Chorismate Mutase from *Streptomyces*. Purification, Properties, and Subunit Structure of the Enzyme from *Streptomyces aureofaciens* Tü 24†

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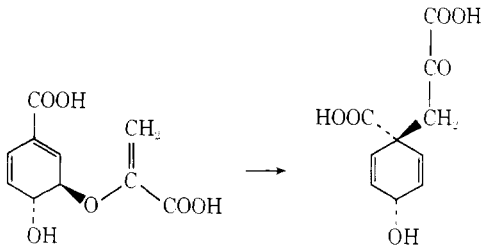
ABSTRACT: Chorismate mutase of *Streptomyces aureofaciens* Tü 24 has been purified 2200-fold and the preparation is homogeneous by the criterion of polyacrylamide gel electrophoresis. To achieve a homogeneous preparation it is essential to treat the enzyme solutions with phenylmethanesulfonyl fluoride after each purification step. Chorismate mutase shows a molecular weight of 51,000 as determined by sucrose gradient centrifugation and of 63,000 as determined by Sephadex gel chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis reveals a single protein band, the molecular weight of which is estimated to be 14,500. Therefore, the native enzyme consists of at least three polypeptides of identical or almost identical molecular weight. These subunits are not linked together by disulfide bridges. The activity of the enzyme does not depend on the presence of metal ions nor are sulfhydryl groups present, which are essential for catalytic activity. The Michaelis constant for its substrate chorismic acid and the properties with respect to feedback inhibition and heat treatment are essentially the same for the purified chorismate mutase as that reported for a partially purified preparation of this enzyme (Görisch, H., and Lingens, F. (1973), *J. Bacteriol. 114*, 645).

The main branching point in the biosynthetic pathway of the aromatic amino acids is the position of chorismic acid. Chorismate mutase, the first enzyme of the terminal steps in the biosynthesis of phenylalanine and tyrosine, catalyzes the conversion of chorismic acid to prephenic acid (Scheme I). The enzyme has been purified from procaryotic organisms (Koch *et al.*, 1970a, 1971a; Davidson *et al.*, 1972) as well as from a eucaryotic one (Weber and Böck, 1970).

In studying the regulation pattern of the aromatic biosynthetic pathway in *Streptomyces* it was found that chorismate mutase of *Streptomyces aureofaciens* reveals a remarkable heat stability, although this organism is not a thermophilic one.

SCHEME I



Materials and Methods

Chemicals. The inorganic chemicals used were of analytical reagent grade. All other chemicals were obtained as the highest purity grade available. Hydroxylapatite Bio-Gel HT was obtained from Bio-Rad Laboratories, Munich, Germany, and antifoam Niax Polyol PPG 2025 was obtained from Brenntag GmbH, Mülheim, Germany. Chorismic acid was isolated according to Gibson (1970).

Organism and Growth Conditions. Streptomyces aureofaciens Tü 24 grown at 29° in a minimal medium containing 10 g of glycerol, 1 g of NH_4Cl, 1 g of K_2HPO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of NaCl, 1 g of $CaCO_3$, and 0.2 ml of antifoam per liter. In the large scale fermentation tap water was used instead of deionized water. *S. aureofaciens* from a submers culture was inoculated into a 1-l. erlenmeyer flask containing 500 ml of the minimal medium and was shaken for 48 hr. The cell suspension then was transferred to a laboratory fermentor containing 10 l. of minimal medium and *S. aureofaciens* was grown with an air flow of 240 l./hr and a stirring velocity of 200 rpm. After 48 hr the culture was transferred to a pilot fermentor containing 500 l. of minimal medium and the organism was grown with an air flow of 10,000 l./hr and a stirring velocity of 300 rpm for an additional 45 hr. *S. aureofaciens* was harvested at the exponential growth phase by a Westfalia-Separator and the wet cell paste was stored at −25°. The yield of the 500-l. batches varied between 5 and 6 kg.

Buffers used were: (A) 0.1 M potassium phosphate (pH 7)–2.5×10^{-4} M EDTA; (B) 0.05 M potassium phosphate (pH 7)–0.05 M $(NH_4)_2SO_4$–2.5×10^{-4} M EDTA; (C) 0.01 M potassium phosphate (pH 7)–2.5×10^{-4} M EDTA; (D) 0.4 M Tris-HCl (pH 8.9)–2.5×10^{-4} M EDTA; (E) 0.025 M Tris-HCl (pH 8.9)–2.5×10^{-4} M EDTA; (F) 0.03 M Tris-HCl (pH 8.9)–2.5×10^{-4} M EDTA.

Enzymatic Test. The activity of chorismate mutase was determined by estimating the prephenate formed from chorismate after 10 min at 30°. The standard reaction mixture contained 2.5 μmol of chorismate and protein in a total volume of 1 ml of buffer B. After incubation 0.5 ml of 2 N HCl was added and incubation continued for a further 10 min at 30°. Following the addition of 1 ml of 3 N NaOH the absorbancy was measured at 320 nm. A molar extinction coefficient of 17,000 was used (Nishioka and Woodin, 1972) to calculate the amount of phenylpyruvic acid formed by the acid treatment of prephenate. Correction for the nonenzymatic conversion of chorismate to prephenate during incubation was made by including an appropriate blank. One unit of enzyme represents the formation of 1 μmol of prephenate/min under standard

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conditions. Specific activity is expressed as units per milligram of protein.

Protein Determination. Protein was determined by the method of Groves *et al.* (1968), using the isoabsorbance wavelengths 224 and 235 nm, which have been determined for a nucleic acid preparation of *S. aureofaciens* in buffer A. This method gave comparable results to that of Lowry *et al.* (1951).

Molecular Weight Determination. CHROMATOGRAPHY ON SEPHADEX G-100. A column 2.5 cm × 90 cm with Sephadex G-100 was prepared according to Andrews (1964) and equilibrated with buffer B at 4°. The sample solutions of 1.5 ml were carefully layered under the buffer solution above the gel. The flow rate was maintained at 25 ml/hr and fractions of 5.4 ml were collected.

SUCROSE GRADIENT CENTRIFUGATION. Sucrose gradient centrifugation was performed according to Martin and Ames (1961) as described previously (Görisch and Lingens, 1973).

Polyacrylamide Gel Electrophoresis. Disc electrophoresis in polyacrylamide gels was performed as described by Ornstein and Davis (1962). Acrylamide gels (7.5%) were made without potassium ferricyanide. Prior to application the enzyme was dialyzed against buffer D. No sample gel was used and the sample was applied directly on top of the concentrating gel. For location of enzymatic activity after electrophoresis the gels were sliced in sections of approximately 1.8 mm by a razor blade. The enzyme was eluted from the sliced gel by 3 ml of buffer D at room temperature for 2 hr. Samples of 500 μl were withdrawn to determine the enzymatic activity in buffer D under standard conditions.

Acrylamide gel electrophoresis in sodium dodecyl sulfate was performed by the method of Weber and Osborne (1969), using two times the amount of cross-linker. Before adding sodium dodecyl sulfate and β-mercaptoethanol to the protein solutions the samples were either heated in a boiling water bath for 3 min or a 2×10^{-2} M solution of $PhMeSO_2F$[1] in 2-propanol was added to a final concentration of 10^{-3} M $PhMeSO_2F$ to prevent the possibility of proteolytic digestion (Pringle, 1970).

Test for Proteolytic Activity. Proteolytic activity was tested in buffers A or B for 30 or 60 min by the casein digestion method according to Laskowski (1955).

Enzyme Purification. All fractionation procedures were carried out at 4°. To reduce proteolytic digestion, the enzyme solutions were treated with $PhMeSO_2F$ after each purification step (Fahrney and Gold, 1963). This was done by adding to the protein solution 0.05 vol of a solution of 2×10^{-2} M $PhMeSO_2F$ in 2-propanol, thus making the resulting solution approximately 10^{-3} M in $PhMeSO_2F$. The procedure will be referred to as adding $PhMeSO_2F$.

STEP I: CRUDE EXTRACT. Wet frozen cells (200 g) of *Streptomyces aureofaciens* were thawed in 700 ml of buffer A and subsequently $PhMeSO_2F$ was added. The suspension was mixed for 1 min in a Waring Blendor and cells were disrupted by ultrasonic treatment. Cell debris was removed by centrifugation for 20 min at 20,000*g*.

STEP II: $(NH_4)_2SO_4$ FRACTIONATION. To the slightly turbid crude extract, solid $(NH_4)_2SO_4$ was added with constant stirring to a final concentration of 50% (Dawson *et al.*, 1969). The resulting precipitate was removed by centrifugation and $(NH_4)_2SO_4$ was added to a final saturation of 70%. The latter fraction contained most of the enzymatic activity and was resuspended in 80 ml of buffer B after centrifugation. $PhMeSO_2F$ was added and the solution was allowed to stand at 4° for 30–60 min.

STEP III: SEPHADEX G-200 CHROMATOGRAPHY. The enzyme solution of step II was applied to the top of a column (10 cm × 90 cm) of Sephadex G-200, which was equilibrated with buffer B. Elution was performed with buffer B with a flow rate of approximately 400 ml/hr. After elution of the bulk of protein with about 4 l. of buffer, fractions of 20 ml were collected. Chorismate mutase was eluted in a symmetrical peak and the active fractions with more than 0.15 unit of enzyme/ml were pooled. The protein was precipitated by adding solid $(NH_4)_2SO_4$ with constant stirring to a final concentration of 75% (Dawson *et al.*, 1969). The precipitate was collected by centrifugation at 20,000*g* for 20 min and redissolved in 20 ml of buffer C. $PhMeSO_2F$ was added and after 30–60 min at 4° the solution was stored at −25°.

STEP IV: HYDROXYLAPATITE CHROMATOGRAPHY. The enzyme solutions of five preparations from step I to III above were combined. After thawing a slight turbidity was removed by centrifugation at 20,000*g* for 20 min. The enzyme was dialyzed for 1–2 hr against 4 l. of buffer C and applied on top of a column (5 cm × 8 cm) of hydroxylapatite, which was equilibrated with 0.01 M potassium phosphate buffer (pH 7). Chorismate mutase was eluted with a linear gradient of 900 ml of 0.01 M potassium phosphate (pH 7) *vs.* 900 ml of 0.15 M potassium phosphate (pH 7) and fractions of 12 ml were collected. The active fractions with more than 0.8 unit of chorismate mutase/ml were combined.

STEP V: SEPHADEX G-100 CHROMATOGRAPHY. The active fractions from the hydroxylapatite column were concentrated to a volume of 15 ml using an Amicon Diaflo ultrafiltration apparatus with a PM-10 membrane. $PhMeSO_2F$ was added and the sample allowed to stand for 30 min at 4°. After dialysis against 500 ml of buffer B for 2 hr the sample was further concentrated as before to a volume of 2 ml. Again $PhMeSO_2F$ was added and after waiting for 30 min the sample was applied on a column (2.5 cm × 90 cm) of Sephadex G-100, which was equilibrated with buffer B. The column was eluted with a flow rate of 20–25 ml/hr and fractions of 6 ml were collected. The active fractions with more than 4.3 enzyme units/ml were combined.

STEP VI: PREPARATIVE GEL ELECTROPHORESIS. The active fractions of the Sephadex G-100 chromatography were concentrated by an ultrafiltration apparatus to a volume of 10 ml. $PhMeSO_2F$ was added and after 30 min the solution was dialyzed against 800 ml of buffer D for 1 hr. The enzyme was further concentrated as before to a volume of 1 ml and subjected to preparative gel electrophoresis. A preparative disc electrophoresis apparatus Model EA 100 from Wissenschaftl.-Technische Werkstätten GmbH, Weilheim, Germany, was used. The separating column had an inner diameter of 2.5 cm and separating gels approximately 5 cm long were used underneath a 1 cm concentrating gel. A 7.5% separating gel was made according to Ornstein and Davis (1962) without ferricyanide. The sample was carefully layered under the electrode buffer on top of the concentrating gel. The system was run at 2° and a constant current of 30 mA was applied, which required 400–500 V. The elution buffer D had a flow rate of 20 ml/hr and fractions of 4.2 ml were collected. The main active fractions with more than 3 units of chorismate mutase per ml were pooled.

STEP VII: SUCROSE GRADIENT ELECTROPHORESIS. To the combined active fractions of the preparative gel electrophoresis $PhMeSO_2F$ was added. After standing 1 hr at 4° the sample was dialyzed against 1 l. of buffer E for 2 hr, with one buffer change after 1 hr. The enzyme was concentrated as before to

[1] Abbreviations used are: $PhMeSO_2F$, phenylmethanesulfonyl fluoride; *p*-HgBzOOH, *p*-hydroxymercuribenzoate.

TABLE I: Summary of Purification Procedure.[a]

Step	Vol (ml)	Total Enzyme Units (U) (μM/min)	Total protein (mg)	Sp. Act. (U/mg)	Yield (%)	Purification
I. Crude extract	4000	1960	35,600	0.055	100	1
II. $(NH_4)_2SO_4$	400	1670	7,600	0.22	85	4
III. Sephadex G-200	2500	980	1,485	0.66	50	12
IV. Hydroxylapatite	360	392	59.5	6.6	20	120
V. Sephadex G-100	30	314	28.5	11	16	200
VI. Preparative disc electrophoresis	25	157	2.86	55	8	1200
VII. Sucrose gradient electrophoresis	21	98	0.81	121	5	2200

[a] The data for the purification scheme were calculated assuming that the procedure was started with 1 kg of wet cell paste of *Streptomyces aureofaciens*. In practice, steps I–III of the purification procedure were performed with 200 g of *S. aureofaciens* and the eluates of five G-200 columns (step III) were combined for the subsequent chromatography on hydroxylapatite (step IV).

a volume of 1 ml and subjected to sucrose gradient electrophoresis. The apparatus was obtained from H. Technik, A. Hölzel, Munich, Germany, and the procedure described by Zillig *et al.* (1966) was followed. Buffer was used to make up the sucrose solutions and the gradient. After 17 hr of electrophoresis with a constant current of 10 mA, which required 500–600 V, fractions of 3.5 ml were collected. The active fractions with more than 2 units of chorismate mutase per ml were combined and dialyzed for 2 hr against 1 l. of buffer B. After concentration to a volume of 2–3 ml by a Diaflo ultrafiltration apparatus and the addition of $PhMeSO_2F$ the purified enzyme solution could be stored at 4° without loss of activity for at least 1 month.

Results

Purification of Chorismate Mutase. Table I summarizes the results obtained following the procedure described under Materials and Methods. Earlier attempts to purify the enzyme without using $PhMeSO_2F$, an inhibitor of serine proteases, resulted in a splitting of chorismate mutase activity into two major fractions when chromatographed on hydroxylapatite. Both fractions showed the same molecular weight of 51,000 as determined by sucrose gradient centrifugation and of 63,000 as determined by chromatography on Sephadex G-100. They both were stable with respect to heat treatment and also showed the same Michaelis constant as the unmodified enzyme. After storage at 4° for 1 month, both enzyme solutions still showed 100% of the original activity.

When the two fractions of chorismate mutase, which had been separated on hydroxylapatite, were subjected to the subsequent purification steps, they both formed broad elution peaks on preparative disc electrophoresis and split again into two or occassionally three not well separated peaks on sucrose gradient electrophoresis. The overall purification of those preparations varied between 1700- and 2000-fold.

Proteolytic activity could easily be demonstrated to be present in crude extracts and after $(NH_4)_2SO_4$ fractionation. But despite the fact that the bulk of these proteases is separated from chorismate mutase activity on Sephadex G-200, it was necessary to use $PhMeSO_2F$ always throughout the purification procedure to achieve a homogeneous preparation and to prevent chorismate mutase from splitting into different activities during the subsequent purification steps.

Homogeneity of Chorismate Mutase. The homogeneity of the enzyme preparation was tested by analytical polyacrylamide gel electrophoresis. As shown in Figure 1 the enzyme migrates as a single component. No further protein band is visible when up to 50 μg of protein is applied to a single gel. On sectioning analytical gels into slices and checking for activity only one peak of chorismate mutase activity was found. Figure 2 shows the correspondence of enzymatic activity and stained protein band in duplicate runs. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate also reveals only a single band of protein, but this protein shows a much lower molecular weight than the intact enzyme (see subunit structure).

Kinetic Properties of Chorismate Mutase. Chorismate mutase shows uncomplicated reaction kinetics under the conditions of assay. Within a limited range of enzyme concentration the reaction rate is a linear function of protein concentration and no disproportional loss of activity occurs with the dilution of extracts. The reaction rate at 30° is maintained for 15 min. The enzymatic activity shows a broad optimum between pH 7 and 8.2.

The initial velocity of prephenate synthesis at various concentrations of chorismate follows regular Michaelis–Menten kinetics. A Lineweaver–Burk plot yields a straight line which results in a K_m of 5.2×10^{-4} M. No deviation of linearity is detected and there is no evidence of cooperative subunit interaction.

The activity of the purified chorismate mutase is not influenced by phenylalanine, tyrosine, or tryptophan, the end products of the aromatic biosynthetic pathway. The metabolites shikimic acid, anthranilic acid, phenylpyruvic acid, and *p*-hydroxyphenylpyruvic acid also show no effect.

The enzyme can be inactivated at the temperature of a boiling water bath and afterward reactivated at 30° to 100% of its original activity. This reactivation process is concentration dependent. Both the native and the reactivated chorismate mutase show the same Michaelis constant. They also show the same molecular weight as determined by sucrose gradient centrifugation.

Factors Influencing the Enzyme Activity. Chorismate mutase from *S. aureofaciens* is not inhibited in the presence of 5×10^{-3} M EDTA whether the crude extract or the purified enzyme is tested and therefore does not require any metal ion for enzymatic activity. Since the enzyme is also not inhibited in the presence of 5×10^{-4} M *p*-HgBzOOH[1] there exists no sulfhydryl group which is essential for the enzymatic conversion of chorismate to prephenate. β-Mercaptoethanol in concentrations of 10^{-3} M does not affect the activity of chorismate mu-

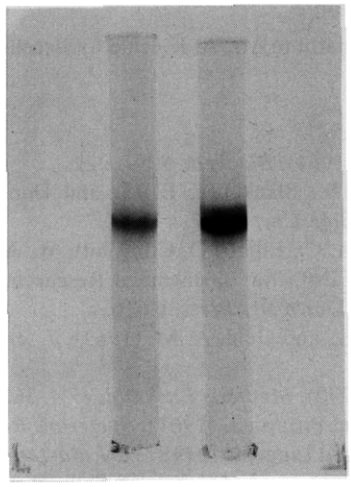

FIGURE 1: Polyacrylamide disc electrophoresis of purified chorismate mutase. Direction of migration is from cathode (top) to anode (bottom); 15 μg (left) and 50 μg (right) of enzyme were applied.

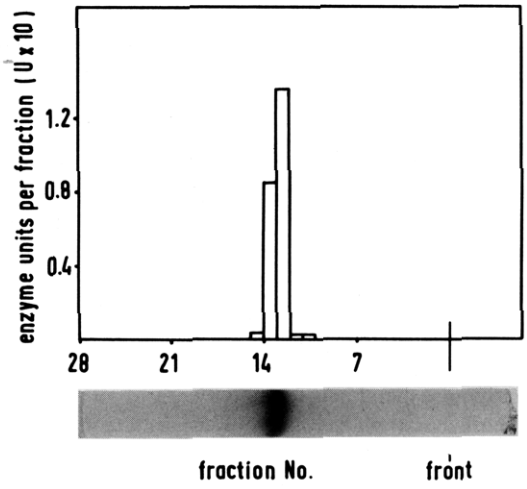

FIGURE 2: Activity of chorismate mutase from analytical polyacrylamide gel; 3 μg of enzyme ($\simeq$0.36 U) was applied. A duplicate gel with 15 μg of chorismate mutase is shown stained for protein. Direction of migration is from left to right.

tase and dithiothreitol in concentrations of 10^{-3} M also does not influence the enzymatic activity whether it is present in the reduced or the oxidized form.

Molecular Weight. The molecular weight of chorismate mutase was determined by Sephadex G-100 gel filtration as described by Andrews (1964). The column was calibrated with proteins of known molecular weights: γ-globulin (bovine) mol wt 160,000; serum albumin (bovine) mol wt 67,000; and chymotrypsinogen (bovine pancreas) mol wt 25,700. Chorismate mutase is eluted in a single symmetrical peak at almost the same position as albumin. From the elution volume corresponding to chorismate mutase activity a molecular weight of 63,000 is calculated.

The molecular weight of the purified enzyme was also determined by sucrose gradient centrifugation according to Martin and Ames (1961), and human hemoglobin was used as a marker. The enzyme showed a Svedberg constant of $s_{20,w} = 3.9$. The molecular weight of the enzyme was calculated to be 51,000 by using the relationship $M_1/M_2 = (S_1/S_2)^{3/2}$, and values of $s_{20,w} = 4.55$ and mol wt (M) 64,000 for hemoglobin.

Subunit Structure from Sodium Dodecyl Sulfate Gel Electrophoresis. In order to learn whether the enzyme is composed of a single polypeptide or whether additional polypeptide chains are present, chorismate mutase was subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate. The enzyme was denatured by sodium dodecyl sulfate in the presence and in the absence of β-mercaptoethanol. In each case a single protein component of low molecular weight was detected, both of which showed the same mobility. To establish a molecular weight–mobility profile on the gel, serum albumin (bovine) (mol wt 67,000), pepsin (hog stomach) (mol wt 35,000), chymotrypsinogen (bovine pancreas) (mol wt 25,700), lysozyme (egg white) (mol wt 14,300), and ribonuclease (bovine pancreas) (mol wt 13,700) were used. The subunit of chorismate mutase showed almost the same mobility as lysozyme and its molecular weight was estimated to be 14,500.

Discussion

Chorismate mutase from *Streptomyces aureofaciens* has been purified 2200-fold. Analytical polyacrylamide gel electrophoresis reveals a single protein band and no additional bands are detectable when up to 50 μg of protein is applied to a single gel. When slices of analytical gels are tested for enzymatic activity only one peak of activity is found. The activity peak is coincident with the protein band stained on a duplicate run. Analytical polyacrylamide gel electrophoresis in sodium dodecyl sulfate also shows only one single protein band. Thus, it is concluded that the preparation of chorismate mutase is homogeneous.

The homogeneous preparation of chorismate mutase could be achieved only by treating the protein solutions with PhMeSO_2F, an inhibitor of serine proteases. Similar observations are reported for the purification of yeast hexokinase. This enzyme could be prepared in a homogeneous form when diisopropyl fluorophosphate as protease inhibitor was used throughout the purification procedure (Lazarus *et al.*, 1966). When chorismate mutase is purified without PhMeSO_2F treatment the activity splits into two major fractions when chromatographed on hydroxylapatite. Thus it seems that proteolytic modification occurs. But this modification is not a severe one because the catalytic properties of the enzymes are not altered. The two different fractions of chorismate mutase obtained after chromatography on hydroxylapatite both show the same Michaelis constant for the substrate chorismic acid as the unmodified enzyme. The molecular weight also is not changed as tested by sucrose gradient centrifugation. Differences in these fractions are revealed when the enzyme is chromatographed on hydroxylapatite or when electrophoretic methods are employed. It is assumed that by the proteolytic digestion small modifications of the enzyme occur which result in species different in net charge but with no cross alteration of the molecular weight or in catalytic properties. In fact no decrease of enzymatic activity was observed when such samples were stored at 4° for a time period of 1 month.

The molecular weight of the intact enzyme was determined by Sephadex G-100 chromatography to be 63,000, whereas by sucrose gradient centrifugation a molecular weight of 51,000 was obtained. The reason for the discrepancy between these two methods of molecular weight determination is not known, but similar differences are reported for chorismate mutase–prephenate dehydratase from *Salmonella typhimurium* (Schmit and Zalkin, 1971). In sodium dodecyl sulfate–polyacrylamide gel electrophoresis chorismate mutase reveals a single protein band with a molecular weight of 14,500. The enzyme therefore consists of subunits which are similar or possibly

identical and the enzymatic active form is built up of at least three subunits. These subunits are not linked together by disulfide bridges because it is not necessary to use a reducing agent like β-mercaptoethanol during subunit dissociation and separation.

Chorismate mutase from *S. aureofaciens* contains no sulfhydryl group which is essential for binding the substrate or for the catalytic function, but which is not necessary for structural reasons since the enzyme is not inactivated by *p*HgBzOOH. The fact that the activity of the enzyme is not inhibited by dithiothreitol either in the oxidized or the reduced form also shows that there is no accessible sulfhydryl group or disulfide linkage in the enzyme, which is essential. Chorismate mutase does not require metal ions for activity.

The most remarkable property of chorismate mutase from *S aureofaciens* is its heat stability. After denaturation at 100° the enzyme can be reactivated. The velocity of this reactivation process is concentration dependent indicating that the rate limiting step is not a simple refolding of a single polypeptide to its native state. With respect to the subunit structure of the enzyme it is assumed aggregation of the inactive subunits to the active enzyme is observed. The Michaelis constant of the purified enzyme for its substrate chorismate is determined to be 5.2×10^{-4} M. The enzyme is not inhibited by the end products nor by intermediates of the aromatic biosynthetic pathway. Thus, the purified and homogeneous preparation of chorismate mutase from *S. aureofaciens* resembles in every aspect the partially purified enzyme described earlier (Görisch and Lingens, 1973).

Compared with the chorismate mutases of other procaryotic organisms, the subunit structures of which have been studied, chorismate mutase from *S. aureofaciens* is quite different. Two chorismate mutase complexes exist in *Escherichia coli* and *Aerobacter aerogenes*. One forms a functional unit with prephenate dehydrogenase and the other with prephenate dehydratase. These bifunctional enzymes show molecular weights of about 80,000 and a subunit molecular weight of 40,000 (Koch *et al.*, 1970a,b, 1971a,b; Davidson *et al.*, 1972). Chorismate mutase of *Streptomyces aureofaciens* with the relatively small molecular weight of about 60,000 is not associated with the activity of prephenate dehydrogenase or prephenate dehydratase; nevertheless, it forms a rather complex molecule built by at least three or perhaps even four small subunits with the molecular weight of 14,500.

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