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PURIFICATION AND PROPERTIES OF THE AROMATIC *(AROM)* SYNTHETIC ENZYME AGGREGATE OF *NEUROSPORA CRASSA*

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SUMMARY

1. Procedures are described for the purification of the wild type *arom* multienzyme complex encoded in the *arom* gene cluster of *Neurospora crassa*.

2. The purified aggregate has all five activities present at high levels and there has been no indication that the five activities are separable during purification.

3. The enzyme is readily inactivated by O_2 even in the presence of thiols.

4. The molecular weight of the purified aggregate is estimated to be approx. 231 000 on the basis of data from equilibrium sedimentation and amino acid analysis.

5. Experiments on disruption of the purified aggregate (which leads to loss of enzymic activities) and on renaturation (which restores three of the five activities), together with independent studies of allelic complementation, suggest that the complex consists of two identical halves bound together by relatively weak non-peptide bonds.

6. Preliminary evidence has also been obtained for the presence of still smaller components, possibly representing the five different polypeptide chains which are thought to be the basis subunits of which the aggregate is composed.

INTRODUCTION

Neurospora crassa possesses a cluster of five structural genes, the *arom* cluster, which encodes an aggregate of five enzymes catalyzing Steps 2 through 6 in the prechorismic acid part of the aromatic synthetic pathway[1–4]. Genetic and biochemical studies have provided a considerable amount of information about the genetical organization of the *arom* region, and about the kinds and consequences of mutational events which occur within this gene cluster. Centrifugation in sucrose density gradients of extracts from wild type and various mutants provided preliminary physical evidence about the multienzyme complex which the *arom* gene cluster encodes. The normal enzyme aggregate from wild type sedimented as an associated complex of five ac-

Abbreviation: DAHP, 3-deoxy-D-arabinoheptulosonate 7-phosphate.

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tivities with an *s* value of 11.4 which provided evidence for a molecular weight of approx. 200 000. These previous studies indicated clearly the need for a further detailed examination of the physical properties of the aggregate based on studies of highly purified aggregate protein from wild type. The present paper reports the current status of such studies.

MATERIALS AND METHODS

Source of arom aggregate protein

A large scale culture of an *arom-3* mutant (Strain 87) served as a source of mycelium for enzyme extraction. This mutant, which lacks chorismate synthetase, was used because *arom-3* mutants typically have relatively high levels of the five activities encoded by the *arom* gene cluster: approx. 3 times the levels found in wild type[1]. The material was grown at 25° with vigorous aeration on Fries minimal supplemented with a limiting level (16 μg/ml) of tryptophan and normal levels of the other aromatic supplements[1]. The harvested mycelium was washed thoroughly with distilled water and lyophylized.

Enzyme assays

Methods of enzyme assay have been described previously[1] except that a modified dehydroquinate synthetase assay was used as follows: The standard assay system had a final volume of 0.6 ml. It was 0.125 M Tris–HCl (pH 7.5) 13 μM NAD^+ and 0.2–0.3 mM 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP). In the presence of dehydroquinate synthetase this system produces dehydroquinate. In the presence of excess dehydroquinase the rate of reaction can be measured by the rate of production of dehydroshikimic acid at 240 nm. If dehydroquinase was not known to be present in a suitable excess it was added as a dehydroquinate synthetase less *arom* complex (from *arom-2* mutant Strain 82) purified to Stage 8 in the procedure given below. This system was stimulated by low levels of phosphate but in 0.1 M potassium phosphate (pH 7.5) it became cobalt dependent as in the original assay procedure[1]. Although the above system appeared to be independent of added metals it was completely inhibited by EDTA and activity could be restored by addition of zinc or cobalt. This standard assay system gave similar measurable activities to the cobalt–phosphate–buffer assay system originally used but it was less subject to interference from thiols used to protect the enzyme.

Extraction and purification procedures

Materials

Stock solutions. 0.1 M dithiothreitol (Calbiochem); 1.0 M potassium phosphate, pH 7.5; 1.0 M $CaCl_2$; 1.0 M KCl; 0.25 M Tris–HCl, pH 7.5; 1.0 M KOH; 0.1 M spermine · 4 HCl (spermine) (Mann Research Laboratories) adjusted to pH 7.0 with KOH; and undiluted liquid α-thioglycerol (K and K Laboratories). The buffers, listed below, and solutions used in the preparation of calcium phosphate gels were prepared by the appropriate dilution of these stock solutions without correcting the pH after dilution unless stated otherwise.

Buffers. All buffers containing sulphydryl reagents (α-thioglycerol or dithiothreitol) were not stored for more than a few hours after the sulphydryl reagent had

been added. When operations, such as dialysis, were carried out over periods longer than 1 h the buffers were kept in tightly stoppered flasks with a N_2 gas phase. Buffer A: 0.01 M Tris–HCl with 0.6 ml of α-thioglycerol per l. Buffer B: 0.01 M Tris–HCl, 0.4 mM dithiothreitol. Buffer C: 0.2 mM spermine, 0.2 mM dithiothreitol. Buffer D: 0.2 mM spermine, 1 mM KCl, 0.4 mM dithiothreitol. Buffer E: 35 mM potassium phosphate, 0.12 M KCl, 0.4 mM dithiothreitol. Buffer F: 175 mM KCl, 10 mM Tris–HCl, 0.4 mM dithiothreitol. Buffer G: 100 mM potassium phosphate, 1 mM dithiothreitol.

Adsorbants and column materials. (1) *Calcium phosphate gel.* The correct amount of calcium phosphate gel for a preparation of this size (190 dry g of Neurospora mycelium) was prepared as follows: 800 ml of 0.1 M potassium phosphate was stirred and 1.0 M $CaCl_2$ was added dropwise interspersed with small additions of 1.0 M KOH so that the pH was held between 7.3 and 7.5 during the $CaCl_2$ addition. A total volume of 40 ml of 1.0 M $CaCl_2$ was added and the pH was set finally at 7.4. The gel was stirred for 10 min and then centrifuged out at low speed, washed by redispersal and centrifugation out of 10 mM potassium phosphate and stored as a suspension in 250 ml 40 mM potassium phosphate. The gel was routinely used approx. 24 h after preparation. Just prior to use it was centrifuged out of this suspension at low speed. (2) K^+ Biorad AG 50W-X8 (Bio-Rad Laboratories). This resin was purchased as the H^+ form. It was washed in 1.0 M KOH, water, and then suspended in 1 mM KCl. The pH of the suspension was adjusted to 7.0 with HCl and then the resin was settled out, drained, and stored wet. (3) DEAE-Sephadex A-50 (Pharmacia Fine Chemicals) was washed with 0.1 M KOH, water, and then dispersed in Buffer E (omitting the dithiothreitol). The pH was adjusted back to pH 7.4 and then the gel was washed with approx. 2 vol. of the same buffer. It was stored as a suspension in the same buffer with a few drops of chloroform as a preservative. The freeze-dried DEAE-Sephadex A-50 used in Step 9 of the procedure below was prepared from the above suspension by washing some gel with 0.1 M KCl, then thoroughly with water and finally freeze-drying the gel. (4) The Sepharose 4B (Pharmacia Fine Chemicals) column was prepared according to the manufacturers instructions and equilibrated with Buffer G. The reservoir containing the Buffer G had a very slow stream of N_2 passing through it to reduce the levels of dissolved O_2.

Method

All operations were carried out at 0–4°. The first centrifugation was 45 min in a refrigerated centrifuge at 13 200–27 300 $\times g$. Subsequent centrifugations, up to Stage 6, were 13 200 $\times g$ for 15 min. After Stage 6 the volumes were smaller and could be centrifuged at higher speeds (*e.g.*, 34 800 $\times g$ for 15 min). The following steps were employed: (1) 190 g of freeze-dried and Wiley-milled Neurospora mycelium was dispersed in 3500 ml of Buffer A. The suspension was stirred for 35 min and centrifuged. (The supernatant often has a hazy lipid layer on top but this can be ignored as it is removed in subsequent steps.) (2) To the supernatant was added spermine (final concn. 5 mM) and was centrifuged. (3) The supernatant was made 90 mM potassium phosphate with the stock potassium phosphate and then the total volume was made 32% saturated with $(NH_4)_2SO_4$ (188 g of solid $(NH_4)_2SO_4$ per l total volume). This was then centrifuged. (4) The supernatant was made 50% saturated with $(NH_4)_2SO_4$ (114 g of solid $(NH_4)_2SO_4$ per l of the supernatant from Step 3). This was centrifuged. (5) The precipitate was carefully drained free of traces of $(NH_4)_2SO_4$ solution and then dissolved in 170 ml of Buffer B. (6) The solution was dialyzed against 3 changes of 6 l

of Buffer C over a period of 36 h under N_2 at 0–4°. The resultant suspension was centrifuged out and the precipitate washed 3 times by redispersal into, and centrifugation out of 75-ml lots of Buffer D. (This precipitate is difficult to disperse and gentle hand homogenization is suggested.) (7) The washed precipitate was then dispersed in 100 ml of Buffer D and stirred with 13 g of K^+ Biorad AG 50W-X8 for 90 min under N_2 at 0–4°. The suspension was decanted from the resin and centrifuged. The supernatant had stock 1.0 M potassium phosphate added to give a final phosphate concentration of 40 mM. (8) The pellet of wet-packed calcium phosphate gel, prepared as described previously, was thoroughly dispersed in the enzyme solution and the suspension was allowed to stand for 5–10 min before centrifugation. (9) The supernatant was made 0.12 M KCl by the addition of 0.136 vol. of stock 1.0 M KCl. This was then poured through a column of DEAE-Sephadex A-50 previously equilibrated with Buffer E. The column contained approx. 50 ml of wet-packed gel and was approx. 4 cm high and 4 cm in diameter. The effluent was collected and then the column was washed with approx. 150 ml of Buffer E. The effluent and washings were bulked and diluted with an equal volume of Buffer B. The whole mixture (approx. 500 ml) was stirred with 110 mg of freeze-dried DEAE-Sephadex A-50 for 3 h under N_2 at 0–4°. (10) The DEAE-Sephadex A-50 was allowed to settle out and then transferred into a small column. This small column was then washed with 25 ml of a mixture of equal parts of buffers E and B. The enzyme was then eluted with 50 ml of Buffer F. The enzyme was concentrated by dialysis against 0.1 M potassium phosphate, 0.4 mM dithiothreitol, containing solid $(NH_4)_2SO_4$ to make the volume of dialysis solution *plus* dialysis bag contents 70% saturated. (47.2 g per 100 ml overall volume). (11) The $(NH_4)_2SO_4$ precipitate from Step 10 was dissolved in approx. 2 ml of Buffer G and loaded onto the column of Sepharose 4B that had been previously equilibrated with N_2-saturated Buffer G. The column contained approx. 200 ml of packed gel and was approx. 44 cm high. The enzyme eluted with a K_{av} of 0.52. It could be concentrated by $(NH_4)_2SO_4$ precipitation, as described in Step 10, and stored dissolved in Buffer G and frozen in N_2-filled tubes. Dehydroshikimic acid reductase activities in different stages of the preparation procedure are shown in Table I.

TABLE I

YIELD OF ENZYME AT VARIOUS STAGES DURING THE PREPARATION PROCEDURE

Dehydroshikimate reductase activity was used to follow the enzyme aggregate. 1 unit of activity is taken as the amount of enzyme that causes a change of $A_{340\ nm}$ of 1.0 in 1 min at 37° in the standard assay mixture.

At end of Stage	*Vol. (ml)*	*Dehydroshikimate reductase*	
		Total activity (units × 10^3)	*Specific activity (units per mg protein)**
1	3100	100	0.6
5	170	50	1.92
7	104	40	22.5
8	100	14	100.0
10	50	2.1	416.0
11	2	2	450.0

* Based on $A_{280\ nm}$.

Sucrose density gradient centrifugation

This procedure employed a linear 5–20% sucrose gradient formed at 4° and has been described previously[1].

Polyacrylamide gel electrophoresis

This procedure employed an apparatus manufactured by Canal Industrial Corporation. All gels were polymerized by riboflavin and light. They were 4 mm in diameter and run at constant current.

Analytical ultracentrifugation and molecular weight determination

Velocity and equilibrium centrifugation experiments were carried out in a Spinco Model E ultracentrifuge, fitted with an ultraviolet scanner. Sedimentation coefficients were determined at 56 000 rev./min at 12 and 8° with the schlieren optical system and corrected for buffer viscosities and densities to the standard conditions of water at 20°.

Procedures to disaggregate the complex

Various procedures were used in attempts to disrupt the aggregate, including: treatment under alkaline-reducing conditions in the presence of high levels of dithiothreitol; polyacrylamide gel electrophoresis in the presence of 7 and 8 M urea, and 5 M urea in 35% acetic acid[5]; treatment with the detergent sodium dodecyl sulfate followed by electrophoresis on acrylamide gels with continuous buffer systems containing sodium dodecyl sulfate[6] or centrifugation of sodium dodecyl sulfate-treated enzyme in a sucrose gradient containing sodium dodecyl sulfate. Various levels of dithiothreitol were used to inhibit aggregations due to thiol oxidation. In one experiment the detergent-treated protein was placed in alkaline-reducing conditions and then alkylated with iodoacetate before electrophoresis.

Procedures for (partial) reactivation of arom enzymic activities after inactivation by detergent, 8 M urea, 6 M guanidine·HCl or heat denaturation

After detergent treatments, which resulted in inactivation, reactivation was attempted by the following procedures: thorough dialysis was carried out over at least 48 h with two changes of dialysis fluid (0.02 M potassium phosphate (pH 7.4) 1 mM dithiothreitol, 0.1 mM EDTA). The EDTA was omitted in the second batch of dialysis fluid. During the removal of the detergent the enzyme protein precipitated. The detergent-denatured (or heat-denatured) protein was redissolved by dialyzing the sample against 8 M urea, 0.1 M glycine, 10 mM dithiothreitol, pH 10.0 for 24–36 h. After this treatment, the following procedure gave partial reactivation of the above material as well as of enzyme that had been inactivated by solution in 8 M urea or 6 M guanidine·HCl: the protein solutions (in 8 M urea or 6 M guanidine·HCl) were dialyzed against 0.1 M glycine, 25 mM dithiothreitol, pH 10.0 for 24–36 h then dialyzed against 0.1 M potassium phosphate pH 7.4, 0.2 mM dithiothreitol, containing a trace of Zn^{2+} (final concentration approx. 0.2 μM $ZnCl_2$). All the above dialysis steps were carried out at 0–4° under N_2.

Amino acid analysis

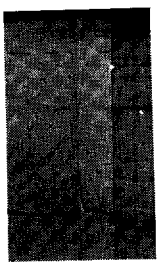
The sample was reduced and carboxymethylated in alkaline 6 M guanidine·HCl

(containing 25 mM Tris–HCl, pH 8.0). After dialysis into water the sample was made 6 M with HCl and hydrolyzed *in vacuo* for 36 h at 100–106°. Analyses were performed on the Beckman–Spinco amino acid analyzer, model 120B (ref. 7). Half-cystine was measured as carboxymethyl cysteine and tryptophan was measured spectrophotometrically in another sample which had not been carboxymethylated[8]. For calculation of $\bar{v}$ the NH_3 measured was assumed to have been derived equally from asparagine and glutamine residues.

RESULTS

Evidence for purity of the arom aggregate protein

The *arom* aggregate protein extracted from Mutant 87 (wild type with respect to the *arom* region) and subjected to the purification procedure described earlier has been tested for purity by several procedures. The aggregate has never shown appre-



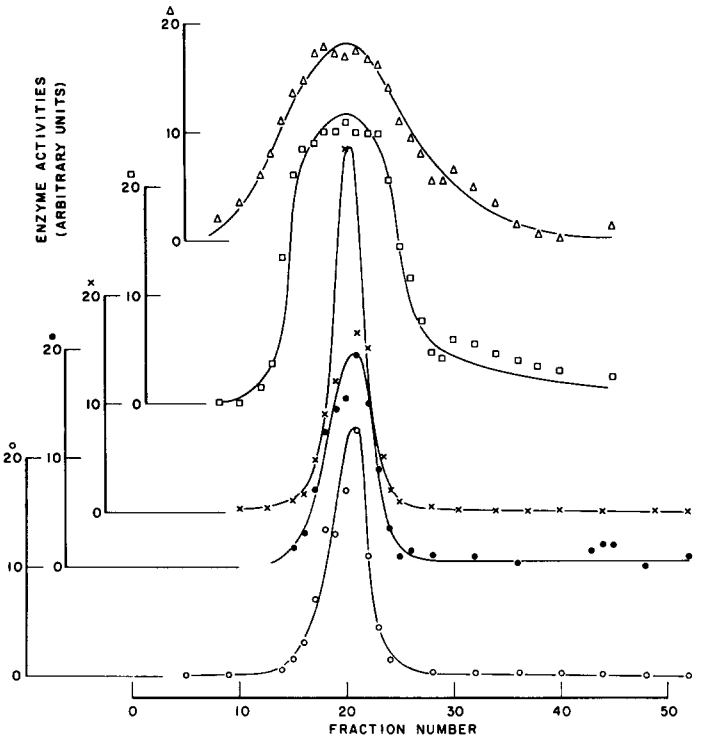


Fig. 1. Sedimentation of the *arom* enzyme aggregate in the analytical ultracentrifuge. A sample of purified material (through Stage 11) dissolved in 0.1 M potassium phosphate (pH 7.4), 0.5 mM dithiothreitol was centrifuged at 56 000 rev./min at 12° in the Beckman–Spinco An-D rotor and observed using schlieren optics. Photographs were taken at 8-min intervals with a bar angle of 60°. A single major sedimenting peak is seen with a sedimentation coefficient of 11.4 S. Small amounts of other species are present but these may be at least partly ,derivatives of this readily oxidized enzyme. Sedimentation is from right to left.

Fig. 2. Distribution, in a sucrose gradient, of the five enzymic activities found in a sample of purified (through Stage 11) wild type *arom* aggregate protein. △—△, shikimate kinase; □—□, 3-enolpyruvyl shikimic acid phosphate synthetase; ×—×, dehydroshikimate reductase; ●—●, dehydroquinase; ○—○, dehydroquinate synthetase.

ciable heterogeneity on acrylamide gel electrophoresis in its native state, and did not show heterogeneity even in urea or sodium dodecyl sulfate. Similarly, centrifugation in detergent (sodium dodecyl sulfate)–sucrose gradients failed to detect gross heterogeneity. Samples of the purified material have also been subjected to high speed sedimentation in the analytical ultracentrifuge and observed using schlieren optics. A single major sedimenting peak is present, although a small amount of a larger aggregate may be indicated by the small peak preceding the major peak (Fig. 1). All the above procedures have shown small traces of possible contaminants. These may be impurities or age-produced derivatives as this protein is very readily oxidized during handling procedures.

The association of enzymic activities during purification

During the purification procedure all five activities run together. At present no definitive statement about constant ratios can be made because some assay procedures still lack the necessary reliable reproducibility. However, all attempts to separate the five enzymic activities have failed, although the following have been tried: (a) centrifugation in sucrose gradients at pH 7.4 (Fig. 2); (b) centrifugation in sucrose gradients at pH 10.0 in the presence of 0.1 M dithiothreitol; (c) centrifugation of detergent-disrupted enzyme in sucrose gradients followed by partial reactivation. Although all enzyme activities could not be followed, the enzyme protein failed to develop significant observable heterogeneity even when subjected to electrophoresis in polyacryl-

TABLE II

AMINO ACID COMPOSITION OF *arom* AGGREGATE PROTEIN

Conditions are described in the text. Values are given as the number of residues per molecule of mol. wt. 231 000.

Amino acid	*Residues per molecule*
Lysine	79
Histidine	31
Arginine	78
Half-cystine*	17
Aspartic acid***	220
Threonine	121
Serine	148
Glutamic acid***	229
Proline	131
Glycine	169
Alanine	238
Valine	149
Methionine	37
Isoleucine	116
Leucine	260
Tyrosine	47
Phenylalanine	53
Tryptophan**	31

* Half-cystine was measured as CM-cysteine.

** Tryptophan was measured spectrophotometrically[8].

*** As all amides appear as NH_3 (164 residues) and free acids, the NH_3 was arbitrarily alloted equally to aspartic and glutamic acid for purposes of calculating $\bar{v}$.

amide gels under conditions that might be expected to disaggregate it (see below). In the electropherograms small amounts of various other species were seen in all cases but the anomalous bands were a very small percentage of the whole.

Amino acid analysis

The results of an amino acid analysis of the purified *arom* multienzyme complex are given in Table II. The amino acid analysis allowed the calculation of $\bar{v}$ for the protein ($\bar{v} = 0.74$). The observed amino acid composition predicts an acid or anionic protein. Electrophoresis and behavior on exchange celluloses agree with this prediction.

Determination of the molecular weight of the aggregate

One equilibrium centrifugation has been carried out on an enzyme solution ($A_{280\ nm} = 0.48$) in 0.1 M potassium phosphate pH 7.4, 1 mM dithiothreitol. The ultracentrifuge cell contained a column of 1.3 mm of solution and a gas phase of N_2. The run was at 12° and 4400 rev./min in the Beckman–Spinco An-D rotor. The resultant plot of ln c against x^2 was close to linear and $d\ln c/dx^2 = 0.26$. Utilizing the value of $\bar{v}$ (= 0.74) based on amino acid analysis (Table II) allowed an evaluation of the molecular weight which was estimated to be 231 000. The $s_{20,w}$ of the complex was calculated to be 11.4 at low protein concentrations ($A_{280\ nm}$ solution = 0.45). This value agrees very well with s values calculated from sucrose density gradient experiments[1]. There is some dependence of s on protein concentration. The behavior of the enzyme on Sepharose 4B is in agreement with the estimate of the molecular weight (above) and the manufacturer's calibration of the gel.

Experiments to disrupt the complex

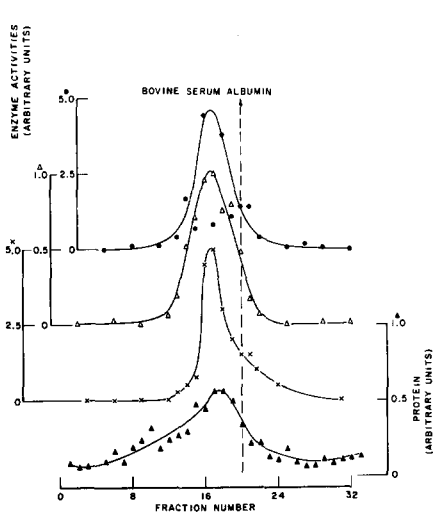
Alkaline-reducing conditions (pH 10.0) in the presence of high concentrations of dithiothreitol (100 mM) failed to lower significantly the sedimentation coefficient of the aggregate as determined from its migration in a sucrose gradient. However, these conditions do allow intragenic complementation "*in vitro*" between dehydroquinate synthetase mutants and as the hybrid complex so formed has a sedimentation coefficient indistinguishable from the normal enzyme it appears that the alkaline dithiothreitol conditions allow exchange of some type of subunits without much enzyme existing as subunits at any one time[9].

Polyacrylamide gel electrophoresis in the presence of 7 M urea, 8 M urea at approx. pH 9.0 in discontinuous buffer systems, and 5 M urea in 35% acetic acid all showed that the aggregate was moving as, essentially, a single species. However, no information was gained on the molecular weight of the migrating species in any of the above urea gels so it is not known whether the urea failed to break the complex or broke it in such a way as to produce very similar, if not identical, fragments.

Following the method of SHAPIRO *et al.*[6] the aggregate was treated with a 1% solution of sodium dodecyl sulfate and subjected to electrophoresis on polyacrylamide gels in 0.1% sodium dodecyl sulfate. To prevent oxidative aggregation, thiols were present in the gels and in one experiment the protein was placed in alkaline-reducing conditions and then alkylated with iodoacetate before electrophoresis. In all cases the protein migrated as essentially one major species. Using lysozyme, bovine serum albumin, and thyroglobulin as standards of known molecular weight and the relation-

ship established by SHAPIRO *et al.*[6], the migrating species was assigned a molecular weight in the region of 100 000.

In other experiments on dissociation, the aggregate protein was treated with detergent (1% sodium dodecyl sulfate) in the presence of dithiothreitol and then centrifuged in a sucrose gradient containing 0.1% sodium dodecyl sulfate and 10 mM dithiothreitol. The enzyme protein moved as a rather broad single peak and sedimented a little faster than bovine serum albumin (mol. wt. = 70 000). The sedimentation rate of the enzyme protein was compatible with a molecular weight in the region of 100 000 and thus supported the estimate from the polyacrylamide gel studies. On these sucrose gradients containing sodium dodecyl sulfate three of the enzymic activities of the protein could be reactivated by the procedure described in MATERIALS AND METHODS and the distribution of activities down the gradient showed a close, although not completely parallel relationship, to the distribution of protein (see Figs. 3 and 4).



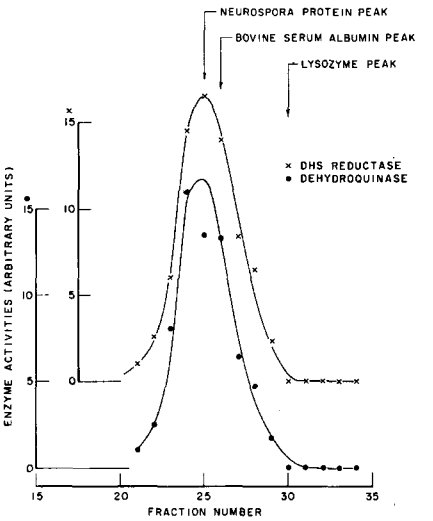


Fig. 3. Distribution, in a sucrose density gradient, of purified (through Stage 11), detergent (sodium dodecyl sulfate)-treated, *arom* aggregate protein and of the three *arom* enzyme activities regenerated after treatment of individual gradient fractions. (The arrow indicates the peak position of the reference protein, bovine serum albumin, mol. wt. 70 000). Details of procedures are given in the text. (Note: In the dehydroquinase assay curve, Points 15, 17, and 19 are badly off the line of best fit. A repeat of this experiment gave a very much closer fit for this activity, as shown in Fig. 4). ▲—▲, protein; ×—×, dehydroshikimate reductase; △—△, shikimate kinase; ●—●, dehydroquinase.

Fig. 4. Repeat of the experiment depicted in Fig. 3. indicating the distribution of regenerated dehydroshikimate reductase and dehydroquinase activities. ×—×, dehydroshikimate reductase; ●—●, dehydroquinase.

Equilibrium centrifugations of reduced and carboxymethylated enzyme in 6 M guanidine·HCl have indicated appreciable heterogeneity with species ranging from mol. wt. 29 000 to 120 000. Satisfactory interpretation of these results must await further studies with a wider variety of conditions and different batches of enzyme.

Reactivation of arom enzymic activities

Reactivation experiments have been performed with various types of inactivated aggregates following the procedures described in MATERIALS AND METHODS. These indicate that reactivation involving the restoration of at least three of the five enzyme activities (all except dehydroquinate synthetase and possibly 3-enolpyruvyl shikimic acid-5-phosphate synthetase) is possible after brief boiling of the enzyme, after urea or guanidine·HCl treatment, and after dissociation by sodium dodecyl sulfate (*cf.* Figs. 3 and 4).

DISCUSSION

Prior studies have established many details concerning the genetic control of the *arom* multienzyme aggregate by the *arom* gene cluster in *Neurospora crassa*[1-4]. This paper describes the purification and some of the properties of this five-enzyme aggregate. The purified aggregate from wild type has all five activities present at high levels and there has been no indication that the five activities are separable during purification.

Equilibrium sedimentation data, together with an amino acid analysis of the purified aggregate protein, have made it possible to estimate the molecular weight of the wild type aggregate as approx. 231 000. The sedimentation coefficient (11.4 S) determined in an analytical ultracentrifuge agrees well with the value previously estimated on the basis of sucrose density gradient centrifugation[1].

Experiments on disruption of the purified aggregate protein from wild type indicate that treatment with detergent (sodium dodecyl sulfate) leads to dissociation of the complex into a single type of subunit with respect to size and electrophoretic mobility, and that this subunit has a molecular weight in the region of 100 000. Treatment with 8 M urea also produces a single type of subunit with respect to electrophoretic mobility in a gel. When the aggregate is reduced and alkylated, dissolved in 6 M guanidine·HCl (pH 6.0) and sedimented in an analytical ultracentrifuge, components with molecular weights between 120 000 and 29 000 are detected. However, these preliminary results must be interpreted with caution, since they indicated that breakdown into units smaller than the half molecular weight aggregate was largely incomplete. This made it difficult to eliminate the possibility that slight proteolytic damage occurring during the purification procedure could explain the results. (More recent comparable experiments, performed under somewhat different conditions, appear to indicate that a more complete dissociation of the aggregate into components ranging in molecular weights from 20 000 to 60 000 can occur (C. W. H. PARTRIDGE and N. H. GILES, unpublished).

The results of the present studies, together with those on allelic complementation[9], lead us to suggest that the *arom* multienzyme complex in wild type *N. crassa* consists of two identical halves bound together by relatively weak non-peptide bonds. The *in vivo* and *in vitro* complementation observed with *arom-2* (dehydroquinate synthetaseless) mutants[9] seems best interpreted as resulting from an exchange ("hybridization") of half-complex subunits, since *in vitro* complementation occurs under relatively mild treatment conditions and disruption of the complex into subunits smaller than the half complex appears to occur only under much more extreme treatment conditions. Presumably these smaller components are the five different poly-

peptide chains which have been postulated to be the basic subunits composing the aggregate[1]. However, additional experimental evidence will be required to establish this point, since present data do not yet exclude the possibility that fewer than five different polypeptide chains are present, some carrying more than one catalytic site.

In the present studies all procedures causing disruption of the wild type *arom* aggregate into subunits (whether of half molecular weight or smaller) have also resulted in the loss of all five enzymic activities. However, studies with the category of pleiotropic ("polarity") mutants in the *arom* gene cluster indicate that these mutants produce *arom* aggregates of reduced molecular weight having only partial enzymic activities[1,10]. These results suggest that it should be possible to find treatment conditions which will cause dissociation of the wild type aggregate into subunits which retain at least partial activity for certain of the five enzymes. This type of evidence, combined with that from the continuing studies of various types of pleiotropic mutants, should help to establish the physical relationships within the aggregate of the five different catalytic sites. Already, in the present studies, the facility with which three (dehydroshikimate reductase, dehydroquinase, and shikimic acid kinase) of the five activities can be regenerated after renaturing treatment indicates that the catalytic sites for these activities can readily reform their correct structure even in the absence of the correct structures at the other two active centers (for 3-enolpyruvyl shikimic acid-5-phosphate synthetase and dehydroquinate synthetase).

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REFERENCES

1 N. H. GILES, M. E. CASE, C. W. H. PARTRIDGE AND S. I. AHMED, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1453.
2 N. H. GILES, C. W. H. PARTRIDGE, S. I. AHMED AND M. E. CASE, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1930.
3 M. E. CASE AND N. H. GILES, *Genetics*, 60 (1968) 49.
4 H. W. RINES, M. E. CASE AND N. H. GILES, *Genetics*, 61 (1969) 789.
5 S. RAZUM AND S. ROTTEN, *J. Bacteriol.*, 94 (1967) 1807.
6 A. L. SHAPIRO, E. VINUELA AND J. V. MAIZEL, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
7 D. H. SPACKMAN, W. H. STEIN AND S. MOORE, *Anal. Chem.*, 30 (1958) 1190.
8 H. EDELHOCH, *Biochemistry*, 61 (1967) 1948.
9 M. E. CASE, L. BURGOYNE AND N. H. GILES, *Genetics*, in the press.
10 M. E. CASE, *Genetics*, 61 (1969) 58 (Abstr.).