate (amino donor or amino acceptor) while the concentration of the other was held constant (Table III). The rank of amino acids with respect to the concentration of substrate required to achieve half maximal velocity

TABLE II

ACTIVITY OF BRANCHED CHAIN AMINO ACID TRANSAMINASE WITH L-AMINO ACIDS

Amino acid	*Specific activity of transaminase with amino acid (μmoles/min per mg protein)*
L-Isoleucine	80
L-Leucine	90
L-Methionine	27
L-Norvaline	38
L-Phenylalanine	20
L-Valine	42
L-Alanine, L-aspartate, glycine, L-serine, L-threonine, L-tryptophane, and L-tyrosine	0

TABLE III

K_m OF SUBSTRATES FOR BRANCHED CHAIN AMINO ACID TRANSAMINASE

Substrate	K_m *(mM)*
2-Oxovalerate	0.26
2-Oxoglutarate	0.40
L-Leucine	1.0
L-Isoleucine	1.2
L-Norvaline	3.0
L-Valine	3.6
L-Glutamate	18

was the same as that reported by AKI *et al.*[19] for the rat liver transaminase and, in fact, the K_m's were quite similar although a different assay was used in the two studies.

pH optima

The pH optima for enzyme activity with the branched chain amino acids and norvaline are listed in Table IV. The curve for activity as a function of pH was bimodal with leucine as the substrate, but was a smooth curve with the other amino acids.

TABLE IV

pH OPTIMA FOR ENZYME ACTIVITY

L-*Amino acid*	*pH optimum*
Leucine	6.8, 8.1
Valine	8.4
Norvaline	8.1
Isoleucine	7.4

DISCUSSION

From the data presented in Tables II and III, it is apparent that the transaminase studied in this report is most active with the branched chain amino acids and that these are the natural substrates for the enzyme. The pseudomonad enzyme therefore is similar to and, is probably the bacterial counterpart, of leucine aminotransferase of pig heart which was highly purified by TAYLOR AND JENKINS[6] and of a similar enzyme which was partially purified from rat liver by AKI *et al.*[19]. Both groups of investigators pointed out that the trivial name "leucine aminotransferase" was insufficient to describe the action of this enzyme and we concur in this view, particularly since AKI et al purified a second transaminase from liver which was active only with leucine. The name branched chain amino acid transaminase seems more correct.

Members of the animal kingdom cannot synthesize branched chain amino acids and therefore their branched chain amino acid transaminase acts only in the deamination of these amino acids prior to oxidation. In the case of an organism such as *P. aeruginosa* which can both synthesize and metabolize the branched chain amino acids, such a transaminase is required in biosynthetic as well as catabolic pathways. Under the conditions used in this study for growth of *P. aeruginosa*, the organism would be obliged to synthesize the branched chain amino acids, but not to catabolize them, and therefore, it follows that branched chain amino acid transaminase functioned only in a biosynthetic capacity. On the other hand, when *P. aeruginosa* was grown with valine as the energy source, L-valine was deaminated by the action of a transaminase and L-glutamate dehydrogenase[21]. At the present time, it has not been established that a single enzyme served both biosynthetic and catabolic functions, but it would appear from the K_m's (Table III) that the enzyme studied in this report should be able to operate in both directions.

ACKNOWLEDGEMENTS

This research was supported by National Science Foundation research Grant 381025 R, Public Health Service Research Grant AM 09750 and Public Health Service Research Career Development Award 2-K3-GM-18, 343 made to J.R.S.

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