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AMINOTRANSFERASES FOR AROMATIC AMINO ACIDS AND ASPARTATE IN *BACILLUS SUBTILIS*

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Summary

Two proteins (form A and form B_2) with aromatic-amino-acid aminotransferase activity were detected in extracts of *Bacillus subtilis*. A histidinol phosphate aminotransferase (protein B_1) with aminotransferase activity for the aromatic amino acids was also present. The aspartate aminotransferase (L-aspartate: 2-oxoglutarate aminotransferase, EC 2.6.1.1) (protein C) also displayed similar activity. Each of the four proteins was isolated free from the others by the successive application of DEAE-cellulose column chromatography and flat-bed isoelectric focusing at pH range 4—6. Form B_2 is the major form of the aromatic-amino-acid aminotransferase (aromatic-amino-acid:2-oxoglutarate aminotransferase, EC 2.6.1.57) and the K_m values of tyrosine and phenylalanine with this form are somewhat lower than with the minor form A. The K_m of tyrosine with histidinol phosphate aminotransferase (protein B_1) is in the same range, but the K_m of phenylalanine with this enzyme is 12—20 times higher than the corresponding values with the two forms of the aromatic-amino-acid aminotransferase. Apparent molecular weights were estimated with Sephadex gel filtration to be approx. 73000, 64000, 54000 and 66000 for form A, form B_2, histidinol phosphate aminotransferase and aspartate aminotransferase, respectively. Form B_2 is being reported for the first time in this communication.

Introduction

The terminal steps in the biosynthesis of phenylalanine and tyrosine in microorganisms are transaminations between the respective keto acids and an appropriate amino group donor (for review see ref. 1). In *Escherichia coli*, two enzymes with the required substrate specificities have been recognized and purified to homogeneity in this laboratory [2,3]. The first is an aspartate aminotransferase (L-aspartate:2-oxoglutarate aminotransferase, EC 2.6.1.1), also known as transaminase A [4], with considerable activity for the aromatic amino acids. The second is an aromatic-amino-acid aminotransferase (aromatic-

amino-acid:2-oxoglutarate aminotransferase, EC 2.6.1.57). In *Bacillus subtilis* the enzymic entities involved in the transamination reactions mentioned above were unknown until recently. In the present study, we report on the presence of proteins displaying transaminating activity for the aromatic amino acids. Since aspartate aminotransferase from animal [5,6], plant [7] and bacterial sources [2,3] displays activity for the aromatic amino acids it was also included in the study.

Materials and Methods

Cells. B. subtilis strain 168 grown to 3/4 log phase in a minimal medium with glucose as the carbon source was obtained as frozen paste from Grain Processing Corporation, Muscatine, Iowa, U.S.A. The cells were thawed in 0.02 M potassium phosphate buffer (pH 7.0), 1.0 mM dithiothreitol, 1.0 mM EDTA, 2.0 mM α-ketoglutarate and 0.2 mM pyridoxal 5′-phosphate. 1 g frozen cells to 2 ml buffer were generally used. The cells were disrupted by sonicating for 5 min in five 1-min bursts when immersed in an ice/salt mixture. The disrupted cells were centrifuged for 30 min at 27000 × *g* and the supernatant fluid was used as the source of the enzymes.

Chemicals. All chemicals were used without additional purification.

Chromatography. DEAE-cellulose column chromatography was performed with Whatman microgranular DE-52, equilibrated with 0.02 M potassium phosphate buffer (pH 7.0) as above, except that pyridoxal phosphate was omitted. Gel filtration on Sephadex G150 and G100 columns (for the estimation of molecular weights) was applied as described in the appropriate legends of figures. The columns were calibrated with proteins of known molecular weight (aldolase, 158000; ovalbumin, 45000; bovine serum albumin, 67000; chymotrypsinogen A, 25000; ribonuclease A, 13700).

Flat-bed isoelectric focusing. The Desaga (Desaga, Heidelberg) apparatus was used. A 20 × 40 cm glass tray was layered with the slurry. The latter was 4% Ultrodex (from LKB) in 220 ml water/2% (w/v) Ampholine. The evaporation limit of 35%, as specified by the manufacturer, was followed in preparing the slurry for the run. The sample (about 2—3 ml), previously dialyzed against 1% glycine/0.2 mM pyridoxal phosphate (pH 7.0), was mixed with the gel removed earlier from the middle of the plate to make a slot about 16 cm long and 0.7—0.8 cm wide. The slot was filled with the mixture and the gel layer was connected with the platinum strip electrodes via filter paper strips impregnated with 1 M H_2SO_4 (anode) and 2 M ethylenediamine (cathode). The run lasted 20—22 h at voltage 800 V. The initial current was about 17 mA and finally 4—5 mA. The gel was collected in sections 1 cm wide along the short dimension of the plate and small portions suspended in 1 ml water for pH measurement. The remainder was suspended in 2—3 ml 0.2 M potassium phosphate buffer (pH 7.3) and the supernatant fluid assayed for enzyme activities. The separated enzymes were recovered from the appropriate gel fractions by elution with 0.05 M potassium phosphate buffer (pH 7.5)/0.2 mM pyridoxal phosphate. Ampholine was removed by dialysis for 17 h against a large volume of the same buffer. The enzyme solution was concentrated to a small volume by ultrafiltration in an Amicon cell (PM-10 membrane).

Enzyme assays. Tyrosine, phenylalanine, tryptophan and aspartate aminotransferase activities were assayed as described previously [3] and the amino acceptor used was always α-ketoglutarate. Histidinol phosphate aminotransferase was assayed by a modification of the method of Ames and Horecker [8] and Chapman and Nester [9]. The enzyme was preincubated for 5 min at 37°C with 0.03 ml 0.12 mM pyridoxal phosphate. Subsequently the following were added: 0.1 ml 0.1 M potassium phosphate buffer (pH 7.6), 0.05 ml 0.1 M potassium arsenate, 0.02 ml 0.3 M α-ketoglutaric acid (neutralized) and the reaction was started by adding 0.01 ml 0.15 M histidinol phosphate. Incubation was continued for 20 min. 0.4 ml concentrated HCl was added and the tubes were placed in a boiling water bath for 30 min. After cooling to room temperature 1 ml 3 M NaOH was added and the product was read at 370 nm after 4 min in a recording spectrophotometer. Units of enzyme activity are expressed as μmol of product formed per min.

Kinetic experiments. Apparent K_m values were calculated by the double reciprocal plot of Lineweaver and Burk [10]. We did not observe inhibition in the range of substrate concentrations used in this study.

Protein analysis. Protein was determined by the method of Lowry et al. [11].

Results and Discussion

Anion-exchange chromatography

Elution of a crude extract with a linear KCl gradient from a DE-52 column resulted in three activity peaks (Fig. 1). Peaks A and B represent aromatic-amino-acid aminotransferase activities and although the individual fractions of

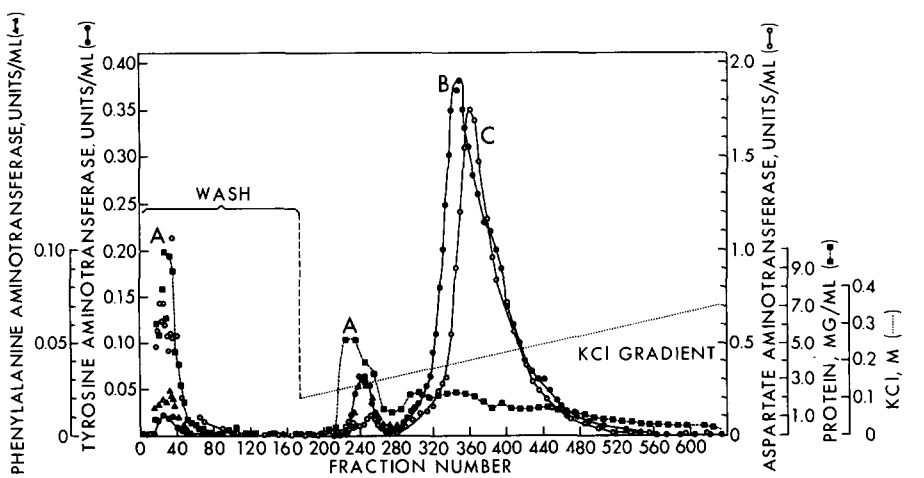

Fig. 1. First DEAE-cellulose column (2.5 X 32 cm) chromatography of a crude extract of *B. subtilis*. The column was packed with DE-52 and equilibrated as described in Materials and Methods. The extract contained 3680 mg protein and elution was carried out with 1500 ml of a linear gradient made from two 750-ml solutions of 0.1 M and 0.35 M KCl in 0.02 M potassium phosphate buffer, pH 7.0, supplemented with 1.0 mM dithiothreitol, 1.0 mM EDTA and 2.0 mM α-ketoglutarate. Fractions of 2.9 ml were collected. The column had been initially washed with 1250 ml of the above buffer to remove unadsorbed protein and during this operation 7-ml fractions were collected.

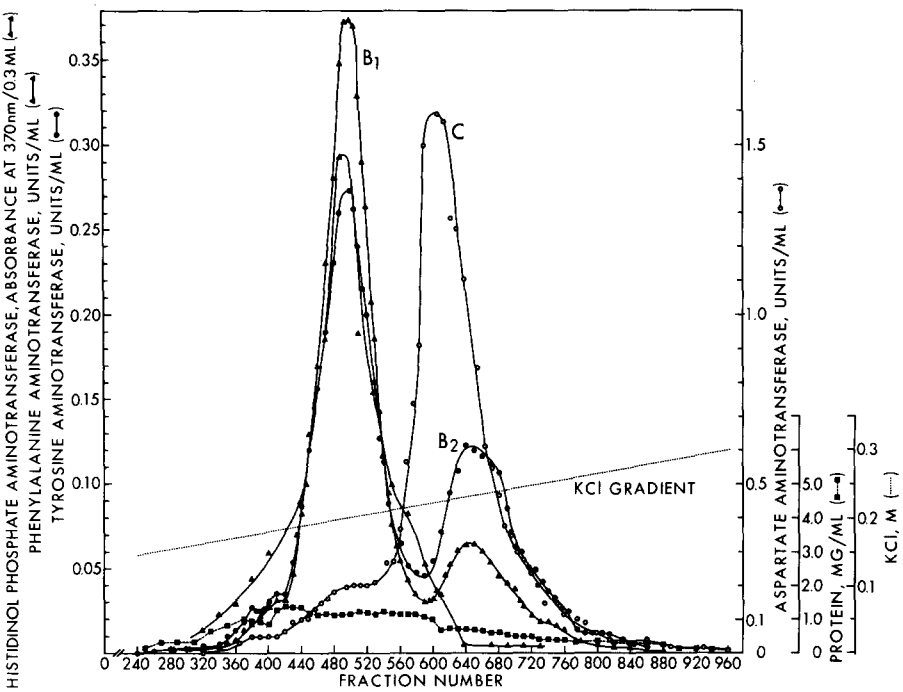

Fig. 2. Second DEAE-cellulose column (2.5 X 30 cm) chromatography. Fractions 281—530 from the first chromatography (Fig. 1) were pooled and applied to a DE-52 column. Total protein applied 776 mg. Elution was carried out as in Fig. 1 except that the KCl gradient extended from 0.1 to 0.3 M. Fractions of 1.3 ml were collected.

peak B were not assayed in this experiment for phenylalanine aminotransferase, the pooled fractions did contain this activity. Peak C largely overlaps with peak B and represents aspartate aminotransferase. This activity accompanies the fractions of peak A also. The wash-through contained the enzymes in very low specific activities. This component (Ao) was not investigated further, except for a heat stability test (which showed the aspartate aminotransferase to be heat labile at 55°C whereas the eluted aspartate aminotransferase (peak C) is heat stable at this temperature, see below). This information suggests that component Ao (Fig. 1) is not due to overloading of the column. Fractions 281—530, which include peaks B and C (Fig. 1), were pooled and rechromatographed on a second DE-52 column (Fig. 2). Fractions were assayed for phenylalanine, tyrosine and aspartate aminotransferase activities. Peak B of Fig. 1 was resolved into peaks B_1 and B_2. Both peaks contained the activities for the two aromatic substrates, but in inverse relative concentrations. The aspartate aminotransferase activity (peak C) was only partially resolved from form B_2 but it clearly represented a protein distinct from those of peaks B_1, B_2. Hence, there seemed to be present in the crude extract at least three proteins (A, B_1, B_2) which catalyse transamination between the two aromatic amino acids and α-ketoglutarate. The fractions also catalyzed transamination between L-tryptophan and α-ketoglutarate (unpublished data). The fourth protein (peak C) represented aspartate aminotransferase with intrinsic aromatic-amino-acid aminotransferase activity. Fractions 421—560 were used as a source of form B_1

and fractions 581—720 as a source of forms B_2 and C for further study.

While our work was in progress, Nester and Montoya [12] reported the separation on a DE-52 column of two proteins from crude extracts of *B. subtilis* with aminotransferase activity for the aromatic amino acids and an elution profile very similar to that of our Fig. 1. Moreover, their major peak (corresponding to our peak B, Fig. 1) was also a histidinol phosphate aminotransferase. We assayed the fractions of Fig. 2 and found that protein B_1, but not protein B_2, exhibited histidinol phosphate aminotransferase (Fig. 2). Shortly after, Weigent and Nester [13] reported the partial purification of the two aminotransferases. However, protein B_2 was not observed by Nester and collaborators [12]. It should be noted that we have observed protein B_2 only upon a second ion-exchange chromatography, involving a relatively low column load and collection of 1-ml fractions (compare Figs. 1 and 2).

Complete separation of B_2 from aspartate aminotransferase

We were able to observe partial resolution of form B_2 from aspartate aminotransferase (peak C) on columns of Sephadex G-100 and G-150 (unpublished data). Complete separation was achieved by flat-bed isoelectric focusing at pH 4—6 (Fig. 3). Aspartate aminotransferase coincided with the minor tyrosine aminotransferase activity peak. In similar experiments, a phenylalanine aminotransferase activity peak also coincided with peak C. We assume that the aspartate aminotransferase of *B. subtilis* possesses aromatic-amino-acid aminotransferase activity, as do other aspartate aminotransferases [2,3,5—7]. Form B_2 is partly contaminated by a minor form of aspartate aminotransferase, which was not studied further. It should be noted that isoelectric focusing resulted in severe losses of activity. Under our conditions, no more than 10—15% of either activity could be recovered. The average apparent isoelectric points from five experiments were 4.6 and 5.1 for form B_2 and aspartate aminotransferase, respectively.

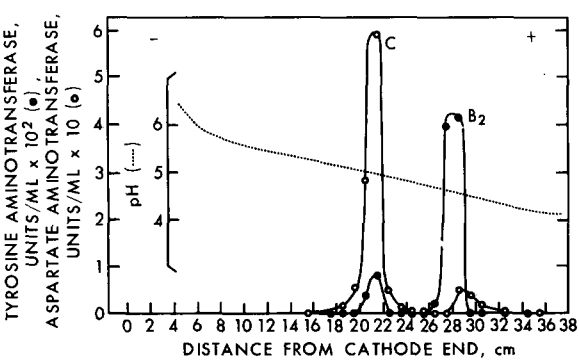

Fig. 3. Flat-bed (20 X 40 cm) isoelectric focusing of a mixture of aspartate aminotransferase (C) and of form B_2 obtained from the appropriate fractions of an experiment similar to that shown in Fig. 2. The thickness of the layer after evaporation (see Materials and Methods) was about 3 mm. The duration of the run was 21 h at 800 V and the current ranged from 16 to 4 mA during this period. The sample (about 10 mg protein) had been recovered from two previous isoelectric focusing experiments in which Sephadex G-75 ultrafine was used and which had resulted in incomplete separation.

TABLE I

ESTIMATION OF MOLECULAR WEIGHTS BY GEL FILTRATION ON SEPHADEX COLUMNS

Forms A and B_1 were obtained from the experiments of Fig. 1 and 2, respectively. Form B_2 and aspartate aminotransferase (C) were applied on the columns as a mixture and were obtained from the experiment of Fig. 2.

Enzymic protein	Sephadex G-100	Sephadex G-150
A	—	73 000
B_1	54 000	54 000
B_2	63 000	65 000
C	65 000	67 000

Complete separation of form B_1 from aspartate aminotransferase

It was possible to free B_1 completely from contaminating aspartate aminotransferase on columns of Sephadex G-100 (unpublished data). Complete separation of form B_1 from accompanying aspartate aminotransferase activity was also accomplished by flat-bed isoelectric focusing in the pH range of 4—6 as in Fig. 3. Large losses of activity were encountered also for this protein. The apparent isoelectric point of form B_1 was 5.3.

Estimation of molecular weights

Apparent molecular weights were estimated in two columns of Sephadex G-100 and G-150 calibrated with the appropriate range of standard proteins (Table I). There is good agreement between the values estimated with the two kinds of Sephadex. Form B_2 of the aromatic aminotransferase has an apparent molecular weight of 64000, only slightly lower than that of the aspartate aminotransferase (about 66000). The histidinol phosphate aminotransferase (protein B_1) displays the lowest molecular weight (54000) whereas form A of the aromatic aminotransferase the highest (73000). The two latter values are higher than those reported for the corresponding proteins by Weigent and Nester [13].

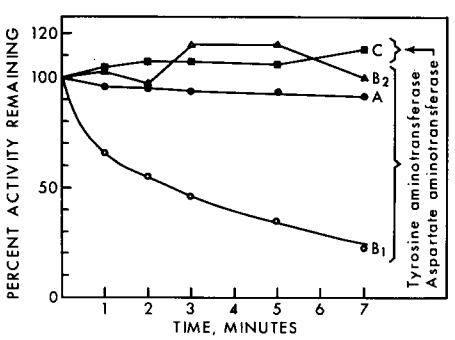

Fig. 4. Heat sensitivity of forms A, B_1 and B_2 and of aspartate aminotransferase (C) at 55°C. Form A was obtained from the experiment of Fig. 1 and form B_1 from that of Fig. 2. Form B_2 and aspartate aminotransferase (C) were tested as a mixture and obtained from fractions of the experiment of Fig. 2. The protein concentration during the heat treatment was 12.7 mg/ml for form A and 0.58 mg/ml for B_1, B_2 and C. The preparations were in 0.2 M potassium phosphate buffer, pH 7.0, supplemented with 0.2 mM pyridoxal phosphate, in volumes of about 1 ml. Stirring was provided by a small spinning bar.

Heat sensitivity tests

It was observed that the only protein sensitive to 55°C was the histidinol phosphate aminotransferase (B_1) whereas the two aromatic aminotransferases (A and B_2) and the aspartate aminotransferase (C) were stable at this temperature (Fig. 4). Weigent and Nester [13] reported similar findings for the histidinol phosphate aminotransferase and their aromatic aminotransferase which chromatographically corresponds to form A of our aromatic aminotransferase. Although the heat stability of B_2 and C were tested in a mixture, the stability was confirmed when the two forms were heat treated individually at 55°C, after separation by isoelectric focusing. In fact, form B_2 was activated 2-fold after 7 min. Aspartate aminotransferase (C) was stable up to 4 min and lost about 25% of its activity between 4 and 7 min.

K_m values

The apparent K_m values are reported in Table II. Our K_m values with form B_1 are in good agreement with those reported by Weigent and Nester [13] for histidinol phosphate aminotransferase. However, on the assumption that our form A (Fig. 1) is very similar or identical with the aromatic aminotransferase of Nester and coworkers [12], our K_m values are significantly lower than those reported by Chapman and Nester for this protein [13]. The K_m values with form B_2 are somewhat lower for tyrosine and phenylalanine than the corresponding values with form A (Table II). With aspartate aminotransferase the K_m for aspartate (about 4 mM) is very similar to that previously reported for the *E. coli* enzyme [3]. It was not possible to calculate the kinetic constant for the aromatic amino acids with this enzyme because of the very low recovery of active enzyme after isoelectric focusing (Fig. 3).

Our study revealed the presence in *B. subtilis* of two forms of aromatic-amino-acid aminotransferase (peaks A and B_2, Figs. 1 and 2). The activity ratio, as tyrosine aminotransferase, of form B_2 to form A in crude extracts is estimated to be about 3 : 1 in favor of B_2; form B_2 is, thus, the major form of the enzyme and has not been reported earlier. A third protein (peak B_1, Fig. 2) is

TABLE II

K_m VALUES FOR THE ENZYMIC PROTEINS A, B_1, B_2 AND C

These are averages of two to four determinations. No significant differences were observed among the individual values. Form A was obtained from the experiment of Fig. 1 and similar experiments; form B_1 from the experiment of Fig. 2 and similar experiments; form B_2 and C from the isoelectric focusing experiment of Fig. 3. The K_m values with forms A, B_1 and B_2 were calculated for α-ketoglutarate while L-tyrosine was kept at 6 mM; for L-tyrosine and L-phenylalanine at 10 mM α-ketoglutarate. With aspartate aminotransferase (C) the K_m for each of the two substrates was calculated at the fixed (α-ketoglutarate, 6.4 mM; aspartate, 50 mM) concentration of the other substrate. Pyridoxal phosphate was present in all cases at 38 μM.

Substrate	K_m (mM)			
	A	B_1	B_2	C
α-Ketoglutarate	3.07	8.70	7.77	1.00
L-Tyrosine	0.58	0.59	0.31	—
L-Phenylalanine	0.14	1.70	0.08	—
L-Aspartate	—	—	—	4.2

the histidinol phosphate aminotransferase of the organism which also displays high aminotransferase activity for the aromatic amino acids. However its K_m with phenylalanine is 12—20 times higher than the corresponding values with forms A and B_2 (Table II) thus suggesting that phenylalanine is a relatively poor substrate for this enzyme.

In the context of aromatic amino acid biosynthesis, the aromatic-amino-acid aminotransferase of Weigent and Nester, which appears to be identical with our form A, is considered as a possibly superfluous gene product, in view of its high K_m values with the aromatic amino acids and other considerations [13]. However, K_m values for the amino acids are of little relevance to the physiological significance of the enzyme, since the amino acids are normally products rather than substrates in vivo. It would appear unlikely that both the minor form (A) and the major form (B_2) of the enzyme are irrelevant to the synthesis of aromatic amino acids, in spite of the presence of a histidinol phosphate aminotransferase with aminotransferase activity for these end products.

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