Characterization of the Functional Activities of the Subunits of 3-Deoxy-D-arabinoheptulosonate 7-phosphate Synthetase-Chorismate Mutase from Bacillus subtilis 168*

(Received for publication, November 9, 1973)

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SUMMARY

The interrelationships between the 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthetase-chorismate mutase enzyme and shikimate kinase were studied. Attempts were made to distinguish between the various catalytic and regulatory activities of this ternary enzyme complex by a variety of techniques. Limited trypsin treatment specifically cleaved the active region of chorismate mutase and the region required for the activation of shikimate kinase activity while most of the catalytic activity of DAHP synthetase remained intact. The loss of catalytic activity of chorismate mutase paralleled the loss of feedback control of DAHP synthetase and substrate binding studies revealed only one site for prephenate binding on each polypeptide chain. Antibody to DAHP synthetase-chorismate mutase specifically inhibited chorismate mutase and shikimate kinase activity but did not inhibit DAHP synthetase activity. These data indicate that DAHP synthetase activity does not depend on a catalytically functional chorismate mutase activity. The data further suggest that the chorismate mutase active site is identical with the feedback inhibition site for DAHP synthetase, thus facilitating feedback control of the aromatic amino acid biosynthetic pathway. The distribution of the catalytic and regulatory activities of this enzyme on one polypeptide chain is discussed.

In the preceding paper (1) the structure of DAHP synthetase-chorismate mutase from Bacillus subtilis was examined. The protein was shown to be an oligomer composed of four identical subunits of molecular weight 38,500. Thus, two different catalytic activities have been shown to be associated with the same polypeptide chain. This polypeptide chain is also responsible for several control functions concerned with aromatic amino acid biosynthesis. DAHP synthetase is feedback inhibited by the substrate and product of chorismate mutase (2). The oligomer has been shown to activate the enzyme shikimate kinase, another enzyme in the pathway which is feedback-inhibited by the same two effectors, chorismate, and prephenate (3). Mutants devoid of chorismate mutase activity suffer simultaneous loss of DAHP synthetase activity and the capacity to activate shikimate kinase (3). One interpretation of these data is that DAHP synthetase activity and shikimate kinase activation require a catalytically functional chorismate mutase. Therefore we re-examined these three activities with regard to the interrelationships required for their catalytic and regulatory functioning. This paper examines the possibility that the catalytic site of chorismate mutase serves as the feedback inhibition site of DAHP synthetase and shikimate kinase.

MATERIALS AND METHODS

Bacterial Strain—WB 2802 derepressed in the synthesis of several enzymes of aromatic amino acid biosynthesis (4) was used throughout this investigation.

Enzyme Assay—DAHP synthetase was assayed as described by Jensen and Nester (5). Chorismate mutase was assayed by the method of Lorence and Nester (6) as modified by Nakatsuksa and Nester (3). The shikimate kinase assay method described by Nakatsuksa and Nester (3) was modified as follows. The amount of [3-14C]shikimic acid used was reduced from 0.04 to 0.01 μCi per ml and the total volume of the reaction mixture was reduced from 1.0 to 0.5 ml. For standard assays, in order to obtain maximum shikimate kinase activity, excess DAHP synthetase-chorismate mutase was included in the reaction mixture. This was accomplished by adding 0.05 ml of DAHP synthetase-chorismate mutase obtained by ammonium sulfate fractionation of a crude extract of Bacillus subtilis as previously described (1). The reaction was stopped by the addition of 2 ml of 1-butanol-glacial acetic acid (100:6). The shikimate 3-phosphate formed during the reaction was isolated by passing the reaction mixture through a 2.5-cm DE51 filter paper disc (Whatman). The filter was washed extensively with a mixture of 1-butanol-glacial acetic acid-water (100:6:25) to remove the shikimate acid. The filter was dried, suspended in 10 ml of toluene-Liquifluor, and counted in a Beckman liquid scintillation counter (model 100) with a counting efficiency of 70%. The 2.5-cm DE51 filter paper discs had a maximum capacity of 7 nmoles of shikimate 3-phosphate. When the specific activity of shikimate kinase was to be determined, the
samples were diluted to permit the synthesis of 1 to 7 nmoles of shikimate 5-phosphate. When activation of shikimate kinase by DAHP synthetase-chorismate mutase was to be studied, the DAHP synthetase-chorismate mutase was added to the reaction mixture. For the activation studies purified shikimate kinase was diluted with 1 mg per ml of bovine serum albumin in 0.05 M Tris-HCl, pH 7.5, containing 10-3 M MgCl2 to a concentration which produced 7 nmoles of shikimate 5-phosphate in 10 min of incubation at 37°C in the presence of excess DAHP synthetase-chorismate mutase. The DAHP synthetase-chorismate mutase preparation added for activation was diluted with 1 mg per ml of bovine serum albumin in 0.05 M Tris-HCl, pH 7.5, containing 10-2 M MgCl2 to a concentration of enzyme which produced 6.5 nmoles of shikimate 5-phosphate in 10 min at 37°C when mixed with the previously established dilution of purified shikimate kinase. Thus, the DAHP synthetase-chorismate mutase concentration was the limiting factor in these reactions and change in the activation of shikimate kinase could be attributed to a change in the DAHP synthetase-chorismate mutase portion of the complex.

Protein Determination—Protein concentration was determined either by the method of Lowry et al. (7) or by the method of Groves et al. (8), with bovine serum albumin as the standard.

Preparation of Antiserum—The preparation of the antiserum to DAHP synthetase-chorismate mutase was carried out by the method of Veron et al. (11) with the following modifications. The trypsin (Sigma) digestion was carried out at 25°C in 0.05 M Tris-HCl buffer, pH 7.5. To stop the proteolysis, sufficient soybean trypsin inhibitor (Sigma) was added to the reaction mixture to obtain a ratio of trypsin to trypsin inhibitor of 1:1 (w/w). With this amount of enzyme, trypsin is limiting, and the reaction is terminated after about 30 min.

Determination of Number of Substrate Binding Sites by Equilibrium Dialysis—The determination of the number of binding sites of phosphoenolpyruvate by DAHP synthetase-chorismate mutase was carried out by a modification of the method of Janin et al. (12). The specific activity of 14C-labeled phosphoenolpyruvate (New England Nuclear) was 6 mCi per mole. The constant flow dialysis cell purchased from TechniLab Instruments, Inc. was used for equilibrium dialysis. Samples were run simultaneously, using leucine blocks which held 0.15 ml of protein solution and 0.25 ml of buffer containing the ligand. Equilibrium was attained in 18 hours at 4°C as determined by preliminary experiments. Competition of [14C]phosphate binding by unlabeled phosphoenolpyruvate was determined by calculating the saturation curve. The protein concentrations used in the phosphate binding studies were 3.8 to 5.5 mg per ml. The specific activity of the [14C]phosphate was 0.65 mCi per mmole.

Purification of Enzyme—The purification procedure for DAHP synthetase-chorismate mutase was described in a previous paper (1). Highly pure DAHP synthetase-chorismate mutase preparations from DEAE cellulose chromatography were used throughout this investigation. The preparation of the purified shikimate kinase used in this study will be described elsewhere.1

1 L. Y. Huang, manuscript in preparation.

Preparation of Antiserum—The preparation of the antiserum to DAHP synthetase-chorismate mutase and the Ouchterlony slide technique used have been described by Nakatsuksa and Nester (3).

Chemicals and Substrates—Chloramycin acid, barium prephenate, and erythrose 4-phosphate were prepared as described by Nakatsuksa and Nester (3). Radioactive barium [14C]prephenate was accumulated and isolated by the procedure of Dayan and Sprinson (14) using the tyrosoe auxotroph of Salmonella typhi murium T9. To achieve maximum incorporation of isotope, the procedure of Dayan and Sprinson (14) was modified as follows; 1 mCi of uniformly labeled [14C]glucose (207 mCi per mmole, New England Nuclear Corp.) was added to 200 ml of accumulation medium to give a final glucose concentration of 0.242%. After the cells were harvested, the supernatant from the accumulation medium was passed through a column (0.9 x 12 cm) of Dowex 1-x8 (chloride form) previously washed with 50 ml of 0.001 M NH4OH. The column then was washed with 50 ml of cold distilled water. The prephenate was eluted from the column with a linear gradient of 125 ml of 0.12 M NH4Br (pH 9.0) and 125 ml of 1.2 M NH4Br (pH 9.0). Fractions of 3 ml were collected and assayed (14). The fractions containing prephenate were pooled, dialyzed 10-fold with cold distilled water, and applied to a second column of Dowex 1-x8 column (0.9 x 12 cm). The column was washed with water and eluted with a 250-mllinear gradient as described above. Fractions of 1 ml were collected and assayed. The fractions containing prephenate were combined and a 6-fold molar excess of 2 M BaBr2 was added. The barium prephenate then was precipitated by slowly adding 2 volumes of chilled methanol followed by 2 volumes of ether. The product was not purified further. Before use the barium prephenate was converted to potassium prephenate by adding 4 mol equivalents of potassium sulfate to a solution of barium prephenate and centrifuging out the barium sulfate. The specific radioactivity of the [14C]prephenate was determined by chemical assay in the solution used to determine the prephenate concentration and by placing samples of the solution in the Beckman scintillation mixture Aquasol and counting them in a Beckman liquid scintillation counter.

Limited Trypsin Treatment—Limited tryptic digestion of DAHP synthetase-chorismate mutase was carried out by the method of Coulson and Evans (15) was adapted to thin layer chromatography as follows. Plastic sheets precoated with cellulose MN300 (Brinkmann Instruments, Inc.) were impregnated with 0.025 m sodium tetraborate, pH 9.0, and dried. After spotting varying amounts of the barium [14C]prephenate and unlabeled barium prephenate (97% pure by weight) on the pretreated sheet ascending chromatography was carried out using 1-butanol-ethanol-0.025 M sodium tetraborate, pH 9.0, and dried. After spotting varying amounts of the barium [14C]prephenate and unlabeled barium prephenate (97% pure by weight) on the pretreated sheet ascending chromatography was carried out using 1-butanol-ethanol-0.025 m sodium tetraborate, pH 9.0 (1:1:1) as the mobile phase. After the chromatogram was dried, spots were detected with a short wavelength UV light (Ultraviolet Products Inc.). Confirmation of prephenate in the spots was obtained by trapping the cellulose MM300 off the plastic sheet, melting the cellulose MM300, and evaporating the extract for prephenate (14). Radioactivity on the chromatogram was detected by cutting a strip from the plastic sheet and passing it through a Vanguard automatic chromatogram scanner model 880. The presence of a single spot of radioactivity, which corresponded in position to the unlabeled prephenate control, was confirmed by cutting the strip into 1-cm pieces and counting these pieces in toluene-Liquifluor in a Beckman liquid scintillation counter model 100. Since no radioactive impurities were found the product was not purified further. Before use the barium prephenate was converted to potassium prephenate by adding 4 molar equivalents of potassium sulfate to a solution of barium prephenate and centrifuging out the barium sulfate. The specific radioactivity of the [14C]prephenate was determined by chemical assay in the solution used to determine the prephenate concentration and by placing samples of the solution in the Beckman scintillation mixture Aquasol and counting them in a Beckman liquid scintillation counter.

1 L. Y. Huang, manuscript in preparation.

Limited Trypsin Treatment of DAHP Synthetase-Chorismate Mutase—Proteolysis by a limited amount of trypsin affects the functions of the DAHP synthetase-chorismate mutase protein in a number of ways (Fig. 1). The first property affected is the ability of DAHP synthetase-chorismate mutase to activate shikimate kinase. With 15 min of trypsin treatment, the DAHP...
The mixture was incubated at 25°C. Samples were taken at 0, 5, 10, 15, 20, 30, and 45 min by pipetting 0.1-ml samples into tubes containing 10 μl of trypsin inhibitor (22.5 μg per ml) sitting in an ice bath. After all the samples were taken the activities were determined as described under "Materials and Methods." ▲—▲, chorismate mutase activity; ■—■, shikimate kinase activity; ●—●, DAHP synthetase activity; ○—○, DAHP synthetase activity in the presence of 1 mM prephenate. 

The DAHP synthetase-chorismate mutase has lost almost 90% of its ability to activate shikimate kinase. In the same time period, approximately 60% of the chorismate mutase activity is lost. Significantly, only 10% of the DAHP synthetase activity is destroyed in this time. However, the ability of 0.1 mM prephenate to inhibit the remaining DAHP synthetase activity is reduced from 74 to 30%. After 30 min of treatment, 100% of the ability to activate shikimate kinase activity is destroyed. In the same time period, over 90% of the chorismate mutase activity is destroyed and the ability of 0.1 mM prephenate to inhibit DAHP synthetase activity is reduced from 74 to 5%, while only 30% of DAHP synthetase activity is destroyed. Further incubation does not affect these activities. These results indicate that it is not possible to distinguish, by limited trypsin digestion, between the sites for chorismate mutase activity and the feedback inhibition of DAHP synthetase by prephenate. However, it seems evident that the site of DAHP synthetase catalytic activity is distinct from the functional sites for chorismate mutase activity, shikimate kinase activation, and feedback inhibition by prephenate. The trypsin digestion kinetic data suggest that the site for shikimate kinase activation may be distinct from, but close to, the site for chorismate mutase activity.

In addition to the kinetic studies of the enzyme activities, following partial digestion by trypsin, the products of proteolysis were examined after electrophoresis both on 7% polyacrylamide gels containing SDS and on 7% polyacrylamide gels lacking SDS. Four 7% polyacrylamide gels lacking SDS were run on each sample, taken after 0, 15, and 45 min of trypsin treatment (Fig. 2). The first polyacrylamide gel was stained for protein with Coomassie brilliant blue and destained, and then the density of the staining was recorded with the aid of a Gilford gel scanner. The second polyacrylamide gel was sliced and the fractions were assayed for DAHP synthetase activity. The slices of the third polyacrylamide gel were assayed for DAHP synthetase activity in the presence of 0.1 mM prephenate. Following gel slicing the final polyacrylamide gel was assayed for chorismate mutase activity. With no trypsin treatment one band of protein was observed which corresponded in position to the chorismate mutase activity and to the DAHP synthetase activity, which was feedback inhibited by prephenate. After 15 min of trypsin treatment three bands of protein were observed. The first band (I) corresponded in position to the original protein and had chorismate mutase activity and prephenate feedback inhibited DAHP synthetase activity. The second (II) and third (III) bands had no chorismate mutase activity and had DAHP synthetase activity which was no longer feedback-inhibited by prephenate. After 45 min of treatment the same three bands were found as observed after 15 min of trypsin digestion. However, the amounts of protein and enzyme activity in Bands I and II had decreased while that found in Band III had increased when compared to the levels that had been observed after 15
Fig. 3. Mild proteolysis of DAHP synthetase-chorismate mutase by trypsin: electrophoresis on 7% polyacrylamide gels containing SDS. Aliquots, 30 μl, of samples taken at 0, 5, 15, and 45 min, as described in Fig. 1, were added to 30 μl of 8 M urea containing 2% SDS and 2% 2-mercaptoethanol. The proteins were denatured by placing the solution in boiling water bath for 1 min. Forty microliters, of samples treated in this manner, were placed on top of 7% polyacrylamide gels containing SDS and electrophoresis was carried out. The gels were stained and destained as described under “Materials and Methods.”

min of treatment. These results indicated that the products of proteolysis exhibit only DAHP synthetase activity which is no longer feedback-inhibited by prephenate.

When the products of proteolysis, sampled after 0, 5, 15, and 45 minutes, were analyzed on polyacrylamide gels containing SDS, the band corresponding to the original polypeptide chain disappeared while two faster moving bands, namely A and B bands, appeared (Fig. 3). The A band appeared in the 5- and 15-min samples and just about disappeared after 45 min of trypsin treatment. The density of the B band, when measured with a Gilford gel scanner, increased 2-fold in the 45 min sample over that observed in the 15-min sample. A correlation might be drawn between the A and B bands found on polyacrylamide gels containing SDS (Fig. 3) and the II and III bands found on the regular polyacrylamide gels (Fig. 2). The A and II bands appear with short times of trypsin treatment and disappear with 45 min of trypsin treatment while the B and III bands increase in staining intensity after 45 min of trypsin treatment. A split polyacrylamide gel containing SDS was used to determine the molecular weights of the A and B bands. A sample of denatured standard proteins of known molecular weights was placed on one side of the polyacrylamide gel containing SDS and a denatured 15-min trypsin-treated sample, to which cytochrome c had been added, was placed on the other side and electrophoresis was carried out as described under “Materials and Methods.” After staining and destaining the mobility of the standard proteins relative to cytochrome c was plotted versus their molecular weights on semilog graph paper (Fig. 4). The molecular weights of the A and B bands and their mobilities relative to cytochrome c were determined from this graph (Fig. 4). The molecular weight of the A band was estimated to be 34,000 and that of the B band, 29,300. Thus, the molecular weight difference between the original polypeptide chain and B band is 9,000. The active region for chorismate mutase activity is probably located within the fragments which give a molecular weight total of 9,000 and that for DAHP synthetase activity is located on the residual polypeptide chain of 29,300 molecular weight. The regions for activation of shikimate kinase and for prephenate feedback inhibition of DAHP synthetase activity are very close to the region of chorismate mutase activity. These results further demonstrate not only that the sites for chorismate mutase activity and DAHP synthetase activity reside on the same polypeptide chain but also that the sites for activation of shikimate kinase activity and prephenate feedback inhibition of DAHP synthetase activity are located on the polypeptide chain and that these latter sites are very close to or identical with the site of chorismate mutase activity.

Effect of Antiserum on DAHP Synthetase, Chorismate Mutase, and Shikimate Kinase Activity—Antibodies prepared against the purified DAHP synthetase-chorismate mutase strongly inhibited the chorismate mutase activity as well as the activation of
The effect of antiserum on DAHP synthetase, chorismate mutase, and shikimate kinase activities. The enzyme preparations used for the assays are as follows: DAHP synthetase-chorismate mutase (purified protein) and shikimate kinase (Sephadex G-200 DAHP synthetase-chorismate mutase and shikimate kinase fractions). Because the final volume of the assay varied from 0.2 ml for the DAHP synthetase to 1.0 ml for shikimate kinase, the amount of antiserum used is expressed as milliliters of antiserum per ml of reaction mixture. In all cases, the antiserum was added to the enzyme preparation before the addition of the respective substrates, but without any preincubation of the enzyme with the antiserum. The activities without inhibitors are as follows: DAHP synthetase, 1.740 absorbance units at 549 nm; chorismate mutase, 0.280 absorbance unit at 320 nm; and shikimate kinase, 4737 cpm.

Fig. 5 (left). The effect of antiserum on DAHP synthetase, chorismate mutase, and shikimate kinase activities. The enzyme preparations used for the assays are as follows: DAHP synthetase-chorismate mutase (purified protein) and shikimate kinase (Sephadex G-200 DAHP synthetase-chorismate mutase and shikimate kinase fractions). Because the final volume of the assay varied from 0.2 ml for the DAHP synthetase to 1.0 ml for shikimate kinase, the amount of antiserum used is expressed as milliliters of antiserum per ml of reaction mixture. In all cases, the antiserum was added to the enzyme preparation before the addition of the respective substrates, but without any preincubation of the enzyme with the antiserum. The activities without inhibitors are as follows: DAHP synthetase, 1.740 absorbance units at 549 nm; chorismate mutase, 0.280 absorbance unit at 320 nm; and shikimate kinase, 4737 cpm.

Phosphoenolpyruvate on DAHP Synthetase-Chorismate Mutase-Determination of Number of Binding Sites for Prephenate and Phosphoenolpyruvate on DAHP Synthetase-Chorismate Mutase—The dialysis rate technique of Colowick and Womack (12) was used to measure the binding of phosphoenolpyruvate to DAHP synthetase-chorismate mutase. The Scatchard plot (16) seen in Fig. 6 was obtained from experiments in which the protein concentration used was 1.02 x 10^-4 m. After eight fractions were collected 5 ml of 5 X 10^-4 m unlabeled phosphoenolpyruvate were added and another eight fractions were collected. Subsequent 5-ml aliquots of 10^-2 m unlabeled phosphoenolpyruvate were added after each set of eight fractions were collected. The fractions were mixed with 10 ml of Triton X-100-toluene-Liquifluor (1:2) in a Beckman liquid scintillation counter. The ratio of moles of bound phosphoenolpyruvate to moles of free phosphoenolpyruvate was plotted versus the moles of phosphoenolpyruvate bound to give this plot.

Fig. 6 (center). Binding of phosphoenolpyruvate to DAHP synthetase-chorismate mutase: Scatchard plot (17). One millilitre of DAHP synthetase-chorismate mutase (3.33 mg per ml) in 0.05 M Tris-HCl buffer, pH 7.0, containing 5 X 10^-4 M CdCl2 was placed in the top cell of a constant flow dialysis apparatus (TechniLab Instruments, Inc.). Tris-HCl buffer, 0.05 M, pH 7.0, containing 5 X 10^-4 M CdCl2 was passed through the bottom cell at a flow rate of 8 ml per min and 2-ml fractions were collected. The experiment was carried out at 4°C and was started by the addition of 10 ml of [IC]phosphoenolpyruvate (specific activity, 6 mCi per mmole) to give a final phosphoenolpyruvate concentration of 4.7 X 10^-4 M. After eight fractions were collected 5 ml of 5 X 10^-4 M unlabeled phosphoenolpyruvate were added and another eight fractions were collected. Subsequent 5-ml aliquots of 10^-2 m unlabeled phosphoenolpyruvate were added after each set of eight fractions were collected. The fractions were mixed with 10 ml of Triton X-100-toluene-Liquifluor (1:2) in a Beckman liquid scintillation counter. The ratio of moles of bound phosphoenolpyruvate to moles of free phosphoenolpyruvate was plotted versus the moles of phosphoenolpyruvate bound to give this plot.

Fig. 7 (right). Binding of prephenate to DAHP synthetase-chorismate mutase: saturation curve. DAHP synthetase-chorismate mutase, 0.15 ml, (3.86 mg per ml) in 0.05 m potassium phosphate buffer, pH 7.0, was placed in each cell on one side of the leucite microdialyzer apparatus (Hoefer Scientific Instruments, Inc.) samples, 0.25 ml, of 0.05 m potassium phosphate buffer, pH 7.0, containing [14C]prephenate (specific activity, 0.67 mCi per mmole) at final concentrations varying from 0.1 to 10 mM prephenate were placed in the cells on the other side of the dialysis membrane. Dialysis was carried to equilibrium at 4°C. Aliquots of the protein solutions and buffer containing ligand solutions were each added to 1.0 ml of water and 10 ml of Triton X-100-toluene-Liquifluor (1:2) were added. The samples then were counted in a Beckman liquid scintillation counter. The ratio of moles of prephenate bound to moles of protein then was plotted versus the concentration of free prephenate in the buffer plus ligand solutions.
of 38,500, was used with concentrations of prephenate from 0.1 to 10 mM to generate the saturation curve shown in Fig. 7. These data indicated that there were 1.02 binding sites on each polypeptide chain of 38,500 molecular weight. To test the prephenate binding observed at the chorismate mutase active site, experiments were performed in which unlabeled chorismate was added to the ^14C-labeled prephenate to compete for the binding sites. Table II indicates that chorismate was an effective competitor of prephenate for the binding, as an equal molar concentration of chorismate reduced the binding of prephenate by 50.4%, thus suggesting that the binding observed was indeed at the chorismate mutase active site. The presence of only one prephenate binding site per polypeptide chain and the evidence that this binding site is the chorismate mutase active site further suggests that the active site for chorismate mutase is the prephenate feedback inhibition site for DAHP synthetase.

### Table I

<table>
<thead>
<tr>
<th>Addition</th>
<th>DAHP synthetase (4 μM nm)</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.489</td>
<td>0</td>
</tr>
<tr>
<td>AS, 20 μl</td>
<td>0.430</td>
<td>12</td>
</tr>
<tr>
<td>AS, 30 μl</td>
<td>0.378</td>
<td>23</td>
</tr>
<tr>
<td>Prephenate, 1 mm</td>
<td>0.049</td>
<td>90</td>
</tr>
<tr>
<td>Prephenate, 1 mm, + 20 μl of AS</td>
<td>0.222</td>
<td>486</td>
</tr>
<tr>
<td>Prephenate, 1 mm, + 30 μl of AS</td>
<td>0.247</td>
<td>356</td>
</tr>
<tr>
<td>Prephenate, 0.1 mm</td>
<td>0.150</td>
<td>69</td>
</tr>
<tr>
<td>Prephenate, 0.1 mm, + 20 μl of AS</td>
<td>0.353</td>
<td>186</td>
</tr>
<tr>
<td>Prephenate, 0.1 mm, + 30 μl of AS</td>
<td>0.381</td>
<td>06</td>
</tr>
</tbody>
</table>

* AS, antiserum.
* Final concentration per reaction mixture.
* Per cent inhibition when compared to reaction mixture containing antiserum alone.

### Table II

**Competition of radioactive prephenate binding by chorismate**

The dialysis was performed with the aid of a leucite apparatus which held 0.15-ml samples of a 8.4 × 10⁻⁴ M solution to DAHP synthetase-chorismate mutase, based on a subunit molecular weight of 38,500. Ligand samples, containing 0.05 M potassium phosphate buffer, pH 7.0, 8 mM prephenate, and each of the chorismate concentrations indicated below, were placed on the other side of the dialysis membrane in leucite cells which held 0.25 ml. Dialysis was carried out with rotation of the cell at 4° for 24 hours. The prephenate concentration of the ligand solutions were determined by chemical assay (14) before and after dialysis to assure that the concentration observed was due to the presence of chorismate and not to the enzymatic or nonenzymatic conversion of chorismate to prephenate.

<table>
<thead>
<tr>
<th>Chorismate concentration (mM)</th>
<th>Prephenate bound (nm)</th>
<th>Decrease in prephenate binding %</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.06</td>
<td>0</td>
</tr>
<tr>
<td>0.8</td>
<td>5.28</td>
<td>25.0</td>
</tr>
<tr>
<td>8.0</td>
<td>3.52</td>
<td>50.4</td>
</tr>
<tr>
<td>40.0</td>
<td>0.97</td>
<td>86.2</td>
</tr>
</tbody>
</table>

Jensen and Nester (2) have shown that the feedback inhibition of DAHP synthetase by prephenate is noncompetitive with phosphoenolpyruvate and erythrose 4-phosphate, thus indicating that the feedback-inhibition site is distinct from the DAHP synthetase active site. The fact that chorismate effectively competes, when present in a 1:1 ratio, with prephenate for binding to the protein suggests that the binding detected is indeed binding to the active site for chorismate mutase. The presence of only one binding site for prephenate per polypeptide chain suggests that the chorismate mutase active site and the prephenate feedback-inhibition site are identical. The concomitant loss of chorismate mutase activity and prephenate feedback inhibition of DAHP synthetase upon mild proteolysis or upon the addition of antiserum further supports this hypothesis. That chorismate is also an allosteric feedback inhibitor of DAHP synthetase is consistent with the feedback-inhibition site being identical to the chorismate mutase active site. The fact that the concentration of chorismate required to inhibit DAHP synthetase is 10-fold higher than the concentration of prephenate can be accounted for by the prephenate having a more pronounced allosteric effect on the protein than chorismate when it is bound to the enzyme.

One of the most interesting functions of DAHP synthetase-chorismate mutase is that it activates the shikimate kinase polypeptide (3). The data from the mild proteolysis experiments suggest that the activation site for shikimate kinase is very close to but distinct from the chorismate mutase active site. The antiserum inhibition of chorismate mutase and shikimate kinase activities are consistent with the idea that two regions of close proximity are involved for manifestation of the two enzyme activities. However, it must be noted that the
inhibition by antiserum cannot distinguish between an identical active site and two active sites in close proximity on the polypeptide chain. Since the effect of antibodies may be due to either direct binding or to steric inhibition of active sites, the facts that prephenate feedback inhibition of shikimate kinase is noncompetitive with the activation of shikimate kinase and that there is only one binding site for prephenate on the enzyme complex also support the presence of two distinct sites for chorismate mutase activity and for activation of shikimate kinase, located in close proximity on the polypeptide chain.

The one observation that is yet to be explained is that all the mutants found to be lacking chorismate mutase also lack DAHP synthetase and shikimate kinase activities and no mutants lacking only chorismate mutase activity have been found. One possible explanation for the lack of DAHP synthetase activity in mutants lacking chorismate mutase may be that folding of the polypeptide chain is required to obtain the DAHP synthetase active site and that this folding is influenced by the region of the chorismate mutase active site. It has been shown that the chorismate mutase active site is the feedback inhibition site for DAHP synthetase and does influence DAHP synthetase activity. Thus, a mutation in the chorismate mutase active site could quite possibly influence DAHP synthetase activity.

In recent years an increasing number of bifunctional enzymes, which carry two active sites on a single polypeptide chain, have been identified (17–22). Gene fusion has been proposed as an important mechanism in the evolution of these complex proteins (23). This theory could account for the facts known about DAHP synthetase-chorismate mutase. It is interesting to note, in contrast to other systems, that the enzyme activities found on this single polypeptide chain are not sequential in the biosynthetic pathway. They are, however, a unit of regulation since we have shown that the chorismate mutase active site is the feedback inhibition site for DAHP synthetase. The facts that shikimate kinase activity is dependent upon the formation of the complex and that it is feedback inhibited by prephenate and chorismate point out another way in which this unit of regulation influences the intermediates formed in the aromatic amino acid biosynthetic pathway of Bacillus subtilis.

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Characterization of the Functional Activities of the Subunits of 3-Deoxy-d-arabinoheptulosonate 7-phosphate Synthetase-Chorismate Mutase from *Bacillus subtilis* 168

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