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SOME PROPERTIES OF THE 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASE ISOENZYMES FROM MUTANT STRAINS OF *NEUROSPORA CRASSA*

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SUMMARY

1. Extracts of mutant strains of *Neurospora crassa* which retain only one 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptulosonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating) EC 4.1.2.15) (DAHP synthase) isoenzyme have been used to test the properties of isoenzymes by direct analysis rather than by the differential inhibition analysis previously used for wild-type preparations. DAHP synthases (Phe) and (Tyr), in the presence of their specific negative effectors, show a 40% decrease in molecular weight as estimated by gel filtration. This is in accord with results obtained with wild-type preparations and is interpreted as indicating the dissociation of a polymer into subunits.

DAHP synthase from mutants in which the remaining isoenzyme is insensitive to inhibition do not dissociate, implicating dissociation as an essential part of the normal inhibition mechanism.

2. DAHP synthase (Trp) has not been found to dissociate in the presence of tryptophan.

3. Evidence of an *in vitro* interaction between DAHP synthases (Phe) and (Tyr) has been obtained. The presence of a specific allele of the *arom-6* locus (resulting in the loss of DAHP synthase (Tyr) activity) is correlated with a change in the gel-filtration characteristics of DAHP synthase (Phe), the product of an unlinked gene (*arom-7*).

INTRODUCTION

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptulosonate D-erythrose-4-phosphate-lyase (pyruvate-phospho-

Abbreviation: DAHP synthase, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase.

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rylating), EC 4.1.2.15) (DAHP synthase) is the first enzyme specific to the pathway of aromatic biosynthesis in *Neurospora crassa*. DAHP synthase activity in *N. crassa* has been shown to consist of three isoenzymes, each sensitive to inhibition by one of phenylalanine, tyrosine or tryptophan[1-9]. There is no evidence for repression of these isoenzymes below wild-type levels by the aromatic amino acids[2,9].

Two classes of mutation affecting each of the DAHP synthase isoenzymes have been isolated and described[7], those affecting enzyme activity (activity mutations) and those affecting the sensitivity of the isoenzyme to its specific negative effector (allosteric inhibition mutations). Activity mutations at the *arom-6* locus result in the inactivation of DAHP synthase (Tyr), at the *arom-7* locus in inactivation of DAHP synthase (Phe) and at the *arom-8* locus in inactivation of DAHP synthase (Trp).

The DAHP synthase isoenzymes from wild-type *N. crassa* can be detected by chromatography on Agarose beads in the presence of the substrate phosphoenolpyruvate[2,6,8]. On the basis of inhibition by the three negative effectors phenylalanine, tyrosine and tryptophan, this activity can be shown to consist of three overlapping peaks, each sensitive to inhibition by a specific negative effector[6,7].

Strains carrying mutant alleles at one or more of *arom-6*, *arom-7* or *arom-8* have been shown by chromatography to lack the appropriate isoenzyme(s). A strain carrying mutant alleles at all three loci has no detectable activity[7].

In the presence of phenylalanine, tyrosine and tryptophan, the elution volumes of DAHP synthases (Phe) and (Tyr) increase, indicating that these two isoenzymes can exist in at least two different forms having different molecular weights[7]. The lower molecular weight forms occur preferentially in the presence of the aromatic amino acids. Despite the technical difficulties, it has been shown that DAHP synthases (Phe) and (Tyr) from wild-type cells, each dissociate to the lower molecular weight form only in the presence of their specific negative effector[6]. These experiments had the advantage of allowing all possible subunit interactions to occur, but the difficulty that recognition of isoenzymes is dependent on measurement of the differential of activity in the absence and presence of the individual inhibitors. At pH 7.4, which favours recovery of enzyme activity, a considerable portion of the activity is non-inhibited under the standard conditions leading to complex distributions and consequent problems of interpretation[6].

In this report, each isoenzyme has been examined using mutant strains which retain only one active isoenzyme, the other two having been inactivated by mutation. This approach has the advantage that the elution profile can be determined directly as total activity instead of being calculated from inhibition data. The results confirm the interpretations[6] of data obtained with preparations from wild-type cells and directly correlate the failure of DAHP synthase (Phe) and DAHP synthase (Tyr) to dissociate, in response to the normal negative effectors, with the presence of the appropriate allosteric mutation. The method also provides evidence that the phenylalanine and tyrosine isoenzymes can interact.

MATERIALS AND METHODS

In general, techniques are similar to those described previously[6,7].

Cultures. The genotypes of all mutant strains used are listed in Table I. The

TABLE I

GENOTYPES OF STRAINS OF *N. crassa* USED

Strain No.	*Genotype*
74A	Wild-type
150	*arom-6*(DH1), *arom-8*(DH8)
152	*arom-7*(DH7), *arom-8*(DH8)
154	*arom-6*(DH1), *arom-7*(DH7)
273	*arom-6*(DH1), *arom-8*(DH8), *arom-7*^{r}(DH25)
274	*arom-6*(DH1), *arom-8*(DH8) *arom-7*^{r}(DH26)
275	*arom-6*(DH1), *arom-8*(DH8) *arom-7*^{r}(DH27)
265	*arom-7*(DH7), *arom-8*(DH8), *arom-6*^{r}(DH22)
267	*arom-7*(DH7), *arom-8*(DH8) *arom-6*^{r}(DH23)
366	*arom-7*(DH7), *arom-8*(DH8) *arom-6*^{r}(DH36)
368	*arom-7*(DH7), *arom-8*(DH8) *arom-6*^{r}(DH37)
280	*arom-6*(DH1), *arom-7*(DH7), *arom-8*^{r}(DH19)
439	*arom-6*(DH34), *arom-8*(DH8)
73	*arom-6*(DH1)
100	*arom-8*(DH8)
462	*arom-6*(DH34), *arom-8*(DH8)
463	*arom-6*(DH34), *arom-8*(DH8)

isolation, mapping and phenotypic characteristics of these have been described previously[7]. Mycelia were grown and harvested by the methods described by HALSALL AND DOY[7].

Preparation of extracts. The mycelium was ground, at 0–4°, with glass powder and 2 vol. of the buffer mixture used in column equilibration and elution. Cell debris was removed by centrifugation at 20 000 × *g* for 15 min. The crude extract (10 ml, containing approx. 150 mg protein) was applied to the column duplex (see below) and washed on with 2 ml of buffer (see also refs. 6, 7).

Gel filtration. Two columns were linked in series so that the protein flowed directly from the first to the second column. Column 1 (22 cm × 15 mm diameter) was packed with Bio-Gel P-2 (200–400 mesh, exclusion limit 1600 molecular weight) to remove small molecules prior to Agarose chromatography[6,7]. Column 2 (94 cm × × 25 mm) was packed with Agarose beads (Bio-Gel A-0.5 m, 100–200 mesh, operating range 10 000–500 000 molecular weight). The columns were preequilibrated with 1.5 bed volumes of the buffer mixture used also for elution: 0.05 M KH_2PO_4–NaOH buffer (pH as specified in the text and figures) containing phosphoenolpyruvate (0.1 mM). Aromatic amino acids were added to the buffer mixture where specified, the concentration (10 μM) was chosen so that, upon dilution into the reaction mixtures, the final concentration (1 μM) would allow detection of inhibitable activity.

Molecular weight estimations are based on a calibration of the duplex with proteins of known molecular weight. An example of a typical calibration, including the shape of a single component distribution, is given in ref. 7.

Protein was eluted at a flow rate of 1 ml/min and fractions were collected over 6 min intervals. Both columns and fractions were maintained at 4°. Fractions (25 μl samples) were assayed for DAHP synthase within 30 min of collection essentially by the method described previously[7].

The concentration of allosteric ligand present (50 μM) during the estimation of the individual isoenzymes was sufficient to inhibit DAHP synthase by at least 98%.

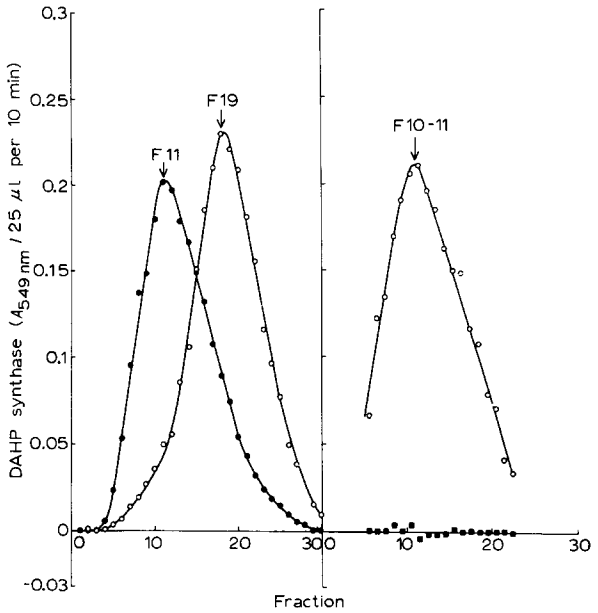

Fig. 1. Elution profiles of DAHP synthase (Phe) activity from (a) strain 150 (*arom*-6(DH1), *arom-8*(DH8)) and (b) strain 273 (*arom-7*^{r} (DH25), *arom-6*(DH1), *arom-8*(DH8)), chromatographed on a Bio-Gel P-2–Agarose column duplex at pH 7.4 (see MATERIALS AND METHODS). ●, in the absence of phenylalanine; ○, in the presence of phenylalanine (10 µM); ■, proportion of activity inhibited by phenylalanine ($A_{549\,nm}$ without phenylalanine *minus* $A_{549\,nm}$ with phenylalanine). A positive departure from zero indicates inhibition, a negative, "activation".

RESULTS

DAHP synthase (Phe)

When DAHP synthase activity from strain 150 (Table I), which retains only DAHP synthase (Phe), is chromatographed in the presence and in the absence of phenylalanine (Fig. 1), an increase in the elution volume of this isoenzyme is observed when phenylalanine is present in the buffer used for column-duplex equilibriation and elution. The molecular weight is estimated to decrease from 100 000–110 000 to 60 000–65 000, based on a calibration similar to that detailed in ref. 7.

The elution profile of DAHP synthase activity from a strain (273), carrying an allosteric inhibition mutation *arom-7*^{r} which results in the loss of the sensitivity of DAHP synthase (Phe) to inhibition by phenylalanine, is shown in Fig. 1. In the presence of phenylalanine there is no increase in the elution volume of DAHP synthase (Phe) from this strain. This result is also obtained with extracts of strains 274 and 275 which carry different *arom-7*^{r} alleles.

DAHP synthase (Tyr)

Results analogous to those found with DAHP synthase (Phe) are obtained when activity from strain 152 (Table I), which retains only DAHP synthase (Tyr), is chromatographed in the presence and absence of the negative effector tyrosine (Fig. 2). In the presence of tyrosine, the elution volume of DAHP synthase activity

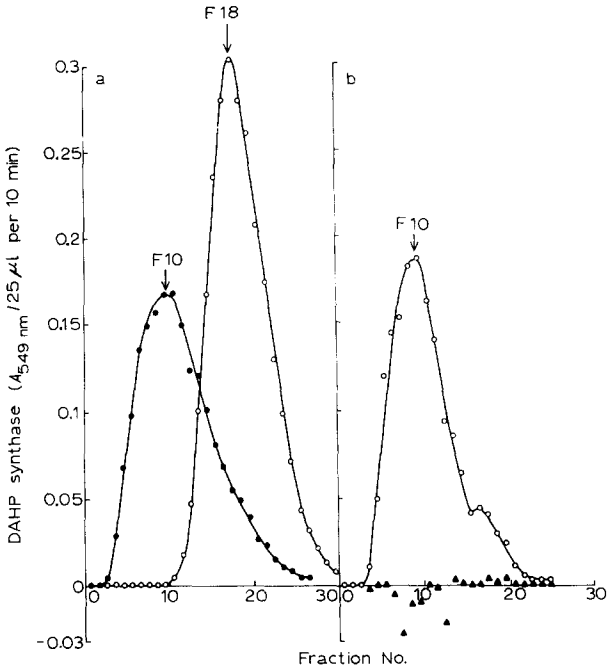

Fig. 2. Elution profiles of DAHP synthase (Tyr) activity from (a) strain 152 (*arom-7*(DH7), *arom-8*(DH8)) and (b) strain 265 (*arom-6*^{r} (DH22), *arom-7*(DH7), *arom-8*(DH8)) chromatographed on a Bio-Gel P-2–Agarose column duplex at pH 7.4 (see MATERIALS AND METHODS). ●, in the absence of tyrosine; ○, in the presence of tyrosine (10 µM); ▲, proportion of activity inhibited by tyrosine ($A_{549\ nm}$ without tyrosine minus $A_{549\ nm}$ with tyrosine). A positive departure from zero indicates inhibition, a negative, "activation".

increases, indicating a decrease in molecular weight from an estimated 110 000–120 000 to 65 000–70 000. The elution profiles of DAHP synthase activity from strains 265, 267, 366 and 368, each carrying a different allosteric inhibition mutation affecting DAHP synthase (Tyr) (*arom-6*^{r} mutations), do not exhibit this change in elution volume in the presence of tyrosine (Fig. 2).

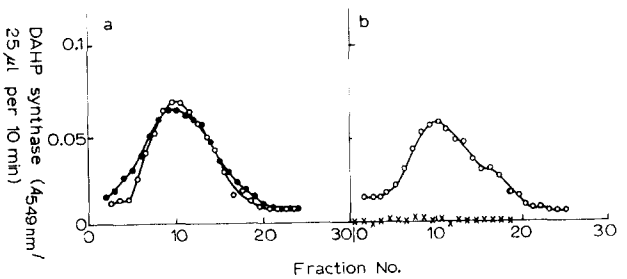

Fig. 3. Elution profiles of DAHP synthase (Trp) activity from (a) strain 154 (*arom-6*(DH1), *arom-7*(DH7)) and (b) strain 280 (*arom-8*^{r} (DH19), *arom-6* (DH1), *arom-7*(DH7)) chromatographed on a Bio-Gel P-2–Agarose column duplex at pH 6.4 (see MATERIALS AND METHODS). ●, in the absence of tryptophan; ○, in the presence of tryptophan (10 µM); ×, proportion of activity inhibited by tryptophan ($A_{549\ nm}$ without tryptophan *minus* $A_{549\ nm}$ with tryptophan.) A positive departure from zero indicates inhibition, a negative, "activation".

DAHP synthase (Trp)

Comparable experiments on DAHP synthase activity from strain 154, which retains only DAHP synthase (Trp), give a different result. The addition of tryptophan to the eluting buffer has no effect on the elution profile of DAHP synthase (Trp) (Fig. 3). In this case, the H^+ concentration of the eluting buffer and of the reaction mixtures was changed to pH 6.4 as the estimation of DAHP synthase (Trp) is unreliable at pH 7.4[6]. (When DAHP synthases (Phe) and (Tyr) were examined at pH 6.4, they showed a change in elution profile, in response to the negative effectors, comparable with that observed at pH 7.4.) Strain 280, carrying an allosteric inhibition mutation which renders DAHP synthase (Trp) insensitive to inhibition by tryptophan, also showed no change in elution profile in the presence of tryptophan. Thus, it would appear that this isoenzyme differs from the other two isoenzymes in the nature of its response to the negative effector.

An effect of different alleles at the arom-6 locus on the elution profile of DAHP synthase (Phe)

DAHP synthases (Phe) and (Tyr) resemble each other in that each is a polymeric protein of similar molecular weight and each dissociates in the presence of its negative effector (refs. 6, 7 and this paper). The possible significance of this similarity is strengthened by the finding that the elution profile of DAHP synthase (Phe) is modified in extracts of cells containing a specific allele of *arom-6*, the locus specifying DAHP synthase (Tyr). This suggests that the two isoenzymes interact.

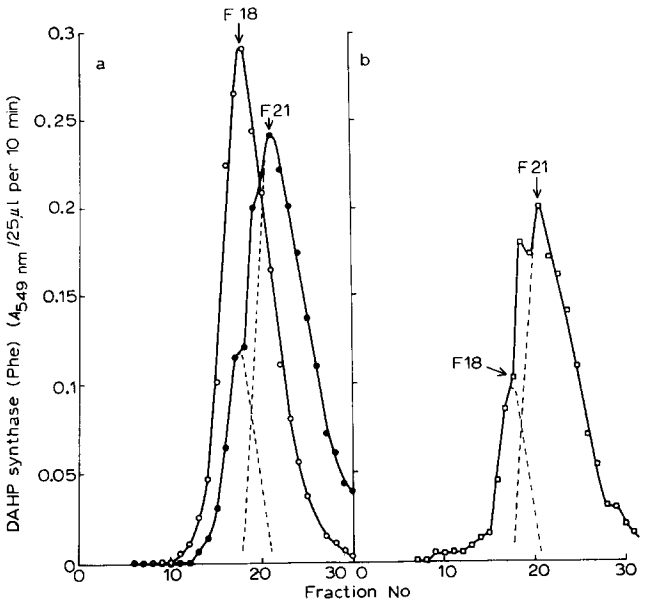

Fig. 4. Elution profiles of DAHP synthase (Phe) activity from (a) strain 150 (*arom-6*(DH1), *arom-8*(DH8)) (○), strain 439 (*arom-6*(DH34), *arom-8*(DH8)) (●); and (b) wild-type 74A (□) chromatographed on a Bio-Gel P-2–Agarose column duplex at pH 6.4 in the presence of phenylalanine (10 µM). When chromatographed in the absence of phenylalanine all profiles shift to higher molecular weight similar to the change illustrated in Fig. 1.

Two strains carrying the *arom-8* (DH 8) mutation and either *arom-6* (DH 1) or *arom-6* (DH 34) were chromatographed at pH 6.4 in the presence of phenylalanine (Fig. 4). The elution profile, as determined by inhibition data, of the phenylalanine sensitive isoenzyme from wild-type cells is shown also for comparative purposes. Chromatography at pH 6.4 results in changes of detail from the pattern observed at pH 7.4. (*cf.* Fig. 1) presumably because of a change in the relative proportion of the components discussed below.

DAHP synthase (Phe) from strain 150 (*arom-6* (DH 1), *arom-8* (DH 8)) gives a single peak of activity whereas the isoenzyme from strain 439 (*arom-6* (DH 34), *arom-8* (DH 8)) gives a complex peak which, for the purposes of analysis, can be resolved into two overlapping peaks (dotted lines in Fig. 4). The values indicated by these lines sum to give the observed value. The inhibition profile of the wild-type isoenzyme closely resembles the activity profile obtained from strain 439. Upon examination, the proposed minor peak of activity from strain 439 and from wild-type coincides with the single peak of activity from strain 150. This would suggest that whereas the native isoenzyme, *in vitro*, can exist in at least two forms in the presence of the specific negative effector (phenylalanine), strain 150 has lost this ability as a result of the DH 1 mutation at the *arom-6* locus.

To confirm that this change in elution profile is associated with the DH 1 mutation, strains carrying the *arom-6* (DH 1) and *arom-8* (DH 8) mutations independently (73 and 100, respectively) and strains containing DH 1 and the *arom-8* mutation derived from independent crosses (462, 463) were also examined. The results showed that the modified elution profile was obtained only when the DH 1 mutation was present in the strain.

These results are consistent with the hypothesis that the change in elution profile of DAHP synthase (Phe) is due to the DH 1 mutation.

Since these experiments show that the elution volume of an isoenzyme can be affected by varying the allele used to inactivate a different isoenzyme, the estimation of molecular weight using mutant strains may be subject to an inherent error resulting from cryptic effects of the inactivating mutations. Hence, the molecular weight estimations given here are for comparative purposes only (see also ref. 6).

DISCUSSION

The results obtained with mutant strains retaining only one active isoenzyme clearly demonstrate the different responses of the three DAHP synthase isoenzymes to their negative effectors. DAHP synthases (Phe) and (Tyr), in the presence of phenylalanine and tyrosine, respectively, show a decrease in molecular weight, as estimated by gel filtration, of approx. 40%. This probably represents the dissociation of a polymeric protein into subunits with an associated change in subunit conformation. As DAHP synthase (Trp) does not show a decrease in molecular weight under conditions comparable to those effective in the dissociation of the phenylalanine and tyrosine isoenzymes, it is probable that the response of this isoenzyme to tryptophan does not involve dissociation. These results are in accord with those obtained by inhibition studies of wild-type preparations[6] showing that similar dissociations occur in mixtures in which the other two isoenzymes are altered by mutation. This suggests that the interactions leading to dissociation are a fundamental property of the indi-

vidual gene products rather than due to possible interactions between them. This conclusion is supported strongly by the results with allosteric inhibition mutations which are allelic with activity mutations.

The allosteric inhibition strains examined, in which DAHP synthase (Phe) or (Tyr) is insensitive to inhibition by the negative effector[7], do not show this decrease in molecular weight. This suggests that dissociation is an essential part of the inhibition mechanism and that failure of the mutant enzyme to dissociate is concomitant with the loss of sensitivity to the negative effector. In the previous observation of the failure of dissociation in the presence of allosteric mutations[6], the correlation was confounded by the presence of mutations used to inactivate the other two isoenzymes. In this paper, it is shown that these activity mutations do not prevent dissociation in the presence of the negative effector; dissociation failure occurs only in the presence of allosteric mutations, and with all alleles tested (three for DAHP synthase (Phe) and four for DAHP synthase (Tyr)).

In Figs. 1 and 2 small departures from a smooth distribution have not been emphasised but it is possible that point to point deviations are significant. Comparison with the profiles of standard proteins used for calibration (see ref. 7) suggests that several components contribute to most, possibly all, the DAHP synthase profiles.

Although dissociation of individual isoenzymes is not prevented by mutations at the loci specifying the other isoenzymes, there is a correlation between the alteration in the elution profile of DAHP synthase (Phe) with the presence of one particular allele of *arom-6* (the locus specifying DAHP synthase (Tyr)). This provides evidence for interaction between DAHP synthases (Phe) and (Tyr). If both isoenzymes are presumed to have evolved from a single enzyme following gene duplication, it is highly likely that there are large regions of homology in the polypeptides coded for by the *arom-6* and *arom-7* genes. If such homologous regions cover the sites involved in subunit polymerisation, then it is feasible that, at least *in vitro*, subunits of DAHP synthases (Phe) and (Tyr) can form heteropolymers. On this hypothesis, since the dissociated form of the tyrosine sensitive isoenzyme appears to have a higher molecular weight than the phenylalanine sensitive isoenzyme, the double peak found with the wild-type enzyme would represent the heteropolymer (minor peak) and the homopolymer (major peak). This would imply that the mutation DH 1 results in a mixed polymer being formed preferentially. In the cell, the polymerisation of isoenzyme subunits may be regulated in such a way that either hetero- or homopolymers are formed exclusively; in this event, the double peak would be an artifact of the *in vitro* system.

The formation of heteropolymers may provide a basis for the observed synergistic inhibition by phenylalanine *plus* tyrosine[2,3,5]. Thus, although the specific ligands are necessary for isoenzyme dissociation the opportunity to form a mixed heteropolymer may lead to more efficient inhibition in the presence of both negative effectors. The existence of heteropolymers would also offer a possible explanation of the complexities of the elution profiles and the non-inhibited activity observed in studies of wild-type preparations (see ref. 6). It seems likely that each major zone of activity (ref. 6) represents a mixture of homo- and heteropolymers in which two strongly inhibitible forms (by assay at pH 7.4) are separated by a poorly inhibited form.

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