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STUDIES ON THE SUBUNIT STRUCTURE OF CHORISMATE MUTASE–PREPHENATE DEHYDROGENASE FROM *AEROBACTER AEROGENES*

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SUMMARY

1. Studies on the properties of dissociated chorismate mutase–prephenate dehydrogenase from *Aerobacter aerogenes* indicated the presence of similar subunits. Estimates of the molecular weight of the subunits following dissociation by sodium dodecyl sulphate or urea yielded a value of about 40 000 which is half the molecular weight of the native enzyme. Two-dimensional chromatography of dansylated tryptic peptides from the protein also indicated that it consisted of two very similar or identical subunits of about 40 000 molecular weight. The amino terminal end group of the protein was found to be methionine.

INTRODUCTION

Evidence for the presence of subunits in the enzyme chorismate mutase–prephenate dehydrogenase was obtained during earlier studies on the enzyme from *A. aerogenes*[1,2]. The nature of these subunits is of considerable interest since the enzyme catalyses two separate reactions, and also has one activity, chorismate mutase, which is common to another bifunctional enzyme, chorismate mutase–prephenate dehydratase. The availability of pure protein[3] has permitted a detailed study on the nature of the subunits of chorismate mutase–prephenate dehydrogenase.

MATERIALS AND METHODS

Preparation of the enzyme

The isolation of the enzyme from *A. aerogenes* poly 3 and the composition of the buffers have been described in the previous paper[3]. Approx. 20 mg of purified enzyme was obtained from 100 g of bacterial paste. The homogeneity of each preparation was checked by disc gel electrophoresis as described previously[3]. In all cases

Abbreviation: dansyl, 1-dimethylaminnoaphthalenesulphonyl.

the protein used was over 95% pure and was kept as an $(NH_4)_2SO_4$ (70% saturation) suspension.

Chemicals

Most of the chemicals used have been described previously[3]. Reagents were of the highest grade obtainable and not further purified. Urea was purchased from Mallinckrodt Chemical Works and sodium dodecyl sulphate was from Matheson, Coleman and Bell, Ohio, N.J. Reagents for acrylamide gel electrophoresis were obtained from Eastman Kodak (Rochester, N.Y., U.S.A.). 1-Dimethylamino-naphthalenesulphonyl (dansyl) chloride was obtained from Calbiochem Laboratories, Los Angeles, U.S.A. Dansyl amino acids were products from Calbiochem (California, U.S.A.); trypsin was obtained from Worthington (Freehold, N.J.) and treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone before use[4]. Silica gel plates were from Merck A.G. (Darmstadt, Germany) and Eastman Kodak (Rochester, N.Y., U.S.A.).

Solvents

The chromatographic solvents used were: Solvent 1, methyl acetate–propan-2-ol–aq. NH_3 (sp. gr. 0.88) (9:7:4, by vol.)[5]; Solvent 2, chloroform–95% aq. (v/v) ethanol–acetic acid (38:4:3, by vol.)[5]; Solvent 3, *n*-butyl acetate–dimethylformamide–propionic acid (50:5:4, by vol.)[6]; Solvent 4, *n*-Butanol–triethylamine–dimethyl sulphoxide–benzyl alcohol (4:2:1:4, by vol.)[6].

Performic acid oxidation

Lyophilised protein was oxidized by the method of HIRS[7]. The oxidised protein was resuspended in 0.1 M Tris–HCl (pH 7.4) containing 8 M urea or 0.5% sodium dodecyl sulphate.

Preparation of denatured enzyme

The $(NH_4)_2SO_4$ precipitate of the enzyme was suspended in Buffer A and dialysed extensively against the same buffer. The protein was denatured by making the solution 8 M with respect to urea or 0.5% with respect to sodium dodecyl sulphate and incubating for 3 h in the presence of 100 mM dithiothreitol. When protein oxidized with performic acid was used, the dithiothreitol was omitted.

Protein assays

The concentration of protein in samples was determined spectrophotometrically using the extinction coefficient of 0.95 absorbance unit/cm per mg at 280 nm (ref. 3), after dialysis to remove traces of dithiothreitol and tyrosine.

Acrylamide gel electrophoresis of denatured enzyme

Polyacrylamide gel electrophoresis was carried out in the buffer system of DAVIS[8], with the following modifications: The sample gel and stacking gel were eliminated, and the sample containing either 40% sucrose or 8 M urea applied directly to the top of the resolving gel. Bromophenol blue (0.0005%) was added to the upper buffer solution. Enzyme denatured with urea was electrophoresed in gels containing 8 M urea which was not added to either the upper or lower reservoir buffers. Electro-

phoresis was carried out in 0.5 cm × 6 cm gels at room temperature with a current of 2.5 mA per tube until the bromophenol blue had migrated to the bottom of the gel. The gels were stained with 1% Amido Black in 7% acetic acid and destained by diffusion in 7% acetic acid.

Protein which had been denatured with sodium dodecyl sulphate was electrophoresed according to the procedure of WEBER AND OSBORN[9]. The protein solution was dialysed overnight at room temperature against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulphate and 0.1% β-mercaptoethanol. Protein samples (50–100 μg) were electrophoresed in 10% gels at a constant current of 4 mA per gel at room temperature. After staining with Coomassie brilliant blue[9] destaining was carried out by diffusion in solutions containing 75 ml acetic acid, 50 ml of methanol and 875 ml of water. Gels were stored in 7% acetic acid solution.

Molecular weight determinations on denatured enzyme

Approximate estimates of the molecular weight of enzyme denatured with urea were obtained from electrophoresis in acrylamide gels containing 8 M urea. Samples (100 μg) of enzyme and several standard proteins were electrophoresed in 6 and 10% acrylamide gels containing 8 M urea and the mobility of the protein in each gel calculated from the ratio of the distance migrated by the protein to that migrated by the marker dye. The relative mobility of each protein was calculated from the ratio of the mobility in the 6% gel to that in the 10% gel. Finally the relative mobility of each protein of known molecular weight was plotted against the log of the molecular weight. The resulting standard curve was used to estimate the molecular weight of the enzyme in 8 M urea.

The molecular weight of the enzyme which had been denatured with sodium dodecyl sulphate was estimated by the method of WEBER AND OSBORN[9]. Samples (10–50 μg) of several proteins were electrophoresed in 10% gels as described above. After electrophoresis the gels were removed and the length of the gel and the distance moved by the dye measured. Staining and destaining were carried out as described above and the length of the gel and the distance moved by the protein recorded. The mobility of each protein was calculated as:

$$\text{Mobility} = \frac{\text{distance of protein migration}}{\text{length after destaining}} \times \frac{\text{length before staining}}{\text{distance of dye migration}}$$

A standard curve was prepared by plotting mobilities against the log of the known molecular weights.

Preparation of peptide maps

The protein (2 mg) was oxidised with preformed performic acid[7] and lyophilised. It was then hydrolysed by trypsin (1% by weight) in aq. NH_4HCO_3 solution (0.5%) at 37° for 4 h. The NH_4HCO_3 was removed by lyophilisation and the hydrolysate dissolved in water (approx. 20 μg/2 ml) and the pH adjusted to 4 by the addition of glacial acetic acid (approx. 20 μl), causing precipitation of "core" material (less than 5% by weight of the protein). The core was centrifuged off, the soluble peptides were lyophilised and used for the peptide maps.

The soluble peptides were dissolved in a minimum of water, applied to a sheet of Whatman 3 MM paper, and subjected to high-voltage electrophoresis at pH 1.9

or 4.7 in tanks designed after MICHL[10] using Varsol as heat exchanger. The relevant strip was cut out, sewn on to a fresh sheet of paper and subjected to a chromatographic separation. The two-dimensional map was stained with ninhydrin.

Peptide mapping of dansyl peptides

Tryptic digestion of the protein was carried out as described above in 0.1 M $NaHCO_3$ buffer. After 4 h incubation the mixture was added dropwise to an equal volume of a 2% (w/v) solution of dansyl chloride in acetone. The mixture was allowed to stand overnight and passed through a 1 cm × 2 cm column of Dowex 50 (X8, 100–200 mesh) equilibrated with 0.01 M acetic acid. The column was washed with about 200 ml of the same buffer and finally eluted with M ammonia in 25% (v/v) aq. acetone. The fractions containing yellow fluorescent material were pooled and the solvent removed by rotary evaporation. The dansyl peptides were dissolved in a minimum amount of 80% (w/v) aq. acetone and subjected to two-dimensional chromatography in Solvent 1 (1st dimension) and Solvent 2 (2nd dimension) using thin-layer silica gel plates (20 cm × 20 cm; 0.25 mm thick).

End group analysis

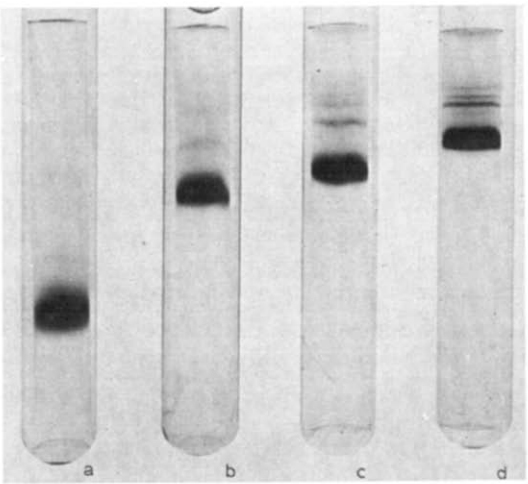
Amino terminal end group analysis was carried out by the dansylation procedure of GROS AND LABOUESSE[11]. Samples of protein (0.5–1 mg) were dissolved in 0.9 ml of 0.06 M phosphate (pH 8.2) containing 4 M urea and 25% (v/v) dimethylformamide, and treated with 0.1 ml of 0.2 M dansyl chloride in acetonitrile. The mixture was allowed to stand overnight and 10 ml of 10% trichloroacetic acid added. The precipitated protein was collected by centrifuging after 3 h standing, washed twice with 1 M HCl and finally suspended in 6 M HCl. After hydrolysis in sealed evacuated tubes for 4 h at 108° the hydrolysates were evaporated, suspended in pyridine–acetate (pH 3.5) and repeatedly extracted with ether. The ether extract was concentrated and chromatographed on thin-layer sheets using Solvent 3. Confirmation of the identity of the end group was established by chromatography in Solvent 4 which separates the dansyl derivatives of the basic amino acids.

RESULTS

Electrophoresis of dissociated enzyme

Electrophoresis of enzyme which had been denatured and reduced in urea was carried out at several gel concentration sin an attempt to detect the presence of charge or size isomers[12]. Fig. 1 shows the results of electrophoresing approx. 100-μg samples of the reduced, denatured enzyme in 8 M urea at a number of gel concentrations. Usually only a single band of protein could be detected when the gel concentration was varied from 5 to 12.5%. In some cases, however, slower moving bands were detectable. On the other hand, when the samples were oxidised with performic acid and electrophoresed under the same conditions only a single band of protein was obtained, indicating that the additional species probably resulted from disulphide-bridged polymers.

Fig. 2 shows the results obtained on electrophoresing a 50-μg sample of the enzyme denatured in sodium dodecyl sulphate in a 10% acrylamide gel containing sodium dodecyl sulphate. Unoxidised enzyme sometimes gave several bands of protein



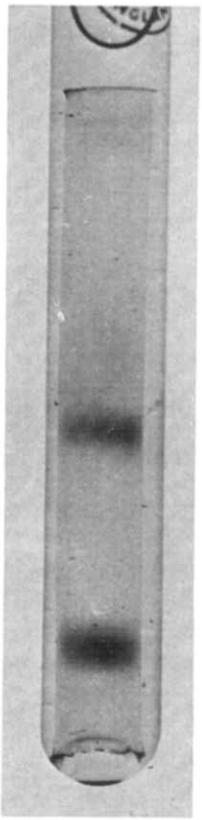


Fig. 1. Polyacrylamide gel electrophoresis of 100-μg samples of chorismate mutase–prephenate dehydrogenase, in the presence of 8 M urea in, a, 5%; b, 7.5%; c, 10%; d, 12.5% polyacrylamide gel.

Fig. 2. Polyacrylamide gel electrophoresis of a 50-μg sample of chorismate mutase–prephenate dehydrogenase in 0.1% sodium dodecyl sulphate using a 10% gel. The lower band represents lysozyme which was used as a marker.

even though the sample was applied in mercaptoethanol. On the other hand, oxidised enzyme gave only a single band on electrophoresis.

The detection of only a single polypeptide species under several dissociating conditions indicates the absence of non-identical subunits in the enzyme.

Molecular weight of dissociated enzyme

The molecular weight of the oxidised enzyme in 8 M urea was determined approximately by electrophoresis in acrylamide gel containing 8 M urea. This method is of limited accuracy and has an uncertainty of the order of 15%. Fig. 3 shows the results of an experiment which yielded a value of 44 000 for the molecular weight of the enzyme in 8 M urea. This value is approximately half of that obtained for the native enzyme[3].

The procedure used by WEBER AND OSBORN[9] for determining the molecular weights of proteins in sodium dodecyl sulphate employing acrylamide gel electrophor-

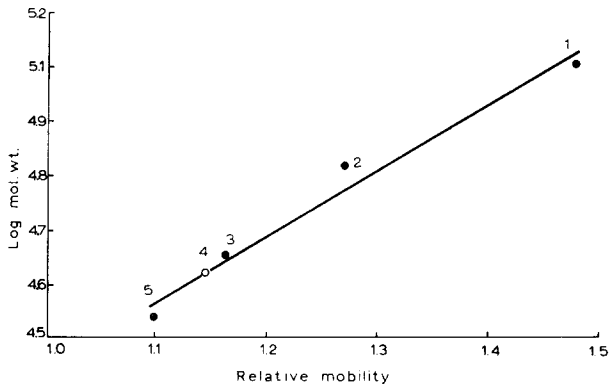


Fig. 3. Estimation of the molecular weight of chorismate mutase–prephenate dehydrogenase in 8 M urea by electrophoresis in polyacrylamide gels. The proteins used (and their molecular weights) were: 1, serum albumin dimer (132 000); serum albumin monomer (66 000); 3, ovalbumin (45 000); 4, chorismate mutase–prephenate dehydrogenase; 5, pepsin (35 000).

esis in the presence of the detergent was used to obtain a more reliable estimate of the molecular weight of the dissociated enzyme. Several runs with standard proteins were performed to determine the error involved in the method. We have found that the uncertainty in this method does not exceed 5% and that the difference in the estimates for oxidised and unoxidised proteins was negligible. Fig. 4 shows the results of an experiment to estimate the molecular weight of the oxidised enzyme in sodium dodecyl sulphate. The data indicate that a value of 39 000 $\pm$ 2000 is a reasonable estimate for the molecular weight of the dissociated enzyme.

Peptide mapping

In order to obtain further information on the identity of the subunits of the enzyme, tryptic digests were studied by two-dimensional mapping procedures. The

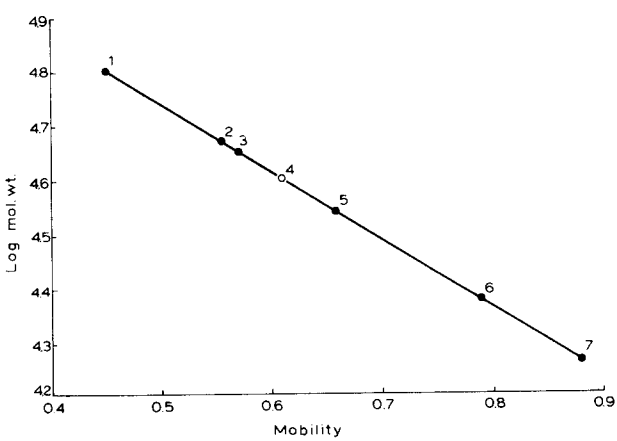


Fig. 4. Estimation of the molecular weight of chorismate mutase–prephenate dehydrogenase in 0.1% sodium dodecyl sulphate by electrophoresis in polyacrylamide gels. The proteins used (and their molecular weights) were: 1, serum albumin monomer (66 000); 2, *Bacillus subtilis* α-amylase (48 000); 3, ovalbumin (45 000); 4, chorismate mutase–prephenate dehydrogenase; 5, pepsin (35 000); 6, chymotrypsinogen (26 000); 7, β-lactoglobulin (18 400).

amino acid composition of the protein[3] reveals the presence of 34 lysine residues and 41 arginine residues per mole of native enzyme. Thus a tryptic digest would be expected to yield a total of 76 peptides. Several attempts to separate and count the tryptic peptides by the conventional two-dimensional procedures involving electrophoresis and chromatography on paper were not successful since the ninhydrin-staining material was extensively smeared. The reason for this smearing is not known but it could be the result of the presence of some contaminant in the preparations.

As an alternative to the conventional procedure for mapping tryptic peptides, we have used the procedure described by ATHERTON AND THOMSON[5] employing dansyl peptides. In contrast to the conventional method, this procedure gave well resolved spots which were relatively easy to count (Fig. 5). A total of 35–40 peptides were detected on the chromatograms which corresponds to approximately half the number expected if the enzyme was composed of a single polypeptide chain of mol. wt. 76 000.

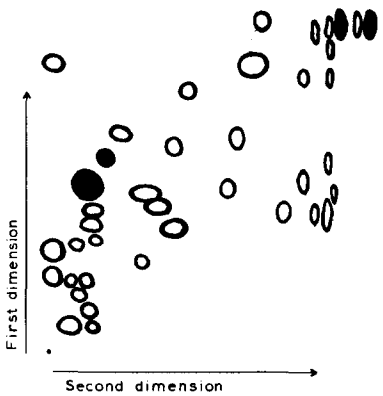


Fig. 5. Dansyl-peptide "map" of a tryptic digest of chorismate mutase–prephenate dehydrogenase. Very faint spots have been omitted. By-products are represented by shaded spots.

End group analysis

The detection and identification of the amino terminal residues in the protein by the dansyl procedure of GROS AND LABOUESSE[11] yielded dansyl methionine, ε-dansyl lysine and O-dansyl tyrosine only. The identity of the dansyl methionine was confirmed by re-running the appropriate spot after elution from the thin-layer plate. It was found that, probably as a result of oxidation on the silica gel, the derivative re-run in the position of dansyl methionine sulphoxide, confirming the identity of the derivative as dansyl methionine. Since the other derivatives observed are products given by amino acids within the peptides it was concluded that methionine was the only detectable amino terminal residue.

Attempts to quantitate the yield of methionine have not been successful. Unlike the findings of GROS AND LABOUESSE[11] the yields of end group from several standard proteins were lower than the calculated values. Furthermore, amino acid analysis of dansylated proteins showed substantial amounts of free lysine and tyrosine, indicating that the reason for the low yields might be a lowered labelling efficiency.

DISCUSSION

Recent studies on several multi-functional proteins consisting of subunits, for example tryptophan synthetase[13], anthranilate synthetase[14], histidinol dehydrogenase[15] and aspartokinase[16] (see also REED AND COX[17]), have shown that they can be placed in one or the other of two categories. Firstly, those consisting of non-identical subunits which might or might not carry the different catalytic sites on the different subunits, *e.g.* tryptophan synthetase. Secondly, those consisting of identical subunits, *e.g.* aspartokinase. Previous studies[2] on the subunits of chorismate mutase–prephenate dehydrogenase from *A. aerogenes* have indicated that this enzyme might belong to the latter category since the subunits appeared to be very similar in size.

In the present work polyacrylamide gel electrophoresis, because of its high resolving power, has been used to study dissociated chorismate mutase–prephenate dehydrogenase. Using several gel concentrations to minimize the possibility that single bands were due to different proteins with compensating size and charge effects[12], a single polypeptide species has been found in the presence of either 8 M urea or sodium dodecyl sulphate. The appearance of additional bands in some instances could be attributed to polymerisation of the monomer by disulphide bridging. It has also been shown previously that the native enzyme has a molecular weight of about 76 000. The molecular weight of the enzyme in either 8 M urea or sodium dodecyl sulphate was found to be about 40 000 suggesting that the native enzyme consists of two identical subunits and that the existence of smaller polypeptides which are not stained on polyacrylamide gels is unlikely.

The estimation of the number of trypsin-sensitive bonds in the protein has also been used to study the identity of the subunits. The detection of only half the number expected for a protein of mol. wt. 76 000 also indicates the absence of subunits with gross differences in primary structure. The detection of methionine as the sole amino terminal end group is also consistent with the other evidence for identical subunits although the finding alone is of limited significance since methionine is found at the amino terminus of most proteins from the closely related species, *Escherichia coli*.

Some reservation must be retained as to whether the subunits are exactly identical since the analytical procedures used might not be capable of resolving relatively small differences in the sequences of proteins. Clearly though, the results do exclude a tryptophan synthetase type model for the enzyme in which the partial reactions are catalysed by two grossly different proteins, and favour an aspartokinase type model in which a bifunctional protein is composed of identical subunits. The earlier finding[2] that the individual subunits are inactive suggests that the active sites may be created by the association of the subunits.

The above data on the structure of chorismate mutase–prephenate dehydrogenase is relevant to the relationship between this enzyme and the analogous enzyme, chorismate mutase–prephenate dehydratase[18], in the phenylalanine pathway. A simple model proposing that one of these enzymes has evolved from the other by a process involving duplication of the chorismate mutase gene and subsequent association of the gene products with prephenate dehydratase or prephenate dehydrogenase, does not appear likely. Such a model requires that the genes are also linked to code for a single polypeptide chain. Further information on the relationship, if

any, between chorismate mutase–prephenate dehydrogenase and chorismate mutase–prephenate dehydratase should be provided by determination of amino acid sequences.

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REFERENCES

1 R. G. H. COTTON AND F. GIBSON, *Biochim. Biophys. Acta*, 147 (1967) 222.
2 R. G. H. COTTON AND F. GIBSON, *Biochim. Biophys. Acta*, 160 (1969) 188.
3 G. L. E. KOCH, D. C. SHAW AND F. GIBSON, *Biochim. Biophys. Acta*, 212 (1970) 375.
4 G. SCHOELLMAN AND E. SHAW, *Biochemistry*, 2 (1963) 252.
5 R. S. ATHERTON AND A. R. THOMSON, *Biochem. J.*, 111 (1969) 797.
6 S. L. PFUELLER AND W. H. ELLIOTT, *J. Biol. Chem.*, 244 (1969) 48.
7 C. H. W. HIRS, *J. Biol. Chem.*, 219 (1956) 611.
8 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
9 K. WEBER AND M. OSBORN, *J. Biol. Chem.*, 244 (1969) 4406.
10 H. MICHL, *Chromatography*, 1 (1959) 11.
11 C. GROS AND B. LABOUESSE, *European J. Biochem.*, 7 (1969) 463.
12 J. L. HEDRICK AND A. J. SMITH, *Arch. Biochem. Biophys.*, 126 (1968) 155.
13 C. YANOFSKY, *Bacteriol. Rev.*, 24 (1960) 221.
14 J. ITO AND C. YANOFSKY, *J. Biol. Chem.*, 241 (1966) 4112.
15 J. YOURNO, *J. Biol. Chem.*, 243 (1968) 3277.
16 P. TRUFFA-BACHI, R. VAN RAPENBUSCH, J. JANIN, C. GROS AND G. N. COHEN, *European J. Biochem.*, 7 (1969) 401.
17 L. J. REED AND D. J. COX, *Ann. Rev. Biochem.*, 35 (1966) 57.
18 R. G. H. COTTON AND F. GIBSON, *Biochim. Biophys. Acta*, 100 (1965) 76.