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Purification and Properties of Aromatic Amino Acid Aminotransferase from *Klebsiella aerogenes*†

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We describe the complete purification of aromatic aminotransferase I, the enzyme responsible for the ability of *Klebsiella aerogenes* to use tryptophan and phenylalanine as sole sources of nitrogen, as well as the partial purification of aromatic aminotransferase IV. An examination of the properties of these enzymes revealed that aminotransferase I had much greater affinity for the aromatic amino acids than aminotransferase IV, explaining the essential role of aminotransferase I in the utilization of exogenously supplied aromatic amino acids. The properties of aminotransferase IV suggest that this enzyme is actually an aspartate aminotransferase (EC 2.6.1.1), corresponding to the product of the *aspC* gene of *Escherichia coli*.

In the accompanying paper we demonstrated the presence of four separate aromatic aminotransferases in cell extracts of *Klebsiella aerogenes*, and we showed that the loss of one of these, aminotransferase I, results in the inability of this organism to utilize tryptophan and phenylalanine as sources of nitrogen (11).

In this paper we describe the complete purification of this enzyme and a partial purification of one of the other aromatic aminotransferases. The properties of these aminotransferases explain their roles in the physiology of *K. aerogenes*.

(Some of the results were taken from a thesis submitted by C.G.P. to the Massachusetts Institute of Technology/Woods Hole Oceanographic Institute Joint Program in Biological Oceanography.)

MATERIALS AND METHODS

Chemicals. Common chemicals were of reagent grade and were used without further purification. Other materials were obtained either as described previously (11) or as follows.

Ammonium sulfate (special enzyme grade) and sucrose (special enzyme grade) were obtained from Schwarz/Mann; protamine was obtained from Calbiochem; DEAE-Sephadex A-50 was obtained from Pharmacia; and hydroxylapatite (special DNA grade) was obtained from Bio-Rad Laboratories. The ampholytes used in isoelectric focusing (pH 4 to 6) were manufactured by LKB and were a gift from Joanne Williamson.

Media. The minimal medium used was W salts medium (14) supplemented with 0.4% glucose and a source of nitrogen (0.2%). Biotin was added to a final concentration of 0.2 μg/ml.

Bacterial strains. Strain MK2 of *K. aerogenes* W-70 was used exclusively. This strain has a single large deletion, *hut*511*, which results in a Gal⁻ Bio⁻ Hut⁻ Chl⁻ P1* phenotype (12).

Procedures for the purification of aminotransferase I. HS buffer and Tris-HS buffer were prepared as described previously (11). For use in the elution of samples from hydroxylapatite, a buffer containing low salt concentrations was used. This buffer (VLS buffer) was essentially HS buffer containing 5 mM potassium phosphate and 2.5 mM EDTA. LS buffer was HS buffer containing 10 mM potassium phosphate and 5 mM EDTA.

Two methods of concentration were used at many stages in the purification and analysis of the aminotransferases of *Klebsiella*. The initial method of concentration was ultrafiltration. The ultrafiltration apparatus used was manufactured by Amicon Corp.; PM10 membranes with a molecular weight cutoff of 10,000 were used. If the volume after ultrafiltration was not reduced sufficiently, the sample was dialyzed against polyethylene glycol (molecular weight, 6,000; either solid flakes or a 20% solution in HS buffer containing 20 mM EDTA). All samples dialyzed against polyethylene glycol were then dialyzed against the buffer of choice before the next operation.

Analytical methods. All assays of aminotransferase activity, both in liquid and in native polyacrylamide gels, were performed as previously described (11). Protein was determined by the method of Lowry et al. (8).

Sodium dodecyl sulfate (SDS) gel electrophoresis of samples in 10% polyacrylamide gels was by the method of Laemmli (7). All SDS electrophoreses were performed at 25°C. The molecular weight standards used to calibrate the gel included phosphorylase A, bovine serum albumin, ovalbumin, chymotrypsinogen A, and RNase. Gels were stained for protein with Coomassie blue dye, as described by Davis (2).

The molecular weight standards used for the cali-

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bration of the Ultrogel AcA44 gel filtration column included aldolase, equine hemoglobin, ovalbumin, chymotrypsinogen A, and RNase.

RESULTS

Isolation and purification of aromatic aminotransferase I. Preliminary experiments indicated that aromatic aminotransferase I was not an integral part of the cell membrane of *K. aerogenes*. During the preparation of crude extracts, the membrane and cell pellet formed by centrifugation after disruption of the cells by sonication or by a Hughes press contained less than 3% of the total activity found in the supernatant solution (data not shown).

Preliminary experiments also showed that aminotransferase I was not located in the periplasmic space. The supernatant solution obtained from cell suspensions subjected to an osmotic shock sufficient to produce spheroplasts (10) possessed no detectable aromatic aminotransferase I activity (data not shown).

This enzyme was isolated from cells of strain MK2 grown at 32°C in glucose minimal medium containing proline as the sole source of nitrogen. The cells were harvested during the midexponential phase of growth and frozen until they were used. A summary of the enzyme purification procedure is shown in Table 1. All steps were carried out at 0 to 4°C, unless otherwise stated. The enzyme was purified 250-fold, and there was an overall yield of 11% relative to the bulk amount of aromatic aminotransferase present in the crude extract.

The following procedures allowed the effective separation of aminotransferase I from the other aminotransferase activities found in the cell extracts. Aminotransferases II and III were precipitated by low concentrations of ammonium sulfate (less than 20% saturation, as calculated at 25°C). Aminotransferase IV was eliminated in two steps; roughly one-half of this enzyme was precipitated at 45% saturation (25°C) of ammonium sulfate, and the remaining aminotransferase IV was adsorbed to a DEAE-Sephadex A-50 column more strongly than aminotransferase I and was separated from aminotransferase I during elution (Fig. 1). The peak of activity that eluted at a concentration of salt that corresponded to a conductivity of 15 mS (120 mM) contained only aminotransferase I. A qualitative assay of each aminotransferase was performed by nondenaturing polyacrylamide gel electrophoresis, followed by activity staining as previously described (11).

Samples of the material from each of the fractionation steps after DEAE-Sephadex chromatography were analyzed to determine the aminotransferase I isoactivity band pattern, as described previously (11) (Fig. 2). There are four

TABLE 1. *Purification of aromatic aminotransferase I*

Step	Fraction	Vol (ml)	Total amt of protein (mg)	Sp act (U/mg)	Yield (%)	Relative purity
1	Cell extract[a]	110	2,930	0.34	100	1.0
2	Protamine[b]	150	2,000	0.54	109	1.6
3	Ammonium sulfate[c]	15.5	263	1.75	47	5.1
4	DEAE-Sephadex A-50[d]	3.5	38	10.6	41	31
5	Ultrogel AcA44[e]	1.9	14	25	35	73
6	Hydroxylapatite[f]	2.9	3.4	64	22	188
7	Isoelectric focusing[g]	5.0	1.3	87	11	253

[a] Frozen cell paste was disrupted in a Hughes press at −20°C, allowed to thaw at 4°C, and mixed with 100 ml of HS buffer; nucleic acids were sheared by brief sonication. Cell debris was removed by centrifugation at 27,000 × *g* for 30 min.

[b] Supernatant after addition of 1.2% protamine.

[c] Solid ammonium sulfate was added and material precipitating between 45 and 57% saturation (calculated for 25°C) was collected by centrifugation and dialyzed against two 2-liter portions of Tris-HS buffer.

[d] The material from step 3 was applied to a column of DEAE-Sephadex A-50 (Fig. 1); fractions containing aminotransferase I activity were pooled, concentrated, and dialyzed against Tris-HS buffer.

[e] The material from step 4 was applied to an Ultrogel AcA44 column (2.3 by 100 cm) which was equilibrated with Tris-HS buffer and eluted with Tris-HS buffer; fractions containing activity were pooled, concentrated, and dialyzed against two 2-liter portions of VLS buffer.

[f] The material from step 5 was applied to an hydroxylapatite column (1.6 by 58 cm) which was equilibrated with VLS buffer and eluted with VLS buffer; fractions containing activity were pooled and concentrated.

[g] The materials from step 6 was mixed with ampholytes and applied to a preparative LKB isoelectric focusing column (110 ml); electrofocusing was carried out at 6°C in a manually formed sucrose gradient for 65 h until power consumption stabilized at 0.5 W (15). Fractions containing activity were pooled and applied to an Ultrogel AcA44 column as described above for step 5 to remove ampholytes. Fractions containing activity were pooled and concentrated.

points to be noted. First, it is clear that the purification procedure outlined in Table 1 selectively separated aminotransferase I from the remaining aromatic aminotransferases originally present (data for original extract not shown) (11). Second, the aminotransferase I material from the final stage of purification contained two isoactivity bands. Third, the isoactivity bands exhibited by the purified preparation were not identical to those found after precipitation with ammonium sulfate; there was a change in the pattern of these isoactivity bands, with no obvious concomitant loss of total activity. Fourth, shifts in isoactivity band patterns which tended toward the regeneration of the patterns observed earlier in the purification occurred as late in the purification as the isoelectric focusing step.

The material present in the ampholyte after the isoelectric focusing step produced two major bands of aminotransferase activity when a native sample was fractionated by gel electrophoresis (Fig. 2, lane 9, bands d and g), but only a single protein band (corresponding to a molecular weight of 42,000) when the sample was subjected to electrophoresis in an SDS-polyacrylamide gel.

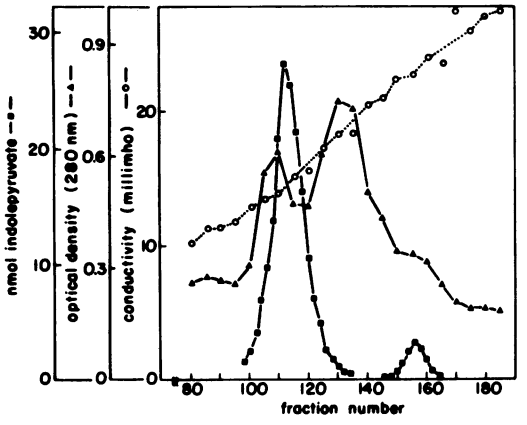

FIG. 1. *Fractionation aromatic aminotransferase by chromatography on DEAE-Sephadex A-50. A sample of the dialyzed (45 to 57%) ammonium sulfate fraction (in Tris-HS buffer, pH 8.0) was applied to a column (3.4 by 23 cm) of DEAE-Sephadex A-50 that had been equilibrated previously with Tris-HS buffer. The column was developed sequentially with the following solutions: (i) 700 ml of Tris-HS buffer and (ii) a linear gradient (1.4 liters) of 0 to 0.5 M sodium chloride in Tris-HS buffer. Fractions (5 ml) were collected at a flow rate of 54 ml/h. Aromatic aminotransferase assays were performed as described in the text. Each reaction mixture contained 5 μl of the fraction to be assayed. Enzyme activity is plotted* (■) *as nanomoles of indolepyruvate formed per minute of incubation. Protein was determined by absorbance at 280 nm* (△). *The position of the gradient was determined by measuring conductivities of selected fractions at 1:100 dilutions* (○).

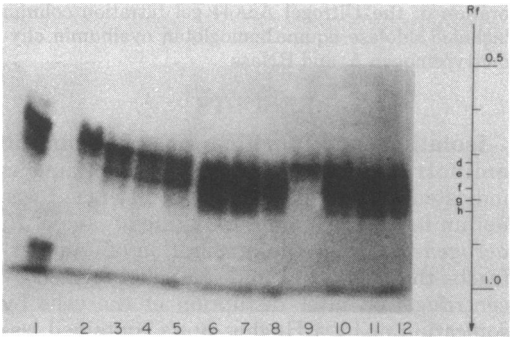

FIG. 2. *Examination of partially purified aminotransferases I and IV by gel electrophoresis. Samples from various steps of the purification procedure described in Table 1 were subjected to electrophoresis and subsequently stained for tryptophan aminotransferase activity (11). The gel was photographed after incubation at 37°C for 45 min. Lane 1, 10 μl of a concentrated pool of fractions containing aminotransferase IV (Fig. 1); lane 2, 4 μl of step 3 fraction (Table 1) (the band corresponding to aromatic aminotransferase IV is too faint to be seen in this photograph); lane 3, 5 μl of fraction 114 (Fig. 1); lane 4, 10 μl of pooled fractions with aminotransferase I activity (Fig. 1); lane 5, 25 μl of a 100-fold-diluted sample of pooled, concentrated fractions having aminotransferase I activity; lane 6, 4 μl of step 5 fraction (Table 1); lane 7, 17 μl of a 100-fold-diluted sample of concentrated material from step 5 fraction (Table 1); lane 8, 40 μl of a 100-fold-diluted sample of concentrated step 6 fraction (Table 1); lane 9, 2 μl of pooled material obtained from the electrofocusing column before removal of ampholytes; lane 10, 20 μl of material from step 7 fraction (Table 1); lanes 11 and 12, 1.5 μl of concentrated step 7 fraction (Table 1). The activity stain in the upper left of the figure is not discussed. The bottom band in all lanes was due to the tracking dye.*

Two isoactivity bands (aminotransferase bands f and h [Fig. 2]) were found in the final purified sample. As estimate of the purity of this preparation of aminotransferase I was made by SDS-polyacrylamide gel electrophoresis. When the purified enzyme was denatured by treatment with SDS in Laemmli sample buffer (7) for 4 min at 100°C and analyzed, a single band was observed, which corresponded to a molecular weight of 42,000. Prolonged treatment of pure aminotransferase I with SDS (overnight incubation at 60°C) resulted in the formation of no new protein bands.

When this sample was analyzed by electrophoresis on nondenaturing gels and stained for protein as described above, the pattern of protein bands observed was identical to the pattern obtained after staining for tryptophan amino-

transferase activity. There appeared to be no other protein bands visible when this sample was run on gels containing 5, 7, and 10.5% acrylamide. This supported the hypothesis that the purification procedure yielded a pure protein which, however, showed two separable activities. We conclude from the data in Fig. 2 and in the accompanying paper (11) that the isoactivity bands of this enzyme arose from a single homogenous protein.

Physical properties of aromatic aminotransferase I. All studies described below were performed with samples of homogeneous aromatic aminotransferase I.

The molecular weight of the native enzyme, as determined by gel filtration on Ultrogel AcA44 in multiple analyses, was 90,000 (6). Differential migration in native polyacrylamide gels of different pore sizes was also used to estimate the native molecular weight of the enzyme (4); this technique yielded a molecular weight in the range of 90,000 to 100,000.

The subunit molecular weight of aminotransferase I, as determined by SDS-polyacrylamide gel electrophoresis, was approximately 42,000. Thus, each native molecule of aromatic aminotransferase I is a dimer composed of two subunits of identical molecular weight. It cannot be concluded that these are identical subunits, in light of the results which showed two activity bands and two protein bands in native gels (Fig. 2).

The isoelectric point of aminotransferase I was pH 4.64.

The activity of aminotransferase I was greatly stabilized during storage at 4°C by the presence of 10% glycerol in all buffers, both in crude extracts and in partially purified preparations. With this addition the enzyme was stable for many months at 4°C in either phosphate or Tris buffer (pH 8.0) containing 0.1 mM dithiothreitol. This stability seemed to be independent of the purity of the protein.

The activity of aminotransferase I was moderately stable to thermal denaturation. When exposed to 55°C (in Tris-HS buffer containing 5 mg of bovine serum albumin per ml), this enzyme lost activity in a linear fashion; 65% of the original activity remained after 10 min.

The optimum pH for aminotransferase I activity was determined in a Tris-glycine-phosphate buffer over the range from pH 5.4 to pH 10.0, using tryptophan as the amino donor. The peak of optimal activity occurred at pH 8.00; however, the peak was broad, and activity dropped to 50% of the maximal value at pH 6.0 and 9.6.

Kinetic parameters of aromatic aminotransferase I. The kinetic parameters of aminotransferase I are summarized in Table 2. At concentrations of phenylalanine greater than 1.0 mM, substrate inhibition occurred. It was on the basis of these data that this enzyme was identified as an aromatic aminotransferase and not purely a tryptophan aminotransferase; the highest affinity of this enzyme was for phenylalanine, and it had very low affinity for histidine.

The K_m for an arylpyruvate as the keto acceptor was determined with glutamate as the amino donor. No estimate of the V_{max} for this couple was made, as the K_m for glutamate was not determined. It was not possible to estimate a K_m for indolepyruvate because of problems with the solubility and stability of this compound.

Evidence for pyridoxal phosphate as cofactor for aminotransferase I. Two observations suggested the presence of pyridoxal phosphate at the active site of aminotransferase I. First, a single band of yellow material was formed during the preparative isoelectric focusing purification step; an analysis of the column fractions revealed that the peak of yellow color corresponded to a peak of both protein and aminotransferase activity. A spectrum of the peak fraction of aminotransferase I activity from the pH gradient showed a small absorption peak at 430 nm, which is consistent with the presence of enzyme-bound pyridoxal phosphate (6).

In addition, purified aminotransferase I was inhibited by 50 μM aminooxyacetate, a known inhibitor of many enzymes whose activities are dependent on the presence of pyridoxal phosphate (5; data not shown).

Aminotransferase IV. The study of aminotransferase IV was simplified by the fact that this enzyme could be obtained free of aminotransferase I activity by chromatography on DEAE-Sephadex A-50. The preparation of aminotransferase IV used in these studies was isolated in the following manner. Fractions contain-

TABLE 2. *Kinetic parameters of aromatic aminotransferase I*

Substrates	K_m (mM)[a]	V_{max} (μmol/min per mg)
2-Ketoglutarate, tryptophan	2.08	82
Oxaloacetate, tryptophan	0.116	932
Tryptophan, 2-ketoglutarate	0.60	79
Phenylalanine, 2-ketoglutarate	0.08	725
Histidine, 2-ketoglutarate	70	82
Phenylpyruvate, glutamate	0.020	ND[b]

[a] The K_m value given is for the first substrate mentioned, as determined with saturating concentrations of the second substrate.

[b] ND, Not determined.

ing aromatic aminotransferase activity from the DEAE-Sephadex column (fractions 150 to 164 [Fig. 1]) were pooled, concentrated, and dialyzed against Tris-HS buffer.

Electrophoresis on polyacrylamide gels revealed the presence of a single band with aminotransferase activity for tryptophan and also for aspartate. Staining for protein allowed us to estimate that this aminotransferase was approximately 20% of the total protein.

The pH optimum for aminotransferase IV activity when tryptophan was the amino donor was 9.0. Activity was reduced approximately one-half at pH 7.5 and 10.2.

The kinetic parameters of aminotransferase IV are summarized in Table 3. The affinity of this enzyme for oxaloacetate was very high; even a concentration of 10 μM produced saturation when tryptophan was used as the amino donor. The product (indolepyruvate) could not be determined accurately below a concentration of 10 μM. Thus, the upper limit for the K_m aminotransferase IV for oxaloacetate is given as 5 μM.

The native molecular weight of this enzyme, as determined by gel filtration with Ultrogel AcA44, was 100,000.

Solutions of aminotransferase IV appeared to be stable when stored at 4°C for approximately 1 month. After this, the enzyme underwent a physical change, which was not reflected in the total aminotransferase activity measured by the borate-arylpyruvate liquid assay. However, the change was visible on native polyacrylamide gels stained for aminotransferase activity. The single band which normally migrated with an R_f of 1.0 in 5% gels became two bands; one band which migrated with an R_f of 1.0 remained, and another band appeared, which migrated with an R_f of 0.95 (Fig. 2, lane 1).

The activity of aminotransferase IV was extremely stable to thermal denaturation at 55°C; samples of aminotransferase IV in Tris-HS buffer containing 5 mg of bovine serum albumin per ml exhibited a 20% increase in activity after incubation for up to 8 min. After that, the activity began to drop slowly, and by 12 min 85% of the activity remained.

TABLE 3. *Kinetic parameters of aromatic aminotransferase IV*

Substrates	K_m (mM)[a]	V_{max} (μmol/min per mg)[b]
2-Ketoglutarate, tryptophan	0.030	ND[c]
Oxaloacetate, tryptophan	<0.005	ND
Tryptophan, 2-ketoglutarate	60	123
Phenylalanine, 2-ketoglutarate	83	132
Histidine, 2-ketoglutarate	121	10
Phenylpyruvate, glutamate	1.6	ND

[a] See Table 2, footnote *a*.

[b] Calculated on the assumption of 20% purity of the enzyme.

[c] ND, Not determined.

DISCUSSION

We have shown in this and the accompanying paper that extracts of *K. aerogenes* contain four distinct aromatic aminotransferases (11). We completely purified the major enzyme, aminotransferase I, and partially purified the second-most-abundant enzyme, aminotransferase IV; we also examined the properties of these enzymes.

Aminotransferase I has a 100-fold-greater affinity for tryptophan and a 1,000-fold-greater affinity for phenylalanine than aminotransferase IV. The great affinities for these aromatic amino acids are appropriate for an enzyme whose function is to catalyze the transfer of the α-amino groups of phenylalanine and tryptophan to 2-ketoglutarate and oxaloacetate. It is this reaction that enables cells to use tryptophan and phenylalanine as sole sources of nitrogen, as shown by our demonstration that a mutation leading to the loss of aminotransferase I results in the loss of this ability (12).

No aminotransferase resembling aminotransferase I in its properties has been found in extracts of *Escherichia coli* K12 (13). This correlates with the inability of this organism to utilize tryptophan and phenylalanine as sources of nitrogen (1). Aminotransferase I can use histidine as an amino donor, although its affinity for this amino acid is very low. An aromatic aminotransferase has been found in extracts of *E. coli* B; however, the formation of this enzyme, in contrast to the formation of aminotransferase I, reportedly required cultivation of the cells in a histidine-containing medium (16, 17).

Aromatic aminotransferase IV is in all likelihood identical to the major aspartate aminotransferase of *K. aerogenes*. These two enzymatic activities were not separated by the procedure that led to the partial purification of aminotransferase IV, nor could they be separated by electrophoresis of cell extracts on polyacrylamide gels (11). Aminotransferase IV enzyme has an extremely high affinity for oxaloacetate and a very low affinity for the aromatic amino acids. It resembles the aspartate aminotransferase of *E. coli* K12 (the product of the *aspC* gene) in being heat stable and in its affinity for phenylalanine (3, 9). The low affinity of this enzyme for the aromatic amino acids precludes it from playing a major role in the utilization of phenylalanine and tryptophan as sources of nitrogen.

Similarly, the low levels of aromatic aminotransferases II and III make it unlikely that these enzymes play any role in the utilization of tryptophan and phenylalanine. One of these enzymes is probably the aromatic aminotransferase responsible for an essential step in tyrosine biosynthesis, corresponding to the product of the *tyrB* gene of *E. coli* (3; unpublished data).

In summary, the results presented here and in the accompanying paper identify the cell components responsible for the ability of *K. aerogenes* to use tryptophan as a source of nitrogen. These components are an uptake system whose formation is activated by nitrogen deficiency and a specific constitutive aromatic aminotransferase with a high affinity for tryptophan and phenylalanine.

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