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PURIFICATION OF THE *AROM* MULTIENZYME AGGREGATE FROM *EUGLENA GRACILIS*

VIRGINIA B. PATEL and NORMAN H. GILES

Genetics Program, Department of Zoology, University of Georgia, Athens, GA 30602 (U.S.A.)

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Summary

The *arom* multienzyme complex that catalyzes steps two through six in the prechorismate polyaromatic amino acid biosynthetic pathway has been purified up to 2000-fold from *Euglena gracilis*. The native *arom* aggregate has a molecular weight of approx. 249 000 based on a sedimentation coefficient of 9.5 and Stokes radius of 60 Å. A comparison between the *arom* aggregates of *Neurospora crassa* and *Euglena gracilis* and the possible phylogenetic relationships between the organisms are discussed.

Introduction

The photosynthetic flagellate, *Euglena gracilis*, may be taxonomically related to both the higher and some of the lower fungi. Vogel [1] has shown that these organisms use the L-α-amino-adipic acid pathway for lysine biosynthesis while bacteria, blue-green and green algae, water molds, and vascular plants use the meso-α, ϵ-diaminopimelic acid pathway. Additional biochemical evidence for a phylogenetic relationship between euglenoids and fungi has come from studies on the organization of enzymes in the tryptophan biosynthetic pathway. Lara and Mills [2] have observed that tryptophan synthetase in *E. gracilis* is similar to the fungal enzyme but not to tryptophan synthetase from bacteria [3,4], blue-green and green alga [5], or higher plants [6].

Further evidence of these relationships comes from studies of the organization of the enzymes involved in pre-chorismic acid polyaromatic amino acid biosynthesis (Fig. 1). In bacteria [7] and several photosynthetic organisms [8], these polyaromatic amino acid biosynthetic enzymes are separable by ultra-

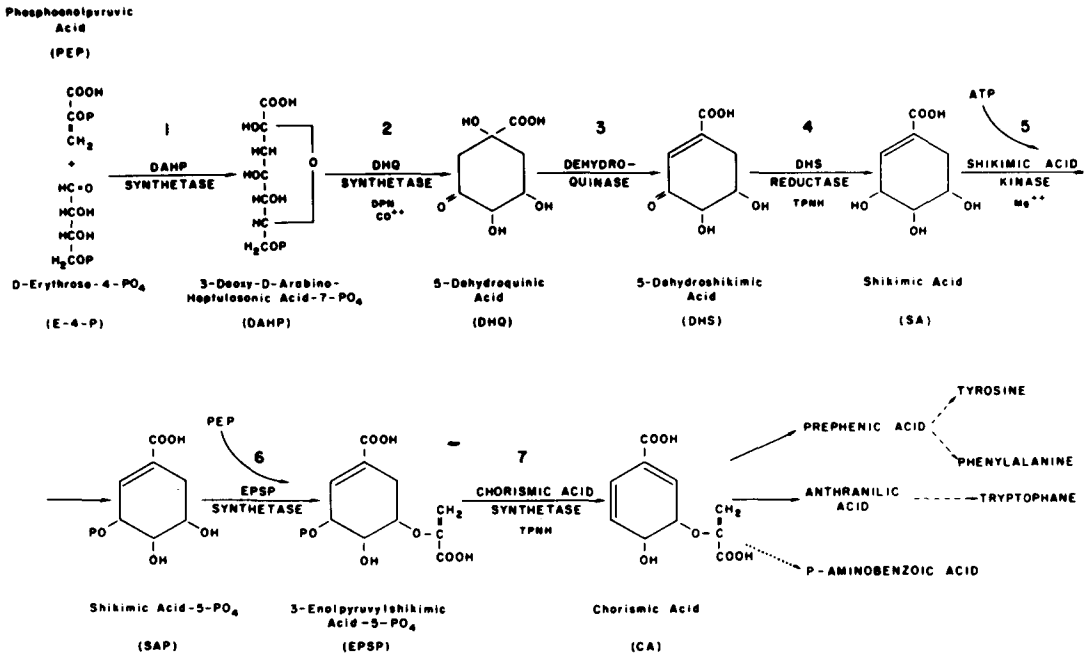

Fig. 1. The biosynthetic pathway for aromatic amino acids. The enzymatic activities catalyzing steps 2—6 are associated in the *arom* aggregate.

centrifugation on sucrose density gradients. There are exceptions in that two of the enzymatic activities, 5-dehydroshikimate reductase and 5-dehydroquinase, remain associated in the green alga, *Chlamydomonas reinhardi*, in the moss, *Physcomitrella patens*, and in the higher plant, *Nicotiana tabacum* [8]. In a more extensive study of these enzymes from *P. patens*, Polley [9] has demonstrated that these two activities are associated with a bifunctional polypeptide having a molecular weight of approx. 45 000. In several fungi, the polyaromatic biosynthetic enzymes for steps two though six are associated in a multifunctional enzyme complex [10]. The *arom* aggregate from *Neurospora crassa* has been extensively studied both in this laboratory [11,12] and in others [13,14]. Among the photosynthetic organisms studied, a striking exception was the apparent association of the enzymes for steps two through six of the prechorismate pathway in *E. gracilis* [8]. Again. these results suggest a phylogenetic relationship between *Euglena* and fungi. The most recent evidence [15,16] indicates that the *arom* complex from *Neurospora* is a homodimer of a pentafunctional polypeptide. This paper describes the purification and characterization of the *arom* multienzyme complex from *E. gracilis* and discusses its similarity to the fungal *arom* aggregate.

Materials and Methods

Strain and growth conditions. Two sources of *Euglena* cells that were used in this study were grown heterotrophically in the presence or absence of light in

Euglena Broth (Difco Laboratories) or in a medium slightly modified from that of Greenblatt and Schiff [17]. *Euglena gracilis* (Z-strain) (Culture Collection of Algae, Indiana University, No. 753) was kindly supplied by Dr. James Rawson (Botany Department, University of Georgia) and *Euglena gracilis* (Z-strain) (ATCC No. 12716), by Dr. Ellis Kempner (National Institutes of Health). After harvesting by centrifugation, the *Euglena* cells were frozen, lyophilized and stored at −70°C until used.

Enzyme assays. The assays for the five activities associated with the *arom* aggregate, 5-dehydroshikimate reductase (shikimate:$NADP^+$ oxidoreductase, EC 1.1.1.25), 5-dehydroquinate dehydratase (5-dehydroquinate hydrolyase, EC 4.2.1.10; also, designated as biosynthetic dehydroquinase), 5-dehydroquinate synthetase, 3-*enol*pyruvyl shikimic acid 5-phosphate synthetase, and shikimic acid kinase, have been described previously [18].

Purification of the arom aggregate. Step 1. Lyophilized *Euglena* cells were ground in a Wiley mill and a 2—4% homogenate was made by suspending the resulting powder in buffer 1 (0.1 M potassium phosphate buffer, pH 7.5, 0.4 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride). The remaining steps of the extraction procedure were carried out at 0°—4°C and, unless otherwise specified, centrifugations were performed at 10 900 $\times g$ for 15—20 min. Dithiothreitol and phenylmethylsulfonyl fluoride in the above concentrations were present in all buffers used for the purification of the *arom* aggregate. The crude homogenate was stirred for 1 h and centrifuged to remove cell debris. The resulting supernatant is referred to as the crude extract fraction.

Step 2. The crude extract fraction was treated with protamine sulfate (0.1 vol. of 1.4% solution), stirred for 1 h and centrifuged. The precipitate at 60% $(NH_4)_2SO_4$ saturation was prepared and dissolved in buffer 2 (10 mM potassium phosphate, pH 7.5) to 1/10 the original volume. It was then exhaustively dialyzed and the precipitate removed.

Step 3. This fraction was adsorbed onto a DEAE-cellulose (Whatman DE-52) column (2.6 cm × 37 cm) equilibrated with buffer 2. The *arom* aggregate was eluted with an 800 ml linear 0—200 mM KCl gradient in buffer 2 (flow rate, 30 ml/h). The active fractions were combined.

Step 4. This fraction was dialyzed against buffer 3 (50 mM potassium phosphate, pH 7.5) and directly adsorbed to a Cibacron-blue F3GA-Sepharose 4B column equilibrated with the same buffer. The size of the column was dependent on the total amount of 5-dehydroshikimate reductase activity. It was determined that approx. 50 ml dye-gel retained an activity equivalent to 64 μmol NADPH generated per min at 37°C and the columns used were usually 2.6 cm × 40 cm. The Cibacron-blue F3GA-Sepharose 4B was prepared in our laboratory as described by Polley [9]. Cibacron-blue F3GA was a gift from Ciba-Geigy Corporation. The *arom* aggregate was eluted with 7—10 mM shikimic acid in buffer 3.

Step 5. The active fractions from Step 4 were combined and applied to a Sephadex G-200 column (1.6 cm × 80 cm) equilibrated and eluted with buffer 1. Active samples were pooled and concentrated in an Amicon ultrafiltration cell using a PM-30 membrane. The purity of the *arom* enzyme aggregate was established by polyacrylamide gel electrophoresis.

Protein concentrations. The protein determinations were based on the

adsorbance at 280 mμ and the assumption that one absorbance unit was equivalent to 1 mg/ml.

Analytical polyacrylamide gel electrophoresis. Electrophoresis was based on a procedure described by Davis [19]. The protein band indicating the *arom* aggregate was detected by 5-dehydroshikimate reductase activity.

Sodium dodecyl sulfate-polyacrylamide electrophoresis. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method described by Fairbanks et al. [20] or by MacGillivray et al. [21].

Sucrose density gradient centrifugation. The method described by Martin and Ames [22] was used. Catalase (Worthington), the *arom* aggregate from *N. crassa*, yeast alcohol dehydrogenase (Worthington), and *E. coli* alkaline phosphatase (Sigma) were used as standards.

Analytical ultracentrifugation. Sedimentation equilibrium analyses were performed in the Beckman model E ultracentrifuge using ultraviolet optics according to the method of Schachman [23].

Results

Purification of the arom aggregate. The purified *arom* complex had a specific 5-dehydroshikimate reductase activity of 79.96 μmol NADPH per min per mg protein. This represents a 2000-fold purification (Table I).

In other extractions, the purified *arom* aggregate has this characteristic specific activity although it may have represented only a 1000-fold purification. This difference is based on the variability in the specific 5-dehydroshikimate reductase activity observed for the crude extract fractions. The average specific activity obtained for 20 crude extractions was 0.05 μmol NADPH generated per min per mg protein at 37°C; however, the range was from 0.04—0.08 μmol of NADPH generated per min per mg protein. The recovery of the 5-dehydroquinase activity is similar to the 5-dehydroshikimate reductase. The other three enzymatic activities were not quantitative analyzed because of the nature of the assays [18]. In these three assays, it was necessary to add *Neurospora* extract. Their activities are determined by the generation of substrates several steps removed in the biosynthetic pathway.

DEAE-cellulose column chromatography. Fig. 2 shows the characteristic elution of the *arom* aggregate that was adsorbed onto a DEAE-cellulose column. Sometimes as much as 10% of the 5-dehydroshikimate reductase activity would elute with the wash; however, it was usually lower. 40—60% of the adsorbed activity was eluted with the linear gradient. As indicated in Fig. 2, the peak of 5-dehydroshikimate reductase activity always appeared to elute at approx. 40 mM KCl. Attempts were made to elute additional activity from the column by introducing higher salt concentrations, up to 1 M KCl; however, no additional activity was recovered. The combined active fractions usually represented 30—40% of the original 5-dehydroshikimate reductase activity of the crude extract fraction.

Cibacron-bleu Sepharose 4-B column chromatography. Fig. 3 shows the elution profile of the *arom* aggregate in response to the application of 7 mM shikimic acid in buffer 3. 54—75% of the adsorbed 5-dehydroshikimate reduc-

TABLE I

PURIFICATION OF THE *AROM* AGGREGATE FROM LYOPHILIZED *EUGLENA* CELLS

Purification steps	Volume (ml)	Protein (mg)	Total 5-dehydroshikimate reductase activity (units *)	Recovery (%)	Specific 5-dehydroshikimate reductase activity (units/mg protein)	Purification
1. Crude extract	2670	27 688	1077	100	0.04	1
2. Protamine sulfate and ammonium sulfate fractionation	333	7 343	803	74	0.11	3
3. DEAE-cellulose column chromatography	52	151	340	32	2.25	58
4. Cibacron-blue Sepharose-4B column chromatography **	8	5.7	247	23	43.66	1122
5. Sephadex G-200 column chromatography **	2.7	1.5	123	11	79.76	2050

* One unit is defined as the generation of 1 μmol NADPH/min at 37°C.

** The data presented were obtained on column fractions that had been combined and concentrated in an Amicon ultrafiltration cell.

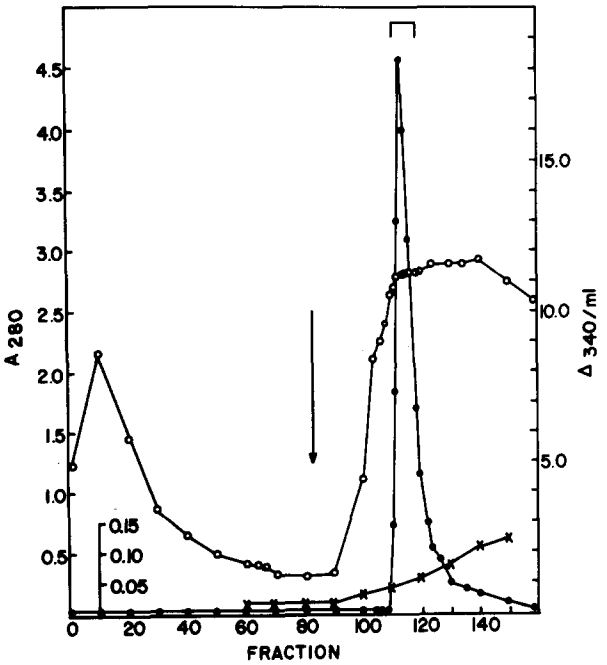

Fig. 2. Chromatography of the *arom* aggregage on DEAE-cellulose (step 3 of the purification scheme). ○———○, A_{280}; ●———●, 5-dehydroshikimate reductase activity (expressed as Δ_{340}/ml); X———X, M KCl. Arrow indicates the introduction of the gradient. Inset ordinate, molarity of KCl.

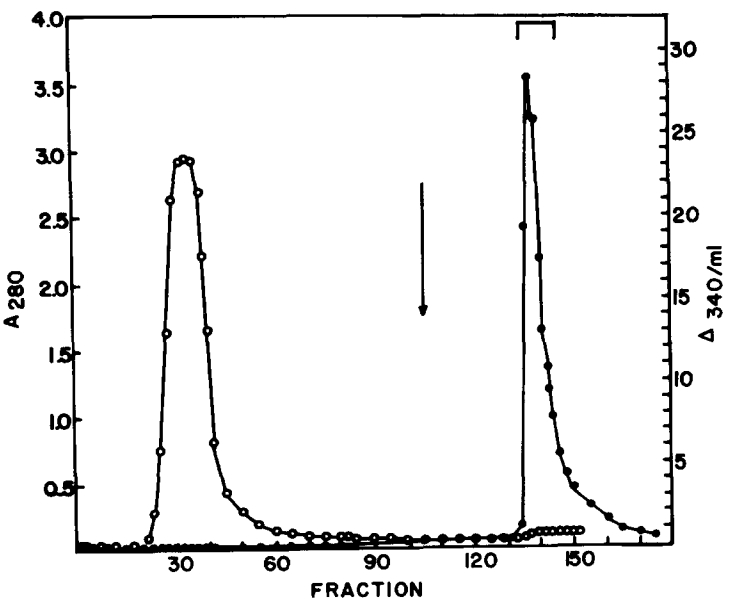

Fig. 3. Affinity chromatography of the *arom* aggregate on Cibacron-bleu Sepharose 4-B (step 4 of the purification scheme). ○———○, A_{280}; ●———●, 5-dehydroshikimate reductase activity (expressed as Δ_{340}/ml). Arrow indicates the application of 7 to 10 mM shikimic acid.

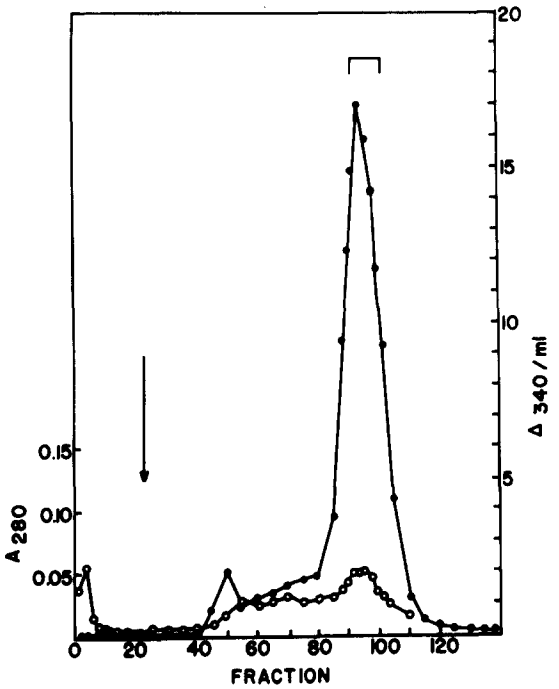

Fig. 4. Chromatogaphy of the *arom* aggregate on hydroxyapatite. *Arom* aggregate in 10 mM potassium phosphate, pH 6.9, was applied to a hydroxyapatite column (0.9 cm X 21 cm) equilibrated with the same buffer. After washing with 2.5 times the bed volume of the column, a gradient of from 10—200 mM potassium phosphate buffer (pH 6.9) was used to elute the *arom* complex. The flow rate was usually 10 ml/h.

tase was eluted by this method. Elution of the *arom* aggregate also occurs by increasing the ionic concentration of the buffer from 50 mM to 200 mM potassium phosphate and results in 52—86% recovery of 5-dehydroshikimate reductase activity. The most active fractions, however, had more protein contaminants, and further purification by hydroxyapatite column chromatography (discussed below) was necessary. Therefore, substrate elution of the *arom* aggregate from Cibacron-blue Sepharose 4-B resulted in a faster and more efficient purification of the enzyme complex. The fact that the *arom* aggregate binds to the chromophore, Cibacron-blue F3GA, indicates that it is a protein that contains a dinucleotide fold [24].

Hydroxyapatite column chromatography. The *arom* aggregate was susceptible to fragmentation by hydroxyapatite column chromatography (Fig. 4). 40—70% of the adsorbed 5-dehydroshikimate reductase was recovered. However, this activity usually eluted as one major peak and several minor ones. The major peak of activity always had the other four enzyme activities characteristic of the *arom* complex and eluted at 150 mM potassium phosphate buffer (pH 6.9). The secondary peaks had various combinations of the enzyme activities. Because of the instability of the *arom* aggregate on hydroxyapatite, other procedures for purification are preferred.

Analytical polyacrylamide gel electrophoresis. Analysis of the highly purified (2000-fold) *arom* aggregate that resulted from Sephadex G-200 column

chromatography (step 5 in Table I) by polyacrylamide gel electrophoresis indicates a few minor bands. When this sample was subsequently rechromatographed on a similar Sephadex G-200 column (1.6 cm × 88 cm), densitometric scans of the gels indicated an increase in concentration of these secondary bands relative to the major band; therefore, they most likely represent degradative products rather than protein contaminants.

Determination of the molecular weight of the arom aggregate. Determination of Stokes radius. Using the method of Porath [25], the Stokes radius of the *arom* aggregate was determined by molecular sieve chromatography. According to Gelotte [26], the partition coefficients (K_d) were determined on standard proteins of known Stokes radii and the *arom* aggregate by separation on a Sephadex G-200 column. A Stokes radius of 60 Å for the *arom* aggregate was then calculated. The *arom* aggregate did not appear to be altered by the purification since a highly purified sample eluted with the same characteristics as the *arom* complex of the $(NH_4)_2SO_4$ fraction.

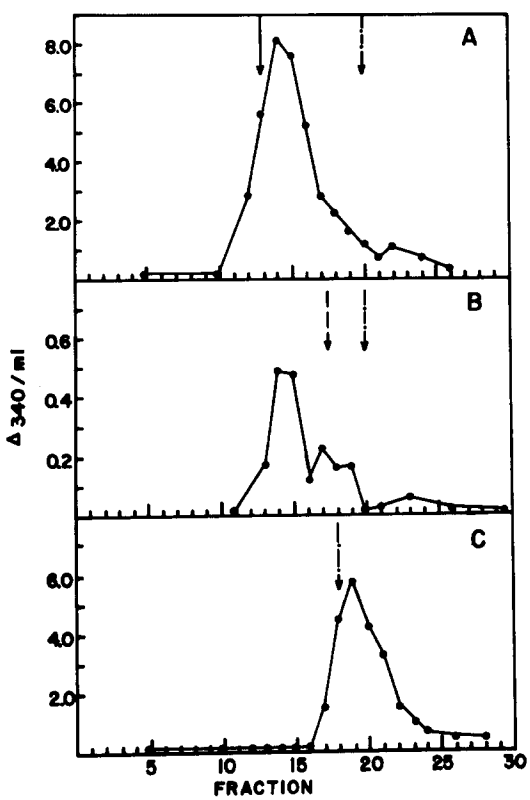

Fig. 5. Sucrose density gradient centrifugation of the 5-dehydroshikimate reductase activity of the *arom* aggregate. A, gradient centrifugation of the native *arom* aggegate with the standards, catalase (11.3 S) and *E. coli* alkaline phosphatase; B, gradient centrifugation of the native *arom* aggregate with the standards, yeast alcohol dehydrogenase (7.4 S) and *E. coli* alkaline phosphatase; C, gradient centrifugation of the *arom* aggregate after sedimentation equilibrium centrifugation with *E. coli* alkaline phosphatase. ●———●, 5-dehydroshikimate reductase activity (Δ_{340}/ml); solid line arrow, catalase; dashed line arrow, yeast alcohol dehydrogenase; and dashed line-closed circle arrow, *E. coli* alkaline phosphatase.

Sucrose density gradient centrifugation. The sedimentation coefficient was calculated from results obtained with sucrose density gradient centrifugation (Fig. 5A and B). The major peak of 5-dehydroshikimate reductase was always the faster sedimenting component and was considered to represent the intact native *arom* complex. This peak was calculated to have a sedimentation coefficient of 9.5.

Sedimentation equilibrium centrifugation. Several attempts were made to obtain data for the determination of the molecular weight of the *arom* aggregate by sedimentation equilibrium centrifugation. However, the results indicated that the *arom* complex was breaking down during the ultracentrifugation. These results were verified by sucrose gradient centrifugation as shown in Fig. 5C. The 5-dehydroshikimate reductase activity of the sample that was recovered from the sedimentation equilibrium centrifugation, sedimented more slowly than *E. coli* alkaline phosphatase (86 000 daltons). The deterioration of the native *arom* aggregate might have been time-dependent [27] or caused by protease activity [28].

Using a sedimentation coefficient of 9.5 S and a Stokes radius of 60 Å, a molecular weight of 249 000 was calculated for the native *arom* aggregate from *Euglena* according to the method of Siegel and Monty [29].

Determination of the frictional coefficient. Using the above calculated Stokes radius of 60 Å (a) and the molecular weight (M) of 249 000, the frictional ratio (f/f_0) of 1.4 was determined for the *arom* complex. The ratio implies that the *arom* aggregate is slightly asymmetric.

SDS-polyacrylamide gel electrophoresis. Attempts to discern the subunit structure of the *arom* complex by gel electrophoresis in the presence of sodium dodecyl sulfate were inconclusive. Multiple banding patterns were obtained that were similar to those observed by Gaertner and Cole [28] and Lumdens and Coggins [14] with sodium dodecyl sulfate-acrylamide gel electrophoresis of the *arom* complex of *N. crassa.* They have indicated that these patterns are artificially produced by protease activity.

Discussion

Biochemical characterization of a highly purified sample of the *arom* aggregate from *E. gracilis* has suggested that it is very similar to the *arom* complex that has been purified from *N. crassa.* The five enzymatic activities characteristic of the *Neurospora* complex co-purify in *Euglena*, and are associated with an aggregate that has a molecular weight of approx. 249 000. Molecular weights reported for the native *arom* aggregate from *N. crassa* are 231 000 [11,12]; 295 000 [13]; and most recently, 330 000 [14]. By the criteria of sucrose gradient density centrifugation, the *arom* complex from *Euglena* is smaller than that from *Neurospora* since it always sediments more slowly. Recent evidence [15,16] strongly supports the hypothesis that the native *arom* from *Neurospora* is a homodimer of a single pentafunctional polypeptide of approx. 150 000 daltons. It is not yet clear if the *Euglena arom* aggregate is also dimeric in structure. It is possible to demonstrate fragments of the *arom* complex having various combinations of the five enzymatic activities. Berlyn et al. [8] observed a pentafunctional aggregate having a molecular weight of approx.

120 000 during sucrose gradient centrifugation of crude extract of *E. gracilis*. It now seems probable that this observation resulted from the dissociation of an original dimeric *arom* complex into identical monomeric subunits during partial purification.

The further fragmentation of the *arom* complex in *Euglena* during purification is most likely the result of protease activity. The problem of proteolysis of the *arom* complex and of tryptophan synthetase in *Neurospora* has been adequately described by Gaertner and co-workers [28,31] and Yu et al. [30], respectively.

The functional significance of the *arom* aggregate encoded in the *arom* "cluster gene" in *Neurospora* appears to be the channeling of intermediates in the biosynthesis of the aromatic amino acids [18,32]. The intracellular sequestering of substrates is necessary because one of the steps (step 3, Fig. 1) in the biosynthetic pathway is common to the inducible quinate catabolic pathway. The existence of these separate catabolic enzymes that are encoded in the *qa* gene cluster [33] has been well established in *Neurospora*. However, there is as yet no evidence for the existence of this inducible catabolic system in *Euglena* [8].

Whether the genes for the *arom* aggregate in *Euglena* are clustered as in *Neurospora* or segregated as in bacteria could be determined conceivably by mutational studies with *Euglena*.

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