Regulation of Aromatic Amino Acid Biosynthesis in Higher Plants

I. EVIDENCE FOR A REGULATORY FORM OF CHORISMA TE MUTASE IN ETIOLATED MUNG BEAN SEEDLINGS

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ABSTRACT

Etiolated mung bean seedlings were examined for chorismate mutase activity. Evidence for the occurrence of two forms of the enzyme (designated CM-1 and CM-2) was obtained by ammonium sulfate fractionation, anion exchange cellulose chromatography, and isoelectric focusing. The two forms showed distinctly different properties, as CM-1 was inhibited by phenylalanine and tyrosine and activated by tryptophan, but inhibition by phenylalanine and tyrosine was reversed by tryptophan. The other form, CM-2, was unaffected by any of the three aromatic amino acids. Isoelectric points of the two forms were CM-1, pH 4.6, and CM-2, pH 5.6. The molecular weights estimated by molecular sieving on Sephadex G-200 were CM-1, 50,000, and CM-2, 36,000.

MATERIALS AND METHODS

Chorismic acid has been established as an important intermediate in the biosynthesis of the aromatic amino acids phenylalanine, tyrosine, and tryptophan in microorganisms (5–7). By action of chorismate mutase, chorismic acid is converted to prephenate, the precursor of phenylalanine and tyrosine. Chorismate serves as a substrate for anthranilate synthetase, the first reaction in a branch pathway leading to the synthesis of tryptophan. Control of chorismate mutase activity would therefore be of prime importance in the regulation of phenylalanine and tyrosine biosynthesis. Multiple forms of chorismate mutase have been found in fungi, bacteria, and blue green algae. Certain of these forms appear to have regulatory properties, and their roles in regulating production of the two amino acids have been investigated in these microorganisms (2, 3, 8–14, 16, 17). However, aside from a report on inhibitable activity in pea seedling extracts (4), chorismate mutase from higher plants has not been investigated.

The aim of the present investigation was to determine if regulatory forms of chorismate mutase occur in higher plants. This communication presents the results of such studies which show that etiolated mung bean seedlings contain at least two forms of chorismate mutase designated CM-1 and CM-2, one of which shows regulatory properties. A preliminary report of these findings has been made (18).

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Plant Material. Etiolated mung bean seedlings (Phaseolus aureus) were purchased from a local retail grocer. Such seedlings germinated in the laboratory and harvested 2 to 3 days after planting gave the same results as those obtained commercially.

Preparation of Acetone Powders. Whole seedlings were added to a Waring Blender and ground for 1 min with acetone precooled to −10 C. The resulting suspension was put on a Büchner funnel and washed with cold acetone until the filtrate was colorless. The acetone powder was air-dried and then stored in a desiccator at 4 C under nitrogen. Powders stored for 2 weeks retained at least 80% of chorismate mutase activity that was present at the time the powders were prepared. Those stored for 1 month possessed only one-half of their original activity.

Preparation of Pulverized Frozen Material. Etiolated mung bean seedlings were frozen in liquid nitrogen and, while frozen, homogenized to a fine powder in a Waring Blender. At least 60 sec at full speed were required to pulverize completely the frozen material. The powder was stored at −10 C until used for enzyme extraction. Powders stored at −10 C for 1 month retained at least 95% of their original chorismate mutase activity.

Enzyme Preparation. Frozen powder was mixed with 20% weight Polyclar AT and 50 mm potassium phosphate buffer, pH 6.8, 5 g fresh weight of powder per ml of buffer. The powder was allowed to thaw and then left suspended in the extracting medium for 30 min at 0 C. Acetone powders were mixed with cold 50 mm potassium phosphate buffer, pH 6.8, (1 g of powder to 7.5 ml of buffer) and Polyclar AT, 1% w/v, and allowed to sit 10 min. With both preparations, the suspensions were filtered through four layers of cotton gauze, and filtrates were centrifuged at 4 C for 20 min at 37,000g. The resulting supernatant liquids were used either as the source of enzyme or for further purification. The precipitates were discarded since they contained less than 1% of the chorismate mutase activity present in the supernatant liquid.

Ammonium Sulfate Fractionation. Solid (NH₄)₂SO₄ was added with stirring to the enzyme preparation from the preceding step. After addition of the desired amount of (NH₄)₂SO₄, the pH of the resulting solution was readjusted to 6.8 by addition of 1 N KOH, and the solution was stirred for an additional 20 min. The suspension then was centrifuged for 10 min at 20,000g, and the pellet was taken up in one-tenth of the initial volume of the enzyme preparation. The solutions were dialyzed by a membrane dialyzer against 10 mm potassium phosphate buffer, pH 6.8, until the conductivity of the dialysate equaled the conductivity of the dialyzing buffer.
Separation by DEAE-Cellulose. The fraction of the crude extract that precipitated between 20 and 90% saturation with (NH₄)₂SO₄ was used for this procedure. The preparation, 40 ml, in 10 mM potassium phosphate buffer, pH 7.2, with 900 to 920 mg of protein and 2400 to 2500 units of chorismate mutase was applied to a column (2.5 × 25 cm) of DEAE-cellulose (C1-form) that had been equilibrated with 10 mM potassium phosphate buffer, pH 7.2. The column was washed with buffer of the same molarity until the absorbance of the eluate at 280 nm was less than 0.10. The column then was eluted with a linear gradient of 0.05 to 0.4 M potassium phosphate, pH 7.2. In a few experiments, as will be specified in the results, DEAE-cellulose was equilibrated with 10 mM potassium phosphate buffer, pH 6.8. Samples were applied to the column in the same buffer and the elution performed stepwise with 0.01 M, 0.1 M, 0.3 M, and 1.0 M potassium phosphate buffer, pH 6.8. In all cases, a constant flow rate of 60 ml/hr was maintained, and the eluate was collected in 4-ml fractions. Chorismate mutase assays and absorbance measurements at 280 nm were performed on all fractions. A conductivity bridge was used to determine the concentration of the potassium phosphate in the eluted fractions. Fractions with chorismate mutase activity then were pooled and concentrated to 3 to 5 ml by a membrane concentrator (BioMed Instruments, Chicago, Ill.) for storage.

Isoelectric Focusing. Isoelectric focusing was carried out by the procedure of Vesterberg et al. (15), with an LKB Model 8101 jacketed column (110 ml capacity). A stabilizing linear sucrose density gradient was formed step-wise as described in the LKB instruction manual. The final average ampholyte (Ampholine carrier ampholyte, LKB) concentration was 2%. The enzyme preparation, 300 to 500 units, specific activity, 30 units/mg, in a solution containing 2% ampholyte was substituted for an equal volume of the “light” ampholyte solution used to prepare the stabilizing density gradient. Electrofocusing was carried out at 4 C for 72 hr with a maximum power output of 2 w. The voltage was increased over a period of 12 hr to a maximum of 600 v. Initial focusing of each isozyme was conducted using a pH 3 to 10 gradient followed by runs at pH 3 to 5 for CM-1 and pH 5 to 7 for CM-2.

Fractions of 2.0 ml were collected and analyzed for protein (absorbance at 280 nm), pH, and chorismate mutase activity. The ampholyte present in the enzyme preparations did not affect the enzyme assay. CM-1 and CM-2 preparations first were separated by ammonium sulfate fractionation and then by DEAE-cellulose chromatography prior to electrofocusing.

Molecular Weight Determinations by Gel Filtration. Determinations were made following the procedure of Andrews (1) on a reverse-flow column (2.5 × 35 cm) of Sephadex G-200 that had been equilibrated with 50 mM potassium phosphate buffer, pH 6.8. Column calibrations were made with gamma globulin, bovine serum albumin, ovalbumin, and chymotrypsinogen A. One-milliliter samples of marker proteins or enzyme preparations (100 units, specific activity 4.0) in 50 mM potassium phosphate buffer, pH 6.8, were applied to the column. Elutions were carried out with the same buffer at a flow rate of 9 ml/hr and a pressure head of 7 cm. Fractions, 1.5 ml, were collected and assayed for absorbance at 280 nm when marker proteins were eluted. Assays for chorismate mutase activity and absorbance measurements at 280 nm were performed on all fractions when enzyme preparations were eluted.

Enzyme Assay Procedure. Chorismate mutase was assayed by a procedure similar to that described by Nester and Jensen (10). Potassium phosphate buffer, pH 6.8, 30 μmole, enzyme preparation, and chorismate, 0.6 μmole in a total volume of 0.6 mL, were incubated for 30 min at 25 C. The reaction was terminated by addition of 0.2 ml of 20% trichloroacetic acid. The precipitated protein was collected by centrifugation for 15 min at 4500 g and saved for the protein analysis described below. The supernatant liquid contained phenylpyruvic acid which was formed from prephenic acid by acidification of the reaction mixture. An aliquot (0.5 ml) of the supernatant liquid then was mixed with 1.5 ml of 2 M sodium arsenate and 1 M sodium borate buffer, pH 6.5. The absorbance at 300 nm was read following a 15-min incubation period at room temperature. The amount of prephenate was calculated by using 9292 as the molar extinction coefficient for the phenylpyruvate-borate complex (9). The reaction rate was constant for 20 min after the start of the reaction and proportional to enzyme preparation concentration under the assay conditions employed. A unit of chorismate mutase activity is defined as the amount of enzyme that catalyzes the formation of one μmole of prephenate/min under the above assay conditions. Specific activity is expressed as units of chorismate mutase activity/mg of protein. For inhibition and activation studies, the chorismate concentration in reaction mixtures was 0.25 mM. Protein in enzyme preparations was determined by the biuret method.

Chemicals. Barium chorismate and chorismic acid were prepared by the method of Gibson (6). Barium chorismate was converted to potassium chorismate by dissolving the barium salt in 50 mM potassium phosphate, pH 6.8, and collecting the precipitate of barium phosphate by centrifugation at 4500 g. The supernatant liquid was used for the source of chorismate. The concentration of chorismate in the supernatant solution was determined from the absorbance at 272 nm (ε₉₀₀ = 2700) (6).

Chromatographic materials were prepared according to the manufacturers’ specifications and were obtained from the following sources: Sephadex G-200, Pharmacia; DEAE-cellulose, Sigma Chemical Co. Polyvinylpyrrolidone (Polyclar AT, industrial grade, GAF Corporation) was washed with water until freed of unidentified water soluble materials that interfered with the chorismate mutase assay. Bovine serum albumin, ovalbumin, gamma globulin, and chymotrypsinogen A were purchased from Mann Research Laboratories. All other chemicals were purchased from commercial sources in the highest grades available and used without further purification.

RESULTS

Separation of Multiple Forms. Crude extracts prepared from acetone powders possessed chorismate mutase activity with a

| Compounds Added                      | Relative Activity | Acetone powder | Frozen powder |
|--------------------------------------|-------------------|----------------|---------------|
| None                                 | 100              | 100            | 100           |
| L-Phenylalanine                      | 30               | 40             | 40            |
| L-Tyrosine                           | 30               | 30             | 30            |
| L-Tryptophan                         | 150              | 250            | 250           |
| L-Phenylalanine, L-tyrosine          | 30               | 30             | 30            |
| L-Phenylalanine, L-tyrosine, L-tryptophan | 140        | 240            | 240           |

1 A relative activity of 100 is assigned to the rate of prephenate formation in the absence of the amino acid which was 10 μmoles per min per ml of enzyme preparation.
specific activity of 2 to 3. The activity was partially inhibited by L-phenylalanine and L-tyrosine, and activated by L-tryptophan (Table I). In addition, L-tryptophan reversed the inhibition by L-phenylalanine and L-tyrosine. Quantitatively similar results were obtained with preparations from frozen powders (Table I).

By ammonium sulfate fractionation chorismate mutase activity in crude extracts was resolved into at least two fractions (Table II). With preparations from acetone powders, one fraction (CM-1) precipitated between 30 and 50% saturation with ammonium sulfate. Chorismate mutase activity in this fraction was inhibited by L-phenylalanine and L-tyrosine and was activated by L-tryptophan. The other fraction (CM-2) precipitated between 60 and 70% saturation with ammonium sulfate. Chorismate mutase activity in this fraction was insensitive to the three aromatic amino acids. To lessen the possibility that the two fractions resulted from artifact production during acetone powder preparation, the same ammonium sulfate fractionations were carried out with preparations from frozen powders. Similar results were obtained using frozen sulfate powders although separation of the two active fractions was observed at slightly different concentrations of ammonium sulfate (Table II).

By gradient elution from DEAE-cellulose, chorismate mutase activity in crude extracts of acetone powders or frozen powders was separated into two fractions. Figure 1 shows the results of such an experiment with frozen powder extracts (20-90% ammonium sulfate fraction). Chorismate mutase activity in peak II was inhibited by phenylalanine and tyrosine and activated by tryptophan. The activity in peak I was insensitive to these amino acids. Of the 2400 units of chorismate mutase activity initially present in the frozen powder extract, 850 units were recovered in peak I and 950 units in peak II.

Since chorismate mutase activity in peak II exhibited proper

### Table II. Ammonium Sulfate Fractionation of Chorismate Mutase Activity from Preparations of Etiolated Mung Bean Seedlings

Methods of fractionation and assay as well as concentration of reactants and amino acids are detailed under “Materials and Methods.” Approximately 75 ± 5% of the total units present in the crude extracts were recovered after ammonium sulfate fractionation. Total units g dry weight were equal either from acetone preparation or from frozen powder. Dry matter yield from the acetone powder was 5 g powder per 100 g fresh weight of tissue.

| Ammonium Sulfate Fractions | Specific Activity | Total Units Recovered | Inhibition by Phenylalanine | Activation by Tryptophan |
|-----------------------------|-------------------|-----------------------|-----------------------------|--------------------------|
|                            | Acetone powders    | Frozen powders        | Acetone powders | Frozen powders | Acetone powders | Frozen powders | Acetone powders | Frozen powders |
| % saturation                | units/mg²          | %                     | units/mg² | % | units/mg² | % | units/mg² | % | units/mg² | % |
| 0-30                        | 0.0               | 1.4                   | 0.0      | 15 |          |     |          |     |          |     |
| 30-40                       | 2.5               | 2.7                   | 80       | 33 |          |     |          |     |          |     |
| 40-50                       | 0.6               | 0.0                   | 4        | 16 |          |     |          |     |          |     |
| 50-60                       | 1.3               | 6.6                   | 17       | 35 |          |     |          |     |          |     |

1 Specific activity is reported in units of activity per mg protein.

2 A 30-50% ammonium sulfate fraction was taken in this experiment.

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**Fig. 1.** DEAE-cellulose chromatography of a crude chorismate mutase preparation (20-90% ammonium sulfate fraction). The preparation, 40 ml, in 10 ml potassium phosphate buffer, pH 7.2, with 900 to 920 mg of protein and 2400 to 2500 units of chorismate mutase activity was applied to a DEAE-cellulose column (2.5 x 30 cm) equilibrated with the same buffer. Elution was accomplished with a linear gradient of 0.05 to 0.40 M potassium phosphate, pH 7.2. The eluate was collected in 4.0-ml fractions and assayed for chorismate mutase activity (▲), absorbance at 280 nm (●), and potassium phosphate concentration (● ● ●).
ties similar to those in the CM-1 preparation (Table II), a sample of the latter prepared by ammonium sulfate precipitation of acetone powder extracts was applied to a DEAE-cellulose column. Chorismate mutase activity from the CM-1 preparation eluted in the same position as peak II from the crude enzyme preparation (Table III). Chorismate mutase activity in both preparations showed identical sensitivity to the two amino acids, thus indicating that the two preparations contained the same molecular form of chorismate mutase. Similarly, a CM-2 preparation when applied to a DEAE-cellulose column was found to be chromatographically indistinguishable from the peak I eluate. Rechromatography of the preparations separated by DEAE-cellulose yielded only that form which was initially applied to the column.

**Behavior on Isoelectric Focusing Column.** Isoelectric points of chorismate mutase as well as concentration of reactants are described under “Materials and Methods.”

### Table III. DEAE-Cellulose Chromatographic Separation of Chorismate Mutase Activity from Preparations of Etiolated Mung Bean Seedlings

| Phosphate Buffer | Preparation Tested |
|------------------|--------------------|
|                  | Crude extract | 30-40% (NH4)2SO4 fraction (CM-1) | 60-70% (NH4)2SO4 fraction (CM-2) |
| mM               | units/mg protein | units/mg protein | units/mg protein |
| 0.01             | 1.3              | 0.0              | 3.5              |
| 0.10             | 0.0              | 0.0              | 0.0              |
| 0.30             | 3.2              | 8.1              | 0.0              |
| 1.00             | 0.0              | 0.0              | 0.0              |

**Fig. 2.** Plot of elution volume, Vₑ, against molecular weight from gel filtration studies with molecular weight marker proteins and chorismate mutase using Sephadex G-200. The system was run in reverse flow configuration using a 2.5 × 35-cm column equilibrated with 50 mM potassium phosphate buffer, pH 6.8. Details of the chromatographic procedure are described under “Materials and Methods.”

**Fig. 3.** Effect of chorismate concentration on chorismate mutase activity of the CM-1 preparation in the presence and absence of L-tryptophan. The enzyme source was a fraction prepared by DEAE-cellulose chromatography. Activity is expressed in μmoles of phenylpyruvate formed/min. L-Tryptophan concentrations were: ●: none; ○: 0.1 mM.

**Fig. 4.** Effect of chorismate concentration on the chorismate mutase activity of CM-2. The source of the enzyme was a preparation from DEAE-cellulose chromatography. Activity is expressed as μmoles of phenylpyruvate formed/min. The inset shows a Lineweaver-Burk plot of the data.

were determined to be pH 4.6 for CM-1 and pH 5.6 for CM-2. Recoveries of chorismate mutase activity by this procedure were greater than 90% for both preparations.

**Effect of Substrate Concentration on Enzyme Activity.** A plot of chorismate mutase activity as a function of chorismate concentration yielded a sigmoid curve with the CM-1 preparation as the enzyme source (Fig. 3). In the presence of 0.1 mM L-tryptophan a parabolic curve was obtained (Fig. 3). The substrate saturation curve for the enzyme in the CM-2 preparation was parabolic and remained unchanged in the presence of L-tryptophan (Fig. 4). The Kₘ value for the enzyme in the CM-2 preparation was 0.33 mM.

**Effect of Various Compounds on Enzyme Activity.** Effects of L-tyrosine and L-phenylalanine on chorismate mutase activity in the CM-1 preparation are shown in Figure 5. Inhibition was over 90% with either 0.1 mM L-tyrosine or 0.1 mM L-phenylala-
nine. As noted earlier, L-tryptophan had an activating effect on CM-1 preparations, which was evident even at concentrations of 10 μM (Fig. 6). In addition, no inhibitory effects of 1 mM L-tyrosine or L-phenylalanine were observed in the presence of 1 mM L-tryptophan using CM-1 as the enzyme source. The activity of CM-2 preparations was unaffected by the presence of aromatic amino acids when tested singularly or in combination at concentrations of 0.01 to 1.0 mM. Other compounds tested at 1 mM and found to be without effect on both CM-1 and CM-2 included D-tryptophan, D-tyrosine, D-phenylalanine, p-aminobenzoic acid, p-hydroxybenzoic acid, L-serine, and indole-3-acetic acid.

Stability. CM-1 preparations separated from acetone powders by ammonium sulfate or by DEAE-chromatography lost as much as 70% activity in 24 hr at 4°C or -20°C. CM-1 activity in preparations from extracts of freeze-ground material, however, was stable for at least 21 days at -10°C in solutions with protein concentrations as low as 1 mg/ml. CM-2 preparations that originated from either source lost no more than 5% of their initial activity after storage at 4°C for 5 days or 60 days at -20°C when stored with protein at 10 mg/ml. During storage at 0°C or -20°C, CM-1 preparations from acetone powders rapidly lost sensitivity to activation by L-tryptophan, but generally did not lose sensitivity to inhibition by L-phenylalanine or L-tyrosine. Those prepared from frozen powder extracts, however, stored for up to 7 days at 0°C or -20°C did not lose sensitivity to activation by L-tryptophan nor sensitivity to inhibition by L-phenylalanine or L-tyrosine.

DISCUSSION

Regulatory patterns of chorismate mutase differ in the microorganisms that have been investigated so far. In *Aerobacter aerogenes*, *Salmonella typhimurium*, and *Escherichia coli*, two forms of chorismate mutase have been found (3, 12). One form is inhibited and repressed by phenylalanine and occurs in an aggregate with prephenate dehydratase. The other form is repressed by tyrosine and occurs aggregated with prephenate dehydrogenase. *Bacillus subtilis* possesses three isoenzymes of chorismate mutase, none of which is affected by the aromatic amino acids but two of which are inhibited by prephenic acid (9). Furthermore, one of the chorismate mutase multiple forms exist in an aggregate with the first enzyme of the shikimic acid pathway, 3-deoxy-d-arabinoheptulosonic acid-7-P synthetase. *Neurospora crassa* (2), *Euglena gracilis* (17), and several green algae (16) were reported to contain a single form of chorismate mutase that is inhibited by phenylalanine and by tyrosine and activated by tryptophan. In contrast, the enzyme from the blue-green alga, *Anacystis nidulans*, did not exhibit *in vitro* regulation by any of the three aromatic amino acids. Preparations from *Claviceps paspali* were chromatographically separated into two distinct fractions with chorismate mutase activity (14). One fraction showed activation by L-tryptophan and inhibition by L-tyrosine and L-phenylalanine. The second fraction showed activation with L-tryptophan but was insensitive to phenylalanine and tyrosine. *Saccharomyces cerevisiae* appears to contain only one form of chorismate mutase. It is inhibited by tyrosine, activated by tryptophan, and unaffected by phenylalanine (8, 13).

More pertinent to our studies is the work of Cotton and Gibson (4), who reported that pea seedlings contain chorismate mutase activity that was inhibited 70% by 4 mM phenylalanine or tyrosine and activated 3-fold by 0.12 mM DL-tryptophan. After chromatography of the pea seedling extract on DEAE-cellulose, only one fraction of chorismate mutase activity was detected. It accounted for 17% of the activity applied to the column and was sensitive to inhibition by phenylalanine and tyrosine and activated by tryptophan.

Our studies indicate that etiolated mung bean seedlings contain at least two forms of chorismate mutase. They do not appear to be artifacts of the extraction procedure because (a) the amounts of chorismate mutase activities in the CM-1 and CM-2 fractions are relatively constant from preparation to preparation, (b) preparations of CM-1 or CM-2 by DEAE-cellulose chromatography yield detectable amounts of only one form of chorismate mutase, and (c) two different extraction procedures (acetone powder and frozen powder) yield the two forms.

Phenylalanine and tyrosine act as feedback inhibitors of CM-1 and the inhibition by either compound is reversed by tryptophan. L-Tryptophan is an activator for CM-1. Thus, CM-1 appears to help regulate phenylalanine and tyrosine biosynthesis in etiolated mung bean seedlings. It is interesting to
note that the CM-1 form of chorismate mutase in plants resembles the chorismate mutase found in green algae, Neurospora, Claviceps, and Euglena.

Although none of the compounds tested serve as effectors for the CM-2 enzyme, we have by no means exhausted the list of other compounds that should be tested. Additional investigations on the enzyme should clarify its role in the synthesis of the aromatic amino acids. To date, we have purified it over 1000-fold and are investigating its properties (J. H. Hill, D. G. Gilchrist, and T. Kosuge, unpublished observations). Such investigations and additional studies on the intracellular distribution of the two forms of chorismate mutase in mung beans might shed further light on their respective roles in aromatic amino acid synthesis.

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