BBA 66160

TYROSINE BIOSYNTHESIS IN *AEROBACTER AEROGENES*

PURIFICATION AND PROPERTIES OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE

G. L. E. KOCH, D. C. SHAW AND F. GIBSON

Biochemistry Department, John Curtin School of Medical Research, Institute of Advanced Studies, Australian National University, Canberra, A.C.T. (Australia)

(Received May 4th, 1970)

SUMMARY

Chorismate mutase–prephenate dehydrogenase from extracts of *Aerobacter aerogenes* was purified to near homogeneity. The molecular weight of the enzyme was determined by gel filtration to be 76 000. The amino acid composition and some kinetic constants of the enzyme are reported. Titration of the sulphydryl groups with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) revealed the presence of four sulphydryl groups in the protein, two of which are reactive towards DTNB only under denaturing conditions. Prephenate protects both activities against inactivation by iodoacetamide.

INTRODUCTION

The terminal stages in the biosynthesis of tyrosine in *Aerobacter aerogenes* and *Escherichia coli* may be represented as in Scheme 1. The conversion of chorismate

$$\text{Chorismate} \xrightarrow{1} \text{prephenate} \xrightarrow{2} \text{4-hydroxyphenylpyruvate} \longrightarrow \text{tyrosine}$$

Scheme 1. The terminal reactions in the biosynthesis of tyrosine in *A. aerogenes*. The enzymic activities are: 1, chorismate mutase; 2, prephenate dehydrogenase (requires NAD^+).

through prephenate to 4-hydroxyphenylpyruvate is carried out by a single protein[1] which has previously been partially purified[2] and some evidence obtained[3] that it consists of two subunits. The chorismate mutase–prephenate dehydrogenase enzyme was previously referred to as the T protein.

The present paper describes the purification of chorismate mutase–prephenate dehydrogenase from *A. aerogenes* and describes some of the properties of the pure protein.

Abbreviation: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)

MATERIALS AND METHODS

Organism

The multiple aromatic auxotroph *A. aerogenes* poly 3 was used as the source of the enzyme. This strain is a multiple aromatic auxotroph derived from the wild type strain of *A. aerogenes* used in previous studies[2]. Growth of this strain in a medium containing limiting tyrosine allowed about a 5-fold derepression of chorismate mutase–prephenate dehydrogenase compared with the level of the enzyme formed by wild type cells grown in minimal medium.

Chemicals

Chorismic acid was prepared using *A. aerogenes* 62-1, and isolated as described elsewhere[4]. Protamine sulphate was obtained from Mann Research Laboratories, N.Y., U.S.A. Sephadex G-100 and DEAE-Sephadex A50 were prepared and regenerated according to the manufacturer's instructions. Hydroxyapatite Bio-Gel HT and Agarose 0.5 M were from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Reagents for acrylamide gel electrophoresis were from Eastman Kodak, Rochester, N.Y., U.S.A. Iodoacetamide was purified by recrystallization from ethyl acetate–petroleum ether and stored in the dark until used. All other reagents were the highest quality available commercially.

Preparation of prephenate

Prephenate was prepared enzymically from chorismic acid by incubation with purified chorismate mutase–prephenate dehydrogenase. Crystalline chorismic acid (100 mg) was dissolved in 4 ml of Tris–HCl buffer (0.1 M, pH 8.0) containing $1 \cdot 10^{-3}$ M EDTA and $1 \cdot 10^{-3}$ M dithiothreitol. Purified chorismate mutase–prephenate dehydrogenase (1000 mutase units) in 1 ml was added and the mixture incubated at 37° for 3 h. The solution was cooled and the protein removed by ultrafiltration in a Diaflo cell, using 5 ml of reaction buffer to wash out the cell. The prephenate preparation was stored frozen in the reaction buffer. The yield of prephenate was over 95%.

Buffer

The main buffer used, Buffer A, contained 0.1 M Tris–HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol and 1 mM tyrosine. The pH was adjusted with HCl at room temperature.

Preparation of cell extracts

Cells were grown in batches of 40 l in a 40-l NBS Fermacell Fermentor in glucose–mineral salts medium[5], supplemented with final concentrations of 0.5% (w/v) glucose, $5 \cdot 10^{-4}$ M L-phenylalanine, $2.5 \cdot 10^{-4}$ M L-tryptophan, $7.5 \cdot 10^{-5}$ M L-tyrosine, $1 \cdot 10^{-5}$ M 4-aminobenzoic acid and $1 \cdot 10^{-5}$ M 4-hydroxybenzoic acid. The inoculum consisted of the growth washed from five slopes of nutrient agar, incubated overnight at 37°. The culture was agitated and aerated (40 l/min) at 37°, and after 16 h the cells were harvested with a Sharples Super Centrifuge. It was found that the whole cell paste could be stored at −20° for several months before the preparation of cell extracts. The yield was about 2.5 g wet weight of cells per l of culture.

Enzyme assays

Chorismate mutase. Chorismate mutase was measured by estimating the prephenate formed from chorismate after 20 min at 37°. The reaction mixture contained 20 μmoles Tris–HCl buffer (pH 7.5), 1 μmole chorismate, 0.2 μmole EDTA and 0.2 μmole dithiothreitol in a final volume of 0.4 ml. After incubation, 0.4 ml of 1 M HCl was added, incubation continued for 10 min at 37°, and then 3.2 ml of 1.0 M NaOH added. The absorbance at 320 nm was recorded within 30 min. A molar extinction coefficient of 17 500 was used to estimate the phenylpyruvic acid formed by the acid treatment of prephenate. Correction for the nonenzymic conversion of chorismate to prephenate during incubation was made by including an appropriate substrate blank.

Prephenate dehydrogenase. Prephenate dehydrogenase activity was measured by spectrophotometric estimation of NADH formed during the conversion of prephenate into 4-hydroxyphenylpyruvate. The reaction mixture contained 20 μmoles Tris–HCl (pH 8.1), 1 μmole prephenate, 0.2 μmole NAD^+, 0.2 μmole EDTA and 0.2 μmole dithiothreitol, in a final volume of 0.4 ml. After incubation at 37° for 20 min, the reaction was stopped by the addition of 3.6 ml of water. The absorbance at 340 nm was recorded within 30 min, during which time there was no change in absorbance. For kinetic experiments, the initial rate of NADH formation was determined spectrophotometrically by measuring the change in absorbance at 340 nm continuously for 2 min. Preheated (37°) reaction mixtures were transferred to a cuvette in the heated compartment (37°) of a Cary Model 14 spectrophotometer. The reaction was started by the addition of the enzyme solution.

One unit of enzyme activity was the formation of 0.1 μmole of product per 20 min at 37°. Specific activities are expressed as units/mg protein.

Protein assays

Protein was estimated in impure preparations using Folin's reagent[6] with bovine serum albumin (Pentex) as standard. Tyrosine and/or dithiothreitol which interfered with the determination were removed by dialysis. Concentrations of pure enzyme were calculated on the basis of the absorbance at 280 nm in pH 8.0 buffer (see RESULTS AND DISCUSSION).

Spectroscopy

Spectra were measured in 1-cm cells using a Cary Model 15 recording spectrophotometer.

Polyacrylamide gel electrophoresis

Polyacrylamide disc gel electrophoresis was performed according to the method of DAVIS[7], with the following modifications. The sample gel and stacking gel were eliminated, and the sample in Buffer A containing 40% sucrose was applied directly to the top of the resolving gel. Bromophenol blue (0.0005%) was added to the upper buffer, and when tyrosine was included in the system, it was added to the sample, gel, and upper buffer at a concentration of $1 \cdot 10^{-3}$ M. The gels (0.5 cm × 6 cm) were run at 4° and 2.5 mA per tube unless otherwise stated.

Protein bands were detected with Amido Black[7] or with 8-anilinonaphthalene sulphonic acid[8]. Prephenate dehydrogenase activity was located in the gels by soaking them at room temperature, in the dark, in a solution containing 0.5 M Tris–HCl

(pH 8.1), $5 \cdot 10^{-3}$ M EDTA, $1 \cdot 10^{-3}$ M prephenate, $1 \cdot 10^{-3}$ M NAD^+, 0.3 mg/ml tetrazolium nitroblue, and 50 μg/ml phenazine methosulphate. Prephenate dehydrogenase activity was detected by the appearance of purple bands after 1 h.

Electrophoresis of protein denatured with urea was carried out in 6 and 10% gels containing 8 M urea, after preincubation of the samples in 8 M urea at 37° for 1 h.

Estimation of molecular weights by polyacrylamide gel electrophoresis was carried out by a modification of the method of HEDRICK AND SMITH[9]. Gels containing 6% (w/v) or 10% (w/v) acrylamide were prepared by the method of DAVIS[7]. Proteins were diluted in Buffer A containing 40% sucrose and 50 μl samples of each protein were electrophoresed at both gel concentrations. Electrophoresis was performed at room temperature with a current density of 2.5 mA per tube. After the run the gels were removed and sliced at the dye front. Gels were stained and destained as described above. Migration of the dye and protein bands was measured from the origin and the results expressed as the ratio of protein migration to dye migration. This ratio represented the mobility of the protein in each gel. The relative mobility (R) of each protein was calculated from the ratio of the mobility in the 6% gel to that in the 10% gel. A standard curve was obtained by plotting the relative mobilities of the proteins against the log of the molecular weight.

Amino acid analysis

Analyses of acid hydrolysates of the protein were performed, essentially by the method of SPACKMAN *et al.*[10] on a Beckman Model 120B amino acid analyzer. Spectrophotometric determination of the tyrosine and tryptophan content was carried out by the method of BEAVAN AND HOLIDAY[11]. Half-cystine was determined as cysteic acid after performic acid oxidation of the protein.

Titration of sulphydryl groups

Titration of sulphydryl groups with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was carried out in 20 mM Tris–HCl buffer (pH 8.1) containing 2 mM EDTA[12]. 20 μl of 2.5 mM DTNB in 20 mM potassium phosphate buffer (pH 7.2) were added to 1-ml samples of protein solution and the change in absorbance at 410 nm recorded continuously until a stable plateau had been reached. Reactions were carried out at room temperature and a sample containing all reagents except the protein was used as the blank. The molar extinction coefficient of the liberated thiol was found to be 14000 (see ELLMAN[13]).

Performic acid oxidation

Performic acid oxidation was performed according to the method of HIRS[14].

Concentration of protein solutions

Protein solutions were concentrated in Diaflo ultrafiltration cells (Amicon Corporation, Mass., U.S.A.). Ultrafiltration was carried out at a pressure of 50 lb/inch^2 of helium.

Estimation of molecular weights by gel filtration

The method of ANDREWS[15] was used to determine the molecular weight of proteins by gel filtration on columns of Agarose 0.5 M (100–200 mesh). A 1.5 cm × 100 cm column of Agarose was poured and equilibrated with Buffer A. Samples of protein

solution (1 ml of 1%, w/v) were applied to the column and eluted by pumping Buffer A through the column at the rate of 4 ml/h. Fractions of about 1.5 ml were collected, the exact volume in each fraction being calculated from the difference in weight of each tube before and after collection. Each protein was applied separately, and the void volume of the column was checked between each application with blue dextran.

RESULTS AND DISCUSSION

Purification of the enzyme

The steps in purification are outlined in Table I and the details are as follows:

Step 1: Preparation of crude extracts. Cells of *A. aerogenes* poly 3 (100 g, stored at −20°) were thawed, suspended in 400 ml of Buffer A and disrupted in a Ribi Cell Fractionator at 20 000 lb/inch². Cell debris was removed by centrifuging at 23 000 × *g* for 30 min. All further steps were performed at 0–4°.

Step 2: Removal of nucleic acid. A solution (1 volume) of protamine sulphate (2%, w/v) in Buffer A was added dropwise to the solution of cell extract (4 volumes). The mixture was stirred for 30 min and the precipitate removed by centrifuging for 20 min at 23 000 × *g*.

Step 3: $(NH_4)_2SO_4$ fractionation. Solid $(NH_4)_2SO_4$ (20.5 g/100 ml) was added to the supernatant from the previous step. After 3 h the suspension was centrifuged for 10 min at 23 000 × *g* and the precipitate discarded. $(NH_4)_2SO_4$ (8.4 g/100 ml) was added to the supernatant, the suspension allowed to stand for 30 min and centrifuged for 10 min at 23 000 × *g*. The supernatant was discarded and the precipitate suspended in Buffer A. Residual $(NH_4)_2SO_4$ was removed by dialysis for 6 h against 2 l of Buffer A.

Step 4: Gel filtration. The dialysate from Step 3 was passed through a 5 cm × 120 cm column of Sephadex G-100 previously equilibrated with Buffer A, and eluted with the same buffer. The activity was eluted from the column between 450 and 600 ml of eluant. The active fractions were pooled and concentrated to about 50 ml in a Diaflo ultrafiltration cell.

Step 5: DEAE-Sephadex chromatography. The concentrated solution from Step 4 was dialysed against 1 l of Buffer A containing 0.05 M KCl, and applied to a 3.5 cm × 20 cm column of DEAE-Sephadex A50 equilibrated with the same buffer. The column was washed with 200 ml of buffer to remove all unadsorbed protein. Protein was then eluted with a linear gradient of KCl (0.05–0.2 M) in a total volume of 1.5 l. The enzyme was eluted at a KCl concentration of 0.15 M. The active fractions were pooled and concentrated to about 50 ml in a Diaflo cell.

Step 6: Hydroxyapatite chromatography. The concentrate from Step 5 was dialysed against 1 l of Buffer A and applied to a 3.5 cm × 6 cm column of hydroxyapatite equilibrated with the same buffer. Protein was then eluted with a linear gradient of sodium phosphate (0–0.1 M) in a total volume of 1 l, in Buffer A. The enzyme was eluted at a phosphate concentration of 0.05 M. Active fractions were pooled and concentrated to about 20 ml in a Diaflo cell.

Storage of purified enzyme

To the purified preparation was added 70% $(NH_4)_2SO_4$ (w/v), and the suspension of enzyme stored at 0–4°.

Biochim. Biophys. Acta, 212 (1970) 375–386

TABLE I

SUMMARY OF THE PURIFICATION OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE

Purification step	*Vol. (ml)*	*Total protein (mg)*	*Enzymic activity*	*Total activity units ($\times 10^3$)*	*Specific activity (units/mg protein)*	*Mutase / Dehydrogenase*	*Yield (%)*
1. Crude cell extract	455	14 600	Mutase	625	42.8	—	100
			Dehydrogenase	—*			
2. Protamine sulphate-treated extract	542	13600	Mutase	610	45.5	—	97.5
			Dehydrogenase	—*			
3. $(NH_4)_2SO_4$ fractionation	40	2310	Mutase	420	182	0.33	67.5
			Dehydrogenase	1260	546		
4. Sephadex G-100 column	214	382	Mutase	275	720	0.33	44
			Dehydrogenase	825	2160		
5. DEAE-Sephadex column	243	87	Mutase	184	2120	0.33	29.5
			Dehydrogenase	552	6300		
6. Hydroxyapatite column	185	31	Mutase	130	4200	0.33	20.8
			Dehydrogenase	390	12600		

* Dehydrogenase activity cannot be quantitated accurately in crude extracts.

Homogeneity of the purified enzyme

Gel filtration of the purified enzyme on columns of Agarose 0.5 M or Sephadex G-200, or chromatography on DEAE-Sephadex yielded a single symmetrical peak of protein. The specific activities of chorismate mutase and prephenate dehydrogenase were constant throughout the peak. When relatively large quantities (100–200 μg) of freshly purified enzyme were electrophoresed on acrylamide gels, one major and a few minor bands of protein were detected (Fig. 1a). Only the major band appeared to possess prephenate dehydrogenase activity. When the enzyme was electrophoresed after storage in Buffer A without tyrosine, the main band was split into two daughter bands (Fig. 1b) both of which possessed prephenate dehydrogenase activity (Fig. 1c). Electrophoresis of either fresh or stored samples in the presence of $1 \cdot 10^{-3}$ M tyrosine gave only one major band of protein which contained all the dehydrogenase activity, and accounted for over 95% of the protein seen. Electrophoresis of performic acid-oxidized protein in acrylamide gels containing 8 M urea resulted in a single major band of protein (Fig. 1d). On the basis of the above criteria, it was concluded that the protein was more than 95% pure.

Stability of the enzyme

It was observed previously[2] that the impure enzyme is very unstable in the absence of a thiol reagent such as mercaptoethanol or dithiothreitol. This observation was confirmed during these studies, but it appears that the thiol requirement is not as strict in the purified protein. Thus, whilst the enzyme in crude extracts loses about 50% activity on standing overnight in the absence of a thiol, the purified enzyme undergoes almost no inactivation under the same conditions. On the other hand, dithiothreitol was found to protect the purified enzyme against heat denaturation. The loss of mutase activity after 30 min at 40° in the absence of dithiothreitol was over 60%, whilst in the presence of $1 \cdot 10^{-3}$ M dithiothreitol the loss did not exceed 20%. When $1 \cdot 10^{-3}$ M tyrosine was also included in the reaction mixture with dithiothreitol the loss in activity was negligible. Consequently both tyrosine and dithiothreitol were included each at a concentration of $1 \cdot 10^{-3}$ M in all buffers used for the purification.

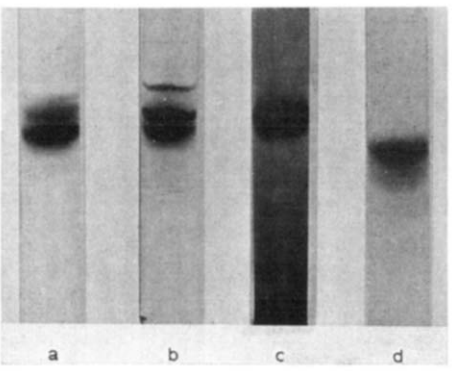

Fig. 1. Polyacrylamide gel electrophoresis of chorismate mutase–prephenate dehydrogenase. a, b and d were stained for protein and c for prephenate dehydrogenase activity. a, freshly purified protein; b and c, protein after standing for 7 days at 4°; d, performic acid-oxidized protein, electrophoresed in 8 M urea.

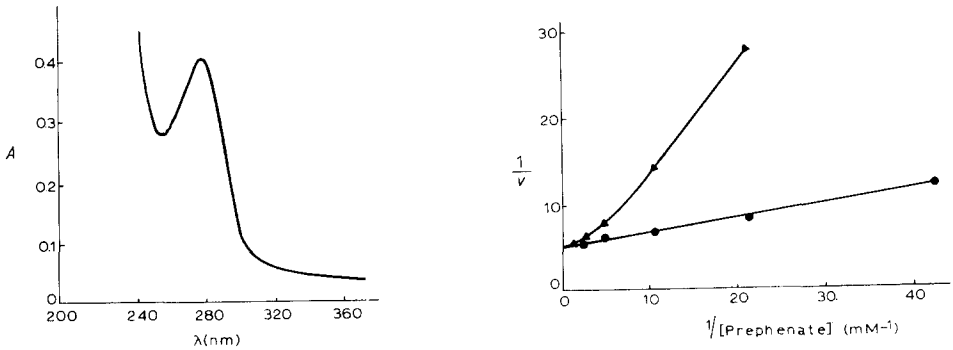

Fig. 2. Ultraviolet spectrum of chorismate mutase–prephenate dehydrogenase (0.40 mg/ml) in Buffer A.

Fig. 3. Determination of the apparent K_m of prephenate dehydrogenase for prephenate from a double reciprocal plot. Assay mixtures contained $2 \cdot 10^{-3}$ M NAD^+ with varying concentrations of prephenate. ●—●, without tyrosine; ▲—▲, with $4 \cdot 10^{-4}$ M tyrosine.

The use of Tris buffer instead of phosphate buffer in the purification was indicated by the observation that the enzyme was more stable in the former. Thus, the half-life of the purified enzyme at 4° in 0.01 M phosphate buffer (pH 7.4) was 6–8 h, whereas that in 0.1 M Tris–HCl buffer (pH 7.4) was 5–6 days. Although the exact cause of this difference is not clear, one possibility appears to be the marked tendency for the purified enzyme to denature and aggregate in the phosphate buffer, as shown by gel filtration and electrophoretic analysis.

The purified enzyme aggregates slowly in Buffer A and consequently was stored as a suspension in $(NH_4)_2SO_4$ under which conditions it was found to be completely stable for at least 10 days.

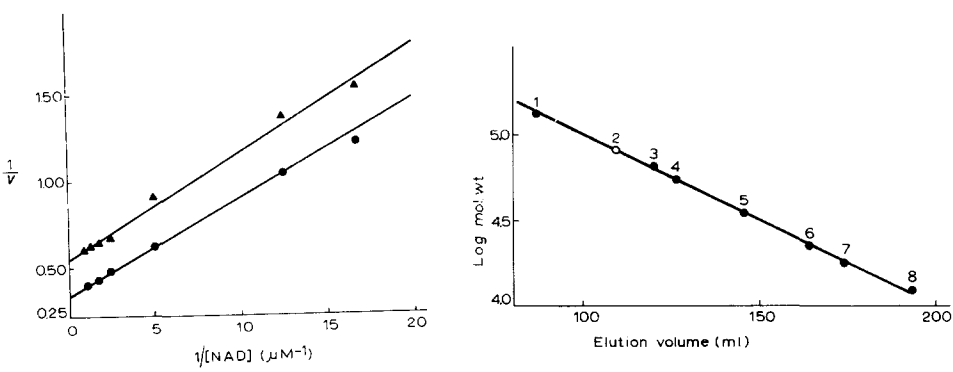

Fig. 4. Determination of the apparent K_m of prephenate dehydrogenase for NAD^+ from a double reciprocal plot. Assay mixtures contained $8 \cdot 10^{-3}$ M prephenate with varying concentrations of NAD^+. ●—●, without tyrosine; ▲—▲, with $4 \cdot 10^{-4}$ M tyrosine.

Fig. 5. Estimation of the molecular weight of chorismate mutase–prephenate dehydrogenase by gel filtration. Blue dextran and 10-mg samples of each protein were applied to a Bio-Gel A 0.5 M column (1.5 cm × 100 cm) and eluted with Buffer A. The proteins used (and their molecular weights) were: 1, serum albumin dimer (132 000); 2, chorismate mutase–prephenate dehydrogenase; 3, serum albumin monomer (66 000); 4, ovalbumin (45 000); 5, pepsin (35 000); 6, chymotrypsinogen (23 000); 7, myoglobin (16 000); 8, cytochrome *c* (13 000).

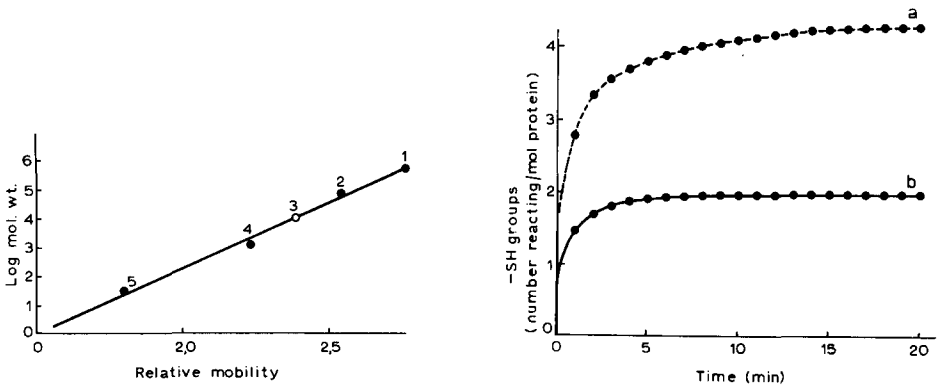

Fig. 6. Estimation of the molecular weight of chorismate mutase–prephenate dehydrogenase by electrophoresis on polyacrylamide gels. The proteins used (and their molecular weights) were: 1, serum albumin dimer (132 000); 2, hexokinase (96 600); 3, chorismate mutase–prephenate dehydrogenase; 4, serum albumin monomer (66 000); 5, ovalbumin (45 000).

Fig. 7. Titration of sulphydryl groups with DTNB. The reaction mixture (1 ml) included 0.5 mg of purified protein and 25 μM DTNB with (Curve a, ●— — —●) and without (Curve b, ●—●) sodium dodecyl sulphate added. For details of the titration see MATERIALS AND METHODS.

Ultraviolet spectrum and extinction coefficient

The absorption spectrum of the purified protein in 0.1 M Tris–HCl buffer (pH 8.0) is shown in Fig. 2. The extinction coefficient at the absorption maximum, 278 nm, was 0.95 absorbance unit/cm per mg. This value was subsequently used for the calculation of the protein concentrations in samples of purified enzyme.

The protein appears to contain a high content of aromatic amino acids as suggested by the shoulder at about 290 nm (Fig. 2) and confirmed by amino acid analysis (see later).

Kinetic studies

The apparent dissociation constant for chorismate was determined from double reciprocal plots using the 20-min assay for chorismate mutase activity. The plots obtained were linear and the apparent K_m for chorismate was estimated to be $1.3 \cdot 10^{-3}$ M. Tyrosine, up to a concentration of $3 \cdot 10^{-3}$ M, had no effect on chorismate mutase. Since the reaction rate of the prephenate dehydrogenase was not uniform over 20 min, initial rates were measured (see MATERIALS AND METHODS). Linear double reciprocal plots were obtained with either prephenate or NAD^+ as variable substrate (Figs. 3 and 4). The apparent K_m for prephenate was $3.5 \cdot 10^{-4}$ M and that for NAD^+ was $6 \cdot 10^{-4}$ M. The inclusion of tyrosine in the reaction mixture when prephenate was the variable substrate rendered the substrate–velocity curve sigmoid with a corresponding curving of the double reciprocal plots (Fig. 3). Tyrosine did not affect the linearity of the double reciprocal plot when NAD^+ was the variable substrate (Fig. 4) and the two lines do not intersect in the first quadrant as previously reported[2]. The reason for this discrepancy is not known but could be related to the difference in the methods used to measure the dehydrogenase activity. One consequence of using the spectrophotometric assay in place of the fluorometric assay has been the need to increase the tyrosine concentration significantly to show inhibition. It should also be noted that

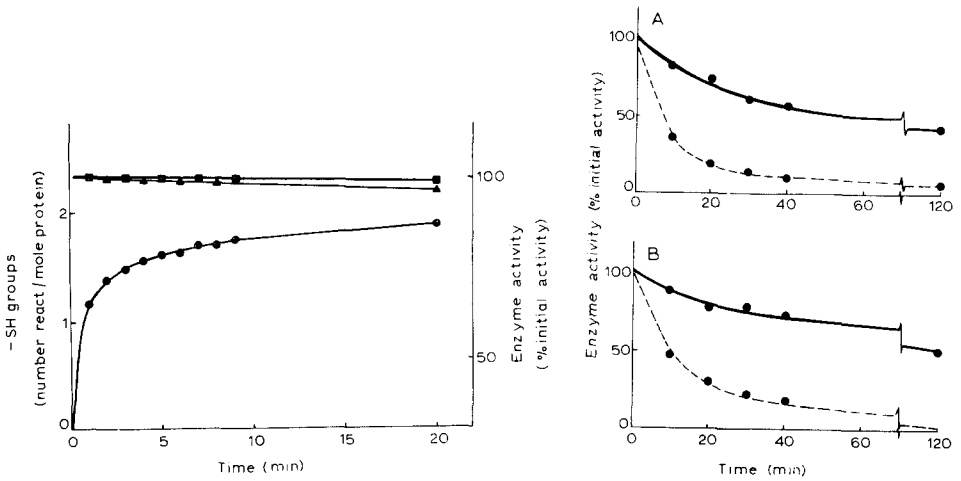

Fig. 8. The effect of titrating the sulphydryl groups with DTNB on enzyme activity. The reaction mixture (1 ml) included 1 mg of purified protein and 25 μM DTNB, and the titration was followed spectrophotometrically (●—●). Samples were taken at appropriate intervals, diluted, and assayed for chorismate mutase (■—■) and prephenate dehydrogenase (▲--▲) as described in MATERIALS AND METHODS.

Fig. 9. Effect of iodoacetamide on chorismate mutase–prephenate dehydrogenase. The purified enzyme (1 mg/ml) was incubated with $2.5 \cdot 10^{-3}$ M iodoacetamide in the presence (●—●) and absence (●.....●) of $1 \cdot 10^{-3}$ M chorismate or prephenate. Samples were assayed for prephenent dehydrogenase (A) and chorismate mutase (B) as described in MATERIALS AND METHODS.

TABLE II

AMINO ACID COMPOSITION OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE

Amino acid	*Residues per mol. wt. 76 000*
Lys	34.1
His	15.9
Arg	41.4
Cya*	7.8
Asp	69.2
Thr**	36.3
Ser**	35.8
Glu	82.0
Pro	34.4
Gly	58.0
Ala	80.3
Val	50.4
Met	16.9
Ile	35.0
Leu	68.6
Tyr	19.9
Phe	26.6
Trp***	8.0

* Cysteic acid was determined from analyses of performic acid-oxidized protein.
** Corrected for losses by extrapolation to zero time.
*** Determined spectrophotometrically.

the apparent K_m values for chorismate, prephenate and NAD^+ reported here are somewhat higher than those obtained previously[2].

Molecular weight of the native enzyme

A molecular weight calibration curve was obtained by gel filtration using an Agarose column (Fig. 5). The molecular weight of chorismate mutase–prephenate dehydrogenase was estimated to be 76 000.

This method of molecular weight determination gives good correlation with the molecular weight of a protein only when the standard and unknown proteins employed have quite similar frictional ratios and partial specific volumes[16]. Consequently a modification of the method of HEDRICK AND SMITH[9] for determining molecular weights of proteins on acrylamide gels was also employed. The validity of the method was checked using published data[9] and found to give linear standard curves when the data were plotted as described above (see MATERIALS AND METHODS). However the accuracy of this method, the results of which are shown in Fig. 6, appears to be somewhat lower than that of the procedure of HEDRICK AND SMITH[9]. The molecular weight of chorismate mutase–prephenate dehydrogenase was estimated from this plot to be 81 000.

Since the molecular weight estimation by the ANDREWS[15] method was considered the more accurate of the two methods used, an approximate molecular weight of 76 000 was assigned to the enzyme.

Amino acid analysis

The amino acid composition of the enzyme is shown in Table II. Analysis of the performic acid-oxidized protein yielded 8 half-cystine residues per 76 000 molecular weight. Titration of the sulphydryl groups with DTNB in the presence of 0.5% sodium dodecyl sulphate yielded a total of 4–5 free sulphydryl groups per 76 000 molecular weight (Fig. 7, Curve a). The difference in the half-cystine estimations could result from the presence of some disulphide bonds in the protein.

The availability and function of sulphydryl groups

Thiols protected chorismate mutase–prephenate dehydrogenase suggesting the possible involvement of one or more sulphydryl groups in the catalytic activity. Consequently the reactivity of the sulphydryl groups in the protein towards DTNB and iodoacetamide was investigated. Titration of the native enzyme with DTNB indicated the presence of 2 reactive sulphydryl groups per 76 000 molecular weight (Fig. 7, Curve b). Titration after incubation with 0.5% sodium dodecyl sulphate, at room temperature overnight, increased the number of reactive sulphydryl groups to about 4 per 76 000 molecular weight (Fig. 7, Curve a). Thus the native enzyme contains at least 4 free sulphydryl groups per mole, of which only 2 are reactive towards DTNB. The possibility that the "exposed" sulphydryl groups are directly involved in the catalytic functions of the enzyme was excluded by the failure of the DTNB to inactivate either chorismate mutase or prephenate dehydrogenase (Fig. 8). Neither chorismate nor prephenate affected the reactivity of the sulphydryl groups in the native enzyme towards DTNB.

In contrast to DTNB, iodoacetamide was an effective inhibitor of both activities of the enzyme (Fig. 9). The presence of either chorismate or prephenate during treatment with iodoacetamide protected the enzyme against inactivation. Iodoacetamide

treatment of enzyme which had been previously fully reacted with DTNB also resulted in complete inactivation.

The above results indicate that the native enzyme possesses two sulphydryl groups which are reactive towards DTNB, but are not directly involved in the catalytic functions of the enzyme. Furthermore, there are probably at least two more sulphydryl groups in the enzyme which are not accessible to DTNB, but are reactive towards the smaller sulphydryl reagent iodoacetamide. The protective action of chorismate and prephenate against inactivation by iodoacetamide suggests that iodoacetamide reacts with one or more groups which are directly involved in the catalytic functions of the enzyme.

During the purification of chorismate mutase–prephenate dehydrogenase to near homogeneity the ratio of the two enzymic activities remains constant, supporting the previous conclusion[1,2] that the one protein catalyses the two reactions. A comparison of this protein with the corresponding protein in *E. coli* is in progress and further studies on the proposed[3] subunit structure of chorismate mutase–prephenate dehydrogenase will be reported separately.

ACKNOWLEDGEMENTS

We wish to thank L. B. James for amino acid analyses and D. Abigail for growing cells and preparing cell extracts.

REFERENCES

1 R. G. H. COTTON AND F. GIBSON, *Biochim. Biophys. Acta*, 100 (1965) 76.
2 R. G. H. COTTON AND F. GIBSON, *Biochim. Biophys. Acta*, 147 (1967) 222.
3 R. G. H. COTTON AND F. GIBSON, *Biochim. Biophys. Acta*, 160 (1968) 188.
4 F. GIBSON, *Biochem. Prep.*, 12 (1968) 94.
5 J. MONOD, G. COHEN-BAZIRE AND M. COHN, *Biochim. Biophys. Acta*, 7 (1951) 585.
6 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
7 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
8. B. K. HARTMAN AND S. UDENFRIEND, *Anal. Biochem.*, 30 (1969) 391.
9 J. L. HEDRICK AND A. SMITH, *Arch. Biochim. Biophys.*, 126 (1968) 155.
10 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
11 G. H. BEAVAN AND E. R. HOLIDAY, *Advan. Protein Chem.*, 7 (1952) 319.
12 G. L. ELLMAN, *Arch. Biochim. Biophys.*, 74 (1958) 443.
13 G. L. ELLMAN, *Arch. Biochim. Biophys.*, 82 (1959) 70.
14 C. H. W. HIRS, *J. Biol. Chem.*, 219 (1956) 611.
15 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
16 L. M. SIEGEL AND K. J. MONTY, *Biochem. Biophys. Res. Commun.*, 19 (1965) 494.