Multiple Molecular Forms of Chorismate Mutase in *Bacillus subtillis**

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ABSTRACT: Two genetically related strains of *Bacillus subtilis* differ in the number of molecular forms of chorismate mutase (CM) they express. Strain 23 and its derivatives have three distinct species separable on DEAE-cellulose, termed CM_1, CM_2, and CM_3. Strain 168 has only the CM_3 form. The locus concerned with CM_3 synthesis has been separated from the locus (*i*) concerned with CM_1 and CM_2 synthesis by genetic recombination. The present data are most compatible with only one locus being concerned with CM_1 and CM_2 formation. Since rechromatography of the CM_2 species (mol wt 140,000) on DEAE-cellulose results in the appearance of the CM_1 moiety (mol wt 75,000), CM_1 and CM_2 may represent different aggregational states involving at least one common subunit. CM_1 and CM_3 differ markedly in a number of physical properties, including molecular weight, heat stability, substrate affinity, and chromatographic properties. Neither CM_1 nor CM_3 activity is inhibited *in vitro* by the aromatic amino acids. The significance of these multiple forms to the biosynthesis of aromatic amino acids is discussed.

The aromatic acid biosynthetic pathway in *Bacillus subtilis* is in part controlled by sequential feedback inhibition. Phenylalanine and tyrosine inhibit prephenate dehydratase and prephenate dehydrogenase, respectively, and tryptophan inhibits anthranilate synthetase activity (Nester and Jensen, 1966) (Figure 1). In turn, the chorismic acid and prephenic acid which accumulate when these enzymes are inhibited, inhibit DAHP[1] synthetase, the first enzyme specific to aromatic acid synthesis (Jensen and Nester, 1965; Jensen and Nester, 1966b). Since at equivalent concentrations prephenic acid inhibits eightfold more than chorismic acid, the control of the synthesis and activity of chorismate mutase, the enzyme converting chorismic to prephenic acid, may regulate the entrance of low molecular weight metabolites into the aromatic amino acid pathway. In addition, since it is the branch point enzyme for tyrosine and phenylalanine biosynthesis, its efficient control is important for the proper routing of chorismic acid into the pathways of tyrosine, phenylalanine, and tryptophan synthesis.

Cotton and Gibson (1965) as well as Pittard and Wallace (1966) demonstrated that *Escherichia coli* and *Aerobacter aerogenes* each possess two molecular species mediating this reaction. One species is complexed with the succeeding enzyme of tyrosine synthesis, prephenate dehydrogenase, and the other form occurs in association with the first enzyme specifically involved in phenylalanine synthesis, prephenate dehydratase. Tyrosine shunts chorismic acid to the tryptophan and phenylalanine pathways by repressing the synthesis and inhibiting the activity of the complex concerned with tyrosine synthesis. Phenylalanine controls the enzyme complex concerned with its synthesis in essentially the same way. In *Neurospora crassa*, tryptophan activates chorismate mutase (Baker, 1966) and inhibits anthranilate synthetase (Lester, 1963; DeMoss, 1965), thereby favoring the synthesis of prephenic acid.

Biochemical and genetic studies of chorismate mutase activity in *B. subtilis* have revealed the presence of multiple enzyme species. However, there is no evidence for association of these molecular forms with the succeeding enzymes of tyrosine and phenylalanine synthesis. A detailed analysis of this system and the biological significance of each of these enzyme species are the subjects of this and the following paper (Nester *et al.*, 1967).

Materials and Methods

Chemicals. All commercially available metabolites of the aromatic amino acid pathway used in this investigation were obtained from Calbiochem. The sources of other chemicals used were protamine sulfate (Calbiochem), DEAE-cellulose (type 20) (Carl Schlei-

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[1] Abbreviations used: DAHP, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate; DHQ, 5-dehydroquinic acid; CM, chorismate mutase; Aro supplementation, phenylalanine, tyrosine, tryptophan, *p*-aminobenzoic acid (PABA), and *p*-hydroxybenzoic acid (POBA); Shk, shikimic acid; PPA, prephenic acid; TSY, trypticase soy broth (BBL) supplemented with 10 g of yeast extract/l.

TABLE I: Description of Strains of *B. subtilis*.

Strain	Genotype	Relevant Enzyme Defect	Sp Act. of Chorismate Mutase[a]	CM Species Expressed
23	Trp^-		High	CM_1^+, CM_3^+
SB 140	His_2^-, Shk^-	DHQ synthetase	Low	CM_3^+
SB 167	Shk^-	DHQ synthetase	Low	CM_3^+
168	Trp_2^-	Indoleglycerol phosphate synthetase	Low	CM_3^+
WB 672	Prototroph (spontaneous revertant 23)		High	CM_1^+, CM_3^+
WB 740	Strain 168 derivative; thienylalanine^r		Low	CM_3^+
WB 746	Strain 168 revertant		Low	CM_3^+
WB 932	Aro^-	CM_1, CM_2, CM_3, DAHP synthetase	None	O
WB 1036	Shk^-	DHQ synthetase	High	CM_1^+, CM_2^+, CM_3^+
WB 2102	Shk^-	CM_3,DAHP synthetase	High	CM_1^+, CM_2^+
WB 2107	Prototroph; derivative of 672 thienylalanine^r		High	CM_1^+, CM_3^+
WB 2130	Prototroph (strain 168 transformant)		High	CM_1^+, CM_3^+

[a] A low specific activity ranges from 1 to 7; high specific activity from 30 to 60, depending on supplementation of the growth medium. Strains of genotype Shk^- respond to shikimic acid. Strains of genotype Aro^- respond to tryptophan, phenylalanine, and tyrosine, but not shikimic acid.

cher & Schuell Co.), Sephadex (Pharmacia) (Uppsala, Sweden), lyophilized catalase (Nutritional Biochemical Corp.), cytochrome C (type V) (Sigma Chemical Corp.), bovine γ-globulin (The Armour Lab), human hemoglobin (two times crystallized) (Pentex, Inc.), alkaline phosphatase (*E. coli*, salt fractionated) (Worthington Biochemical Corp.), lysozyme (Worthington Biochemical Corp.), crystallized bovine plasma albumin (Armour Pharmaceutical Corp.), *N*-methyl-*N'*-nitrosoguanidine (Aldrich Chemical Co.), and α-ketophenylalanine (sodium salt) (Cyclo Chemical Corp.).

Chorismic acid was isolated from culture supernatants of *A. aerogenes* (strain 62-1) according to the method of Gibson (1964) as modified by Edwards and Jackman (1965). Our preparations ranged from 92 to 100% pure as calculated from an extinction coefficient of 2440 at 274 mμ (F. Gibson, personal communication). The amount of chorismic acid used as substrate in enzyme assays was corrected for its purity.

Bacterial Strains. Table I describes the strains of *B. subtilis* used in this investigation. Auxotrophic mutants were obtained following ultraviolet irradiation or *N*-methyl-*N'*-nitrosoguanidine treatment (Mandell and Greenberg, 1960; Adelberg *et al.*, 1965). Cells were grown to the late-log phase in 5 ml of TSY, centrifuged, washed once in a modified Spizizen's minimal medium (Spizizen, 1958) (lacking phosphate, citrate, and glucose, and containing 0.05 M Tris, pH 7.4), and resuspended in 5 ml of the same medium. After 15-min incubation on the rotary shaker at 37°, *N*-methyl-*N'*-nitrosoguanidine (final concentration, 200 μg/ml) was added and the cells were allowed to incubate with aeration at 37° for 30 min. The culture was then centrifuged, washed once in Spizizen's medium plus 0.5% glucose, and resuspended in Spizizen's medium plus glycerol (5%). The cells were plated on minimal medium (Davis and Mingioli, 1950) supplemented with limiting amounts of the aromatic amino acids, phenylalanine and tyrosine (0.5 μg/ml each), tryptophan, PABA and POBA (0.1 μg/ml), and shikimic acid (0.5 μg/ml) at a dilution to give approximately 200 colonies/plate. After incubation for 18 hr at 45°, small colonies were picked with sterile toothpicks to nutrient agar plates and mutants were selected by replica plating to various singly and multiply supplemented plates.

Genetic markers were routinely introduced into the desired genetic backgrounds through DNA-mediated transformation essentially by the procedure of Nester *et al.* (1963). Since competent cells very often take up and integrate several molecules of DNA, it was convenient to select for prototrophic transformants at one locus, and examine these for their acquisition of the desired auxotrophic marker at another locus. The desired recombinant class could then be identified by replica plating to a selective medium lacking the nutrient. When testing for the linkage of two genetic

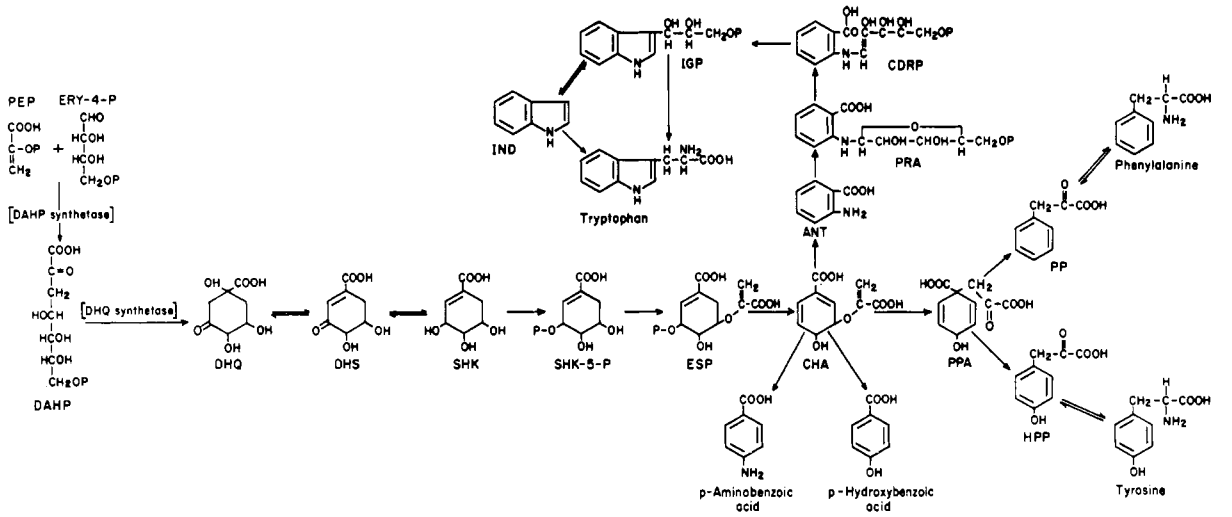

FIGURE 1: Aromatic amino acid biosynthetic pathway. The following abbreviations are used: ERY-4-P, D-erythrose-4-phosphate; PEP, phosphoenol pyruvate; DAHP, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate; DHQ, 5-dehydroquinic acid; DHS, 5-dehydroshikimic acid; Shk, shikimic acid; Shk-5-P, shikimic acid 5-phosphate; ESP, 3-enol pyruvylshikimic acid 5-phosphate; CHA, chorismic acid; CM, chorismate mutase; ANT, anthranilic acid; PRA, *N*-(5′-phosphoribosyl)anthranilic acid; CDRP, 1-(*O*-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate; IGP, indoleglycerol 3-phosphate; IND, indole; PPA, prephenic acid; PP, phenylpyruvic acid; HPP, *p*-hydroxyphenylpyruvic acid.

loci, limiting concentrations of donor DNA were used to eliminate simultaneous transformation by several pieces of DNA.

Preparation of Extracts. Cultures were routinely grown, beginning with a small inoculum, in a liter flask containing 200 ml of Spizizen's minimal salts medium with 0.5% glucose and appropriate supplements when required (shikimic acid, 100 μg/ml; all aromatic supplements 20 μg/ml, except where otherwise noted). When larger quantities of cells were required, a 2800-ml Fernbach flask containing 800 ml of Spizizen's medium was used. Cultures were vigorously aerated at 37° on a rotary shaker and cells harvested during the log or late-log phase of growth before any lysis of the cells was visible. Cells were centrifuged, washed once with Spizizen's medium, and extracts were either prepared immediately or the pellet was frozen and stored at −20° until use.

Lysozyme extracts were prepared unless otherwise noted by lysing the cells at 44° according to the procedure described by Nester and Jensen (1966). Extracts were prepared in the French pressure cell by resuspending the washed cells in glycine buffer (pH 8.9) and freezing the concentrated suspension in a Dry Ice–ethanol bath. The frozen pellet was then introduced into the chamber of the cell and subjected to 15,000 psi. The extruded cell extract was then centrifuged at 0° for 30 min at 25,000*g*. Nucleic acids were routinely removed prior to chromatography on DEAE-cellulose and Sephadex or centrifugation in a sucrose gradient. Approximately 0.1 ml of a 2% solution of protamine sulfate was added for every 10 mg of protein in the crude extract and after allowing the solution to stand at 0° for 15 min, the precipitate was removed by centrifugation at 25,000*g* for 30 min. There was no further purification of the extracts unless otherwise noted.

Protein Assay. Protein concentration was determined by the method of Lowry *et al.* (1951) using crystallized bovine plasma albumin as a standard.

Enzyme Assays. 1. CHORISMATE MUTASE. The reaction mixture (0.5 ml) contained 0.1 ml of chorismic acid (2 μmoles/ml), 0.1 ml of enzyme, and 0.3 ml of buffer, either 0.05 M glycine (pH 8.9) (300 μmoles) for high-activity strains or 0.05 M Tris-maleate (pH 6.4) (300 μmoles) for low-activity strains. Enzyme activities are optimal at these pH values for the high- and low-activity strains, respectively. When strains of unknown activity were assayed, the 0.05 M glycine buffer (pH 8.9) was used. Chorismic acid solutions were prepared fresh each day at 10 μmoles/ml in the assay buffer. After 30-min incubation in a 37° water bath, the reaction was terminated with 0.15 ml of 20% trichloroacetic acid and the tubes were then centrifuged for 15 min in a swinging-bucket centrifuge (International) at room temperature. The supernatant solution (0.25 ml) was added to 1.5 ml of 2 M arsenate–1 M borate solution (pH 6.5) (Lin *et al.*, 1958). The optical density was measured with a Beckman DU spectrophotometer at 300 mμ after the tubes were mixed and allowed to stand at room temperature for 15 min. Under these conditions, enzyme activity was proportional to the amount of enzyme present to an optical density of 0.18. A blank was run for each assay and included

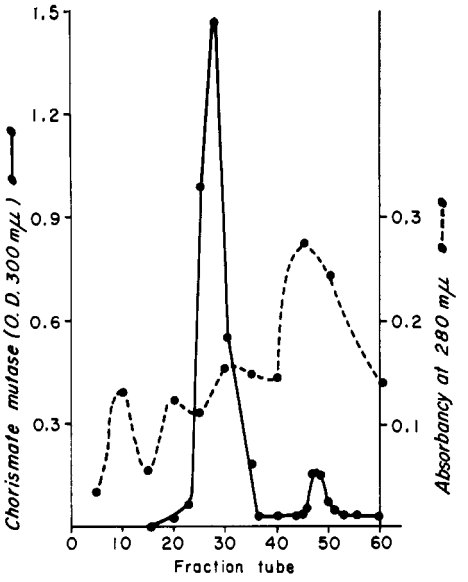

FIGURE 2: DEAE-cellulose chromatography of chorismate mutase activity in an extract from WB 2107. An extract (2.5 ml) containing 15 mg of protein/ml was applied to the column (2 × 20 cm). After the bed had reached incipient dryness, 5 ml of the starting buffer (0.05 M glycine, pH 8.9) was added and elution was begun. Protein was eluted by a linear gradient as described in Methods. Fractions (3 ml) were collected.

all of the components of the reaction mixture except that the enzyme was added after the trichloroacetic acid.

A molar extinction coefficient for phenylpyruvate in arsenate–borate solution at 300 mμ was calculated to be 9292, in close agreement with the value of 9150 obtained by Lin *et al.* (1958). The specific activity of chorismate mutase is defined as the millimicromoles of prephenic acid formed in 1 min/mg of extract protein.

2. PREPHENATE DEHYDROGENASE, PREPHENATE DEHYDRATASE, AND DAHP SYNTHETASE were assayed according to Nester and Jensen (1966) and Jensen and Nester (1966a), except that prephenate dehydratase was assayed at 32°. This enzyme is extremely heat labile (J. C. Coats and E. W. Nester, unpublished observations).

DEAE-cellulose Chromatography. DEAE-cellulose was processed and columns were packed as described by Peterson and Sober (1962). A maximum of 2.5 ml of crude extract or 40 mg of protein was applied to the column (2 × 20 cm) and a linear gradient was formed by eluting with 125 ml of 0.05 M glycine buffer (pH 8.9) in the mixing chamber and the same volume of glycine buffer containing 0.5 M NaCl in the reservoir. A flow rate of 25 ml/hr was maintained. Columns were run at 4° and the fractions were assayed within 18 hr of the beginning of each run.

G-100 Sephadex Chromatography. A 2.5 × 100 cm column was packed with Sephadex G-100 according to the procedure outlined in technical data sheet no. 6 (Pharmacia Fine Chemicals, Inc.). The procedure of Andrews (1964) was used to calibrate the column using cytochrome C (mol wt 24,800 in the dimer form), human hemoglobin (mol wt 64,500), bovine γ-globulin (mol wt 160,000), and lyophilized catalase (mol wt 250,000). For molecular weight determinations, a maximum of 5 ml of crude extract or 100 mg of protein was applied to the column. The density of the extract was increased with sucrose prior to application and injected under the buffer layer. The protein was eluted with 0.05 M phosphate buffer (pH 7.5) and 2-ml samples were collected at 4°. The flow rate was approximately 1 drop every 15–20 sec and the usual run could be collected in 12 hr. Samples were assayed for enzyme activity immediately following the completion of each run.

Sucrose Density Gradients. A 4.5-ml linear sucrose gradient (from 5 to 20%) was prepared in 0.05 M glycine buffer (pH 8.9) according to the technique of Martin and Ames (1961), and 0.2 ml of enzyme was layered on the top. Tubes were centrifuged in a SW-39L swinging-bucket rotor in the Model L Spinco centrifuge at 150,000*g* for 14 or 16 hr at 4°. Each tube was punctured with a small gauge needle and 8-drop fractions were collected in a total of 44–48 tubes. Each fraction was diluted to a final volume of 0.5 ml, and 0.2 ml was assayed for 60 min for chorismate mutase activity. Internal standards of human hemoglobin and bacterial alkaline phosphatase were used in each gradient. The hemoglobin concentration was measured on the DU spectrophotometer by its absorbance at 410 mμ. The procedure of Garen and Levinthal (1960) was used for the assay of alkaline phosphatase activity.

If greater resolution was required, a 13-ml linear sucrose gradient from 10 to 30% was prepared according to Martin and Ames (1961) in 0.05 M phosphate buffer (pH 7.5) and 0.2 ml of enzyme sample was layered on the top. The tubes were centrifuged in an SB-269 swinging-bucket rotor in the B-60 International centrifuge at 150,000*g* for 24 hr. Fractions of 12 drops were collected in a total of 84–86 tubes. Chorismate mutase activity was assayed for 60 min in each tube following the addition of 0.25 ml of 0.05 M glycine buffer (pH 8.9) and 0.1 ml of 10 μmoles/ml of chorismic acid solution to each tube. Human hemoglobin served as the internal standard.

TABLE II: Specific Activity of Chorismate Mutase in Strains 168 and WB 2107.[a]

Strain	Sp Act.
Strain 168	2.8
WB 672	35

[a] Strain 168 was grown on minimal medium supplemented with 5 μg/ml of L-tryptophan. WB 672 was cultured on unsupplemented medium. Extracts were prepared as described.

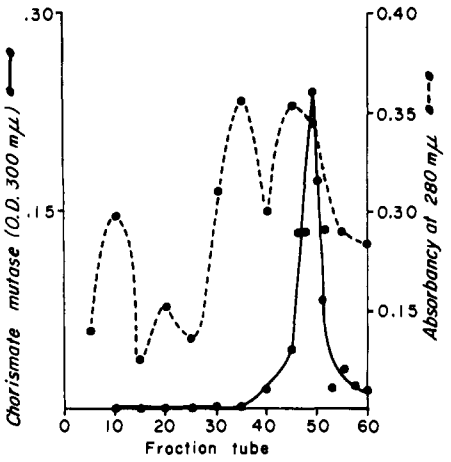

FIGURE 3: DEAE-cellulose chromatography of chorismate mutase in an extract from strain 167. An extract (2.5 ml) containing 13.0 mg of protein/ml was applied to the column (2 × 20 cm). After the sample had entered the bed, 5 ml of the starting buffer (0.05 M glycine, pH 8.9) was added and elution was begun. Protein was eluted by a linear gradient as described in Methods. Fractions (3 ml) were collected.

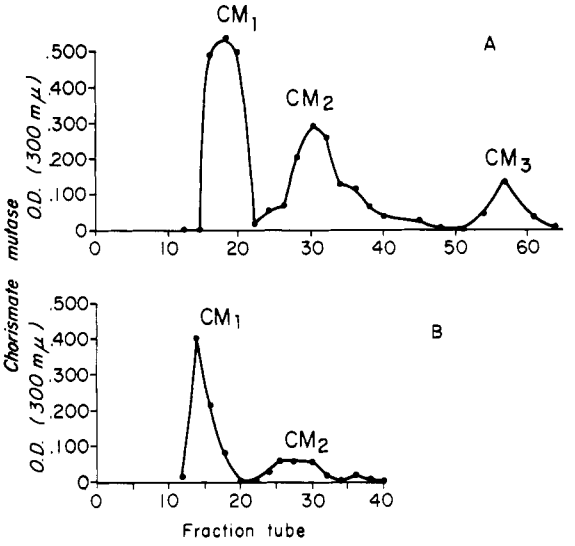

FIGURE 4: DEAE-cellulose chromatography of chorismate mutase activity in an extract from WB 1036 grown under conditions of depression (A) and repression (B). (A) An extract (2.0 ml) containing 22 mg of protein/ml was applied to the column (2 × 20 cm). After the sample had entered the bed, 5 ml of the starting buffer (0.05 M glycine, pH 8.9) was added and elution was begun. Protein was eluted by a linear gradient as described in Methods. Fractions (2 ml) were collected. WB 1036 was grown in a minimal medium supplemented with shikimic acid (100 μg/ml). Conditions of extract preparation were as described in Methods. (B) Cells were grown with shimikic acid (100 μg/ml) and phenylalanine, tyrosine, and tryptophan (50 μg/ml each). An extract (2.5 ml) containing 16.0 mg of protein/ml was applied to the column (2 × 20 cm). After the sample had entered the bed, 5 ml of the starting buffer (0.05 M glycine, pH 8.9) was added and elution was begun. Protein was eluted by a linear gradient as described in Methods. Fractions (2.4 ml) were collected.

Results

The specific activity of chorismate mutase in a random sampling of approximately 40 strains of *B. subtilis*, mostly mutants requiring aromatic amino acids, showed a bimodal distribution. All of these strains are derivatives of either strain 168 or strain 23 (Spizizen, 1958). The specific activity of one class was approximately ten times higher than the other (Tables I and II). Without implying any mechanism, strains possessing the former activity are designated as high-activity strains (represented by WB 672) and those having the latter specific activity as low-activity strains (represented by strain 168). Although the enzyme levels of both high- and low-activity strains varied depending upon the supplementation of the growth medium, in no case was this variation sufficient to confuse the assignment of a strain to the high- or low-activity category.

Chromatography of Chorismate Mutase Activity on DEAE-cellulose. In an attempt to discover the enzymological basis of this disparity, crude extracts of a high-activity (WB 2107) and a low-activity strain (SB 167) were chromatographed separately on DEAE-cellulose. On this adsorbent, chorismate mutase activity in strain 2107 could be resolved into two distinct peaks (Figure 2). The first peak (CM_1) had more than ten times the enzyme activity of the second peak (CM_3). We shall refer to the former peak (CM_1) as the high-activity species, and the latter (CM_3) as the low-activity species.[2] In contrast, an extract of strain 167 revealed only a single peak of activity, corresponding in position and approximate level of enzyme activity to CM_3 (Figure 3).

In an attempt to determine whether derepression of the enzymes of aromatic acid synthesis might reveal additional enzymes species in the high-activity strain, mutant strain WB 1036 (DHQ synthetase less) was examined. This strain, like 167, is derepressed for a number of enzymes of aromatic acid synthesis when shikimic acid serves as the source of the aromatic amino acids. This intermediary metabolite is poorly utilized for the synthesis of the aromatic amino acids and presumably acts as a chemical chemostat (Jensen and Nester, 1965). Chromatography of an extract of 1036 prepared from cells grown on shikimic acid

[2] The designation high- and low-activity species refers only to their quantitative activity when assayed and is not meant to imply that one species is necessarily enzymatically more active than the other. The apparent activity of the high-activity species may be a consequence of the fact that this species is being synthesized at higher levels than the low-activity species.

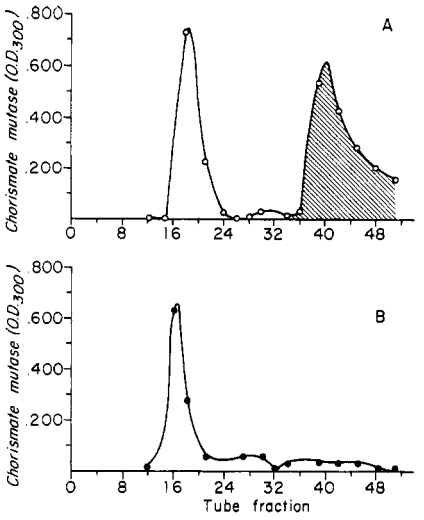

FIGURE 5: Rechromatography of CM_2. Strain 2102 was grown in minimal medium supplemented with shikimic acid (100 μg/ml). The extracts were prepared in the usual manner and 40 mg of protein was applied to a DEAE column (2 × 20 cm). A linear gradient was established between 0.0 and 0.5 M NaCl in glycine buffer (pH 8.9). Samples were collected, and the following day the distribution of chorismate mutase activity was determined. (A) The tubes containing CM_2 activity, indicated by the hatched area, were pooled and bovine serum albumin was added to give a concentration of 4 mg/ml. Solid ammonium sulfate was slowly added with continuous mixing to give a 70% saturated solution. The solution was allowed to stand for 15 min after all of the ammonium sulfate had been added, and then centrifuged at 25,000*g* for 15 min. The pellet was resuspended in 3.5 ml of the solution in which the cells were lysed, and dialyzed against 1 l. of this same solution for 60 min at 4°. The extract was applied to a new DEAE column (2 × 15 cm), and elution was carried out as previously described for the initial chromatography. The distribution of enzyme activity was determined the same day as the chromatography was initiated (B).

revealed a third peak of chorismate mutase activity (CM_2) eluting between CM_1 and CM_3 (Figure 4a). If this strain was grown under conditions which repress enzymes of aromatic acid synthesis, CM_2 activity was greatly diminished (Figures 4b). Thus, high- and low-activity strains have one chorismate mutase enzyme species which has identical chromatographic properties on DEAE-cellulose. In addition, high-activity strains have either one or two additional enzyme species depending on the supplementation of the growth medium. Since the enzyme activity eluting as CM_1 and CM_2 was greater than ten times the total enzyme activity eluting as CM_3, this accounts for the difference in specific activity in crude extracts of high- and low-activity strains. In order to gain some insight into the relationship between CM_1, CM_2, and CM_3, we attempted to determine whether CM_1, CM_2, and CM_3 were specified by one or more than one genetic locus.

Genetic Analysis of Chorismate Mutase Enzyme Species

Since CM_3 exists in the absence of CM_1 and CM_2, at least two loci must be concerned with the synthesis of CM_1, CM_2, and CM_3. A genetic analysis by DNA transformation readily separated two unlinked loci, one controlling the synthesis of CM_1 and CM_2, the other, CM_3. We have no evidence that CM_1 and CM_2 are specified by more than one genetic locus. Thus far we have been unable to separate a locus involved in CM_1 from a locus involved in CM_2 synthesis by genetic recombination.

Linkage of CM_1 to Aro Loci. The locus concerned with CM_1 synthesis is linked to a region of the *B. subtilis* genetic map previously shown to include eight loci specifying enzymes of aromatic acid biosynthesis (aro loci) (Anagnostopoulos and Crawford, 1961; Nester *et al.*, 1963). Thus, when DNA isolated from strain 672 ($Trp_2^+CM_1^+CM_2^+CM_3^+$) was used to transform 168 ($Trp_2^-CM_1^-CM_2^-CM_3^+$) to Trp_2^+, a high proportion (approximately 60%) of the Trp_2^+ transformants were also CM_1^+, judged by their high chorismate mutase enzyme activity. The CM_1 locus has not yet been ordered within the linkage group.

Nonlinkage of CM_3 to Aro Loci. The cross described in Table III tested for linkage of a gene coding for the enzyme CM_3 with the Aro region of the *B. subtilis* chromosome. DNA isolated from strain 168 ($Trp_2^-CM_1^-CM_2^-CM_3^+$) was added to WB 932 ($Trp_2^+CM_1^-CM_2^-CM_3^-$) recipient cells. In the succeeding paper (Nester *et al.*, 1967), we present data that WB 932 is also defective in DAHP synthetase. However,

TABLE III: Linkage of CM_3 to Trp_2.[a]

Medium Supplementation for Primary Selection	Transformant Class (per 247 colonies)		Cotransfer Index
	Trp_2^+-CM_3^+	Trp_2^--CM_3^+	
Tryptophan	245	2	0.0040

[a] CM_3^+ transformants (247) selected on tryptophan supplemented medium were picked to nutrient agar and replica plated to minimal medium to determine the number which were Trp^+. DNA concentration was 0.2 μg/ml of recipient culture.

$$\text{Cotransfer index} = \frac{CM_3^+Trp_2^-}{2(CM_3^+Trp_2^+) + CM_3^+Trp_2^-}$$

Cross: strain 168 ($Trp_2^-CM_3^+$) --x WB 932 ($Trp_2^+CM_3^-$)

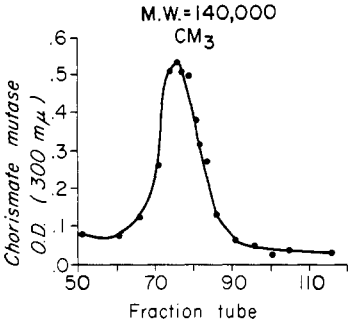

FIGURE 6: G-100 Sephadex chromatography of chorismate mutase activity in an extract from SB 167. Cells were grown in a minimal medium supplemented with shikimic acid (100 μg/ml) and the extract was prepared as described in Methods. An extract (4.0 ml) containing 12.0 mg of protein/ml was applied to the column and the protein was eluted as described in Methods. Fractions (2 ml) were collected. No enzymatic activity was detected in any tube prior to tube 65. The following standards were used to calibrate the column: cytochrome C (peak tube, 162), hemoglobin (peak tube, 112), and catalase and γ-globulin (peak tube, 70).

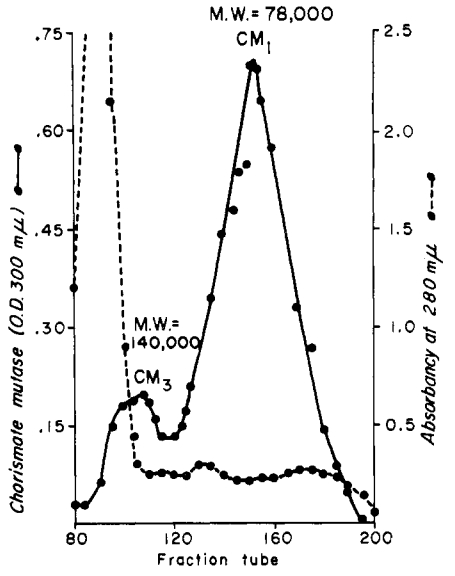

FIGURE 7: G-100 Sephadex chromatography of chorismate mutase activity in an extract from WB 2107. An extract (2.5 ml) containing 38.5 mg of protein/ml was applied to the column and chromatographed as described in Methods. Fractions (2 ml) were collected. The cells were broken by passage (once) through a French pressure cell. Cellular debris was removed by centrifugation at 25,000 *g* for 30 min. Other conditions of extract preparation and nucleic acid treatment are as described in Methods. No enzyme activity was detected in any tube prior to tube 86. The following standards were used to calibrate the column: cytochrome C (peak tube, 245), hemoglobin (peak tube, 155), and catalase and γ-globulin (peak tube, 88).

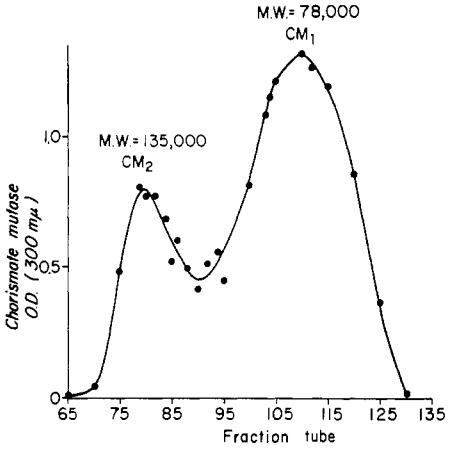

FIGURE 8: G-100 Sephadex chromatography of chorismate mutase activity in an extract from WB 2102. An extract (5.0 ml) containing 13.1 mg of protein/ml was applied to the column and chromatographed as described in Methods. Fractions (2 ml) were collected. Cells were grown on shikimic acid (100 μg/ml) and the extract was prepared as described in Methods. No enzyme activity was detected in any tube prior to tube 70. The following standards were used to calibrate the column: cytochrome C (peak tube, 162), hemoglobin (peak tube, 112), and catalase and γ-globulin (peak tube, 70).

since the loci controlling CM_3 and DAHP synthetase are closely linked genetically, this linkage analysis of the locus specifying CM_3 synthesis to the Aro loci is valid. Selection for CM_3^+ (and $DAHP^+$) transformants was made on minimal agar supplemented with tryptophan. A total of 247 colonies was picked to nutrient agar and replica plated to minimal medium and tryptophan-supplemented plates. All 247 colonies grew with tryptophan, and all but two grew on minimal medium. Therefore, less than 1% of the transformants which had acquired the CM_3 (and DAHP) locus simultaneously received the Trp_2^- marker. This genetic analysis clearly shows that the gene coding for CM_3 is unlinked to the Trp_2 locus, and therefore, is unlinked to CM_1. The two recombinants of genotype $Trp_2^-CM_3^+$ probably resulted from the simultaneous transfer of two molecules of DNA into the same recipient cell.

Linkage of CM_1 and CM_2. A $His_2^+Shk^-$ recombinant (WB 1036) selected from the cross 672 ($His_2^+Shk^+CM_1^+CM_2^+CM_3^+$) (donor DNA) --x SB 140 ($His_2^-Shk^-CM_1^-CM_2^-CM_3^+$) (recipient cells) had all three chorismate mutase enzyme species (Figure 4). Since the CM_1, CM_2, and His_2 loci were transferred simultaneously, they must be closely linked genetically.

So far extensive attempts by genetic recombination have failed to separate CM_1 from CM_2. Thus, 12 high-activity transformants from the cross of a high-activity ($CM_1^+CM_2^+$) with a low-activity strain ($CM_1^-CM_2^-$) simultaneously acquired both molecular species.

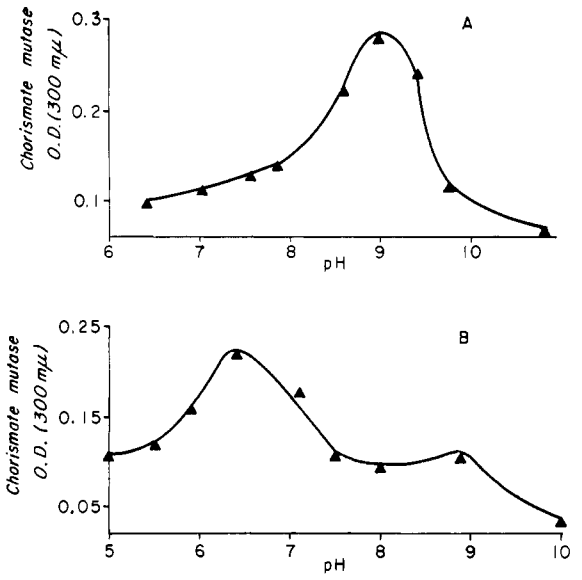

FIGURE 9: Variation of reaction velocity (OD_{300}) with hydrogen ion concentration. Crude extracts of WB 672 (A) and strain 168 (B) were prepared and assayed for enzyme activity by the usual procedure. Each extract was diluted sufficiently to give an optical density change proportional to protein concentration. Buffers used: pH 5.0–6.4, 0.05 M Tris-maleate; pH 7.0–8.0, 0.05 M Tris-HCl; and pH 8.5–10.8, 0.05 M glycine buffer.

Physical Properties

Relationship between CM_1 and CM_2. The observation that extracts prepared from cells grown under conditions of derepression yield both CM_1 and CM_2 enzyme species, whereas cells grown under conditions of repression, express mainly CM_1 activity, is similar to the observations made on β-galactosidase by Appel *et al.* (1965). They concluded that the seven molecular species of this enzyme detected in extracts prepared from induced cells represented different aggregational forms of its two subunits (Steers, 1965). The possibility that CM_1 and CM_2 also represent different aggregational forms having at least one subunit in common was suggested by the following experiment. If CM_2 activity from a DEAE-cellulose column is precipitated with ammonium sulfate, dialyzed, and rechromatographed on DEAE-cellulose, the preponderance of chorismate mutase activity now shifts to the position of CM_1 (Figure 5). In other experiments, we have been able to apparently aggregate CM_1, so that upon rechromatography of CM_1, CM_2 appears (unpublished observations). We have not yet made a systematic study of the conditions which promote association and dissociation *in vitro* as well as *in vivo*, and we certainly do not understand the basis of the relationship between CM_1 and CM_2. However, this does not obscure the strong suggestion that CM_1 and CM_2 are related to each other, and suggests that a single gene locus is responsible for the appearance of both CM_1 and CM_2.

Molecular Weight Determinations of G-100 Sephadex.

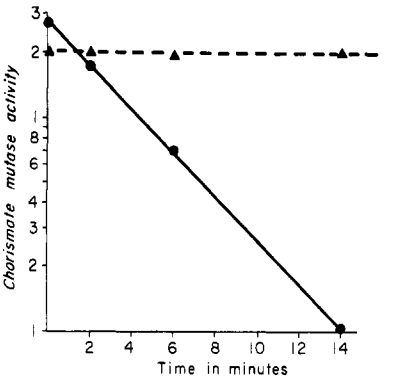

FIGURE 10: Heat sensitivity of chorismate mutase activity in crude extracts from strain 168 and WB 2107 at 60°. Plotted as the logarithm of enzyme activity *vs.* time of heating in minutes. Conditions of growth and extract preparation were as described in Methods. (— —) strain 168. (●—●) strain WB 2107. Comparable levels of protein (approximately 10 mg/ml) were used in the heating.

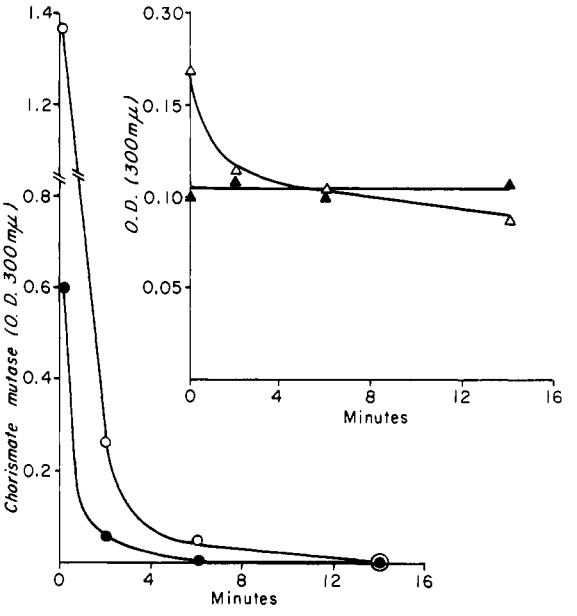

FIGURE 11: Sensitivity to heating at 60° of chorismate mutase activity eluted from G-100 Sephadex column. Condition of extract preparation and column chromatography was described in Methods. (O—O) CM_1 peak tube. (●—●) WB 2107 control. (Δ—Δ) CM_3 peak tube. (▲—▲) strain 168 control.

The suggestion from DEAE-cellulose chromatography that the high- and low-activity strains have an enzyme species in common was reinforced when extracts of SB 167 and 2107 were chromatographed on G-100 Sephadex. The chorismate mutase activity of strain 167 eluted in a single symmetrical peak having an estimated molecular weight of 140,000 (Figure 6).

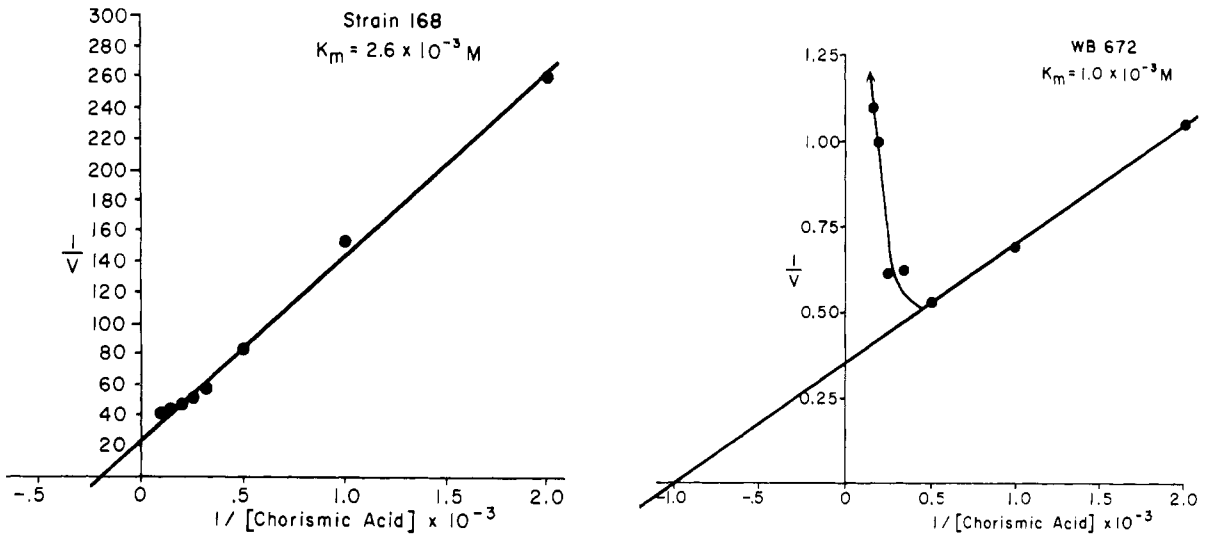

FIGURE 12: Double-reciprocal plot of chorismate concentration and initial reaction velocity in strain 168 and 672. The velocity is expressed as the optical density change at 300 mμ. K_m values and strain employed are indicated on the figures.

The activity of strain WB 2107 was resolved into two activities: one identical with CM_3 of 167 in its molecular weight and its relatively low activity, and another with a molecular weight of 78,000, which must be CM_1 (Figure 7). The activity eluting as CM_1 was approximately ten times greater than the activity eluting as CM_3.

Strain WB 2102, which possesses only CM_1 and CM_2 as shown by DEAE-cellulose chromatography, gave two separable peaks of chorismate mutase activity on Sephadex. One species, presumably CM_2 eluted at a position corresponding to a molecular weight of 135,000 approximately the same as the CM_3 species (Figure 8).

The chromatographic data can be summarized as follows. High-activity strains, which are not nutritionally derepressed, have two molecular species of chorismate mutase. One species (CM_1) has an estimated molecular weight of 70,000 and is not adsorbed to DEAE-cellulose at pH 8.9. The seconds species (CM_3) has an estimated molecular weight of 140,000 represents less than 10% of the total enzyme activity of CM_1, and binds strongly to DEAE. If the high-activity strain is grown under conditions of derepression, a third activity appears (CM_2). Low-activity strains have only one molecular species, which corresponds in charge, molecular weight, and total enzyme activity to CM_3.

Since CM_1 accounts for more than 90% of the enzyme activity in WB 2107, as judged from DEAE chromatography, the properties of chorismate mutase in extracts of this strain compared with strain 168 reflect mainly the properties of CM_1 and CM_3, respectively. Some of the physical properties of CM_1 and CM_3 were compared by studying crude extracts of either 2107 (CM_1 activity) or 168 (CM_3 activity).

pH Optima. Enzyme activity in high-activity strain WB 672 is most active at pH 8.9 (Figure 9A). In comparison, chorismate mutase from low-activity strain 168 has a pH optimum of 6.4 (Figure 9B). Since two peaks of activity were demonstrable by chromatography in extracts of 672, one would expect two peaks of activity in the pH *vs.* activity curve corresponding to CM_1 and CM_3 activity. Inability to observe a peak of activity at pH 6.4 most likely results from the fact that the activity of CM_3 is no longer detectable when the extract is diluted in order to obtain an optical density reading proportional to the concentration of CM_1.

Stability Properties. Crude extracts of strain 168 and 672 are completely stable to storage at 4° over a pH range of 7.0–8.9, and can be kept frozen for 1 month with no loss in activity. The partially purified enzyme in column eluates is somewhat labile, and therefore, column fractions were assayed as soon as possible.

Effect of Heating on Enzyme Activity. The chorismate mutase activity of strain 168 is significantly more heat stable than the enzyme activity of WB 2107. Figure 10 compares the enzyme activity which remained after extracts of strain 168 and 2107 had been heated at 60° for varying periods of time. There was no loss in activity of strain 168 after heating at 60° for 14 min. In contrast, the chorismate mutase activity of extracts of 672 was reduced to 10% of its initial activity by heating at 60° for 10 min. This gross difference in heat sensitivity served as a convenient criterion for distinguishing between CM_1 and CM_3 in eluates from Sephadex and DEAE-cellulose columns. If our inference that the low-activity peak of a high-activity strain is the same enzyme species as the single enzyme species of low-activity strains, their heat sensitivities should be similar.

When an extract of 2107 was chromatographed on G-100 Sephadex and the eluates from the peak

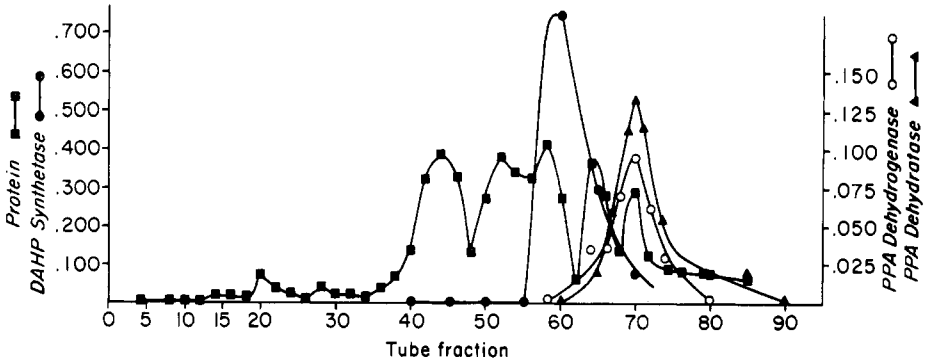

FIGURE 13: DEAE-cellulose chromatography of DAHP synthetase, prephenate dehydrogenase, and prephenate dehydratase in an extract from WB 740. An extract (30 ml) prepared by sonic disruption as described by Nester and Jensen (1966) containing 16.0 mg of protein/ml was applied to the column and allowed to enter the bed. The starting buffer (5 ml) (0.05 M glycine, pH 8.9) was added and elution was begun. Protein was eluted by a linear gradient as described in Methods. Fractions (2 ml) were collected.

tubes of chorismate mutase activity were pooled and heated at 60°, the results of Figure 11 were obtained. The heat sensitivity of CM_3 activity corresponds to the heat sensitivity of extracts of strain 168. In both cases there was no significant loss of enzyme activity. The slight initial decline in activity of the column fraction probably resulted from the loss of some CM_1 activity which overlapped the CM_3 activity peak. The CM_1 species, like crude extracts of WB 672, was labile at this temperature. These data support the previous chromatographic evidence which suggested that CM_3 is the only enzyme species in strain 168.

Effect of Substrate Concentration on Enzyme Activity. The enzyme from strain 168 displayed standard Michaelian kinetics. A K_m value of 2.6×10^{-3} M was calculated from the double-reciprocal plot in Figure 12. In comparison, WB 672 was inhibited by chorismic acid at concentrations greater than 2×10^{-3} M. An extrapolation of the straight-line portion of the curve to the abscissa gives an approximate K_m value of 1.0×10^{-3} M (Figure 12).

To test the possibility that high levels of chorismic acid might be inhibiting CM_1 activity by aggregating the molecule to a less active species, an extract of 2107 was centrifuged in a sucrose gradient in the presence of 15 μmoles/ml of chorismic acid. If CM_1 did aggregate, then a shift in the peak of chorismate mutase activity should be seen. The fractions from two duplicate tubes were pooled and precipitated by the addition of solid ammonium sulfate to 90% saturation. The fractions were centrifuged, and the precipitate was resuspended in 0.05 M glycine buffer (pH 8.9) and assayed for enzyme activity. There was no change in molecular weight of either CM_1 or CM_3 compared with a control centrifuged in the absence of chorismic acid. Further, both CM_1 and CM_3 activities were present in the same relative amounts in the presence and absence of chorismic acid in the gradient.

Chromatography of PPA Dehydrogenase and PPA Dehydratase. In *E. coli* and *A. aerogenes*, Cotton and Gibson (1965) and Pittard and Wallace (1966) observed two separable enzyme species possessing chorismate mutase activity. One species is complexed with the succeeding enzyme of phenylalanine synthesis (PPA dehydratase) and the other with the enzyme of tyrosine synthesis (PPA dehydrogenase). To explore the possibility that in *B. subtilis* the low-activity species might be associated with either of the succeeding enzymes of tyrosine or phenylalanine synthesis, a crude extract of low-activity strain WB 740 was chromatographed on DEAE-cellulose and column fractions were assayed for DAHP synthetase activity, as well as PPA dehydrogenase and PPA dehydratase. The evidence that the CM_3 species and DAHP synthetase are in a molecular complex which is maintained during chromatography is presented in the accompanying paper. DAHP synthetase activity was assayed to mark the position of the complex since it is a more sensitive assay than the chorismate mutase. The results of Figure 13 show that the chromatographic properties of DAHP synthetase, and therefore, CM_3 differ from both PPA dehydrogenase and PPA dehydratase. Since CM_1 and CM_2 both elute much earlier than CM_3 on DEAE-cellulose, we can infer that they are not complexed with either PPA dehydrogenase or PPA dehydratase. These conclusions are consistent with the analysis of chorismate mutase activity in a number of phenylalanine- and tyrosine-requiring mutants lacking either PPA dehydratase or PPA dehydrogenase. There is no loss of high activity (CM_1) in four mutants, two requiring tyrosine and two phenylalanine, nor is CM_3 activity in two phenylalanine and two tyrosine auxotrophs of strain 168 lower than the parent strain. Further, the specific activities of PPA dehydrogenase and PPA dehydratase are the same in the chorismate mutase-less mutant, WB 932 ($CM_1^-CM_2^-CM_3^-$), as in the prototrophic parent.

Regulation of Chorismate Mutase

Studies on Possible Feedback Inhibition of Chorismate

TABLE IV: Specific Activity of Chorismate Mutase in Extracts Grown under Conditions of Repression and Derepression.[a]

Strain	Mol Forms Expressed	Supplementation	Sp Act. of CM	% Repression
SB 167	CM_3	Shk	7.2	
	CM_3	Shk + phenylalanine + tyrosine + tryptophan	1.2	83
WB 2107	CM_1 CM_3	Shk	37	
	CM_1 CM_3	Shk + phenylalanine + tyrosine + tryptophan	21	43
WB 1036	CM_1 CM_2 CM_3	Shk	67	
	CM_1 CM_3 $CM_2\pm$	Shk + phenylalanine + tyrosine + tryptophan	26	61
WB 2102	CM_1 CM_2	Shk	66	
	CM_1 $CM_2\pm$	Shk + phenylalanine + tyrosine + tryptophan	28	58

[a] Conditions of growth: cultures were grown on minimal medium supplemented with shikimic acid (100 μg/ml) and each of the aromatic amino acids (50 μg/ml) wherever indicated. The molecular species was determined by chromatography on DEAE-cellulose, under the conditions described in the Methods section. Conditions of extract preparation and assay of chorismate mutase activity were as described in Methods. The per cent repression is calculated as (specific enzyme activity of Shk grown/specific enzyme activity of Shk + aro grown) × 100 for the particular train studied.

Mutase. No evidence has been found for feedback inhibition of either CM_1, CM_2, or CM_3 activity by the aromatic amino acids phenylalanine, tyrosine, or tryptophan when these amino acids are present singly or in all possible combinations. Inhibition studies have been run at normal and at one-tenth the saturating levels of substrate. These results are in agreement with those previously published by Nester and Jensen (1966). Further, no significant activation by tryptophan, as has been reported in *Neurospora* (Baker, 1966), could be demonstrated. However, both CM_1 and CM_3 are inhibited to 10% of their initial activity by 3.5×10^{-4} M prephenic acid. Inhibition by prephenic acid was not determined for the CM_2 species.

Repression of Chorismate Mutase. Table IV indicates the range of repression and derepression possible in strains 167, 2102, 1036, and 2107. The specific activity of 167 (CM_3 activity) is approximately sixfold higher when the strain is grown on shikimic acid (100 μg/ml), than when it is grown on excess phenylalanine, tyrosine, and tryptophan (50 μg/ml of each).

CM_1 and CM_2 synthesis is also apparently repressed by the aromatic amino acids. Thus, an extract of 1036 prepared from cells grown on excess aromatic amino acids and chromatographed on DEAE-cellulose showed a significant loss of the CM_1 and CM_2 enzyme species (Figure 4A,B). The same repression pattern was observed in an extract of WB 2102 prepared from cells grown on excess levels of the aromatic amino acids (50 μg/ml).

Physiological Significance of CM_1 and CM_2. CM_1 does not influence the growth rate of the organism. The doubling time of the prototrophic strains 2130 (high activity) and 746 (low activity) was almost identical in minimal medium (2130, 87 min; 746, 90 min). The colony sizes of these two strains were identical on minimal medium. Supplementation of low-activity strains with phenylalanine and tyrosine did not increase the growth rate as would be expected if chorismate mutase were limiting tyrosine and phenylalanine synthesis. This observation is not surprising since the specific activities of the two succeeding enzymes in the pathway, prephenate dehydrogenase and prephenate dehydratase, are in the same range as the specific activity of CM_3 (E. W. Nester, unpublished observations). The doubling time of high-activity strains was not decreased by supplementation of the medium with tryptophan, indicating that the high activity of CM_1 was not pulling a disproportionate amount of the chorismic acid to prephenic acid, thereby starving the cell for tryptophan.

Discussion

Chromatographic, biochemical, and genetic evidence all suggest the presence of multiple enzyme species of chorismate mutase in *B. subtilis*. High-activity strains (strain 23 and derivatives) possess three species designated CM_1, CM_2, and CM_3, although CM_2 is only routinely observed when the strain is

grown under conditions of derepression for enzymes of aromatic acid synthesis. Low-activity strains (strain 168 and derivatives) possess only CM_3 even under conditions of maximal derepression. These observations reinforce the contention that although strains 23 and 168 are closely related genetically, nevertheless, they differ in so many of their properties (Spizizen, 1958; Sueoka and Yoshikawa, 1963; Young, 1965; Glaser *et al.*, 1966; Nester and Jensen, 1966) that they should be considered distinctly different strains, perhaps analogous to the K-12 and B strains of *E. coli*.

The data strongly suggest that CM_1 and CM_2 are different aggregational states which have at least one polypeptide in common. Thus, it is clear that it is possible to dissociate CM_2 into CM_1 merely by chromatographying, precipitating with ammonium sulfate, and dialyzing. We do not as yet understand the basis for the apparent association–dissociation phenomenon of CM_1 and CM_2 we observe *in vitro*, but all these data point up the fact that CM_1 and CM_2 are closely related.

Our inability to genetically separate the loci of CM_1 and CM_2 synthesis strongly suggests that one genetic locus is concerned with the appearance of both CM_1 and CM_2. Our data are consistent with the hypothesis that two CM_1 units aggregate to form one CM_2 molecule. However, it will be necessary to study the aggregation–disaggregation phenomenon on highly purified preparations of the CM species before we can state with certainty that another polypeptide is not involved in the aggregated form.

A major question our data pose concerns the relationship between CM_1–CM_2 and CM_3. We feel that the most reasonable interpretation is that strain 168 has a point mutation or a loss of part or all of the structural gene responsible for the synthesis of the polypeptides possessing CM_1–CM_2 activity. Our data do not distinguish between these possibilities.

Another possible explanation is that the gene locus responsible for the appearance of CM_1–CM_2 is a regulatory gene which specifies the level of production of the CM_3 polypeptide. The accompanying paper (Nester *et al.*, 1967) presents evidence that CM_3 is complexed with other polypeptides concerned with DAHP synthetase and possibly shikimate kinase activity. Thus, if CM_3 were produced in excess of the amount necessary for aggregation with these latter polypeptides, the unaggregated units might catalyze the conversion of chorismate to prephenate, but possess properties distinct from CM_3. This explanation seems unlikely, however, since data presented in the accompanying paper (Nester *et al.*, 1967) indicate that the CM_1–CM_2 polypeptides cannot apparently aggregate with the polypeptide concerned with DAHP synthetase activity, and produce a functional complex possessing DAHP synthetase activity.

It is not at all clear why two genes specifying the same enzyme activity should have evolved, especially since the "superfluous" gene locus is responsible for an enzyme activity more than tenfold higher than the level required for optimal growth. It is also surprising that this locus maps in a linkage group concerned exclusively with aromatic acid and histidine synthesis, if it plays no significant role in aromatic acid biosynthesis. We feel that it is not inconceivable that the CM_1–CM_2 activity that we observe is only of secondary importance and that this polypeptide has another function in the synthesis of the aromatic amino acids (or possibly histidine) as yet unrecognized.

Acknowledgment

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An Enzyme Aggregate Involved in the Biosynthesis of Aromatic Amino Acids in *Bacillus subtilis*. Its Possible Function in Feedback Regulation*

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ABSTRACT: Chromatographic and genetic evidence indicates that two enzymes of aromatic amino acid synthesis, 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate (DAHP) synthetase and chorismate mutase, occur in a single aggregate in *Bacillus subtilis*. The possibility that a third enzyme of this pathway, shikimate kinase, is also a part of this aggregate was suggested by chromatographic data. All three enzyme activities are eluted in the same fractions following chromatography on DEAE-cellulose and filtration through Sephadex G-100. A single-step mutant was isolated which lacks both chorismate mutase and DAHP synthetase activities but does have shikimate kinase activity. Since DAHP synthetase and shikimate kinase are feedback inhibited by chorismate and prephenate, we suggest that this aggregate has a functional significance either in localization of inhibitors with control enzymes or in the utilization of a catalytic site for one enzyme as a regulatory site for another enzyme.

Another organism, *Staphylococcus epidermidis*, in which the DAHP synthetase is inhibited by prephenate and chorismate, may also have this enzyme and chorismate mutase in a complex.

Biochemical and genetic analyses of mutationally induced structural alterations of proteins have experimentally demonstrated the requirement for interaction between polypeptide chains for the expression of many enzyme activities (Reithel, 1963; Reed and Cox, 1966). These interactions may involve identical polypeptide chains, presumably coded by the same cistron. The enzyme alkaline phosphatase of *Escherichia coli* falls into this category (Schlesinger, 1964). In other cases, nonidentical subunits interact to form an enzymatically active protein. A well-studied example of this is the tryptophan synthetase of *E. coli* (Yanofsky, 1960). This enzyme consists of an A protein coded by one cistron and a B protein coded by an adjacent cistron. Neither protein by itself has any activity for the terminal physiological step of tryptophan synthesis, but the complex of two molecules of A protein to one molecule of B protein is enzymatically active (Wilson and Crawford, 1965). An enzyme complex or aggregate may possess more than one catalytic activity. Thus, Munkres (1965) has shown that the malate dehydrogenase and aspartate aminotransferase activities in *Neurospora crassa* are associated with the same protein. The subunit components of this complex are dictated by two unlinked genetic loci. Loper *et al.* (1964) have reported that two nonsequential enzymes of histidine biosynthesis are catalyzed by the same protein. In this system, however, only one genetic locus specifies the synthesis of the protein.

DeMoss and Wegman (1965) have shown that in *N. crassa* the activities of three enzymes of tryptophan biosynthesis are associated with a single enzyme aggregate, which thus far cannot be separated into smaller units which possess enzyme activity. Ito and Yanofsky (1966) and Bauerle and Margolin (1966) have shown interactions between enzymes of tryptophan biosynthesis in *E. coli* and *Salmonella*, respectively. Giles *et al.* (1965) have shown that five of the enzymes common to the synthesis of tryptophan, tyrosine, and phenylalanine are closely linked genetically in *Neurospora* and may occur in a single aggregate (N. H. Giles and C. W. H. Partridge, personal communication).

In the course of an investigation on the molecular

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