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Purification and Properties of Chorismate Mutase-Prephenate Dehydratase and Prephenate Dehydrogenase from *Alcaligenes eutrophus*

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Chorismate mutase and prephenate dehydratase from *Alcaligenes autrophus* H 16 were purified 470-fold with a yield of 24%. During the course of purification, including chromatography on diethylaminoethyl (DEAE)-cellulose, phenylalanine-substituted Sepharose, Sephadex G-200, and hydroxyapatite, both enzymes appeared in association. The ratio of their specific activities remained almost constant. The molecular weight of chorismate mutase-prephenate dehydratase varied from 144,000 to 187,000 due to the three different determination methods used. Treatment of electrophoretically homogeneous mutase-dehydratase with sodium dodecyl sulfate dissociated the enzyme into a single component of molecular weight 47,000, indicating a tetramer of identical subunits. The isoelectric point of the bifunctional enzyme was 5.8. Prephenate dehydrogenase was not associated with other enzyme activities; it was separated from mutase-dehydratase by DEAE-cellulose chromatography. Chromatography on DEAE-Sephadex, Sephadex G-200, and hydroxyapatite resulted in a 740-fold purification with a yield of 10%. The molecular weight of the enzyme was 55,000 as determined by sucrose gradient centrifugation and 65,000 as determined by gel filtration or electrophoresis. Its isoelectric point was pH 6.6. In the overall conversion of chorismate to phenylpyruvate, free prephenate was formed which accumulated in the reaction mixture. The dissociation of prephenate allowed prephenate dehydrogenase to compete with prephenate dehydratase for the substrate.

The first reaction specific to the synthesis of phenylalanine and tyrosine is catalyzed by chorismate mutase (EC 5.4.99.5). The enzyme converts the main branch-point intermediate, chorismate, to prephenate. Prephenate is further converted either to phenylpyruvate by prephenate dehydratase (EC 4.2.1.51) or to hydroxyphenylpyruvate by prephenate dehydrogenase (EC 1.3.1.12) (Fig. 1). The biosynthetic steps of the aromatic amino acid pathway appear to be the same in bacteria, fungi, and higher plants. However, several patterns of control mechanisms and enzyme organization have been described in prokaryotic as well as eukaryotic systems.

In enteric bacteria, two distinct chorismate mutases are involved; one is associated with prephenate dehydratase, whereas the other one forms a bifunctional enzyme with prephenate dehydrogenase (6, 8, 27). Multiple molecular forms of chorismate mutase have been separated from extracts of *Bacillus subtilis* (22). Another strain of the same organism has a single chorismate mutase that resides with 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase, the first enzyme of the aromatic amino acid pathway, on a bifunctional protein (17, 18). No associations with succeeding enzymes have been found in *Neurospora crassa* (2), *Saccharomyces cerevisiae* (29), *Streptomyces aureofaciens* (15), and *Euglena gracilis* (33).

The present paper describes the purification of chorismate mutase, prephenate dehydratase, and prephenate dehydrogenase from cell-free extracts of the hydrogen bacterium *Alcaligenes eutrophus* strain H 16. The properties of the enzymes concerned have been studied with respect to their organization.

MATERIALS AND METHODS

Growth of bacteria. *A. eutrophus (Hydrogenomonas eutropha)* strain H 16 (ATCC 17699, DSM

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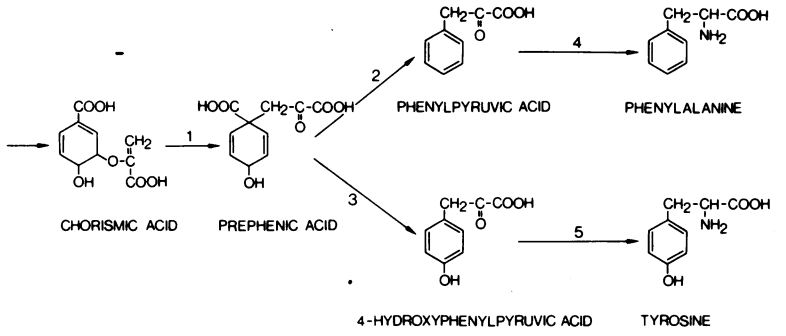

FIG. 1. *Biosynthetic pathway of phenylalanine and tyrosine. Trivial names of enzymes: (1) chorismate mutase; (2) prephenate dehydratase; (3) prephenate dehydrogenase; (4) phenylalanine transaminase; (5) tyrosine transaminase.*

428) was grown heterotrophically in a mineral medium described by Schlegel et al. (25) with 0.5% fructose as carbon source. Cells grown in a 100-liter batch fermenter (AG f. Biologische Verfahrenstechnik, Basel) were harvested in the late exponential phase of growth, washed with 0.05 M phosphate buffer (pH 7.0), suspended (4 ml of buffer per 1 g [wet weight]), and stored in the same buffer at −20 C.

Preparation of crude extracts. A 740-ml amount of a thawed cell suspension of *A. eutrophus* H 16 was sonicated with a 600-W ultrasonic disintegrator (Schoeller and Co., Frankfurt) for 5 h in a continuous-flow cell. The temperature was kept below 10 C by cooling the cell with ethanol at −5 C. Cell debris was removed by centrifugation at 170,000 × *g* for 60 min at 2 C.

Protein was determined according to Lowry et al. (23), using crystalline bovine serum albumin as standard.

Protein solutions were concentrated in Diaflo TCF 12 and 52 cells (Amicon Corp., Lexington, Mass.), using XM 50 and PM 10 membranes. Concentration of small volumes (less than 4 ml) was achieved by ultrafiltration using Minicon-A 25 concentrators (Amicon Corp.).

Enzyme assays. One enzyme unit catalyzes the conversion of 1 μmol of substrate per min. The specific activity is expressed as units per milligram of protein.

Chorismate mutase. In crude extracts and partially purified enzyme preparations, chorismate mutase was determined by using an end-point assay according to the procedure described by Cotton and Gibson (6). The reaction mixture contained, in a final volume of 0.4 ml: 1 mM potassium chorismate, 50 mM potassium phosphate (pH 7.8), and approximately 10 μl of enzyme (at most 0.2 mg of protein). After incubation for 5 min at 37 C the enzyme reaction was terminated by the addition of 0.4 ml of 1 N HCl. During further incubation for 10 min at 37 C, the prephenate formed was chemically converted to phenylpyruvate. Then 3.2 ml of 1 N NaOH was added and the absorbance was measured at 320 nm. Controls were run by including an appropriate substrate blank. The concentration of prephenate was calculated from the absorbance of phenylpyruvate at 320 nm, using a molar extinction coefficient of 17,500.

Prephenate dehydratase. The assay described by Cotton and Gibson (6) was slightly modified. The reaction mixture contained, in a final volume of 0.5 ml: 1 mM potassium prephenate, 50 mM potassium phosphate (pH 7.8), and limiting amounts of enzyme. Reactions were carried out at 37 C and terminated after 20 min by the addition of 1.5 ml of 1 N NaOH. Under these conditions, the amount of phenylpyruvate formed was linear with respect to the concentration of enzyme and the time of incubation. The concentration of phenylpyruvate, measured at 320 nm, was calculated by using a molar extinction coefficient of 17,500. Corrections for the nonenzymatic conversion of prephenate were always included.

Prephenate dehydrogenase. The assay was carried out according to the procedure of Champney and Jensen (5). It was based on the increase of fluorescence of reduced nicotinamide adenine dinucleotide (NADH) formed at 30 C. The reaction mixture contained, in a final volume of 1.0 ml: 0.5 mM potassium prephenate, 1 mM NAD, and 100 mM potassium phosphate (pH 7.8). The reaction was initiated by the addition of enzyme. Initial velocities were determined from the slopes traced on a recorder of an Eppendorf filter fluorimeter (model 1030, Netheler and Hinz GmbH, Hamburg, West Germany) with an excitation wavelength of 313 nm and an emission wavelength of 400 to 3,000 nm. The reaction rate was linear for at least 5 min. The amount of NADH formed was calculated from a known NADH standard.

Determination of molecular weights. (i) Gel filtration. The molecular weights of the purified enzymes were determined by elution through a column (1.5 by 87 cm) of Sephadex G-200 equilibrated with 50 mM phosphate buffer (pH 7.4) according to the method of Andrews (1). A sample of 1.0 ml containing purified chorismate mutase-prephenate dehydratase and prephenate dehydrogenase as well as standard proteins was layered onto the gel surface. The standard proteins were identified as follows: ferritin (molecular weight, 540,000) by reading the

column effluent at 425 nm; and ovalbumin (molecular weight 45,000) and chymotrypsinogen A (molecular weight, 25,000) by absorbance at 280 nm. Rabbit muscle aldolase (molecular weight, 158,000) was determined according to Taylor (31), with the exception that arsenate was replaced by adenosine 5'-triphosphate. The proteins were eluted with 50 mM phosphate buffer (pH 7.4) at a flow rate of 8.5 ml/h at 4 C and collected in fractions of 1 ml.

(ii) Sucrose gradient centrifugation. Linear 5 to 20% sucrose gradients (12.8 ml) were prepared with 50 mM phosphate buffer (pH 7.4) and allowed to stand for about 4 h at 4 C. Protein samples of 0.15 ml containing purified chorismate mutase-prephenate dehydratase or prephenate dehydrogenase and one of the standard proteins were layered on the top of the gradients.

Tubes were centrifuged at 40,000 rpm in a swinging-bucket rotor of a Christ centrifuge (model Omega) for 14 h at 2 C. After centrifugation, the tubes were punctured and 5-drop fractions were collected and assayed for enzyme activity. The $s_{20,w}$ values and the approximate molecular weights of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase were estimated with respect to rabbit muscle aldolase and yeast alcohol dehydrogenase according to Martin and Ames (24). The $s_{20,w}$ value of aldolase was taken as 7.8S, with a corresponding molecular weight of 158,000 (19). The $s_{20,w}$ value and molecular weight for alcohol dehydrogenase were taken as 7.6S and 150,000, respectively (1).

(iii) Gel electrophoresis. According to the method of Hedrick and Smith (16), samples of the purified enzymes and several standard proteins (50 μg of each) were subjected to electrophoresis in gels varying from 6 to 12% (wt/vol) monomer. To each sample (50 μl), sucrose was added to increase its density, and bromophenol blue was taken as a dye marker. Electrophoresis was carried out at 10 C with a current of 3 mA/tube. After the run, the gels were removed and sliced at the dye front. Protein bands were developed by staining with Coomassie brilliant blue according to Weber and Osborn (34). The relative mobility of each protein *(R)* was calculated from the ratio of distance migrated by the protein to that migrated by the dye marker. The log of R was plotted against the concentration of polyacrylamide for each protein, resulting in a straight line. A standard curve was obtained by plotting the slope of each line versus the respective molecular weight. As reference proteins we used rabbit muscle aldolase (molecular weight, 158,000), hexokinase (molecular weight, 97,000), creatine kinase (molecular weight, 81,000), bovine serum albumin (molecular weight, 66,000), and ovalbumin (molecular weight, 45,000).

Polyacrylamide electrophoresis. (i) Analytical electrophoresis. The degree of purification was routinely controlled by polyacrylamide gel electrophoresis in a 7.5% acrylamide gel according to the method of Davis (9). Electrophoresis was carried out in an apparatus of WTW (Munich, West Germany), using a current of 3 mA/gel. Glass tubes with an inner diameter of 0.6 cm and a length of 14 cm were filled with 2 ml of gel solution. Staining and destaining were performed as described (34).

To localize chorismate mutase and prephenate dehydratase activity, a duplicate gel not treated with dye was cut into slices of about 2 mm. The gel slices were homogenized by ultrasonic disruption, and the enzyme was eluted with 0.5 ml of 50 mM phosphate buffer (pH 7.4). The activities of chorismate mutase and prephenate dehydratase were determined from the supernatant of this homogenate by standard assay.

Prephenate dehydrogenase activity was located as a purple band in the gel by treatment with nitroblue tetrazolium and phenazine methosulfate in the presence of the substrates as described by Koch et al. (20).

(ii) Electrophoretic purification of chorismate mutase-prephenate dehydratase. For electrophoretic purification, about 100 μg of protein from step 7 of the purification procedure was applied to a standard gel as described by Bender and Gottschalk (3). Six tubes were used for one run. The protein of one of the gels was stained in order to localize the position of mutase-dehydratase. The corresponding regions of the other gels were cut out. Then the gel pieces were transferred to a tube containing only 0.5 ml of gel solution. The tube was connected to a dialysis bag, which was filled with 1 ml of buffer. The enzyme was eluted electrophoretically overnight into the dialysis bag.

(iii) Sodium dodecyl sulfate electrophoresis. Sodium dodecyl sulfate electrophoresis was carried out in an apparatus described by Stegemann (30) according to the method of Weber and Osborn (34). The electrophoretically purified chorismate mutase-prephenate dehydratase and the reference proteins were incubated in 0.1 M sodium phosphate buffer (pH 7.1) containing 1% sodium dodecyl sulfate and 1% mercaptoethanol at 37 C for 2 h. The protein solutions were mixed with glycerol and bromophenol blue. Then approximately 30 μg of protein of each sample was applied to vertical gel slabs containing 10% polyacrylamide and 0.1% sodium dodecyl sulfate. Electrophoresis was performed at a current of 80 mA for about 4 h. Staining and destaining were carried out as described (34).

Isoelectric focusing. Isoelectric focusing was performed as described by Vesterberg et al. (32). A linear gradient was prepared from (i) a light solution consisting of 59.25 ml of water and 0.75 ml of a mixture of Ampholine carrier ampholytes (pH 3.5 to 10; no. 1809-101, LKB), and (ii) a heavy solution containing 39.75 ml of water, 2.25 ml of Ampholine (pH 3.5 to 10), and 28 g of sucrose. The enzyme was dialyzed against 0.5% Ampholine before it was applied to the gradient. Focusing was performed at 7 C for 45 h in a 110-ml apparatus (LKB) with a final potential of 310 V. Fractions of 2 ml were collected, and the pH of each was determined. The respective enzymes were located by standard assays.

Enzymes and chemicals. The reference proteins for molecular weight determinations as well as NAD and NADH were obtained from Boehringer Mannheim GmbH (Mannheim, Germany). Diethylaminoethyl (DEAE)-cellulose (DE-52) was obtained from Whatman; Sephadex G-200 from Pharmacia; and hydroxyapatite (Bio-Gel HT) from Bio-Rad Labora-

tories. Phenylalanine- and tyrosine-substituted Sepharose were prepared according to the general method of Cuatrecasas et al. (7) as described (26). Chorismic acid was prepared according to the method of Gibson (13). Barium prephenate was prepared by using the auxotrophic mutant strain 6B-1 of *A. eutrophus* H 16 as previously described (11).

RESULTS

Purification of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase. Starting with crude extract (see step 1), chorismatase mutase-prephenate dehydratase and prephenate dehydrogenase were copurified within the first four steps. After the separation of mutase-dehydratase from dehydrogenase activity, each enzyme followed its individual purification steps (Tables 1 and 2). All procedures were carried out at 0 to 4 C.

Step 2: Precipitation of nucleic acids. The crude extract was treated with a 2.5% (wt/vol) solution of cetyltrimethylammonium bromide, resulting in a final concentration of 0.25%. After stirring for 15 min, the precipitate was removed by centrifugation at 42,000 × *g* for 20 min.

Step 3: Ammonium sulfate fractionation. The supernatant solution from step 2 was adjusted to 55% saturation by the addition of 211 g of powdered ammonium sulfate with stirring. The slow stirring was continued for 15 min. After centrifugation at 42,000 × *g* for 20 min, the supernatant was discarded and the pellet was dissolved in 20 mM phosphate buffer, pH 7.4, and dialyzed for 16 h against two changes of the same buffer.

Step 4: Chromatography on DEAE-cellulose. The dialyzed extract was centrifuged at 42,000 × *g* for 20 min to remove insoluble material. Then the clear supernatant was applied to

TABLE 1. *Purification of chorismate mutase-prephenate dehydratase from A. eutrophus H 16*

Purification step	Vol (ml)	Total protein (mg)	Total activity		Sp act		Recovery of mutase activity (%)	Ratio mutase/dehydratase
			Mutase (U)	Dehydratase (U)	Mutase (U/mg)	Dehydratase (U/mg)		
1. Crude extract	660	14,480	1,129	623 (26.1)[a]	0.078	0.043[b] (0.0018)	100	1.81 (43.3)
2. CTAB[c] supernatant	662	10,016	1,022	611 (30)	0.102	0.061 (0.003)	91	1.67 (34)
3. Ammonium sulfate fractionation	125	5,548	860	558 (61)	0.155	0.101 (0.011)	76	1.54 (14.1)
4. DEAE-cellulose	67	385	689	428 (48.1)	1.789	1.111 (0.125)	61	1.61 (14.3)
5. Phenylalanine-Sepharose	41	42	474	347 (40.9)	11.409	8.267 (0.974)	42	1.38 (11.7)
6. Sephadex G-200	16.5	22	373	247 (28.1)	17.055	11.22 (1.277)	33	1.52 (13.4)
7. Hydroxyapatite	31	7.4	271	184 (17.5)	36.622	24.913 (2.358)	24	1.47 (15.5)

[a] Values in parentheses represent activities of the enzyme determined in the absence of tyrosine, i.e., nonactivated.

[b] Prephenate dehydratase activity was measured in the presence of 0.1 mM tyrosine.

[c] CTAB, Cetyltrimethylammonium bromide.

TABLE 2. *Purification of prephenate dehydrogenase from A. eutrophus H 16*

Purification step	Vol (ml)	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Recovery (%)
1. Crude extract	660	14,480	167	0.012	100
2. CTAB[a] supernatant	662	10,016	140	0.014	84
3. Ammonium sulfate fractionation	125	5,548	117	0.021	70
4. DEAE-cellulose	63	311	56	0.180	34
5. DEAE-Sephadex	62	64	31	0.481	18
6. Sephadex G-200	31	23	21	0.919	13
7. Hydroxyapatite	8	1.9	17	8.875	10

[a] CTAB, Cetyltrimethylammonium bromide.

a column of DEAE-cellulose (5 by 36.5 cm) that had been equilibrated with 20 mM phosphate buffer, pH 7.4. The column was washed with 250 ml of the same buffer. Most of the protein was eluted with a 900-ml linear gradient from 20 mM phosphate buffer, pH 7.4, to the same buffer containing 500 mM NaCl. Fractions of approximately 9 ml were collected at an average flow rate of 60 ml/hour. The eluant was tested for absorbance at 280 nm and for mutase, dehydratase, and dehydrogenase activities. A typical elution profile is demonstrated in Fig. 2. There was only one peak of chorismate mutase activity that had been associated with prephenate dehydratase, whereas prephenate dehydrogenase activity was completely separated.

Chorismate mutase-prephenate dehydratase—individual purification steps. Step 5: Chromatography on phenylalanine-substituted Sepharose. The active fractions from step 4 were pooled, dialyzed against 100 mM phosphate buffer, pH 7.4, and concentrated by ultrafiltration as described in Materials and Methods. Then the extract was passed through a column (2.5 by 33 cm) of phenylalanine-substituted Sepharose equilibrated with 100 mM phosphate buffer (pH 7.4). The column was washed with 150 ml of equilibration buffer. This was followed by 100 ml of buffer solution containing 50 mM phosphate (pH 7.4) and 75 ml of buffer solution containing 25 mM phosphate (pH 7.4). Mutase and dehydratase were eluted from the column in a single peak of activity with 5 mM phosphate buffer (pH 7.4) without the addition of phenylalanine (Fig. 3). When phenylalanine was added to the elution buffer

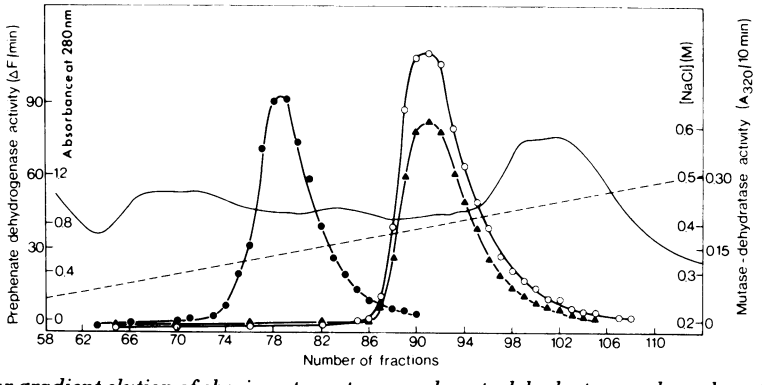

FIG. 2. *Linear gradient elution of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase from DEAE-cellulose (DE-52). Experimental details are described in the text. Symbols: (○) Chorismate mutase activity; (▲) prephenate dehydratase activity; (●) prephenate dehydrogenase activity; (——) absorbance measured at 280 nm; (- -) sodium chloride gradient.*

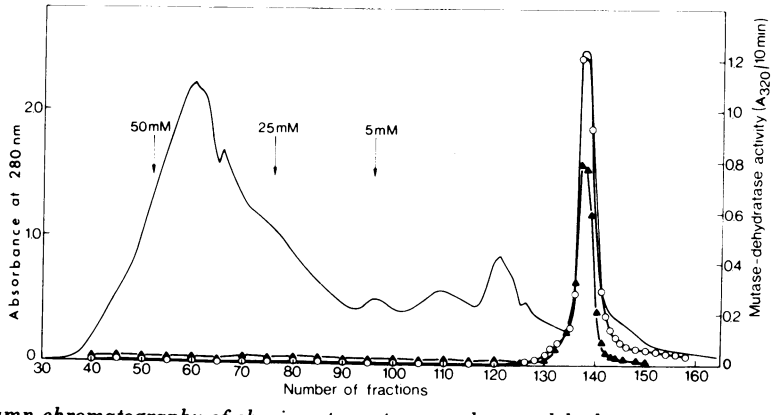

FIG. 3. *Column chromatography of chorismate mutase-prephenate dehydratase on phenylalanine-substituted Sepharose. The protein was eluted in a stepwise declining phosphate gradient. The arrows indicate the phosphate concentration added to the column at these points. Experimental details are described in the text. Symbols: (○) Chorismate mutase activity, (▲) prephenate dehydratase activity; (——) absorbance measured at 280 nm.*

in a concentration of 1 mM, prephenate dehydratase activity could not be detected because it was strongly inhibited by this amino acid. Fractions of 4 ml were collected at an average flow rate of 15 ml/h. The peak fractions were pooled, dialyzed against 50 mM phosphate buffer, pH 7.4, and concentrated by ultrafiltration to a volume of 2 ml.

Step 6: Chromatography on Sephadex G-200. The extract from the previous step was applied to a Sephadex G-200 column (1.5 by 27.5 cm) equilibrated with 50 mM phosphate buffer, pH 7.4. The protein was eluted with the same buffer. Fractions of 2 ml were collected at an average flow rate of 6.0 ml/h. The peak fractions containing chorismate mutase as well as prephenate dehydratase activity were combined, dialyzed against 5 mM phosphate buffer, pH 7.4, and concentrated by ultrafiltration.

Step 7: Chromatography on hydroxyapatite. The concentrated extract from step 6 was applied to a column (1.6 by 14.5 cm) of hydroxyapatite that had been equilibrated with the dialysis buffer. The protein was eluted with 300 ml of phosphate buffer (pH 7.4), starting with a concentration of 5 mM and increasing in a linear gradient to 300 mM. Fractions of 3 ml were collected at an average flow rate of 15 ml/h. Chorismate mutase and prephenate dehydratase activity were eluted simultaneously at 160 mM phosphate. The active fractions were pooled, dialyzed against 50 mM phosphate buffer (pH 7.4), and concentrated with a Diaflo XM 50 membrane to a protein concentration of approximately 1 mg/ml.

These procedures resulted in a 470-fold purification of chorismate mutase-prephenate dehydratase with a yield of 24% (Table 1). During the course of purification, the ratio of the specific activities of mutase and dehydratase varied from 1.3 to 1.8. Where prephenate dehydratase activity was determined in the absence of the activator tyrosine (Table 1, values in parentheses), the apparent purification amounted to 1,310-fold, and the total activity increased within the first purification steps more than twofold. This effect was probably due to a partial inhibition of prephenate dehydratase in the crude extract that was reversed by tyrosine.

Prephenate dehydrogenase—individual purification steps. An attempt to purify prephenate dehydrogenase by affinity chromatography failed. Although the enzyme was adsorbed onto tyrosine-substituted Sepharose, only 6 to 8% of the total activity was recovered after elution. This poor yield remains as yet unexplained.

Step 5: Chromatography on DEAE-Sephadex. The peak fractions from step 4 were pooled, dialyzed against 100 mM phosphate buffer, pH 7.4, and applied to a column of DEAE-Sephadex A-50 (2.5 by 25 cm) equilibrated with the dialysis buffer. The protein was eluted with a 300-ml linear gradient from 100 mM phosphate buffer, pH 7.4, to the same buffer containing 400 mM NaCl. Fractions of 3 ml were collected at an average flow rate of 15 ml/h. After concentrating the prephenate dehydrogenase-containing fractions to a volume of 2.8 ml by ultrafiltration, the extract was dialyzed against 50 mM phosphate buffer, pH 7.4.

Step 6: Chromatography on Sephadex G-200. The extract from the previous step was applied to a Sephadex G-200 column (2.5 by 40 cm) that had been equilibrated with 50 mM phosphate buffer, pH 7.4. The enzyme was eluted with the same buffer. Fractions of 2 ml were collected at an average flow rate of 13 ml/h. The peak fractions were combined and dialyzed against 5 mM phosphate buffer, pH 7.4.

Step 7: Chromatography on hydroxyapatite. The extract obtained from gel filtration was applied to a column of hydroxyapatite (1.6 by 11 cm) equilibrated with the dialysis buffer. Prephenate dehydrogenase was eluted with a 200-ml linear gradient from 5 to 300 mM phosphate buffer, pH 7.4, at 150 mM. Fractions containing the enzyme activity were pooled, dialyzed against 50 mM phosphate buffer, pH 7.4, and concentrated with a Diaflo PM 10 membrane to a protein concentration of approximately 0.7 mg/ml. This preparation contained 740-fold purified enzyme with a yield of 10%.

Stability of purified enzymes. The purified enzymes were found to be stable for at least 10 months when stored in 50 mM phosphate buffer (pH 7.4) at −20 C as a concentrated solution (at least 0.7 mg of protein per ml). When storing diluted enzyme preparations, glycerol was added to 60% to increase the stability.

Homogeneity of purified enzymes. Polyacrylamide electrophoresis of purified chorismate mutase-prephenate dehydratase (step 7) revealed one main band of protein containing both enzyme activities, whereas the few contaminating protein bands contained neither mutase nor dehydratase activity (Fig. 4A). The contaminating proteins were removed by polyacrylamide electrophoresis as described in Materials and Methods. The resulting enzyme preparation was electrophoretically homogeneous.

When prephenate dehydrogenase was subjected to electrophoresis on polyacrylamide gels, three protein bands appeared (Fig. 4C), one of which contained prephenate dehydrogenase activity as could be demonstrated by assay (Fig. 4B). After storage for 6 months in 50 mM

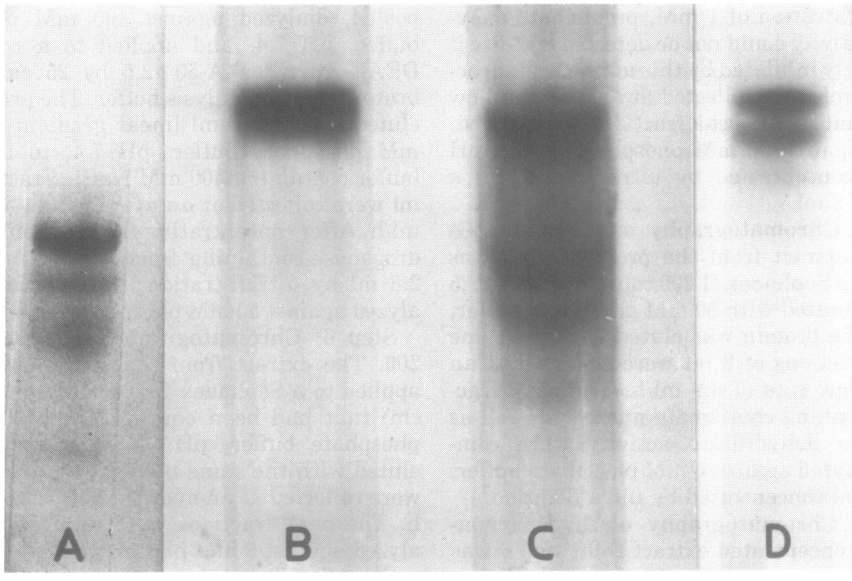

FIG. 4. *Polyacrylamide gel electrophoresis of highly purified chorismate mutase-prephenate dehydratase (A) and prephenate dehydrogenase (B-D). (A) 50-μg sample of chorismate mutase-prephenate dehydratase, purified about 500-fold (step 7); (B) 100-μg sample of freshly purified prephenate dehydrogenase (700-fold) stained for enzyme activity and (C) stained for protein; and (D) prephenate dehydrogenase activity after storage for 6 months at −20 C.*

phosphate buffer containing 60% (vol/vol) glycerol at −20 C, the same enzyme preparation revealed two catalytically active protein bands (Fig. 4D). An attempt to remove the contaminating proteins by electrophoresis failed because prephenate dehydrogenase lost activity under these conditions.

Molecular weight. The molecular weights of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase were determined by three different methods: gel filtration (1), sucrose gradient centrifugation (24), and electrophoresis (16).

Filtration on Sephadex G-200 resulted in a molecular weight of 187,000 for mutase-dehydratase, whereas the value determined for prephenate dehydrogenase was 65,000 (Fig. 5).

Lower molecular weights were obtained when the purified enzymes were subjected to sucrose gradient centrifugation. Relating the peak fractions of chorismate mutase-prephenate dehydratase as well as prephenate dehydrogenase to those of aldolase ($s_{20,w}$ = 7.8S) or yeast alcohol dehydrogenase ($s_{20,w}$ = 7.6S), $s_{20,w}$ values of 7.4S and 3.9S were calculated, corresponding to molecular weights of 144,000 and 55,000, respectively. The sedimentation constant for chorismate mutase-prephenate dehydratase increased to 7.8S when centrifugation was conducted in the presence of the inhibitor phenylalanine (1 mM) or the activator tyrosine

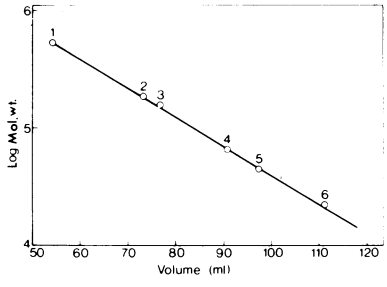

FIG. 5. *Determination of the molecular weight of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase by gel filtration. (2) 0.7 mg of chorismate mutase-prephenate dehydratase as well as (4) 0.5 mg of prephenate dehydrogenase and the following reference proteins (molecular weights in parentheses): (1) 1.4 mg of ferritin (540,000); (3) 1.1 mg of aldolase (158,000); (5) 2.7 mg of ovalbumin (45,000); and (6) 1.9 mg of chymotrypsinogen A (25,000) were applied to a column of Sephadex G-200 and eluted as described in the text.*

(1 mM). Under all conditions, the activity profiles remained exactly coincident.

A molecular weight of 160,000 to 180,000 for chorismate mutase-prephenate dehydratase was estimated by electrophoresis according to Hedrick and Smith (16) as described in Materials and Methods. Using this technique, a value of 65,000 for freshly prepared prephenate dehydrogenase was determined (Fig. 6). However,

after storage of the enzyme in 60% glycerol (vol/vol), an approximately twofold higher molecular weight (116,000) was obtained (not shown).

Sodium dodecyl sulfate-polyacrylamide electrophoresis. Electrophoretically homogeneous chorismate mutase-prephenate dehydratase was treated with 1% sodium dodecyl sulfate in the presence and absence of mercaptoethanol. After electrophoresis, only a single band of protein appeared. Its electrophoretic mobility corresponded to a molecular weight of 47,000, referring to the mobilities of catalase, aldolase, ovalbumin, and chymotrypsinogen (Fig. 7). An additional treatment of the enzyme with 8 M urea did not influence this result, which indicates that mutase-dehydratase is composed of identical or at least similar subunits.

Isoelectric point. An extract containing a mixture of purified chorismate mutase-prephenate dehydratase as well as prephenate dehydrogenase was subjected to isoelectric focusing in a gradient ranging from pH 3.5 to 10. The isoelectric point for prephenate dehydrogenase was found to be pH 6.6, whereas the activities of mutase and dehydratase banded at pH 5.8 (Fig. 8). Even by this method, based on a different behavior of proteins in a pH gradient, no separation of chorismate mutase from prephenate dehydratase activity occurred.

Effect of metal ions and thiol reagents on the enzyme activities. The activity of chorismate mutase-prephenate dehydratase was inhibited in the presence of the divalent metal ions Ni^{2+}, Hg^{2+}, Cu^{2+}, and Fe^{2+}. However, the dehydratase activity was apparently more sensitive to the cations than chorismate mutase and was additionally affected by Mg^{2+}, Co^{2+}, and Zn^{2+} (Table 3). Prephenate dehydrogenase was inhibited by Mg^{2+}, Hg^{2+}, Cu^{2+}, and Fe^{2+} (Table 3).

None of the enzyme activities assayed in this study were found to be stimulated by the addition of thiol reagents such as mercaptoethanol and dithiothreitol.

Formation of prephenate in the overall conversion of chorismate to phenylpyruvate. Because of the association of mutase and dehydratase, the question arose of whether prephenate is an enzyme-bound or a dissociable intermediate in the overall reaction leading from chorismate to phenylpyruvate. To solve this problem, purified chorismate mutase-prephenate dehydratase was incubated with chorismate, and the formation of prephenate and phenylpyruvate was measured simultaneously by taking aliquots at different time intervals from one sample. It was obvious (Fig. 9) that chorismate was instantaneously converted to prephenate,

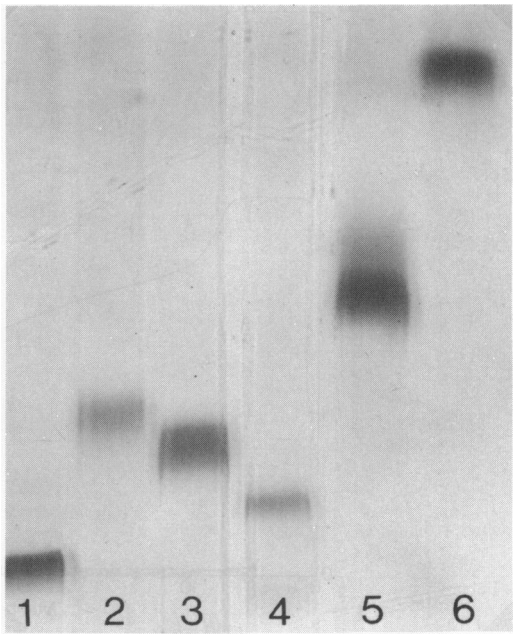

FIG. 7. *Electrophoresis of chorismate mutase-prephenate dehydratase in a gel containing 0.1% sodium dodecyl sulfate and 10% polyacrylamide. The proteins were treated with 1% sodium dodecyl sulfate for 2 h at 37 C in the presence of 1% mercaptoethanol. Then 30 μg of each sample was applied to vertical gel slabs. The bands represent the following proteins and their corresponding minimum molecular weights (in parentheses): (1) catalase (60,000); (2) aldolase (40,000); (3) ovalbumin (45,000); (4) chorismate mutase-prephenate dehydratase; (5) chymotrypsinogen A (25,000); (6) cytochrome c (11,700).*

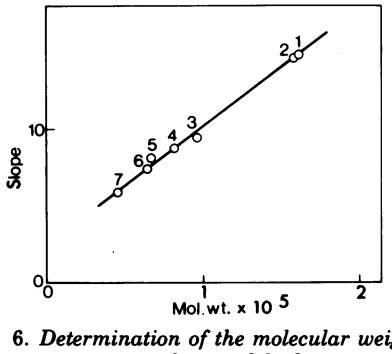

FIG. 6. *Determination of the molecular weight of chorismate mutase-prephenate dehydratase and prephenate dehydrogenase by electrophoresis on polyacrylamide gels (19). The following proteins were used (molecular weights in parentheses): (1) Chorismate mutase-prephenate dehydratase, (2) aldolase (158,000), (3) hexokinase (97,000), (4) creatine kinase (81,000), (5) bovine serum albumin (66,000), (6) prephenate dehydrogenase, (7) ovalbumin (45,000).*

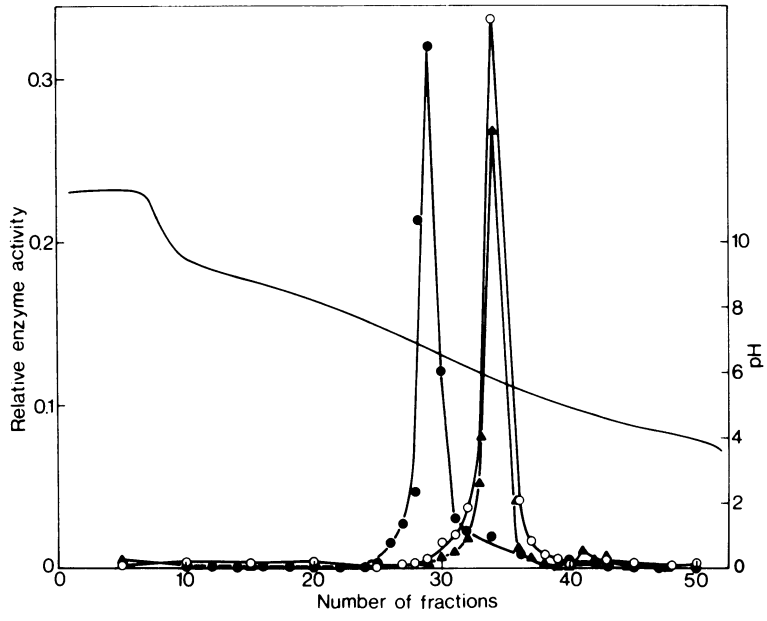

FIG. 8. *Isoelectric focusing. Fractions (2 ml) were assayed for chorismate mutase (○), prephenate dehydratase (▲), and prephenate dehydrogenase (●) activity by the standard procedure as described in the text. The pH (——) of each fraction was determined.*

whereas the formation of phenylpyruvate started after a period of 8 min. This time is obviously necessary for prephenate to be accumulated up to a certain concentration before it allows prephenate dehydratase activity to become evident.

DISCUSSION

Chorismate mutase from *A. eutrophus* H 16 has never been separated into more than one enzyme species. No abrupt decay of its total activity has ever been observed that would indicate the loss of an isoenzyme. With respect to this property, chorismate mutase is similar to the enzyme characterized in *Neurospora crassa* (2), *Saccharomyces cerevisiae* (29), *Streptomyces aureofaciens* (15), and *Euglena gracilis* (33). However, chorismate mutase from *A. eutrophus* H 16 is associated with prephenate dehydratase activity and is therefore different from the protein in the above-mentioned organisms.

A great number of proteins catalyzing two or more different reactions have been discovered in microorganisms (14). As shown for enteric bacteria, there are two distinct chorismate mutases. One of them is associated with prephenate dehydrogenase, and the other is linked to prephenate dehydratase activity (6, 8, 27). Both proteins are bifunctional enzymes (8, 20). In *Pseudomonas aeruginosa* two prephenate dehydratase isoenzymes were found, one of which is complexed with chorismate mutase (4). As reported for *Bacillus subtilis* 168, chorismate mutase is present as a bifunctional protein in association with 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthetase (17, 18).

The following data clearly show that chorismate mutase and prephenate dehydratase ac-

TABLE 3. *Effect of divalent cations on chorismate mutase-prephenate dehydratase and prephenate dehydrogenase activity*[a]

Cation	Enzyme activity (%)		
	Chorismate mutase	Prephenate dehydratase	Prephenate dehydrogenase
–	100	100	100
CO^{2+}	94	67	100
Cu^{2+}	35	0	0
Fe^{2+}	21	15	52
Hg^{2+}	59	6	0
Mg^{2+}	95	30	10
Mn^{2+}	ND[b]	ND[b]	85
Ni^{2+}	62	17	100
Zn^{2+}	91	42	86

[a] The assays were done under standard conditions except that metal ions were added as chloride salts at a final concentration of 0.1 mM. The enzyme activities refer to values measured in the absence of the inhibitors, set as 100%.

[b] ND, Not determined.

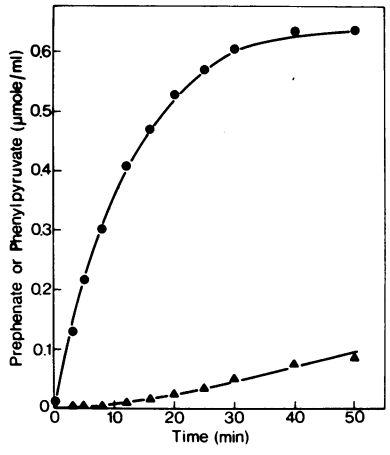

FIG. 9. *Formation of prephenate and phenylpyruvate from chorismate catalyzed by chorismate mutase-prephenate dehydratase. The reaction mixture contained in a final volume of 8 ml: 0.6 mM potassium chorismate, 50 mM potassium phosphate (pH 7.8), and purified enzyme (0.35 unit of chorismate mutase activity). Aliquots of 0.2 ml were removed for determination of prephenate (●) and phenylpyruvate (▲).*

tivity in *A. eutrophus* H 16 are at least tightly associated in a protein complex. However, some of the evidence strongly indicates a bifunctional protein consisting of identical polypeptide chains. Furthermore, it is very likely that the tyrosine-synthesizing enzyme, prephenate dehydrogenase, is not linked to any other protein of the pathway.

(i) Mutase and dehydratase activity were separated by neither DEAE-cellulose, Sephadex G-200, nor hydroxyapatite. Both activities were adsorbed on phenylalanine-substituted Sepharose and co-eluted from the column. Prephenate dehydrogenase was easily separated from the bifunctional enzyme by ion exchange chromatography or gel filtration.

(ii) The ratio of the specific activity of chorismate mutase to prephenate dehydratase remained relatively constant. However, it must be noticed that this could only be achieved by estimating prephenate dehydratase activity in the presence of its activator, tyrosine. In crude extracts, the dehydratase had a remarkably low affinity for prephenate which changed during the purification, resulting in an apparent increase of the total activity. Tyrosine maintained the K_m for prephenate at an almost constant value, thus preventing this effect. From the regulation studies (12), it is likely that prephenate dehydratase is partially inhibited in the crude extract. By removing the inhibitor it gains activity.

(iii) The isoelectric points of mutase-dehydratase were identical: both activities banded at pH 5.8. The isoelectric point of prephenate dehydrogenase was found to be pH 6.6.

(iv) Mutase and dehydratase were located in the same band of protein in polyacrylamide gels.

(v) The molecular weights of native chorismate mutase and prephenate dehydratase were identical, but varied with respect to the method applied, ranging from 144,000 to 187,000. The reason for this discrepancy has not been investigated, but similar deviations are reported for mutase-dehydratase from *Salmonella typhimurium* (28) and chorismate mutase from *Streptomyces aureofaciens* (15). Prephenate dehydrogenase had a much lower molecular weight of approximately 60,000.

Electrophoresis of homogeneous mutase-dehydratase in sodium dodecyl sulfate-polyacrylamide gel revealed a single protein band corresponding to a molecular weight of 47,000. With respect to the native molecular weight, the enzyme is composed of four similar or identical subunits.

(vi) Single-site mutants have been isolated that lack chorismate mutase as well as prephenate dehydratase activity (11). Both activities were simultaneously regained by reversion.

Chorismate mutase-prephenate dehydratase as a bifunctional enzyme and prephenate dehydrogenase as a separate protein without association represent a new type of enzyme organization in phenylalanine and tyrosine biosynthesis. Prephenate, whose formation in enteric bacteria is guaranteed by two distinct bifunctional chorismate mutases (6, 8, 27), is synthesized in *A. eutrophus* H 16 by only one enzyme, which concomitantly catalyzes its further conversion to phenylpyruvate. The problem appeared of whether prephenate sticks to the enzyme or dissociates during the overall reaction. In fact, prephenate accumulated in the reaction mixture, thus allowing prephenate dehydrogenase to compete for the substrate. Such a complex biosynthetic system requires a highly sensitive control in order to function. This is subject to a separate investigation, which will also consider the fact that *A. eutrophus* H 16 is able to form tyrosine from phenylalanine by enzymatic hydroxylation (10).

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