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PURIFICATION AND STABILITY OF THE MULTIENZYME COMPLEX ENCODED IN THE *AROM* GENE CLUSTER OF *NEUROSPORA CRASSA*

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SUMMARY

An improved method is described for the purification of the *arom* multi-enzyme complex from *Neurospora crassa*. The product of purification is an aggregate of approximately 230 000 mol. wt having five enzyme activities which function in the common pathway in aromatic amino acid biosynthesis. Conditions promoting dissociation of the enzyme complex have been determined. Alkaline pH values, storage in low ionic-strength buffers, and high temperature all promote dissociation. The aggregate fragments resulting from dissociation are quite labile and have not been thoroughly characterized. However, in all but one of the fragments observed 5-dehydroshikimate reductase (shikimate:$NADP^+$ oxidoreductase, EC 1.1.1.25) and 5-dehydroquinate dehydratase (5-dehydroquinate hydro-lyase, EC 4.2.1.10) activities are associated. These two activities have been found alone or in association with one of the three additional activities. A presumptive dimer of 3-enolpyruvylshikimic acid 5-phosphate synthetase has also been observed. To explain the variety of fragments observed, the minimum number of polypeptides present is postulated to be 4. Thus, the possibility that the 115 000 mol. wt subunits of the complex are a single multifunctional polypeptide has been excluded.

INTRODUCTION

The *arom* gene cluster in *Neurospora crassa* encodes five enzymes which catalyze steps 2–6 in the biosynthetic pathway for aromatic amino acids[1] (Fig. 1). An important feature of these five activities (5-dehydroshikimate reductase (shikimate: $NADP^+$ oxidoreductase, EC 1.1.1.25), 5-dehydroquinate dehydratase (5-dehydroquinate hydro-lyase, EC 4.2.1.10), 5-dehydroquinate synthetase, 3-enolpyruvyl-

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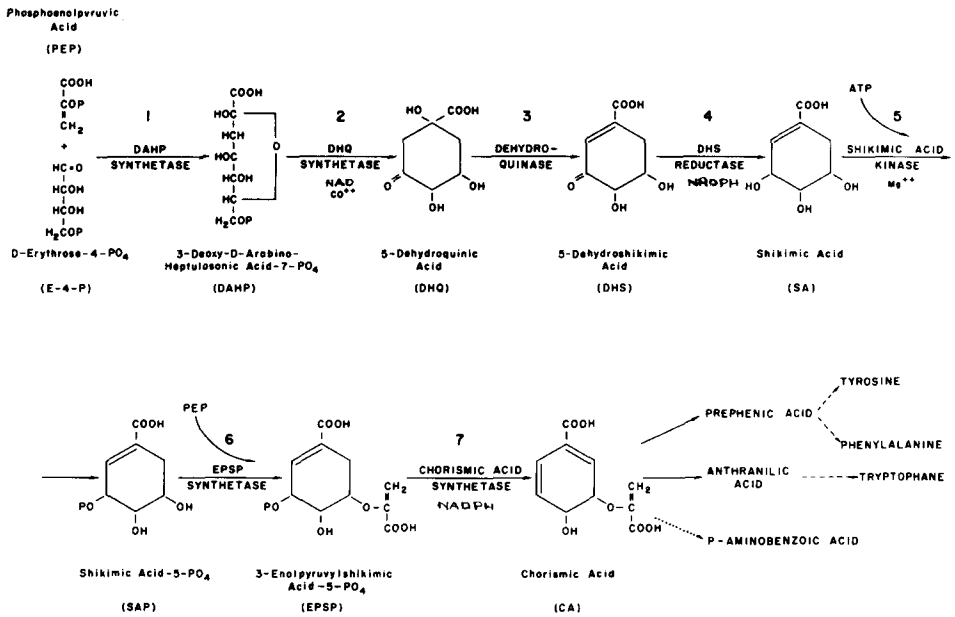


Fig. 1. The biosynthetic pathway for aromatic amino acids in *Neurospora crassa*. The enzymes catalyzing steps 2–6 are associated in a multi-enzyme complex.

shikimic acid 5-phosphate synthetase and shikimic acid kinase) is that they are physically associated in an enzyme aggregate with an approximate mol. wt of 230 000. Two purification procedures for the aggregate have been reported previously[2,3]. In this laboratory the aggregate was previously purified from a strain lacking chorismate synthetase, Strain 87, which has a 3-fold higher level of the enzyme activities. The enzyme complex was recovered in low yield, approx. 2%, and was very stable. After treatment with dissociating agents and conditions a half molecular weight component (approx. mol. wt 115 000) could be recovered following sucrose density-gradient centrifugation.

The extraction procedure was re-examined in an effort to increase the overall yield. All modifications of the procedure resulted in approximately the same yield. Wild type, Strain 74-A, was taken through the same purification procedure to determine if the problems encountered were due to the mutant strain used. The yield with wild type was as low as with the mutant strain. Therefore, the procedure was abandoned and a new extraction procedure, reported here, was developed using the wild type strain.

At various stages during the development of the new purification procedure, dissociation of the aggregate occurred with the concomitant reduction in enzyme activities. Since this dissociation had to be controlled before purification could be achieved, the conditions promoting dissociation were investigated. This study shows that dissociation is promoted by: alkaline pH, low ionic-strength buffers and length and temperature of storage. Minimizing the use of these conditions allowed an 80-fold purification with recovery of 20–40% of the starting activity.

MATERIALS AND METHODS

Strain and growth conditions

Wild type Strain 74-A (74-OR23-1A) was used as a source of the *arom* aggregate. Media and growth conditions have been described previously[1]. After harvesting, mycelial pads were stored at —18 °C until used.

Enzyme assays

All enzyme assays have been described previously[1].

Buffers

100 mM potassium phosphate buffer, pH 7.5, was used for extraction. Dithiothreitol was present in all buffers at a concentration of 0.4 mM. The proteolytic inhibitor phenylmethylsulfonyl fluoride was present at a concentration of 0.1 mM in all buffers prior to preparative gel electrophoresis.

Column chromatography

DEAE-cellulose (Whatman DE-52) column chromatography was carried out in a 2.5 cm × 30 cm column in 20 mM potassium phosphate buffer, pH 7.5, *plus* the above additives. The enzyme was eluted with a 1-l linear gradient of from 0 to 350 mM KCl at a flow rate of 40 ml/h. Sephadex G-200 gel filtration columns (2.5 cm × 90 cm) were equilibrated and run at 27 ml/h with the standard extraction buffer. All chromatography was done at 4 °C.

Preparative polyacrylamide gel electrophoresis

Preparative electrophoresis was carried out on a Buchler poly-prep (Model 200). The small-pore separating gel, 7% acrylamide, and the large-pore stacking gel, 2.5% acrylamide, were prepared according to the method described by Gaertner and DeMoss[3]. 80 ml of separating gel was normally used with 20 ml of the stacking gel. The gel was run for 15 min with 2 ml of thioglycolic acid in 15% sucrose followed by 2.5 ml of bromphenol blue in 20% sucrose. The marker dye was allowed to reach the interface of the two gels before the sample in 30% sucrose was layered on the surface of the stacking gel. Sample volume was between 2 and 10 ml with a maximum protein concentration of 200 mg. Amperage was maintained at 50 mA throughout the run while the voltage increased from 250 to 550 V. Samples were collected at 30-min intervals with an 18 ml/h flow rate.

Analytical polyacrylamide gel electrophoresis

Progress during purification was followed by analytical gel electrophoresis using the method of Davis[4]. When a single band was observed, several electrophoretic conditions were used to check sample purity. Gels were run in the system of Davis[4] using acrylamide concentrations of 5 and 7.5%. Systems of pH 7.5 (ref. 5) and of pH 4.2 (ref. 6) were also used. Samples were layered on the gels in 30% sucrose and started at 1 mA per tube for 1 h and then completed at 2 mA per tube. A sample was considered pure when all systems gave a single protein band on electrophoresis. The protein band was correlated with an enzyme activity band which detects 5-dehydroshikimate reductase[7]. The reaction mixture was made up in 50 mM Tris–glycine

buffer, pH 10.6, and contained 4 mM shikimic acid, 0.32 mM NADP, MTT tetrazolium (3(4,5-dimethylthiazolyl 2)-2,5-diphenyltetrazolium bromide) (1 mg/ml), and phenazine methosulfate (0.1 mg/ml). The activity band developed within minutes after placing the gel in the reaction mixture.

Sucrose density gradient centrifugation

Sucrose density-gradient centrifugation was carried out according to the method of Martin and Ames[8] using gradients of 5–20% and a Beckman SW 65 rotor. Molecular weight markers used were beef liver catalase, mol. wt 244 000 (Worthington), *Escherichia coli* alkaline phosphatase, mol. wt 80 000 (Sigma) and horseradish peroxidase, mol. wt 40 000 (Worthington).

Concentration of samples

All samples were concentrated by pressure dialysis in 1/4 inch dialysis tubing using a positive nitrogen pressure of 10 lb/inch^2. This resulted in a 90% reduction in volume in 18 h.

Biochemicals

$(NH_4)_2SO_4$ (ultra-pure) was purchased from Mann Research Chemical Co., dithiothreitol from Calbiochem, phenylmethylsulfonyl fluoride from Sigma, and acrylamide monomer from Eastman Chemical Co. Deoxyribonuclease and ribonuclease were purchased from Sigma.

RESULTS

Purification

Mycelium of Strain 74-A was broken by grinding lyophilized material in a Wiley mill. The powder obtained from 100 g dry weight of mycelium was dispersed by stirring slowly in 1700 ml of extraction buffer at 4 °C for 30 min. Cell debris was removed by centrifugation (all centrifugation was carried out at 10 000 × *g* for 15 min). After deoxyribonuclease and ribonuclease were added to the supernatant solution to a final concentration of 5 μg/ml, the extract was maintained at 37 °C for 2 h. After nuclease treatment, solid $(NH_4)_2SO_4$ was slowly added with stirring at 4 °C to a final concentration of 25% saturation. The resulting precipitate was removed by centrifugation. Solid $(NH_4)_2SO_4$ was added to the supernatant to give 50% saturation. The resulting precipitate contained the *arom* aggregate and was recovered by centrifugation. The precipitate was resuspended in the extraction buffer. The $(NH_4)_2SO_4$ was removed by desalting on a column (5 cm × 90 cm) of Sephadex G-25 in 20 mM potassium phosphate buffer, pH 7.5. The sample was adsorbed to a DEAE-cellulose column and the activity eluted with a linear KCl gradient. The fractions containing 5-dehydroshikimate reductase activity were combined, concentrated and passed through a Sephadex G-200 sieving column. The combined fractions from the G-200 column were concentrated and applied to a preparative polyacrylamide gel electrophoresis column. The sample was divided into two or three samples which were run separately so that less than 200 mg was applied to a single gel column. After electrophoresis, fractions containing 5-dehydroshikimate reductase activity were combined, concentrated and checked for purity by analytical gel electrophoresis.

TABLE I

PURIFICATION OF THE *arom* AGGREGATE FROM 100 g DRY WEIGHT OF MYCELIUM

Purification steps	*Volume (ml)*	*5-Dehydroshikimate reductase activity (units**)*	*Protein* (mg)*	*Specific activity (units/mg protein)*	*Recovery (%)*
1. 50% $(NH_4)_2SO_4$	77	921	6360	0.15	100
2. Sephadex G-25	175	906	3760	0.24	98.4
3. DEAE-cellulose	75	656	1340	0.47	71.2
4. Sephadex G-200	120	710	1010	0.70	77.1
5. Preparative polyacrylamide gel electrophoresis	85	334	28	11.92	36.3

* A_{280} assuming 1 absorbance unit = 1 mg/ml.
** 1 unit = utilization of 1 μmole substrate per min at 37 °C.

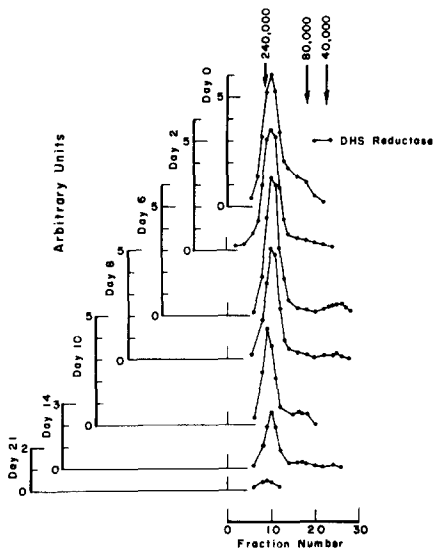
Samples were dialyzed for a minimum of 6 h against the starting extraction buffer *minus* phenylmethylsulfonyl fluoride. After dialysis, the samples were sterilized using a Millipore 0.45 μm filter and stored at 4 °C.

A summation of the purification is shown in Table I. During purification the *arom* aggregate was followed by assaying 5-dehydroshikimate reductase. The presence of the four remaining activities was demonstrated at all steps of the purification as well. The final product of the purification had all five activities and an apparent mol. wt of 230 000 by sucrose density centrifugation. The recovery of each of the other four activities was approximately the same as that for 5-dehydroshikimate reductase. In a few purification attempts, the 3-enolpyruvylshikimic acid 5-phosphate synthetase activity was lost. It is not known whether this represents an inactivation of that activity, or a physical separation of the 3-enolpyruvylshikimic acid 5-phosphate synthetase subunit from the complex.

Stability of the aggregate

While developing the purification procedure, dissociation of the *arom* aggregate was observed. To facilitate purification it was necessary to prevent this dissociation. Therefore, the conditions which promoted dissociation were determined. The first two parameters tested were the effects of pH and temperature. A partially purified preparation of aggregate, post DEAE-cellulose chromatography, was subjected to four treatments. Samples were dialysed at 4 °C against a buffer solution containing 0.4 mM dithiothreitol and 0.1 mM phenylmethylsulfonyl fluoride and (1) 100 mM potassium phosphate, pH 6.5; (2) 100 mM potassium phosphate, pH 7.5; (3) 100 mM Tris–HCl buffer, pH 9.0. The fourth sample was filter-sterilized and stored at room temperature (approx. 25 °C). The buffer was 50 mM Tris–HCl, pH 9.0, containing 160 mM KCl, 0.4 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride. At intervals samples were removed and analyzed by sucrose density gradient centrifugation.

The majority of the 5-dehydroshikimate reductase activity in pH 6.5 buffer (Fig. 2) remained in the 230 000 mol. wt region for the 21 days of the experiment. In the day 0 control a small amount of activity was present in the 80 000 mol. wt region of the gradient. With time this activity shifted to the 20 000 mol. wt region. By day



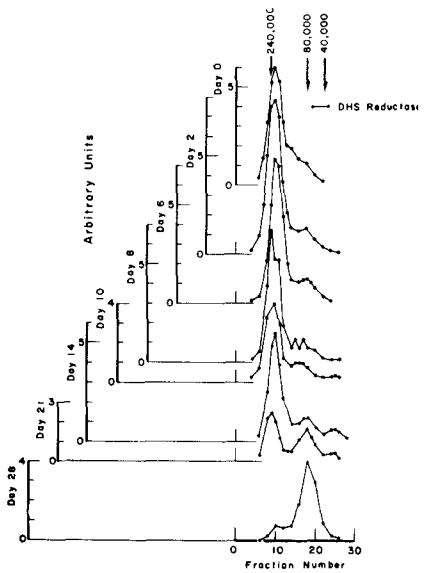
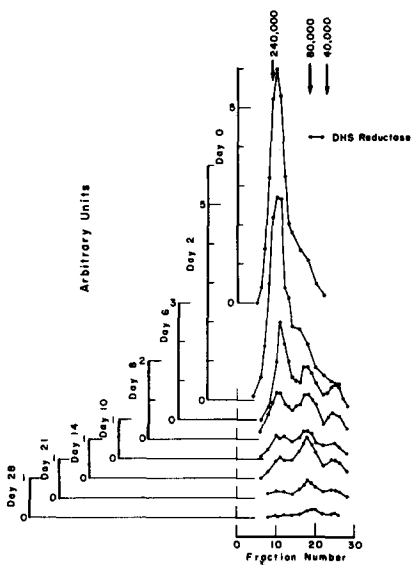


Fig. 2. Distribution, after centrifugation in sucrose density gradients, of 5-dehydroshikimate (DHS) reductase activity. Before centrifugation the enzyme complex was dialyzed against 100 mM potassium phosphate buffer (pH 6.5) at 4 °C for the times indicated. The positions of molecular weight markers run under similar conditions are shown. The conditions of dialysis and centrifugation are described in Materials and Methods and Results.

Fig. 3. Distribution, after centrifugation in a sucrose density gradient, of 5-dehydroshikimate (DHS) reductase activity. Before centrifugation the enzyme complex was dialyzed against 100 mM potassium phosphate buffer (pH 7.5) at 4 °C for the times indicated. The positions of molecular weight markers run under similar conditions are shown. The conditions of dialysis and centrifugation are described in Materials and Methods and Results.

10 a small amount of activity re-appeared in the 80 000 mol. wt region indicating that some dissociation was taking place. Although less than 10% of the activity remained at the termination of the experiment, protein profiles indicated that little dissociation had taken place. At pH 7.5 (Fig. 3), the aggregate remained relatively stable for 10 days but then started to dissociate. By day 14 a decrease in the activity in the 230 000 mol. wt peak and an increase in the light peak activity, especially the 20 000 mol. wt peak, indicated that dissociation was taking place. By the termination of the experiment the 80 000 mol. wt region contained the most activity. At pH 9.0 (Fig. 4), the sample started to dissociate by 6 days. By 14 days the majority of the activity was in the 80 000 mol. wt region. At the termination of the experiment there was residual activity in all three regions of the gradient. The pH 9.0 sample left at room temperature gave dramatic results. After 1 day dissociation was complete with activity remaining only in the 80 000 mol. wt region of the gradient (Fig. 5). By 14 days less than 5% of activity remained in the 80 000 mol. wt region and the experiment was terminated. Whenever dissociation was indicated, protein profiles, although complex because of the partially purified sample, showed a shift of protein to the lower molecular weight regions of the gradient.

A third parameter investigated was the effect of buffer concentration on aggregate stability. Three enzyme samples were dialyzed against three volumes of potassium phosphate buffer (pH 7.5) at 4 °C. The buffer concentrations used were 500, 100



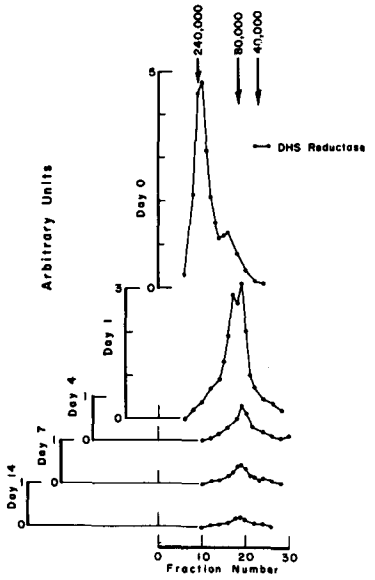


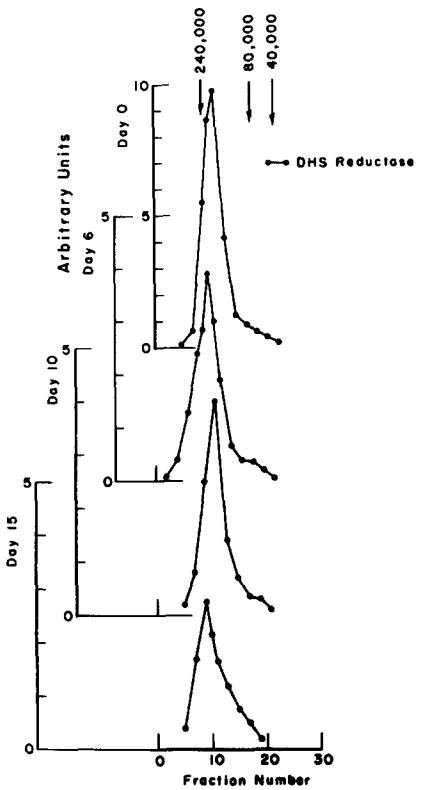
Fig. 4. Distribution, after centrifugation in sucrose density gradients, of 5-dehydroshikimate (DHS) reductase activity. Before centrifugation the enzyme complex was dialyzed against 100 mM Tris–HCl buffer (pH 9.0) at 4 °C for the times indicated. The positions of molecular weight markers run under similar conditions are shown. The conditions of dialysis and centrifugation are described in Materials and Methods and Results.

Fig. 5. Distribution, after centrifugation in sucrose density gradients, of 5-dehydroshikimate (DHS) reductase activity. Before centrifugation the enzyme complex in 50 mM Tris–HCl buffer (pH 9.0) was filter-sterilized and left at room temperature for the times indicated. The positions of molecular weight markers run under similar conditions are shown. The conditions of treatment and centrifugation are described in Materials and Methods and Results.

and 10 mM. At various intervals samples were removed and analyzed by sucrose density gradient centrifugation. In 500 mM buffer (Fig. 6), the 5-dehydroshikimate reductase activity remained in the 230 000 mol. wt region throughout the experiment, although inactivation occurred. In 100 mM buffer (Fig. 7), the sample was stable for 6 days but by day 10 it was partially dissociated. After 15 days the activity was primarily in the 80 000 mol. wt region. In 10 mM buffer (Fig. 8), the sample was partially dissociated by 6 days. The activity remaining at the 80 000 mol. wt region was much reduced by the termination of the experiment.

The remaining four aggregate activities were assayed in all sucrose gradients. All four additional activities were present in the 230 000 mol. wt region of the gradients. 3-enolpyruvylshikimic acid 5-phosphate synthetase was only slightly active in these enzyme preparations and was not detected in any other region of the gradient. The 5-dehydroquinate dehydratase activity paralleled the 5-dehydroshikimate reductase in the 230 000 and 80 000 mol. wt regions. It also was detected in the 20 000 mol. wt region. However, the activity was so low, that the reliability of the assay was questionable. Both shikimic acid kinase and 5-dehydroquinate synthetase were sometimes detectable in the 80 000 mol. wt region. 5-Dehydroquinate synthetase was very labile and was only detected in the early stages of the experiments.

Analytical polyacrylamide gels of samples taken from the 80 000 mol. wt region



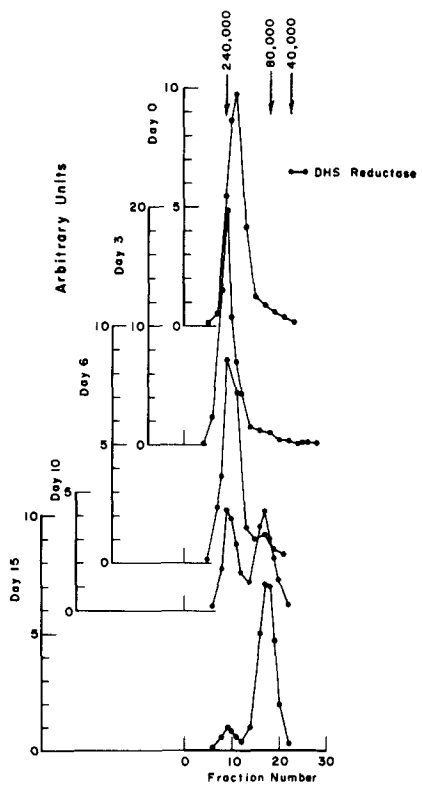


Fig. 6. Distribution, after centrifugation in sucrose density gradients, of 5-dehydroshikimate (DHS) reductase activity. Before centrifugation the enzyme complex was dialyzed against 500 mM potassium phosphate buffer (pH 7.5) at 4 °C for the times indicated. The positions of molecular weight markers run under similar conditions are shown. The conditions of dialysis and centrifugation are described in Materials and Methods and Results.

Fig. 7. Distribution, after centrifugation in sucrose density gradients, of 5-dehydroshikimate (DHS) reductase activity. Before centrifugation the enzyme complex was dialyzed against 100 mM potassium phosphate buffer (pH 7.5) at 4 °C for the times indicated. The position of molecular weight markers run under similar conditions are shown. The conditions of dialysis and centrifugation are described in Materials and Methods and Results.

showed up to three bands having 5-dehydroshikimate reductase after staining for that activity. Two samples of 80 000 mol. wt material from other extractions were checked further by separation on preparative polyacrylamide gel electrophoresis. One sample had 5-dehydroshikimate, 5-dehydroquinate dehydratase, and shikimic acid kinase activities. When subjected to electrophoresis two peaks having 5-dehydroshikimate reductase and 5-dehydroquinate dehydratase were detected (Fig. 9). The shikimic acid kinase, however, was associated with only the slower 5-dehydroshikimate reductase–5-dehydroquinate dehydratase peak. On re-analysis of these separate peaks by sucrose density-gradient centrifugation, the first 5-dehydroshikimate reductase–5-dehydroquinate dehydratase peak had an apparent mol. wt of 73 500 while the peak associated with shikimic acid kinase had an apparent mol. wt of 83 200. A second sample of 80 000 mol. wt material had 5-dehydroshikimate reductase, 5-

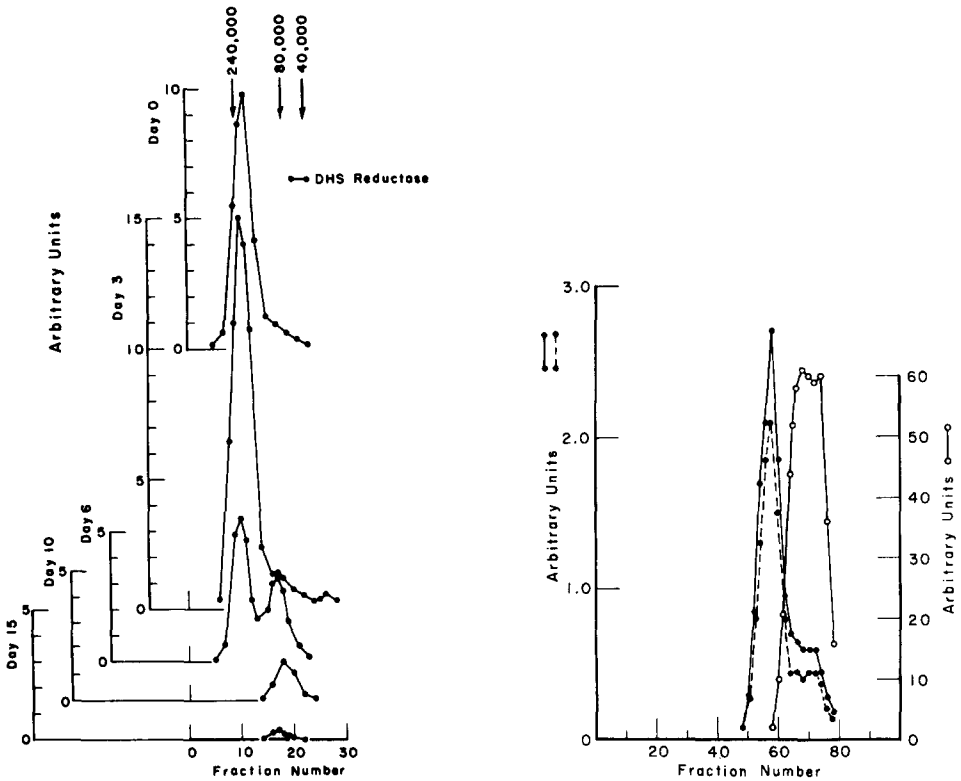


Fig. 8. Distribution, after centrifugation in sucrose density gradients, of 5-dehydroshikimate (DHS) reductase activity. Before centrifugation the enzyme complex was dialyzed against 10 mM potassium phosphate buffer (pH 7.5) at 4 °C for the times indicated. The positions of molecular weight markers run under similar conditions are shown. The conditions of dialysis and centrifugation are described in Materials and Methods and Results.

Fig. 9. Preparative polyacrylamide gel electrophoresis of dissociation products of the *arom* complex. The three enzyme activities present are 5-dehydroshikimate reductase (●—●), 5-dehydroquinate dehydratase (●---●), and shikimic acid kinase (○—○). The conditions of electrophoresis are described in Materials and Methods.

dehydroquinate dehydratase, and 3-enolpyruvylshikimic acid 5-phosphate synthetase activities. Preparative polyacrylamide gel electrophoresis of this sample again revealed two components having both 5-dehydroshikimate reductase and 5-dehydroquinate dehydratase activity; only one of these was associated with 3-enolpyruvylshikimic acid 5-phosphate synthetase.

DISCUSSION

Purification of the *arom* aggregate has been achieved with recovery of 20–40% of the total 5-dehydroshikimate reductase activity. The other four activities were also recovered in an aggregate with an approximate mol. wt of 230 000 which is similar if not identical to the complex previously reported[2]. All activities are recovered in approximately the same ratio. The variability seen may reflect the difficulties present

in the assay systems. Since the assay of any one enzyme requires the binding or release of substrates and products which are normally retained in the aggregate, all the assays may be very sensitive to subtle changes in aggregate structure on environment. Evidence has been obtained that the aggregate will preferentially utilize precursors in the biosynthetic pathway rather than those intermediates supplied externally[9].

The loss of 3-enolpyruvylshikimic acid 5-phosphate synthetase activity from some extractions may result from loss of the 3-enolpyruvylshikimic acid 5-phosphate synthetase subunit. During one extraction a 60 000 mol. wt component was recovered which contained only 3-enolpyruvylshikimic acid 5-phosphate synthetase. This weight is similar to that seen for D polar mutants having only this activity[10]. It seems possible, therefore, that the 3-enolpyruvylshikimic acid 5-phosphate synthetase activity can be relatively easily dissociated from the other aggregate activities. Based on the minimal mol. wt of approx. 30 000 observed with several species of bacteria[11] and photosynthetic organisms[12], the 60 000 mol. wt species may be a dimer of the 3-enolpyruvylshikimic acid 5-phosphate synthetase subunit which is formed by reaggregation after it is dissociated from the wild-type complex.

Further dissociation of the aggregate into smaller components can be achieved by treatment with alkaline pH values, length and temperature of storage, low ionic strength, or a combination of these. It has thus far been difficult to stabilize the resulting components. Dissociation occurs with an overall reduction in 5-dehydroshikimate reductase activity and varying reduction of the other activities. The composition of the components produced by dissociation is as yet unclear. However, the 80 000 mol. wt region seems to contain at least three components which are made up of various combinations of the five activities. The components in the 20 000 mol. wt region contain both 5-dehydroshikimate reductase and 5-dehydroquinate dehydratase. No evidence is available, however, as to whether these activities represent two distinct peptides of similar molecular weight, two aggregated peptides, or a single bifunctional peptide. It is of interest that these two activities remain associated in a variety of photosynthetic organisms: *Chlamydomonas reinhardti*; *Nicotiana tabacum*; a moss, *Physcomitrella patens*[12]; and oak, *Quercus pendunculata*[13]. A 57 000 mol. wt species of 5-dehydroquinate dehydratase has also been observed[14].

After dissociation of the complex, the 5-dehydroshikimate reductase and 5-dehydroquinate dehydratase activities are easily detected. These two associated activities are present in multi-molecular weight forms and appear to be reasonably stable. The remaining three activities can also be detected but they are less stable and become inactivated on storage. This is in direct contrast to the types of partial aggregates seen in the pleiotropic mutants[10]. In pleiotropic mutants partial aggregates with 5-dehydroshikimate reductase and 5-dehydroquinate dehydratase have never been recovered. Partial aggregates having 3-enolpyruvylshikimic acid 5-phosphate synthetase alone or associated with shikimic acid kinase are seen and some are sufficiently stable to allow at least partial purification. In three classes of pleiotropic mutants, 5-dehydroquinate synthetase can be detected *in vivo* by complementation but cannot be detected in cell extracts[1,10]. In pleiotropic mutants arising from nonsense mutations at sites proximal to the *arom-9* gene no 5-dehydroquinate reductase or 5-dehydroquinate dehydratase would be expected. However, only complete polar mutants of type E which lack all five activities have been shown to be suppressed by

super suppressors[15]. Furthermore, experiments designed to recover suppressors of the other pleiotropic mutants have given only negative results (M. E. Case, unpublished results). Therefore, it seems unlikely that all the pleiotropic mutants are nonsense mutants. It seems peculiar that apparent missense mutants which interrupt aggregation have lost two or more activities. The lack of these activities may reflect a requirement for the formation of the aggregate as the polypeptides are synthesized. Without this aggregation the individual peptides may never form the active conformation. Thus, peptides synthesized after a missense mutation which interrupts aggregation would lack activity and not be detected. However, in wild type, once the active conformation is achieved, it remains reasonably stable even after the aggregate is dissociated.

The number of unique polypeptides present in the *arom* complex has not yet been definitely established, however a minimum of four polypeptides must be present if the 5-dehydroquinate synthetase activity seen in the 80 000 mol. wt region is real. The possibility that the 115 000 mol. wt subunit of the complex is a single multifunctional polypeptide has thus been excluded. No evidence has been obtained to exclude the possibility that the peptides present are synthesized as a single polypeptide which is cleaved by proteolytic enzymes after synthesis. The cleavage could occur during or immediately following synthesis of the polypeptide. Association would follow cleavage in a manner similar to that reported for polio virus[16]. Conversely the formation of the correct conformation could expose specific regions of the polypeptide to proteolytic action giving rise to the individual polypeptides as is the case with chymotrypsinogen[17]. Either of these schemes is feasible since genetic evidence indicates that the genes of the *arom* cluster are translated from a single mRNA[1,15]. The absolute polarity of the completely non-complementing *arom* mutants supports the single polypeptide hypothesis as suggested for the *his-4* region in yeast[18,19].

The *arom* complex is believed to consist of two identical 115 000 mol. wt subunits each of which contains all five activities[2,10,20]. Each half mol. wt subunit is postulated to contain five different polypeptide chains, although the exact number of peptides present remains uncertain. However, to explain the variety of fragments observed a minimum number of four polypeptides is required. Under the conditions used the 115 000 mol. wt subaggregate is less stable than was previously observed. Although activities were present in the 115 000 mol. wt region of many gradients, they represented a minor species which rapidly dissociated into smaller subunits. Even though somewhat labile, all activities except 5-dehydroquinate synthetase may be active in the partial aggregates smaller than 115 000 mol. wt. The 5-dehydroquinate synthetase, although observed briefly in an 80 000 mol. wt component, is extremely sensitive to dissociation and may require the aggregated state for activity. This is supported by the observation that in pleiotropic mutants 5-dehydroquinate synthetase, if present, can only be detected by complementation *in vivo* and cannot be detected in cell extracts[1,10].

Attempts are now being made to isolate the various polypeptides present in the aggregate in order to verify the presence of five unique polypeptides as is suggested by the genetic data. These polypeptides will be compared to each other and to polypeptides isolated from partial aggregates found in several classes of pleiotropic mutants and to homologous enzymes isolated from other organisms which lack *arom*

aggregates[11,12]. Such comparisons may reveal evolutionary homologies among the peptides involved in the aggregate.

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