The Subunit Structure of the *arom* Multienzyme Complex of *Neurospora crassa* A POSSIBLE PENTAFUNCTIONAL POLYPEPTIDE CHAIN

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A new procedure for the purification of the *arom* multienzyme complex from *Neurospora crassa* is presented. Important factors are the inactivation of proteinases by phenylmethanesulphonyl fluoride and the use of cellulose phosphate as an affinity adsorbent. A homogeneous enzyme, with a specific shikimate dehydrogenase activity of 70 units/mg of protein, is obtained in 25% yield. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate, combined with cross-linking studies using dimethyl suberimidate, suggest that the complex is composed of two subunits of molecular weight 165 000. Glycerol-density-gradient centrifugation indicates a molecular weight for the intact complex of about 270 000. Evidence for the effects of proteolysis, both during the preparation and on storage of the purified complex, is presented, and previous reports in the literature of the occurrence of multiple subunits are discussed in this light.

The *arom* multienzyme complex of *Neurospora crassa* contains five distinct enzyme activities which together catalyse the conversion of 3-deoxy-D-arabino-heptulosonate 7-phosphate into 5-enoylpyruvoylshikimate 3-phosphate in the biosynthesis of chorismic acid, the common precursor of the three aromatic amino acids, tyrosine, phenylalanine and tryptophan (Giles *et al.*, 1967). Genetic studies showed that the five structural genes coding for these enzymes occurred as a cluster (Gross & Fein, 1960; Giles *et al.*, 1967), and the occurrence of pleiotropic mutants lacking two or more enzyme activities (Giles *et al.*, 1967; Case & Giles, 1968; Rines *et al.*, 1969) suggested that the cluster was normally transcribed in a polarized fashion as a polycistronic mRNA molecule.

The *arom* multienzyme complex was first purified to apparent homogeneity by Burgoyne *et al.* (1969), who reported a mol.wt. of 231 000 for the intact complex. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate indicated a single type of subunit of mol.wt. about 100 000, but equilibrium centrifugation in the presence of guanidine hydrochloride gave a picture of 'appreciable heterogeneity with species ranging in molecular weight from 29 000 to 120 000'. Burgoyne *et al.* (1969) concluded that the complex consisted of identical halves each made up of an unknown number of smaller components representing the constituent enzymes; however, they also commented on the difficulties of eliminating the possibility of proteolytic damage to the complex during purification. Subsequently, a different purification scheme was devised in the same laboratory (Jacobson *et al.*, 1972) and a study was

made of the dissociation of the complex under various conditions of pH, ionic strength and temperature. Several labile 'subassemblies' with mol.wts. in the range 20 000–80 000 were observed by sucrose-density-gradient centrifugation, and it was concluded that the complex consisted of at least four distinct polypeptide chains. The possibility that the 115 000-mol.wt. 'half-complex' was a single multifunctional polypeptide was discounted.

In an independent study, Gaertner (1972), by careful sedimentation-equilibrium analyses, obtained a mol.wt. of 293 000 for the intact complex. It appeared to be composed of four distinct subunits, for which mol.wts. of 54 000, 63 000, 84 000 and 95 000 were determined by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Gaertner and his colleagues have reported several novel kinetic properties of the purified complex (Gaertner *et al.*, 1970; Welch & Gaertner, 1975, 1976).

Our attempts to follow the published purification schemes met with little success (J. M. Jamieson, J. Lumsden & J. R. Coggins, unpublished work). Very large losses of enzyme activity invariably occurred during the first few steps. A new and more rapid procedure has been devised which pays due attention to the problems of proteolysis (Pringle, 1975) and takes advantage of the discovery by Cole & Gaertner (1975) that cellulose phosphate is an effective affinity adsorbent for the *arom* complex. In the present paper, we describe the details of the procedure and some properties of the resulting purified *arom* complex.

Materials and Methods

Chemicals

The sources of chemicals used in this work were as follows: dithiothreitol from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Nitro Blue Tetrazolium, phenazine methosulphate, oxaloacetic acid and all chemicals for polyacrylamide-gel electrophoresis from BDH Chemicals, Poole, Dorset, U.K.; benzamidine hydrochloride, 1,6-dicyanohexane, shikimic acid and quinic acid from Aldrich Chemical Co., Gillingham, Dorset, U.K.; NADP⁺, phosphoenolpyruvate and D-erythrose 4-phosphate from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K.; phenylmethanesulphonyl fluoride from Eastman-Kodak, Kirkby, Liverpool, U.K.; DEAE-cellulose (DE52) and cellulose phosphate (P11) from Whatman Biochemicals, Maidstone, Kent, U.K.; Coomassie Brilliant Blue G250 from Serva Feinbiochemica, Heidelberg, West Germany; Sephadex G-200 (superfine grade) from Pharmacia (G.B.) Ltd., London W.5, U.K.; Ultrogel AcA34 from LKB Instruments Ltd., South Croydon, Surrey, U.K.

Proteins and enzymes

Deoxyribonuclease-1 (type DN-CL), ox liver catalase (type C-100) and ovalbumin (grade V) were obtained from Sigma, rabbit muscle aldolase was from Boehringer and bovine serum albumin was from Armour Pharmaceutical Co., Eastbourne, Sussex, U.K. DNA-dependent RNA polymerase from *Escherichia coli* was a gift from Dr. A. D. B. Malcolm, Department of Biochemistry, St. Mary's Hospital Medical School, London W2 1PG, U.K.

Growth of cells

Neurospora crassa strain 74-OR23-1A (F.G.S.C. no. 987 obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA 95521, U.S.A.) was grown in stirred cultures with vigorous aeration on Vogel's medium N supplemented with 2% (w/v) sucrose (Davis & De Serres, 1970). Cells were harvested after 24 h by filtration, freeze-dried and stored dry at -20°C. The dried cells were powdered before extraction in a Waring blender.

Preparation of buffers

Buffers used for the enzyme purification were prepared as 1 M stock solutions and the pH was adjusted at room temperature (20°C). Just before use these stock solutions were diluted into water at 4°C with no further adjustment of pH, and solid dithiothreitol and a fresh solution of phenylmethanesulphonyl fluoride [6 mg/ml in 95% (v/v) ethanol] were added.

Purification of the arom multienzyme complex from *Neurospora crassa*

All steps were carried out at 4°C unless otherwise stated.

Step 1: extraction and centrifugation. *N. crassa* powder (20 g) was added to 300 ml of 0.1 M-Tris/HCl, pH 7.5, 0.4 mM-dithiothreitol, 1.2 mM-phenylmethanesulphonyl fluoride, 5.0 mM-EDTA, stirred for 1 h and then centrifuged for 30 min at 25000g. The conductivity of the supernatant was measured and adjusted to 4.0 mmho by using a few ml of 1 M-KCl.

Step 2: passage through DEAE-cellulose. The crude extract was applied to a column (6.5 cm × 4.4 cm) of DEAE-cellulose, previously equilibrated with 50 mM-Tris/HCl (pH 7.5)/75 mM-KCl and freshly washed with the same buffer containing 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride. The flow rate was 300 ml/h and 12.5 ml fractions of the material that passed unretarded through the column were collected; all fractions containing shikimate dehydrogenase activity were pooled.

Step 3: treatment with deoxyribonuclease. This (5 µg/ml) was added to the pooled material and the mixture was incubated for 2 h at 37°C and then centrifuged for 45 min at 30000g.

Step 4: fractionation with (NH₄)₂SO₄. The clear supernatant was made 1 mM in benzamidine and powdered (NH₄)₂SO₄ was added slowly, with stirring, to give a concentration of 242 g/litre starting volume. The solution was stirred for 15 min and the precipitate removed by centrifugation for 20 min at 25000g. More (NH₄)₂SO₄ was added to the supernatant to give a concentration of 63 g/litre starting volume and, after stirring for 15 min, the precipitate was collected by centrifuging for 30 min at 25000g. The supernatant was discarded and the precipitate dissolved in 50 mM-Tris/HCl (pH 7.5)/0.4 mM-dithiothreitol/1.2 mM-phenylmethanesulphonyl fluoride and dialysed overnight against 500 ml of the same buffer.

Step 5: chromatography on DEAE-cellulose. The enzyme was applied to a column (13 cm × 1 cm) of DEAE-cellulose, previously equilibrated with 50 mM-Tris/HCl, pH 7.5, and freshly washed with the same buffer containing 0.4 mM-dithiothreitol and 1.2 mM-phenylmethanesulphonyl fluoride. The column was then washed with this buffer containing 30 mM-KCl until the A₂₈₀ of the eluate was less than 0.1. The enzyme was eluted with a linear gradient (150 ml) of KCl (30–300 mM) in the same buffer. The flow rate was 15 ml/h and 6 min fractions of the eluate were collected and assayed for shikimate dehydrogenase activity. The active fractions were pooled, and, at this stage, were normally stored overnight at 4°C.

Step 6: chromatography on cellulose phosphate. The enzyme was dialysed for 1–2 h against 10 mM-potassium phosphate (pH 6.5)/0.4 mM-dithiothreitol/1.2 mM-phenylmethanesulphonyl fluoride and then applied to a column (42 cm × 1 cm) of cellulose phos-

phate, previously equilibrated with 10mM-potassium phosphate, pH 6.5, and freshly washed with the same buffer containing 0.4mM-dithiothreitol and 1.2mM-phenylmethanesulphonyl fluoride. The column was washed with this buffer until the A_{280} of the eluate was zero; then the enzyme was eluted with 0.2M-potassium phosphate (pH 6.5)/0.4mM-dithiothreitol/1.2mM-phenylmethanesulphonyl fluoride. The column was operated at 7.5 ml/h and 1.5 ml fractions were collected, except during the wash with 10mM buffer (15 ml/h and 3 ml fractions). The fractions with the highest specific activity were pooled and dialysed against 50mM-sodium phosphate (pH 7.5)/0.4mM-dithiothreitol/1.0mM-benzamidine. For long-term storage, an equal volume of glycerol was then added and the enzyme was kept at -20°C .

Preparation of substrates

Ammonium dehydroquininate was prepared from quinic acid by the method of Grewe & Haendler (1968). 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate was prepared by incubating D-erythrose 4-phosphate and oxaloacetate in the presence of CoCl_2 (Herrmann & Poling, 1975). The required product was isolated by paper chromatography.

Enzyme assays

Spectrophotometric assays were performed at 37°C in a total assay volume of 1.0 ml by using a Unicam SP.8000 spectrophotometer with a slave recorder attachment. During purification, the *arom* complex was followed by assaying for shikimate dehydrogenase activity (i.e. the reverse reaction of enzyme 3 of the complex, dehydroshikimate reductase, shikimate-NADP⁺ oxidoreductase, EC 1.1.1.25) by following the reduction of NADP⁺ at 340 nm ($\epsilon = 6.18 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). The reaction mixture contained 0.1 M- Na_2CO_3 , pH 10.6, 4mM-shikimic acid and 2mM-NADP⁺. Dehydroquinase (5-dehydroquininate hydrolyase, EC 4.2.1.10) was assayed by a modification of the method of Mitsuhashi & Davis (1954). The reaction mixture contained 0.1M-Tris/HCl, pH 7.5, and 0.33mM-ammonium dehydroquininate; production of dehydroshikimate was followed at 234 nm ($\epsilon = 1.2 \times 10^4 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$). Dehydroquinase synthase was detected by coupling to the dehydroquinase reaction as described by Welch & Gaertner (1976). Shikimate kinase (EC 2.7.1.71) was assayed by the method of Fewster (1962) and 5-enoylpyruvoylshikimate 3-phosphate synthase by the method of Levin & Sprinson (1964), except that in both cases shikimate was determined as described by Gaitonde & Gordon (1958).

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was carried out as described by Shapiro & Maizel (1969) or Laemmli

(1970). To improve mechanical stability, 3% (w/v) acrylamide gels were made 6M in urea. Protein samples for electrophoresis were made 1% (w/v) in sodium dodecyl sulphate and 1% (v/v) in 2-mercaptoethanol and immediately placed in a boiling-water bath for 5 min. A portion (50 μl) of the sample, 10 μl of 0.05% (w/v) Bromophenol Blue and 10 μl of glycerol were applied to each gel, and after electrophoresis the position of the dye was marked with a thin wire. Gels were stained for protein by incubation for 1 h at 40°C in 0.1% (w/v) Coomassie Brilliant Blue G250 in methanol/acetic acid/water (5:1:4, by vol.) and destained, also at 40°C , in methanol/acetic acid/water (1:1:8, by vol.). Gels were scanned at 600 nm in a Gilford spectrophotometer fitted with a model 252 gel scanner.

Electrophoresis in the absence of sodium dodecyl sulphate, when the gels could be stained for enzyme activity as well as protein, was by the method of Davis (1964) as modified by Hayes & Wellner (1969). Gels were pre-run for 1 h at 2 mA per gel before electrophoresis of the enzyme at the same current. Shikimate dehydrogenase activity was detected by a modification of the general method of Gabriel (1971). The reaction mixture contained 0.25M-Tris/HCl, pH 9.0, 0.5mM-NADP⁺, 0.5mM-shikimic acid, Nitro Blue Tetrazolium (0.5 mg/ml) and phenazine methosulphate (5 μg /ml). The reaction was stopped by incubating the gel briefly in 10% (w/v) trichloroacetic acid. R_f values were in all cases expressed relative to the mobility of the Bromophenol Blue marker.

Cross-linking with dimethyl suberimidate

Dimethyl suberimidate dihydrochloride was prepared from 1,6-dicyanohexane by the method of Davies & Stark (1970). The reagent, which is hygroscopic and unstable at room temperature, was stored in a tightly stoppered bottle at -20°C . Stock solutions (0.1M) were prepared immediately before use in a solvent made by mixing 1 vol. of 1.0M-triethanolamine hydrochloride (pH 8.0) and 1 vol. of 0.4M-NaOH; if necessary, the final pH was adjusted to 8.0 with 1.0M-NaOH or -HCl. The *arom* complex was dialysed exhaustively against 0.1M-triethanolamine hydrochloride (pH 8.0)/0.4mM-dithiothreitol/1.0mM-benzamidine. Cross-linking was carried out at 20°C with final concentrations of 0.16 mg of enzyme/ml, 0.1M buffer and 10mM-dimethyl suberimidate; NaCl concentration was maintained at 0.04M ('low salt') or 0.44M ('high salt'). After 1 h, samples were removed for assay of shikimate dehydrogenase activity, and the cross-linking reaction was terminated by the addition of 1M- NH_4HCO_3 to a final concentration of 0.05M. After a further 30 min at 20°C , 0.1 vol. of 10% (w/v) sodium dodecyl sulphate and 0.01 vol. of 2-mercaptoethanol were added and the samples were heated at 100°C for 5 min.

The denatured protein solutions were dialysed against 0.1M-sodium phosphate (pH 6.5) / 0.1% sodium dodecyl sulphate/0.01% 2-mercaptoethanol for 2h at 20°C before electrophoresis. Cross-linked proteins were prepared for use as molecular-weight standards: ovalbumin by the method of Carpenter & Harrington (1972), aldolase and RNA polymerase by the method described above (J. R. Coggins, J. Lumsden & A. D. B. Malcolm, unpublished work).

Density-gradient centrifugation

Glycerol-density-gradient centrifugation was carried out in a Beckman-Spinco model L2-65 preparative ultracentrifuge, with a SW41 rotor. A linear 5–20% (v/v) glycerol density gradient (8.6cm in length; 11.5ml in volume), containing 50mM-Tris/HCl, pH 7.5, 0.4mM-dithiothreitol, 1.0mM-EDTA, 1.0mM-benzamidine was used. The *arom* complex and the marker enzymes, aldolase and catalase, in a total volume of 0.3ml of the same buffer, were centrifuged on to the gradients for 9h at 41000 rev./min. The temperature was kept at 5°C. After centrifugation, the gradient was harvested by displacement, by using an MSE gradient harvester, and 10-drop fractions were collected. Catalase was assayed by an u.v. method as described by Aebi (1974); aldolase was assayed by the method of Gibbons & Perham (1974).

Analytical methods

Conductivity measurements were made on a Radiometer conductivity meter, type CDM2e (Radiometer, Copenhagen, Denmark).

Protein was assayed by the method of Lowry *et al.* (1951). For crude fractions, protein was precipitated by boiling in 5% (w/v) trichloroacetic acid and resuspended in 0.5M-NaOH for assay. More highly purified fractions were dialysed to remove the dithiothreitol and assayed directly. Crystalline bovine serum albumin was used as a standard.

Results

Purification of the *arom* multienzyme complex from *Neurospora crassa*

The purification procedure described in the Materials and Methods section is summarized in Table 1. The following points are noteworthy.

Step 1. The yield of enzyme activity in crude extract was increased 10-fold by the inclusion of the proteinase inhibitor phenylmethanesulphonyl fluoride (Gold, 1967). This indicates that the intracellular serine endopeptidase reported by Yu *et al.* (1973) was very active in these preparations. EDTA was also included at this stage to inactivate bivalent-metal-ion-dependent aminopeptidases (Siepen *et al.*, 1975).

Step 2. It appeared probable, from the studies by Siepen *et al.* (1975) on five intracellular proteinases from *N. crassa*, that four of these enzymes would be adsorbed by DEAE-cellulose at pH 7.5 and low ionic strength. Under these conditions the *arom* complex is not bound. This step was therefore included as part of a general anti-proteinase strategy.

Step 3. Unless DNA is hydrolysed by nuclease treatment, the subsequent $(\text{NH}_4)_2\text{SO}_4$ fraction was too viscous for the chromatography steps.

Step 4. Benzamidine is an effective reversible inhibitor of proteinases with trypsin-like specificity (Mares-Guia & Shaw, 1965); it was added at this point because, unlike phenylmethanesulphonyl fluoride, it is not salted out of aqueous solution at high concentrations of $(\text{NH}_4)_2\text{SO}_4$. It is desirable to have a proteinase inhibitor present during the $(\text{NH}_4)_2\text{SO}_4$ fractionation because proteinases initially present as complexes with proteinase inhibitor may be activated by exposure to high salt concentrations.

Step 5. The results of the DEAE-cellulose chromatography are shown in Fig. 1. Fractions 26–33 were pooled. The *arom* complex was quite stable at this stage, but was still only a minor component.

Step 6. It is likely that an alkaline endopeptidase

Table 1. *Purification scheme for the arom multienzyme complex from N. crassa*

The results presented below are taken from a typical preparation starting with 20g dry wt. of cells. Protein was determined by the method of Lowry *et al.* (1951); for the purified *arom* complex (after step 6) $A_{1\text{cm}}^{1\%}$ at 280nm is 6.8. Shikimate dehydrogenase activity was determined as described in the Materials and Methods section; 1 unit is defined as the amount of enzyme that catalyses the formation of 1 μmol of NADPH/min at 37°C.

Step	Stage	Vol. (ml)	Protein concn. (mg/ml)	Activity (units/ml)	Total activity (units)	Yield (%)	Specific activity (units/mg)	Purifi- cation (fold)
1	Extraction and centrifugation	274	7.4	0.9	243	100.0	0.12	1
2	First DEAE-cellulose	275	5.2	0.8	222	91.4	0.16	1.3
3	Deoxyribonuclease	270	5.2	0.8	218	89.7	0.16	1.3
4	$(\text{NH}_4)_2\text{SO}_4$	7.2	30.0	23.9	172	70.6	0.80	6.7
5	Second DEAE-cellulose	10.4	2.0	9.4	98	40.2	4.7	39.2
6	Cellulose phosphate	4.5	0.195	13.8	62	25.5	70.5	587.5

Fig. 1. *Chromatography of the arom multienzyme complex on DEAE-cellulose (step 5 of the purification scheme)* Enzyme from step 4 (7.2ml, containing 23.9 units of activity/ml) was applied to the column (13cm×1cm) of DEAE-cellulose equilibrated with 50mM-Tris/HCl, pH 7.5, containing 0.4mM-dithiothreitol and 1.2mM-phenylmethanesulphonyl fluoride. The column was developed with a 150ml linear gradient of KCl (30–300mM) in this buffer. The flow rate was 13.6ml/h and 6 min fractions were collected. Fractions are numbered from the application of the gradient. ○, A_{280} ; ●, shikimate dehydrogenase activity; ----, conductivity (mmho).

Fig. 2. *Chromatography of the arom multienzyme complex on cellulose phosphate (step 6 of the purification scheme)* Enzyme from step 5 (10.4ml, containing 9.4 units of activity/ml) was applied to the column (42cm×1cm) of cellulose phosphate equilibrated with 10mM-potassium phosphate, pH 6.5, containing 0.4mM-dithiothreitol and 1.2mM-phenylmethanesulphonyl fluoride. At the arrow, 0.2M-potassium phosphate (pH 6.5)/0.4mM-dithiothreitol/1.2mM-phenylmethanesulphonyl fluoride was applied. The flow rate was 7.5ml/h and either 3 ml (fractions 1–18) or 15 ml (fractions 19–69) fractions were collected.

in the form of an inactive complex with a specific polypeptide inhibitor is still present at this stage (Yu *et al.*, 1974). Dissociation of the proteinase-inhibitor complex would be favoured by the use of pH 6.5 buffer in this step (Yu *et al.*, 1974) and the proteinase itself would bind to cellulose phosphate under these conditions (Siepen *et al.*, 1975). It was therefore important that both the enzyme preparation and the cellulose phosphate column contained fresh phenylmethanesulphonyl fluoride. Cellulose phosphate was first used as an affinity adsorbent for the

arom complex by Cole & Gaertner (1975). The elution conditions have been simplified and the *arom* complex is obtained, in a highly reproducible manner, as a sharp peak, greatly retarded with respect to the 0.2M-phosphate front (Fig. 2). Only the three fractions of highest specific activity were pooled; there was a sharp fall in specific activity in the two fractions on the right-hand side of the peak. Sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms of these fractions showed, in addition to the native *arom* complex band, eight to ten additional

Table 2. *Enzyme activities during the purification of the arom complex*

Enzyme assays were performed as described in the Materials and Methods section; 1 unit is defined as the amount of enzyme that catalyses the conversion of 1 μ mol of substrate/min at 37°C.

Stage in purification	Enzyme activities (units/mg of protein)			Ratio of activities (A/B/C)
	Shikimate dehydrogenase (A)	Dehydroquinase (B)	Shikimate kinase (C)	
(NH ₄) ₂ SO ₄	0.80	0.13	0.026	30.8:5.0:1
Second DEAE-cellulose	4.70	0.76	0.14	33.6:5.4:1
Cellulose phosphate	70.50	10.90	2.10	33.6:5.2:1

faster-moving bands, the most prominent of which had a mol.wt. 117000. It seems probable that this material consists of proteolytically damaged *arom* complex.

Enzyme activities of the purified complex

The activities of the enzymes shikimate dehydrogenase, dehydroquinase and shikimate kinase at three stages during the purification are presented in Table 2. A constant ratio of enzymic activities was obtained. Because of technical difficulties (for example high absorbance at 234nm and the presence of phosphatases) assays on the initial extract are not presented. The presence of the other two enzymes has also been demonstrated qualitatively in the purified material.

Purity of the arom complex

The purified complex exhibited only one protein band (R_F 0.18) on polyacrylamide-gel electrophoresis in the absence of sodium dodecyl sulphate, and this coincided with a band of shikimate dehydrogenase activity. The enzyme activity band had the same R_F value in crude extract and throughout the purification; however, in crude fractions two minor fast-moving bands of activity (R_F 0.31 and 0.34) were also detectable; the latter was lost after step 4 and the former after step 5.

Molecular weight and subunit composition of the arom complex

A single band of protein was obtained on polyacrylamide-gel electrophoresis of the purified complex in the presence of sodium dodecyl sulphate, whether a phosphate or a Tris/glycine buffer system was used (Plate 1, gels A and C). Various sample loadings from 0.5 to 50 μ g were used with the same result; only at the highest loadings were a number of faint, diffuse, contaminant bands detectable and these might be attributed to proteolysis.

Dimethyl suberimidate-treated proteins (aldolase, ovalbumin and RNA polymerase) were used for molecular-weight calibration. It had been shown (Carpenter & Harrington, 1972; Hucho *et al.*, 1975; J. R. Coggins & J. Lumsden, unpublished work) that the resulting cross-linked species are reliable

Fig. 3. Plot of logarithm of molecular weight against R_F for various species of cross-linked proteins on electrophoresis in 3% polyacrylamide gels containing sodium dodecyl sulphate

Gels were run by the method of Shapiro & Maizel (1969). Proteins were cross-linked with dimethyl suberimidate as described in the Materials and Methods section. $\square$, Aldolase; Δ , ovalbumin; $\circ$, RNA polymerase. The vertical bars indicate the R_F values of control (1) and cross-linked (2) *arom* complex.

molecular-weight standards in the gel-electrophoresis system used. From the resulting calibration (Fig. 3), a subunit mol.wt. of 165000 was obtained for the *arom* complex.

Cross-linking of the *arom* complex with dimethyl suberimidate, whether at low or high salt concentration, led to the conversion of the *arom* subunit into a single, slower-moving, species (Plate 1, gel B) of mol.wt. 330000 (Fig. 3). This suggests that the complex has a dimeric structure. The shikimate dehydrogenase activity of the cross-linked complex was 140% of that of the control enzyme.

Fig. 4 shows the profiles of enzymic activities of rabbit muscle aldolase, ox liver catalase and the *arom* complex as a function of fraction number (equivalent to distance of migration) after centrifugation in a glycerol density gradient. The activity peak of the *arom* complex migrated very close to that of ox liver catalase. By using the method of Martin & Ames (1961) and by assuming that the enzymes have the same partial specific volume, values for the mol.wt. of the *arom* complex of 282000

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis of purified arom complex preparations in the presence of sodium dodecyl sulphate
 Polyacrylamide gels were run in either a phosphate (Shapiro & Maizel, 1969) or a Tris/glycine (Laemmli, 1970) buffer system. Gel (A), 16 μ g of *arom* complex in a 3% polyacrylamide gel run in the phosphate buffer system; gel (B), 16 μ g of dimethyl suberimidate-cross-linked *arom* complex run as in gel (A); gel (C), 8 μ g of *arom* complex in a 5% polyacrylamide gel run in the Tris/glycine buffer system; gel (D), 12 μ g of *arom* complex (stored for 19 days in 50% glycerol at -20°C) in a 4% polyacrylamide gel run in the phosphate buffer system; gel (E), 33 μ g of *arom* complex [stored for 19 days at 4°C in 50mM-sodium phosphate (pH 7.5)/0.4mM-dithiothreitol/1.0mM-benzamidine] run as in gel (D).

Fig. 4. Glycerol-density-gradient centrifugation of aldolase, catalase and *arom* complex

The mixture of enzymes (0.3ml) was layered on a linear 5–20% glycerol density gradient containing 50mM-Tris/HCl, pH 7.5, 0.4mM-dithiothreitol, 1.0mM-EDTA and 1.0mM-benzamidine and centrifuged in an SW-41 rotor at 41000 rev./min for 9 h at 5°C. After centrifugation, fractions of 10 drops were collected and enzymes were assayed as described in the Materials and Methods section. Fraction 42 was the bottom of the gradient. The activities were normalized with respect to the peak tubes. ○, Aldolase activity; □, catalase activity; ●, shikimate dehydrogenase activity.

(relative to aldolase as standard) and 255000 (relative to catalase as standard) were obtained. Martin & Ames (1961) have pointed out that such variation is to be expected because of differences in the shapes of the standard enzymes used.

Attempts to obtain a value for the molecular weight of the native complex by gel filtration (on Sephadex G-200 or Ultrogel AcA34) resulted in complete loss of enzyme activity, despite the inclusion of benzamidine in the elution buffer. Two possible reasons for this are (a) denaturation at low protein concentration and (b) activation of a trace proteinase contaminant (Yu *et al.*, 1973).

Stability and storage of the *arom* complex

The purified complex, stored in 50% glycerol at –20°C, was stable for at least 1 month as judged by shikimate dehydrogenase activity and polyacrylamide-gel electrophoresis. However, if the enzyme was stored for 19 days in the absence of glycerol and at 4°C, a dramatic change in subunit structure was observed (Plate 1, gels D and E). Up to ten

fragments, with mol.wts. in the range 30000–125000, were produced, presumably as a result of proteolysis. Although none of the original 165000-mol.wt. subunit remained, the shikimate dehydrogenase activity was still present at 85% of its original value. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate showed that the fragments obtained were different from those observed in the low-specific-activity fractions from the cellulose phosphate column.

Discussion

The *arom* multienzyme complex as prepared in the present study differs both in specific activity and subunit composition from the same complex isolated by other workers (Burgoyne *et al.*, 1969; Jacobson *et al.*, 1972; Gaertner, 1972). Comparisons with published values of specific activity for the shikimate dehydrogenase component cannot be made directly but, if corrections are made for our measured $A_{1\text{cm}}^{1\%}$ value of 6.8 at 280nm, for the molar extinction coefficient of NADPH and for differences caused by pH and temperature of assay (Gaertner, 1972), then specific activities of 70 (present paper), 49.5 (Burgoyne *et al.*, 1969), 8.1 (Jacobson *et al.*, 1972) and 11.0 (Gaertner, 1972) units/mg of protein are obtained.

Under denaturing conditions the purified enzyme described in the present paper appears to be homogeneous with respect to polypeptide-chain size. No heterogeneity was detected by electrophoresis or by glycerol-density-gradient analysis under non-denaturing conditions. The subunit mol.wt. was 165000, and this, together with the mol.wt. of 330000 for the cross-linked enzyme and the estimated mol.wt. of 255000–282000 for the native enzyme, indicates that the *arom* multienzyme complex is a dimer of similar and probably identical subunits. We believe that the smaller subunits reported by other workers (Gaertner, 1972; Jacobson *et al.*, 1972) were formed by proteolytic cleavage of the native complex by intracellular proteinases present during the purification (Siepen *et al.*, 1975).

In a comprehensive review, Pringle (1975) has recorded several instances of enzymes purified from yeast (a system that has many similarities to *N. crassa*) in which the reported subunit molecular weights showed a steady increase in magnitude as more recognition was given to the necessity of keeping proteolysis under control. Two relevant examples are tryptophan synthase (EC 4.2.1.20) (Matchett & De Moss, 1975) and anthranilate synthase (EC 4.1.3.27) (Hulett & De Moss, 1975) from *N. crassa*. The latter has mol.wt. of 330000 [previously reported as low as 240000 by Gaertner & De Moss (1969)] and has an $\alpha_2\beta_2$ subunit structure. Earlier work indicating the presence of three different subunits (Arroyo-Begovich

& De Moss, 1973) was shown to be the result of a proteolytic artifact, and, in fact, a progressive degradation of the purified complex into smaller subunits could be demonstrated on freeze-thaw treatment or storage at 4°C (Hulett & De Moss, 1975). We have noted a similar effect on storage of the purified *arom* complex, and the molecular weights of the resulting proteolytically damaged subunits are similar to those reported for the complex isolated in other laboratories (Jacobson *et al.*, 1972; Gaertner, 1972).

In this connexion, the persistence of most of the shikimate dehydrogenase activity, despite the total loss of the 165000-mol.wt. native subunit, requires comment. It has been proposed that multi-functional polypeptides evolve as a result of gene fusion (Bonner *et al.*, 1965); indeed such a situation has been brought about by mutational events in the intercistronic region of the histidine operon of *Salmonella typhimurium* (Yourno *et al.*, 1970). A frequent observation with such proteins is that limited proteolysis results in the physical separation of the individual enzyme components, often with minimal loss of enzymic activity (Goldberg, 1969; Kohno & Yourno, 1971; Veron *et al.*, 1972; Setlow *et al.*, 1972; Hulett & De Moss, 1975). Multienzyme complexes of this type are believed to retain the distinct folding domains of the component enzymes and to be linked together with exposed loops of polypeptide chain, probably representing the translation product of the original intercistronic regions. These exposed loops are very susceptible to proteolytic attack.

It has not yet been determined whether the *arom* complex is a dimer of identical subunits or is composed of two non-identical subunits of the same molecular weight, but genetic evidence strongly favours the former possibility. Allelic complementation has been observed for two of the *arom* enzymes, namely dehydroquinase synthase and dehydroshikimate reductase (Giles *et al.*, 1967). For the former enzyme, complementation has also been demonstrated *in vitro* by mixing the mutant complexes at alkaline pH (Case *et al.*, 1969). This is good evidence that the *arom* complex contains two copies of these enzymes, each located on different polypeptide chains (Crick & Orgel, 1964). The genes for these two enzymes are located at the proximal and distal ends respectively of the *arom* cluster, which is believed to be transcribed as a single mRNA molecule (Giles *et al.*, 1967; Jacobson *et al.*, 1972). The only model for the *arom* complex consistent with our subunit structure and the genetics is one consisting of a dimer of identical subunits.

It therefore appears that the subunits of the *arom* multienzyme complex are pentafunctional polypeptides. This represents an extreme example of a type of multienzyme complex containing multi-

functional polypeptides which seems to be common among the enzymes of aromatic amino acid biosynthesis. For example, 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase-chorismate mutase of *Bacillus subtilis* (Huang *et al.*, 1974), chorismate mutase-prephenate dehydratase of *Escherichia coli* (Davidson *et al.*, 1972), indol-3-ylglycerol phosphate synthase (EC 4.1.1.48) from *E. coli* (Creighton & Yanofsky, 1966) and tryptophan synthase of *N. crassa* (Matchett & De Moss, 1975) all contain bifunctional polypeptides, and anthranilate synthase of *N. crassa* contains a trifunctional polypeptide (Hulett & De Moss, 1975).

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