Multispecific Aspartate and Aromatic Amino Acid Aminotransferases in Escherichia coli*

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SUMMARY

Two aminotransferases from Escherichia coli were purified to homogeneity by the criterion of gel electrophoresis. The first (enzyme A) is active on L-aspartic acid, L-tyrosine, L-phenylalanine, and L-tryptophan; the second (enzyme B) is active on the aromatic amino acids. Enzyme A is identical in substrate specificity with transaminase A and is mainly an aspartate aminotransferase; enzyme B has never been described before and is an aromatic amino acid aminotransferase. The two enzymes are different in the \( V_{\text{max}} \) and \( K_m \) values with their common substrates and pyridoxal phosphate, in heat stability (enzyme A being heat-stable and enzyme B being heat-labile at 55°C) and in pH optima with the amino acid substrates. They are similar in their amino acid composition, each enzyme appears to consist of two subunits, and enzyme B may be converted to enzyme A by controlled proteolysis with subtilisin. The conversion was detected by the generation of new aspartate aminotransferase activity from enzyme B and was further verified by identification by acrylamide gel electrophoresis of the newly formed enzyme A. The two enzymes appear to be products of two genes different in a small, probably terminal, nucleotide sequence.

We have reported recently the extensive purification of transaminase A of Escherichia coli by isoelectric focusing, gel filtration in Sephadex, and acrylamide gel electrophoresis into two forms designated IA and IB with apparent isoelectric points of about 4.55 and 4.60 and molecular weights of 82,000 and 88,000, respectively (1). The two forms catalyzed the transamination of L-tyrosine, L-phenylalanine, L-tryptophan, and, marginally, L-methionine with either \( \alpha \)-ketoglutarate or oxalacetate as co-substrates. Form IA was not affected, but form IB was repressed by growth of the organisms in the presence of tyrosine. An aspartate: \( \alpha \)-ketoglutarate aminotransferase activity coincided with form IA in electrophoresis columns; however, we were not prepared to claim that this activity resided in the same protein with the aromatic activities, especially in view of conflicting reports claiming the nonidentity (2) and identity (3) of the aspartate and aromatic amino acid components. In the present study, forms IA and IB were recognized to represent different enzymes rather than forms of one enzyme and are abbreviated for convenience to enzyme A and enzyme B, respectively.

EXPERIMENTAL PROCEDURE

Materials—Frozen Escherichia coli Crookes (ATCC No. 8739) were obtained from General Biochemicals. The organisms had been grown at \( \phi H \) 6.5 to late log phase in a minimal medium with sodium succinate as the carbon source. Amino acid substrates, pyridoxal phosphate, \( \alpha \)-ketoglutaric acid, purified glutamate dehydrogenase, malate dehydrogenase, and subtilisin Carlsberg type were from Sigma Chemical Co. Acrylamide for electrophoresis was from Eastman Kodak and Ampholine carrier ampholytes. The enzymes were obtained from KLB Produkter AB through Fisher Scientific.

Enzyme Purification—The purification procedure was as described previously (1) and involved the following steps: ammonium sulfate fractionation, heat treatment at 55°C, adsorption on calcium phosphate gel, DEAE-cellulose column chromatography, and isoelectric focusing. The last step also effected the separation of the two enzymes. In the present study isoelectric focusing was performed in both the small (110 ml) and the large (440 ml) columns and the gradient was stabilized with sucrose. The narrow \( \phi H \) range used to achieve the highest possible resolution was obtained by an initial focusing of carrier ampholytes, \( \phi H \) 4 to 6, and subsequent collection of the fractions approximately in the \( \phi H \) range of 4.3 to 4.8. In the final focusing with the enzyme preparation from the previous purification step, the concentration of carrier ampholytes was about 1%. Other details are given in the appropriate legend. Throughout purification enzyme activity was monitored as tyrosine:2-oxoglutarate aminotransferase (EC 2.6.1.5). In one electrophoresis experiment the fractions were assayed also for aminotransferase activity toward the \( l \)-aromatic amino acids and L-aspartic acid. The carrier ampholytes were removed from selected pooled fractions in an Amicon ultrafiltration unit (membrane filter PM-10) by passage of 2 to 3 liters of 0.05 M potassium phosphate buffer, \( \phi H \) 7.0, containing 1 mM EDTA, 1 mM dithiothreitol, 2 mM \( \alpha \)-ketoglutarate, and 0.2 mM pyridoxal phosphate. The enzyme solutions then were concentrated to a volume of 3 to 5 ml and kept at 20°C. No noticeable loss of activity was observed after several weeks. Protein was determined by the method of Lowry et al. (4) during purification. Protein determination in the purified enzyme solutions by adsorption at 280 nm (5) was unreliable in the presence of the substances added to protect against inactivation. For this reason protein was approximately estimated from the amino acid analyses (see below) and the molecular weights, assuming 100% amino acid recovery after acid hydrolysis and an average molecular weight of 110 per amino acid residue.

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Enzyme Assays—Tyrosine-2-oxoglutarate aminotransferase was assayed by the method of Diamondstone (6). The reaction mixture contained in 3.2 ml the following: 0.2 M potassium phosphate buffer (pH 7.3), 6.0 mM L-tyrosine, 9.4 mM α-ketoglutarate, 38 mM NADH, 10 units of malate dehydrogenase, and enzyme. The mixture was incubated at 94° for 10 min and the reaction was stopped with 0.2 ml of 10 N NaOH. After 30 min at room temperature the optical density was read against a control to which NaOH had been added prior to α-ketoglutarate. A molar extinction coefficient of 19,900 M⁻¹ cm⁻¹ was used.

Phenylalanine aminotransferase was assayed as the tyrosine aminotransferase except that tyrosine was replaced by 6 mM phenylalanine and the optical density was read at 315 nm. A molar extinction coefficient of 17,600 M⁻¹ cm⁻¹ was used.

Tryptophan aminotransferase was assayed also as the tyrosine aminotransferase except that tyrosine was replaced by 6 mM L-tryptophan and the optical density was read at 335 nm. A molar extinction coefficient of 6.2 X 10³ M⁻¹ cm⁻¹ was used.

Aspartate aminotransferase was assayed by the method of Karmen (7) by coupling with malate dehydrogenase at 24°. The reaction mixture contained in 3.0 ml the following: 0.1 M potassium phosphate buffer (pH 7.8), 178 mM L-aspartate, 6.4 mM α-ketoglutarate, 38 mM pyridoxal phosphate, 0.24 mM NADH, 10 units of malate dehydrogenase, and enzyme. The reaction was started by adding the enzyme and was followed by the fall of optical density at 340 nm. A molar extinction coefficient of 6.2 X 10³ M⁻¹ cm⁻¹ was used.

One enzyme unit is defined as that amount of enzyme which catalyzes the conversion of 1 μmol of substrate to product per min at the temperature of the assay.

Acrylamide Gel Electrophoresis—The method of Davis (5) was used with an acrylamide concentration of 10% in a Polyacrylamide electrophoresis apparatus (Buchler Instruments) at 1-2°. The gels were prepared in glass tubes (75 or 100 X 5 mm) at 3 ma per gel, with bromophenol blue as the tracking dye. Proteins were visualized with Coomassie brilliant blue R. Aminotransferase activity was detected by incubating the gels in the mixture used by Ryan et al. (9) for 20 to 30 min. Purple rings were formed at the position of transaminase activity. Staining for aspartate aminotransferase was poor compared with the excellent staining obtained for the aromatic aminotransferases, and we prolonged the incubation with aspartate to 45 to 60 min which practice also resulted in much darker backgrounds. Inferior staining in the presence of L-tyrosine, L-phenylalanine, and L-tryptophan and single protein bands with acrylamide concentrations varying from 5 to 11% (not shown). Therefore, by the criterion of gel electrophoresis, the two preparations, A and B, were considered to be pure.

Results

Purification and Resolution of Two Enzymes on Preparative Isoelectric Focusing Column—Purification and resolution by focusing in the small (110 ml) column resulted in partially overlapping bands so that it was possible to obtain only small amounts of enzyme from either band without cross-contamination. In the large (440 ml) preparative column, which could be loaded with 5 to 6 times more enzyme units, superior resolution was obtained and sufficient amounts of enzymes were recovered for kinetic and other experiments. Fig. 1 shows the separation obtained in the large column, and this profile was reproducible. The intermediate activity fractions were rejected and only the fractions of the highest activity from each peak were pooled, dialyzed, and concentrated by ultracentrifugation and tested for protein and enzyme activity bands by gel electrophoresis. Each of the two enzymes gave a single protein band and a single enzyme activity band when stained in the presence of L-tyrosine, L-phenylalanine, and L-tryptophan and single protein bands with acrylamide concentrations varying from 5 to 11% (not shown). Therefore, by the criterion of gel electrophoresis, the two preparations, A and B, were considered to be pure.
were obtained free of each other and foreign proteins. These preparations were used for the study of subunit structure, heat sensitivity tests, kinetic experiments, amino acid composition, and conversion experiments with subtilisin.

**Substrate Specificity of Fractions Resolved by Isoelectric Focusing—**Isoelectric focusing, not shown here, on a small (110 ml) column, resolved an enzyme preparation from DEAE-cellulose column chromatography into two peaks, A and B (enzymes A and B), when the fractions were assayed for transaminase activity with L-tyrosine, L-phenylalanine, and L-tryptophan. Assaying for aspartate aminotransferase resulted in a single peak completely coincident with Peak A. The coincidence of aspartate and aromatic amino acid activities in enzyme A was further confirmed by gel electrophoresis of enzyme A. Staining the gels for protein, aspartate aminotransferase, and a mixture of enzyme A than with enzyme B as indicated by their high B:A ratios. The $K_m$ values for α-ketoglutarate and pyridoxal phosphate were 35- and 40-fold higher with enzyme B than with enzyme A, respectively, indicating significantly higher affinities of the keto acid and the co-factor for enzyme A than for enzyme B (Table I). The lower $K_m$ value of the co-factor with enzyme A is in agreement with its tighter physical binding during dialysis in our experiments for apoenzyme formation. The same relationship, qualitatively, was found in the $K_m$ values of the other keto acid substrate oxaloacetate (Table I).

**Subunit Structure—**Each enzyme on acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate yielded a single protein band which corresponded to a molecular weight of 42,000 to 45,000 (range of five experiments). Since the molecular weights of enzyme A and B were estimated to be about 82,000 and 88,000, respectively (1), each appears to consist of two subunits of equal size. In two experiments, one of which is shown in Fig. 3, we were able to calculate a slightly higher subunit molecular weight for enzyme B (43,000) than for enzyme A (42,000), in

![Fig. 2. Polyacrylamide gel electrophoresis (in tubes, 100 × 5 mm) after isoelectric focusing, dialysis, and concentration of enzyme A. The gels were prerun for 1 hour and then were overlaid with 0.48 μg (left gel) and 1.92 μg (middle and right gels) of enzyme A and run for 4.5 hours at 3 mA per gel. The left gel was stained for protein (Prot.), the middle gel for aspartate aminotransferase, and the right gel for both tyrosine and aspartate aminotransferases.](http://www.jbc.org/)

**Kinetic Experiments—**Table I lists the $K_m$ and $V_{max}$ values and their B:A ratios for enzymes A and B. The $V_{max}$ values clearly indicate that the major activity toward the aromatic amino acids resides in enzyme B, the B:A ratio varying from about 14 to 25. In the presence of equimolar amounts of the two enzymes, a maximum of only about 7% of the total $V_{max}$ toward phenylalanine and only a few percent toward tryptophan and tyrosine would be contributed by enzyme A. On the other hand, the major activity of enzyme A is toward aspartate as attested to by the high $V_{max}$ value for this amino acid compared to the $V_{max}$ values for the aromatic amino acids (Table I). Since aspartate aminotransferase was assayed at 25°C, whereas the aromatic aminotransferases were assayed at 37°C, the relative magnitude of $V_{max}$ for aspartate is actually underestimated in Table I by a factor of about 2 (if about 2.0 be considered the value of temperature coefficient $Q_{10}$ in this instance). The $V_{max}$ values for the two keto acids and pyridoxal phosphate (in the presence of tyrosine as the amino acid substrate) were also higher with enzyme B than with enzyme A as indicated by their high B:A ratios. The $K_m$ values for α-ketoglutarate and pyridoxal phosphate were 35- and 40-fold higher with enzyme B than with enzyme A, respectively, indicating significantly higher affinities of the keto acid and the co-factor for enzyme A than for enzyme B (Table I). The lower $K_m$ value of the co-factor with enzyme A is in agreement with its tighter physical binding during dialysis in our experiments for apoenzyme formation. The same relationship, qualitatively, was found in the $K_m$ values of the other keto acid substrate oxaloacetate (Table I).

![Fig. 3. Estimation of the molecular weights of enzymes A and B by gel electrophoresis in the presence of sodium dodecyl sulfate. The standard proteins used were: 1, bovine serum albumin; 2, ovalbumin; 3, aldolase; 4, chymotrypsinogen A.](http://www.jbc.org/)
keeping with the higher molecular weight of enzyme B. The failure to calculate consistently a higher subunit molecular weight for enzyme B than for enzyme A is due to the short distance traveled by proteins of so similar size in the sodium dodecyl sulfate gels (about 1.7 cm in 5 hours at 8 ma per gel).

**pH Activity Curves**—Fig. 4 illustrates the dependence of activity of the two enzymes on pH. The pH optima for enzyme A with its four substrates vary from 8.0 (toward aspartate) to 9.0 (toward tyrosine) with 8.5 being the pH optimum toward phenylalanine and tryptophan (Fig. 4A). For enzyme B the pH optimum toward tyrosine drops to 8.0 with 7.5 and 8.0 being the optimum toward phenylalanine and tryptophan (Fig. 4B). In view of the physical evidence of homogeneity, the differences in the pH optima of each enzyme with its substrates reflect the differences in the pK values of the substrates rather than the existence of different enzymes.

**Heat Sensitivity Test**—The time courses of activity loss of the two enzymes toward their substrates (tryptophan was not included) at 55° are shown in Fig. 5. Enzyme B rapidly lost activity with tyrosine and phenylalanine as substrates so that, after 10 min, less than 10% of the original activity was left.

![Fig. 4. Dependence of activity on pH of the purified enzymes A and B. The following buffers were used: pH 6.0 to 6.5, citrate-phosphate; pH 7.0 to 9.0, Tris HCl; pH 9.5 to 11.0, glycine-NaOH. The enzymes were assayed with tyrosine (△-△), phenylalanine (△-△), tryptophan (□-□), and enzyme A in addition with aspartate (●-●). The standard abbreviations of amino acids followed by the letter A indicate the respective aminotransferases.](image)

![Fig. 5. Heat stability of the purified enzymes A and B at 55°. Enzyme A was assayed with aspartate (Asp), phenylalanine (Phe), and tyrosine (Tyr) and enzyme B was assayed with phenylalanine and tyrosine.](image)

Enzyme A, however, was relatively heat stable, with 52 and 64% of the original activity remaining after 10 min with tyrosine and phenylalanine, respectively, as substrates. For each enzyme the initial rate of inactivation as well as the final activity loss (especially for enzyme A) was more pronounced with tyrosine than with phenylalanine as substrate and this was verified in another experiment (not shown). More striking was the almost complete absence of inactivation of enzyme A with aspartate as substrate seen against its partial loss of activity with the two aromatic amino acids (Fig. 5). Kinetic evidence (to be reported in detail elsewhere) indicates that of the two ionizing groups at the active site of enzyme A, only one is affected by the binding of aspartate, but both are affected by the binding of tyrosine. The less stringent requirement for aspartate binding most probably accounts for the heat stability of the enzyme versus this substrate. A heat sensitivity test such as ours applied to a crude or semipurified preparation containing the two enzymes would yield a heat-stable aspartate aminotransferase, a more labile phenylalanine aminotransferase, and an even more labile tyrosine aminotransferase and it would be misinterpreted to suggest the existence of one enzyme specific for aspartate and one enzyme (and possibly two) specific for the aromatic amino acids. Such a plot, derived from an experiment with a crude extract, was published recently (2) in support of the nonidentity of the two types of activity.

**Amino Acid Composition**—The amino acid composition of the two enzymes was looked into with the purpose of obtaining some insight into the structure of the two enzymes. Their similarity in size and especially in charge suggested that they might be similar proteins. The data of Table II do indeed indicate that their amino acid compositions are very similar. The significant differences in the number of amino acid residues appear to be limited to glutamic acid, glycine, alanine, valine, and possibly to lysine and phenylalanine. This suggested to us that extensive homology might exist between the two proteins and we were successful in converting enzyme B to enzyme A by controlled proteolysis.

**Conversion of Enzyme B to Enzyme A by Subtilisin Treatment**—Incubation for 30 min of enzyme B at 37° in the presence of

![Table II: Amino acid composition of enzymes A and B](image)

The figures indicate the nearest integral number of amino acid residues per molecule of enzyme. Approximately 40 μg of enzyme A and 18 μg of enzyme B were hydrolyzed.

| Amino acid | Enzyme A | Enzyme B | Difference (enzyme B - enzyme A) |
|------------|----------|----------|---------------------------------|
| Asp        | 86       | 83       | -3                              |
| Thr        | 42       | 30       | -3                              |
| Ser        | 37       | 40       | +3                              |
| Glu        | 89       | 109      | +20                             |
| Pro        | 33       | 39       | +6                              |
| Gly        | 61       | 77       | +16                             |
| Ala        | 87       | 101      | +14                             |
| Val        | 50       | 63       | +13                             |
| Met        | 15       | 14       | -1                              |
| Ile        | 30       | 34       | +4                              |
| Leu        | 74       | 71       | -3                              |
| Tyr        | 12       | 14       | +2                              |
| Phe        | 38       | 30       | -8                              |
| His        | 12       | 14       | +2                              |
| Lys        | 35       | 46       | +11                             |
| Arg        | 43       | 37       | -6                              |
subtilisin; AspA, aspartate aminotransferase; TyrA, tyrosine aminotransferase.

Aliquots (0.5 ml) were withdrawn at the indicated times and assayed for enzyme activities. Control, in the absence of subtilisin; AspA, aspartate aminotransferase; TyrA, tyrosine aminotransferase.

Control, TyrA

FIG. 6. Generation of aspartate aminotransferase activity from enzyme B by limited proteolysis with subtilisin. Enzyme B (1.8 units) was incubated at 37° in 4.0 ml of 0.2 M Tris-HCl buffer, pH 8.0, in the presence of bovine serum albumin (0.3 mg/ml) and 0.2 μg of subtilisin. Aliquots (0.5 ml) were withdrawn at the indicated times and assayed for enzyme activities. Control, in the absence of subtilisin; AspA, aspartate aminotransferase; TyrA, tyrosine aminotransferase.

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Control, TyrA

FIG. 7. Generation of enzyme A (upper band) after limited proteolysis of enzyme B (lower band). Enzyme B was incubated in the presence of subtilisin exactly as described in legend of Fig. 6. Aliquots were withdrawn after 5, 10, and 15 min of incubation and were pooled and the pooled mixtures were subjected to acrylamide gel electrophoresis for 4 hours at 3 ma per gel. The gels then were stained for tyrosine aminotransferase activity. Gel 1, enzyme B (zero time); Gel 2, enzyme B after incubation with subtilisin; Gel 3, mixture of authentic enzymes A and B; Gel 4, enzyme B after incubation in the absence of subtilisin; Gel 5, enzyme B incubated in the presence of subtilisin plus authentic enzyme A. About 0.15 μg of original (before incubation) enzyme B, 1 μg of authentic enzyme A, and 0.3 μg of enzyme B were applied on the gels.

Detection of Enzymes in Fresh Crude Extracts of Other Strains—In addition to detecting the enzymes in the fresh crude extracts of E. coli B by polyacrylamide gel electrophoresis and the staining technique (1) we have clearly detected them by the same technique in the fresh crude extracts of E. coli Crookes and of the tyrosine auxotroph MS83-8 (a gift from Dr. B. D. Davis, Harvard Medical School).1

DISCUSSION

The present study demonstrated differences in the following properties of enzymes A and B: (a) substrate specificity, enzyme A being specific mainly for aspartate and enzyme B for the aromatic amino acids; (b) $K_m$ and $V_{max}$ values (Table I); (c) heat sensitivity with the two aromatic amino acids as substrates

1 Unpublished experiments.
been described before in E. coli and its "dissection" from enzyme A has some important consequences regarding the actual aspartate and aromatic amino acid activities in enzyme A. We propose multispecific aspartate aminotransferase as the recommended name (14) to replace the name transaminase A.

Enzyme B, an aromatic amino acid aminotransferase, has never been described before in E. coli and its "dissection" from enzyme A has some important consequences regarding the actual aminotransferase involved in the regulation of tyrosine and phenylalanine biosyntheses. It has been reported (15) that transaminase A of E. coli is specifically repressed by L-tyrosine and is enzyme B of the present study (1). The enzyme, assayed only as tyrosine and phenylalanine aminotransferases, was found in crude extracts to be heat-labile and to account for most of the activity toward the two aromatic amino acids (15). The heat lability and substrate specificity conformed with the properties of our enzyme B (Table I, Fig. 5). It is now apparent that, by assaying only for aromatic aminotransferase activities, the above investigators (15) were observing in the crude extracts the repression not of transaminase A but of the new enzyme B. They were led to their erroneous conclusion because of the omission to assay for aspartate aminotransferase. Accordingly, references in the literature on the repression by tyrosine of transaminase A assayed as tyrosine aminotransferase (which is the usual practice) reflect the repression of enzyme B, not of transaminase A which is insensitive to repression by tyrosine.

In the same study (15) evidence was presented purporting to show that there exist in E. coli a specific phenylalanine aminotransferase distinct from transaminase A with the following properties studied in crude extracts: (a) relatively heat-stable at 60°C (25 to 30% loss of activity after 10 min) (Fig. 1 of Ref. 15); (b) nonrepressible by tyrosine or any other amino acid; (c) it contributed about 20% of the total activity toward phenylalanine; (d) its resolution from the activity toward tyrosine could not be achieved by ammonium sulfate fractionation and DEAE-cellulose chromatography. These are properties of our enzyme B (transaminase B) which: (a) is relatively heat-stable with phenylalanine (about 35% loss of activity at 55°C, Fig. 5); (b) is not repressed by tyrosine (1); (c) contributes little to the total activity toward phenylalanine (Table I); and (d) was separated with some difficulty from the strictly aromatic activity of enzyme B, with techniques not used or unavailable to the previous investigators (15). It must be emphasized that we are not claiming that a distinct phenylalanine aminotransferase does not exist in E. coli, only that the earlier studies (15), rather than providing evidence for such an enzyme, merely described properties of transaminase A itself against the background of the unsuspected enzyme B. In the absence of rigorous enzymological evidence and in the light of the present data its occurrence in these organisms must remain in doubt. Consequently, and until a specific phenylalanine aminotransferase is unequivocally established, it is enzyme B that appears to be involved in the terminal step of phenylalanine, as well as of tyrosine, biosynthesis.

The conversion of enzyme B to enzyme A by controlled proteolysis in the presence of subtilisin is of interest. The conversion experiments were suggested by, and are in agreement with, the similarity in amino acid composition of the two enzymes. It appears likely that cleavage of a terminal polypeptide tract from each subunit of enzyme B is responsible for the generation of enzyme A under our conditions of limited proteolysis. It is unlikely that the process is of physiological significance. Since enzyme B is selectively repressed by L-tyrosine whereas enzyme A is unaffected (1) the two enzymes are products of two genes different only in a small, probably terminal, nucleotide sequence. The enzymological implications of the conversion are of interest also. It seems clear that cleavage of polypeptide(s) from enzyme B is sufficient to generate a new activity, aspartate aminotransferase, typical of enzyme A. In this respect, the conversion is reminiscent of the long known zymogen-enzyme relationships. The very low aspartate aminotransferase activity of enzyme B noted in Fig. 6 in the absence of subtilisin may mean that masking of the activity in enzyme B is not 100% complete or that enzyme B was slightly contaminated with enzyme A during isolation from the isolotic focusing column. To the best of our knowledge this is the first instance of an enzyme being converted to another distinct, physiologically occurring enzyme by controlled proteolysis.

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