IN VIVO AND *IN VITRO* COMPLEMENTATION BETWEEN DHQ SYNTHETASE MUTANTS IN THE *AROM* GENE CLUSTER OF *NEUROSPORA CRASSA*

MARY E. CASE, LEIGH BURGOYNE,* AND NORMAN H. GILES

Department of Biology, Kline Biology Tower, Yale University, New Haven, Connecticut 06520

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THE *arom* gene cluster in *Neurospora crassa* consists of five structural genes which encode an aggregate of five aromatic synthetic enzymes catalyzing steps two through six in the pre-chorismic acid part of the polyaromatic synthetic pathway. Allelic complementation has been detected between certain pairs of mutants within two of the five genes in the cluster—the *arom-2* gene which encodes dehydroquinic acid (DHQ) synthetase and the *arom-1* gene which encodes dehydroshikimic acid (DHS) reductase (GILES, CASE, PARTRIDGE and AHMED 1967; RINES, CASE and GILES 1969).

Allelic complementation is usually considered to involve molecular hybridization between two differentially defective, inactive, multimeric enzyme proteins leading to the restoration of enzyme activity (CRICK and ORGEL 1964; FINCHAM 1966). However, the occurrence of allelic complementation between mutants within a gene encoding an enzyme which is part of a multienzyme complex (aggregate) raises interesting questions concerning the mechanism of complementation in such mutants. This paper discusses studies of both *in vivo* complementation in heterocaryons between *arom-2* mutants and *in vitro* complementation produced in mixtures of partially purified *arom* aggregate proteins obtained from *arom-2* mutants lacking DHQ synthetase activity.

MATERIALS AND METHODS

The origin of the *arom-2* mutants used in these studies, together with procedures for complementation tests, genetic mapping, sucrose density-gradient centrifugation, and enzyme assays have been presented previously (GILES *et al.* 1967). The assay procedure for DHQ synthetase used in the present studies, which is somewhat different from that initially employed, is described by BURGOYNE, CASE and GILES (1969).

In vitro complementation tests were performed with partially purified extracts from various pairs of *arom-2* mutants. Purification was carried out to stage eight of the procedure used to purify the *arom* aggregate from wild type (BURGOYNE, CASE and GILES (1969)). Approximately equal amounts (calculated on the basis of their DHS reductase activities) of *arom* aggregate protein from two different *arom-2* mutants were mixed together. The mixture was dialyzed against 20 volumes of 0.1 M glycine, plus 0.025 M dithiothreitol (DTT) (Calbiochem) with KOH added to give a pH of 10.0. Dialysis was performed at 0–4°C under an atmosphere of nitrogen for 24 to 30 hrs. The dialysis bag containing the enzyme mixture was then transferred into a

* Present address: Department of Biology, Flinders University, Bedford Park, South Australia 5042

large volume (e.g., 1500 ml) of 0.1 M potassium phosphate, pH 7.4 plus 4×10^{-4} M DTT, and dialyzed at 0–4°C, under an atmosphere of nitrogen for 24 hrs. During the whole procedure care was taken to avoid lengthy exposures to air and to avoid trapping air inside dialysis bags.

RESULTS

Complementation map of the arom-2 *gene.*—Numerous pairwise complementation tests were performed with *arom-2* mutants to establish the occurrence and pattern of complementation among these mutants. From these initial tests a set of mutants exhibiting different patterns of complementation was selected. This set of testers was then used to check the complementation responses of a large number of additional *arom-2* mutants. In this way a complementation map of the *arom-2* gene has been constructed (Figure 1). It should be emphasized that this map is preliminary in that not all possible pairwise combinations of the mutants used have been tested, and many additional *arom-2* mutants have not been tested at all. However, the present map already indicates a moderately complex pattern of complementation and has served to identify mutant pairs for further biochemical studies. Of interest is the low frequency of *arom-2* mutants which exhibit no allelic complementation, but complement with mutants in other genes in the *arom* cluster. On the map as presented in Figure 1 mutant *82* appears to be un-

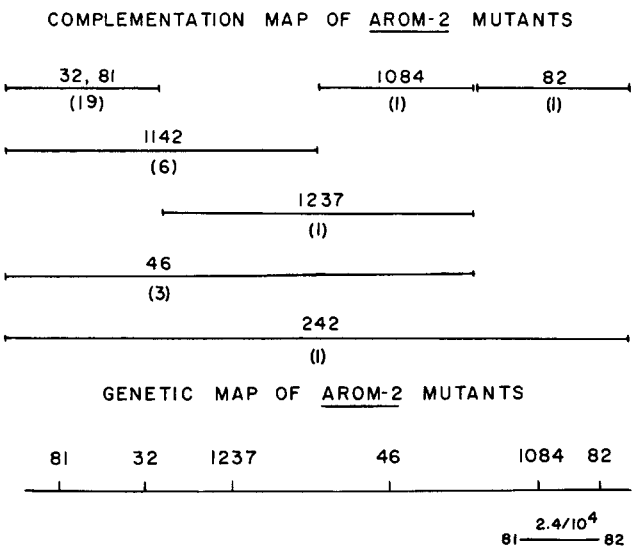

FIGURE 1.—Preliminary complementation map (above) and genetic map (below) of the *arom-2* gene in the *arom* gene cluster of *Neurospora crassa*. The complementation map is based on heterocaryon tests. The numbers above the segments on the map indicate specific mutant tester strains exhibiting a particular pattern of complementation. The numbers in parentheses below the segments indicate the total number of mutants of a given type on the basis of tests to date. The positioning of mutants on the genetic map is based on relative prototroph frequencies in viable ascospores plated in various crosses (e.g., in crosses of mutants *81* × *82*, the average prototroph frequency, excluding pseudowild types which can be distinguished phenotypically, is 2.4 per 10^4 total viable ascospores plated).

usual in that it complements with all but one (mutant *242*) of the other *arom-2* mutants tested. This is true in tests to date of *arom-2* mutants derived directly from wild type. However, several *arom-2* mutants induced as reversions in a noncomplementing mutant (*M25*) which maps within the *arom-2* gene (CASE, GILES and PARTRIDGE 1967) fail to complement with mutant *82* although they do complement with other *arom-2* mutants.

At the bottom of Figure 1 is shown, for comparison with the complementation map, a genetic map indicating the relative locations within the *arom-2* gene of six mutants.

Biochemical studies of the DHQ synthetase produced by in vivo *complementation in heterocaryons.*—Previous studies indicated that *arom-2* mutants lack detectable DHQ synthetase activity, but have essentially normal levels of the other four enzyme activities encoded in the *arom* gene cluster. Furthermore, the sedimentation coefficients of these activities determined in sucrose density-gradients indicated that the four activities are associated in an aggregate having a normal molecular weight (*ca.* 230,000) equivalent to that of the aggregate in wild type (GILES *et al.* 1967; BURGOYNE, CASE and GILES 1969). It thus became of considerable interest to determine the physical characteristics of the DHQ synthetase produced in complementing heterocaryons.

For these comparisons, and also for subsequent *in vitro* tests, four *arom-2* mutants were utilized: numbers *32*, *46*, *81*, and *82*. Additional sucrose gradients, run in both Tris and phosphate buffers, confirmed earlier evidence that the four *arom* enzyme activities present in these mutants are associated in an aggregate of normal molecular weight (Table 1 and Figure 2). Comparable gradients from complementing *arom-2* heterocaryons have been assayed for all five *arom* enzyme activities with the results shown in Table 1 and Figure 3. The data indicate quite clearly that in the three heterocaryons tested the restored DHQ synthetase activity sediments together with the other four *arom* activities as part of an aggregate having a normal molecular weight.

Characteristics of the DHQ synthetase produced by in vitro *complementation:* —Using the procedure described in MATERIALS AND METHODS, tests were performed for the restoration of DHQ synthetase activity by *in vitro* complementation with all possible pairwise combinations of the four mutant strains *32*, *46*, *81*, and *82*. As controls, the individual mutants and wild type were subjected to the same procedure and tested for activity. As indicated previously, the DHS reductase activities of individual mutant preparations were used as a basis for mixing equal amounts of *arom* aggregate protein for each pair of mutants, and for comparing the levels of restored DHQ synthetase activity with that in wild type. The results of these tests for *in vitro* complementation are presented in Table 2. When compared with Figure 1, these data indicate an exact correlation in this sample of *arom-2* mutants between the occurrence or nonoccurrence of complementation *in vivo* and *in vitro*.

Following these tests for the restoration of DHQ synthetase by *in vitro* complementation, the behavior of the restored activity during centrifugation in sucrose density-gradients was studied. The results of such experiments are given in

TABLE 1

Sedimentation coefficients ($s_{20,w}$) of arom *enzyme activities from* arom-2 *mutants and from* in vivo *and* in vitro *complementation between* arom-2 *mutants*

Strain / Mutants	Enzyme activities: DHQ-ase	DHS-reductase	SA-kinase*	EPSP-synthetase*	DHQ-synthetase	Gradient conditions and enzyme preparation**
46	11.4	11.4	11.4	11.4	—	Tris, $(NH_4)_2SO_4$ cut
*46****	11.5	11.5	—	—	—	Phosphate, stage no. 8
*32****	11.5	11.5	—	—	—	Phosphate, stage no. 8
*81****	11.6	11.6	11.6	11.6	—	Tris, stage no. 8
*82****	11.4	11.4	11.4	11.4	—	Tris, stage no. 8
In vivo complementation (in heterocaryons)						
32 + *82*	11.4	11.4	—	—	11.4	Phosphate, $(NH_4)_2SO_4$ cut
46 + *82*	11.4	11.4	11.4	11.4	11.4	Tris, $(NH_4)_2SO_4$ cut
81 + *82*	11.7	11.7	—	—	11.7	Phosphate, $(NH_4)_2SO_4$ cut
In vitro complementation (in mixed extracts)						
32 + *82****	11.5	11.5	—	—	11.5	Phosphate, stage no. 8
81 + *82****	11.3	11.3	—	—	11.3	Phosphate, stage no. 8

* SA-kinase and EPSP-synthetase assays cannot be obtained from phosphate gradients, since phosphate interferes with these assays.

** 0.1 M KPO_4, pH 7.4 + 2 × 10^{-3} M DTT, or 0.1 M Tris HCl, pH 7.4 + 2 or 4 × 10^{-4} M DDT, 32–50% ammonium sulfate material or stage No. 8 material from partially purified enzyme preparations (BURGOYNE, CASE and GILES 1969).

*** S-values estimated from comparable gradients on which *N. crassa* MDH ($s_{20,w} = 4.77$) was used as the standard.

TABLE 2

Tests for the restoration of DHQ synthetase activity by in vitro *complementation between partially purified* arom *aggregate proteins from various pairs of* arom-2 *mutants*

Mutant or mutant pair	DHQ synthetase activity (as percent of wild type=100)
32	0.0
46	0.0
81	0.0
82	0.0
32 + *46*	0.0
32 + *81*	0.0
32 + *82*	30.0
46 + *81*	10.0
46 + *82*	4.6*
81 + *82*	26.0

* This value is probably low because strain *46* grew poorly in this particular experiment and yielded an extract with a low protein content.

Details of procedures and the basis for calculating levels of restored activities, are described in the text.

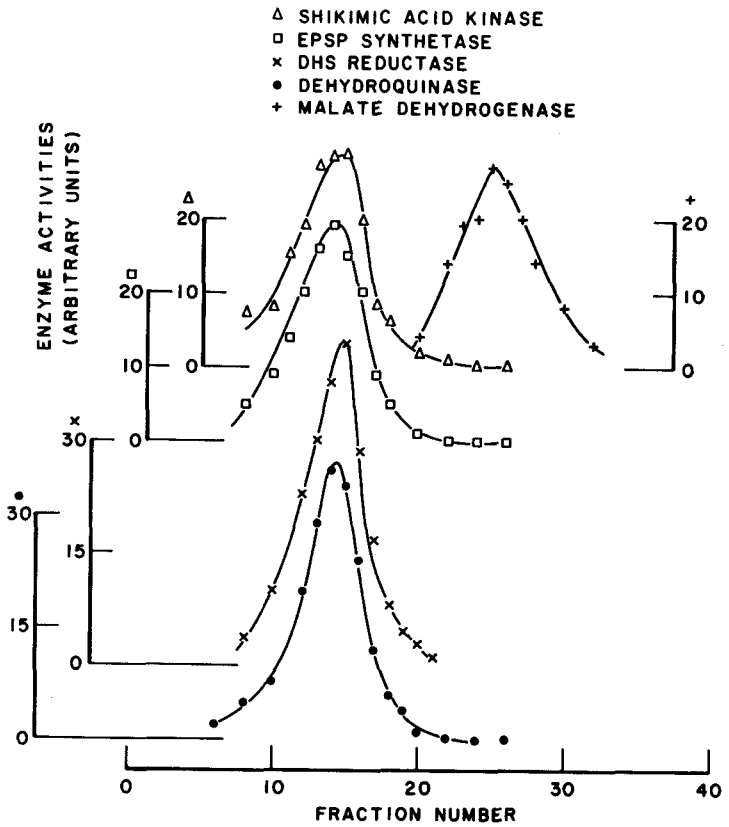

FIGURE 2.—Distribution, in a sucrose density-gradient, of the four *arom* enzyme activities present in an extract from *arom-2* mutant *81*. Very similar gradients were obtained from three additional *arom-2* mutants tested (Nos. *32*, *46*, and *82*). None of these mutants contain detectable DHQ synthetase activity.

Table 1 and Figure 4. As in cases of *in vivo* complementation, restored DHQ synthetase activity has always been found to sediment as part of an *arom* aggregate having a normal molecular weight.

DISCUSSION

The present studies of the *arom-2* mutants used for *in vivo* and *in vitro* complementation support previous conclusions (GILES *et al.* 1967) that these mutants can be most easily interpreted as resulting from missense mutations. On this view such mutations cause the substitution of a single incorrect amino acid in the presumptive polypeptide chain carrying the active site for DHQ synthetase and thus lead to the formation by these *arom-2* mutants of intact *arom* aggregates of normal molecular weight lacking DHQ synthetase activity. However, such *arom-2* aggregates have essentially normal active sites for the four enzymes

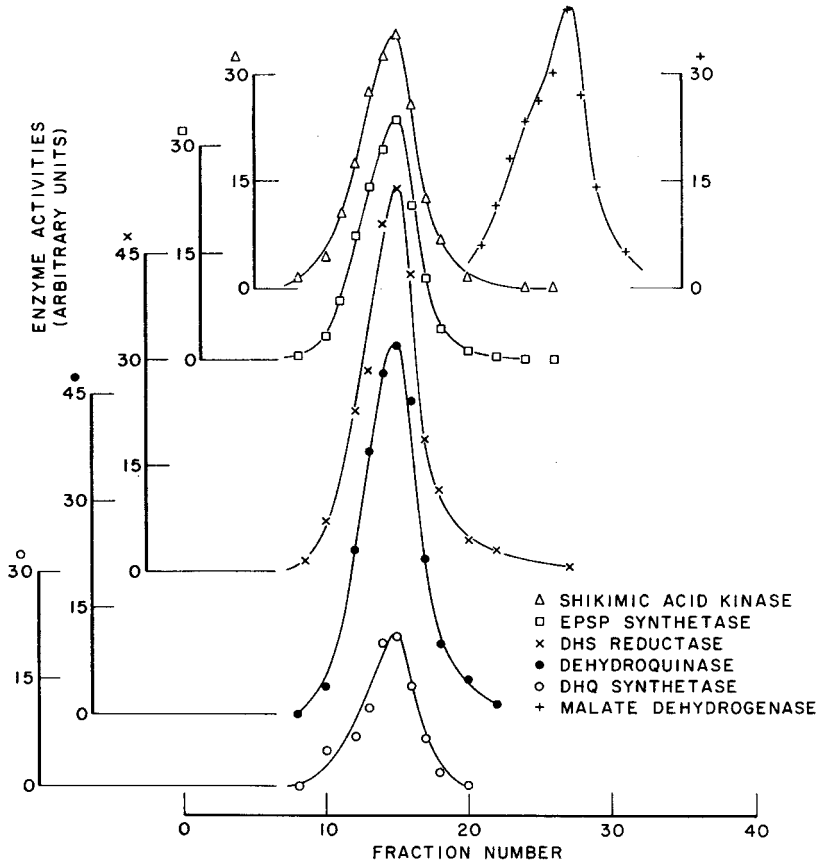

FIGURE 3.—Sedimentation behavior of the DHQ synthetase activity resulting from *in vivo* complementation in a heterocaryon between two *arom-2* mutants (*46* and *82*) in *N. crassa*. The diagram indicates the distribution, in a sucrose density-gradient run in Tris buffer, of the five aromatic synthetic activities encoded in the *arom* gene cluster (and of the reference activity—Neurospora malate dehydrogenase).

which are associated with the presumptive four different polypeptide chains encoded by the four other genes in the *arom* gene cluster.

The *in vivo* complementation studies indicate that the restored DHQ synthetase activity formed in heterocaryons sediments along with the four other *arom* enzyme activities as part of an *arom* aggregate of normal size. These results strongly support the view that a hybrid enzyme aggregate responsible for the restored DHQ synthetase activity is being formed in such heterocaryons. More direct evidence for this conclusion has been obtained from the *in vitro* experiments. In these experiments partially purified *arom* aggregate protein from single mutants and from mixtures of mutant pairs was exposed to highly alkaline conditions in the presence of high levels of DTT. The preparations were then dialyzed against normal buffer, after which assays indicated a substantial restoration of DHQ synthetase activity in mixtures of mutant pairs which complemented *in*

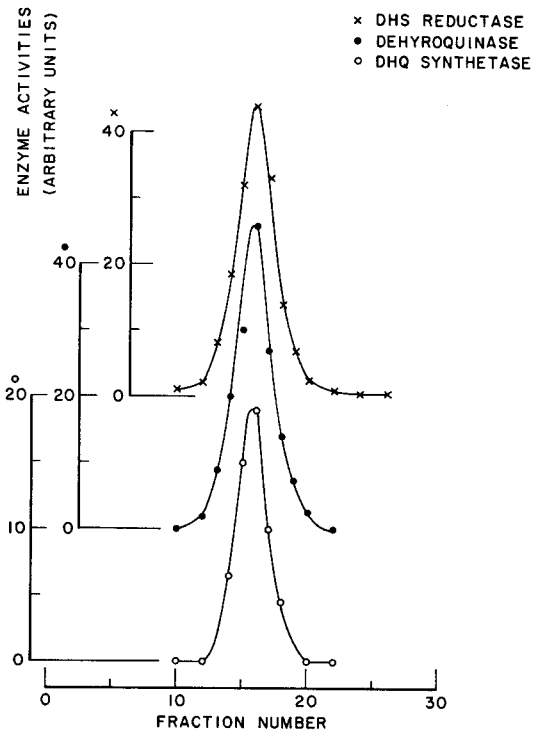

FIGURE 4.—Sedimentation behavior of the DHQ synthetase activity resulting from *in vitro* complementation between partially purified extracts of two *arom-2* mutants (*32* and *82*) in *N. crassa*. The diagram indicates the distribution, in a sucrose density-gradient run in phosphate buffer, of three of the five aromatic synthetic activities encoded in the *arom* gene cluster. The methods used to produce *in vitro* complementation are described in the text.

vivo. By contrast, no DHQ synthetase activity was detected in preparations from single mutants (parental strains) or from mixtures of mutant pairs which did not complement *in vivo*, when subjected to the same treatment. The DHQ synthetase activity regenerated *in vitro* was also found to sediment with an S-value identical with that for the wild type aggregate. These results make it highly probable that the partial restoration of DHQ synthetase activity by complementation results from the dissociation of each of two types of differentially defective aggregates (inactive for DHQ synthetase) present in the two parental strains, followed by a reaggregation of subunits from each of the two different strains to form enzymically active hybrid aggregates of normal molecular weight.

At the present time there is no direct evidence from experiments with *arom-2* mutants as to the nature of the aggregate subunits involved in dissociation and reaggregation. However, direct evidence for dissociation of wild-type *arom* aggregates into halves has come from studies with purified wild-type *arom* aggregate protein treated with detergent (sodium dodecyl sulfate) or with 6 M guanidine hydrochloride (BURGOYNE, CASE and GILES 1969). On the basis of these observations it appears reasonable to conclude that the most likely subunits

involved in complementation between *arom-2* mutants are those of half the normal size. However, sucrose gradient centrifugation of the wild-type aggregate under alkaline-DTT conditions that give *in vitro* complementation shows that, even under these conditions, the complex exists largely as an aggregate of normal size (BURGOYNE, CASE and GILES 1969). Thus it appears probable that some type of equilibrium can exist between the normal aggregate and the half-sized subunits, and that alkaline-DTT conditions allow exchange of subunits to occur without much of the aggregate being dissociated at any one time.

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SUMMARY

Certain pairs of *arom-2* (DHQ synthetaseless) mutants in the *arom* gene cluster of *Neurospora crassa* can complement in heterocaryons to restore DHQ synthetase activity. Preliminary complementation and genetic maps of the *arom-2* gene have been constructed on the basis of *in vivo* complementation and genetic recombination studies. Complementation has also been produced *in vitro* with mixtures of partially purified *arom* aggregate proteins from pairs of *arom-2* mutants which complement *in vivo*. The overall results, including sucrose density-gradient centrifugation studies of restored DHQ synthetase activity, are interpreted as indicating that allelic complementation between *arom-2* mutants in the *arom* gene cluster involves the formation, both *in vivo* and *in vitro*, of hybrid *arom* aggregates of normal molecular weight having partially restored DHQ synthetase activity. On the basis of comparative studies of wild-type, *arom* aggregate protein it is suggested that these *arom-2* hybrid aggregates are formed from the dissociation of mutant parental aggregates of normal molecular weight into half-sized subunits followed by the reaggregation of these subunits.

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