STRUCTURAL GENES FOR DAHP SYNTHASE ISOENZYMES IN *NEUROSPORA CRASSA*

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3 -DEOXY-D-*arabino*-heptulosonate 7-phosphate synthase (DAHP synthase) is the first enzymic function specific to aromatic biosynthesis. In *Neurospora crassa*, there are three DAHP synthase isoenzymes, each subject to feedback inhibition by one of the three aromatic amino acids (DOY 1968, 1970; JENSEN and NASSER 1968): phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp). Mutations inactivating each isoenzyme separately have been isolated (HALSALL and DOY 1969): *arom-6* (aromatic-6) affecting DAHP synthase (Tyr), *arom-7* affecting DAHP synthase (Phe), and *arom-8* affecting DAHP synthase (Trp). The *arom-7* and *-8* genes are loosely linked and unlinked to *arom-6* (HALSALL and DOY 1969). A second class of mutation rendering each isoenzyme, separately, insensitive to allosteric inhibition has been found to map within one centiMorgan of the corresponding activity mutation (HALSALL and DOY 1969).

As the two classes of mutation affecting one isoenzyme, in each case, fail to complement both *in vivo* and *in vitro* (HALSALL and DOY 1969), the allosteric inhibition mutants are considered to be alleles of *arom-6*, *-7*, or *-8* and are distinguished from the activity mutations by a superscript "r." By rendering an isoenzyme insensitive to feedback inhibition, the allosteric mutants alter a property of the catalytic species. Thus, assuming the test for allelism to be adequate, *arom-6*, *-7*, and *-8* can be considered to be the structural genes for DAHP synthase (Tyr), DAHP synthase (Phe), and DAHP synthase (Trp), respectively (HALSALL and DOY 1969). This hypothesis is strengthened by the finding of a new class of mutations which simultaneously confer insensitivity to feedback inhibition and reduced stability of the catalytic site. Some of these mutations affect DAHP synthase (Phe) and others affect DAHP synthase (Tyr).

MATERIALS AND METHODS

Neurospora strains 150 and 152 have been described previously (HALSALL and DOY 1969; HALSALL, CATCHESIDE and DOY 1971). Cell culture, preparation of cell-free extracts, assay of DAHP synthase, and the preparation and analysis of forced heterocaryons were as described by HALSALL and DOY (1969), with the exception that the standard extraction buffer, 0.05M KH_2PO_4 -NaOH (pH 7.4), was supplemented with 1.0 mM phosphoenolpyruvate (PEP) where required.

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RESULTS AND DISCUSSION

Allosteric inhibition mutants of DAHP synthase (Phe) were selected by plating conidia irradiated with ultraviolet light on medium containing phenylalanine. The irradiated strain (150) contained mutations inactivating the other two isoenzymes, *arom-6* (DH1) and *arom-8* (DH8); (HALSALL and DOY 1969). Since phenylalanine inhibits the only remaining DAHP synthase, further mutation is necessary to permit growth. Mutations affecting DAHP synthase (Phe) were screened from revertants of *arom-6* and *arom-8*, and from any other classes relieving growth inhibition by exogenous phenylalanine, by examining DAHP synthase activity *in vitro*. Mutants containing DAHP synthase (Phe) which had lost sensitivity to phenylalanine were found. These include not only strains similar to the previously described *arom-7^r* (DH25) mutation (HALSALL and DOY 1969), in which the now phenylalanine-insensitive DAHP synthase (Phe) is present at an activity level essentially the same as that in the parental strain (150), but also other strains with a considerably reduced specific activity for the isoenzyme. In extreme cases (DH32 [strain 219] and DH33 [strain 220]), no DAHP synthase activity could be detected in extracts prepared by the standard method.

A similar spectrum of mutants able to grow on tyrosine-supplemented medium and affecting DAHP synthase (Tyr) were selected from a strain (152) [*arom-7* (DH7), *arom-8* (DH8)] which retains only DAHP synthase (Tyr). Again, in the extreme cases (DH24 [strain 453], DH41, and DH42), no activity could be detected in standard extracts.

Since the extracts were made from cells able to grow on unsupplemented medium, it seemed probable that *in vivo* activity was unstable to the extraction technique. PEP is known to increase the stability of the (Phe) and (Tyr) isoenzymes during agarose chromatography (HALSALL and DOY 1969; DOY 1970; HALSALL, CATCHESIDE and DOY 1971) and during purification (P. J. HOFFMANN,

TABLE 1

Allosteric inhibition insensitive DAHP synthase (Phe) and DAHP synthase (Tyr) in strains containing DH32 or DH33^p and DH24^p, respectively

Strain number	Mutations affecting DAHP synthase			Percent inhibition of DAHP synthase by:
	(Tyr)	(Phe)	(Trp)	
				phenylalanine
150	<i>arom-6</i> (DH1)		<i>arom-8</i> (DH8)	98
219	<i>arom-6</i> (DH1)	(DH32)	<i>arom-8</i> (DH8)	0
220	<i>arom-6</i> (DH1)	(DH33 ^p)	<i>arom-8</i> (DH8)	0
				tyrosine
152		<i>arom-7</i> (DH7)	<i>arom-8</i> (DH8)	97
453	(DH24 ^p)	<i>arom-7</i> (DH7)	<i>arom-8</i> (DH8)	1

Each strain contains only one isoenzyme, the other two being inactivated by the mutations DH1 and DH8 or DH7 and DH8. Cells were grown on minimal medium and extracted with buffer containing 1.0 mM PEP.

personal communication). The inclusion of PEP in the extraction buffer enabled recovery of activity. As expected, DAHP synthase from each of the mutants examined (DH32, DH33, and DH24) proved insensitive to the normal inhibitors (Phe, Phe, and Tyr, respectively); see Table 1. In the presence of PEP, the mutant enzymes were essentially as stable as those from the parental cell lines (150 and 152). However, following the removal of PEP on a Bio-Gel P-2 column, the activity of both the mutant (Phe) and (Tyr) isoenzymes decayed rapidly at 37°C, in contrast to the initial activation and slow decay of the corresponding isoenzyme prepared from the appropriate parental strain by the same method (Figure 1).

Hence, for both the (Phe) and the (Tyr) isoenzymes, a single mutational event (DH33 and DH24, respectively) appears to alter both the allosteric inhibition site and the stability of the catalytic site. This implies, independently of the inability of allosteric mutants to complement activity mutants, that one polypeptide is concerned with both functions of the isoenzyme in each of DAHP synthase (Phe) and (Tyr). The possibility that the pleiotropic effects of DH33 and DH24 are due to double mutations is reduced, for DH24, by the occurrence of a spontaneous reversion in a genetically marked stock. The revertant, which retained the adenine-8 marker present in the DH24 stock, simultaneously regained a DAHP synthase (Tyr) which is sensitive to tyrosine and stable to extraction in buffers lacking PEP. DH24 and DH33 are regarded as

FIGURE 1.—Effect of DH24^P and DH33^P on the stability of DAHP synthase (Tyr) and (Phe), respectively.

- A: ● DAHP synthase (Tyr) from strain 152 [*arom-6*⁺]
 ○ DAHP synthase (Tyr) from strain 453 [*arom-6* (DH24^P)]
 B: ● DAHP synthase (Phe) from strain 150 [*arom-7*⁺]
 ○ DAHP synthase (Phe) from strain 220 [*arom-7* (DH33^P)]

Each strain retains only one active isoenzyme, the other two being inactive by appropriate mutations (see Table 1).

Cells were grown on minimal medium and extracted with 0.05M KH_2PO_4 -NaOH buffer (pH 7.4) containing 1.0 mM PEP. Extracts were incubated at 37°C following the removal of PEP on a Bio-Gel P-2 column equilibrated with 0.05M KH_2PO_4 -NaOH (pH 7.4).

TABLE 2
Complementation tests between pleiotropic mutations and activity mutations affecting
the same isoenzyme

Strain number	Mutations affecting DAHP synthase			DAHP synthase	
			Mutations for forcing heterocaryons	Specific activity*	Percentage inhibition by:
238	<i>arom-6</i> (DH1)	<i>arom-7</i> (DH7)	<i>arom-8</i> (DH8)	0	tyrosine
353	<i>arom-6</i> (DH34)	<i>arom-7</i> (DH35)	<i>arom-8</i> (DH20)	0	..
332	(DH24 ^p)	<i>arom-7</i> (DH7)	<i>arom-8</i> (DH8)	9.1	2
	heterocaryon 238 + 332			3.7	4
	heterocaryon 353 + 332	(nuclear ratio, 4:1)		2.5	3
293	<i>arom-6</i> (DH1)	<i>arom-7</i> (DH7)	<i>arom-8</i> (DH8)	0	phenylalanine
457	<i>arom-6</i> (DH1)	(DH33 ^p)	<i>arom-8</i> (DH8)	7.3	..
	heterocaryon 353 + 457			9.7	0
	heterocaryon 293 + 457	(nuclear ratio, 1:4)		4.3	7†
					0

* n moles DAHP formed/min/mg protein.

† Stimulation, not inhibition.

Complementation is expected to increase the sensitivity of the single remaining DAHP synthase to the specific negative effector. Heterocaryons were grown on minimal medium, heterocaryon components on media supplemented with adenine (3mM), nicotinamide (80 µM), or pantothenic acid (50 µM) where appropriate. 238, 293, and 353 were also supplemented with aromatic amino acids (1 mM each) and *p*-aminobenzoic acid (1 µM). Cell extracts were made with buffer containing 1.0 mM PEP. Nuclear ratios of heterocaryons were estimated by scoring the phenotype of growth from a number of individual conidia.

pleiotropic mutations and are henceforth designated by a superscript "p."

If complementation occurred between DH24^p or DH33^p and mutations inactivating the corresponding isoenzyme, it would be expected to yield a hybrid isoenzyme which had regained at least partial sensitivity to the specific negative effector (cf. HALSALL and DOY 1969). Forced heterocaryons between *arom-6* (DH1 or DH34) and DH24^p and between *arom-7* (DH7 or DH35) and DH33^p do not show complementation (Table 2), indicating that DH24^p is an *arom-6* allele and DH33^p is an *arom-7* allele. Thus for each isoenzyme, representatives of each of the three classes of mutation i.e., allosteric inhibition, activity, and pleiotropic, appear to be allelic (HALSALL and DOY 1969 and this paper).

A limited number of other mutants have been tested for complementation by the forced heterocaryon technique and no complementation has been observed. Seven mutants affecting DAHP synthase (Tyr), *arom-6* (DH[1, 34, 24^p, 22^r, 23^r, 36^r, and 37^r]) do not show complementation in the following combinations: DH(1 + 34, 1 + 24^p, 1 + 22^r, 1 + 23^r, 1 + 36^r, 1 + 37^r, and 34 + 24^p). Six mutants affecting DAHP synthase (Phe), *arom-7* (DH[7, 35, 33^p, 25^r, 27^r, and 29^r]) do not show complementation in the combinations: DH(7 + 35, 7 + 33^p, 7 + 25^r, 7 + 27^r, 7 + 29^r, and 35 + 33^p). Only three mutants affecting DAHP synthase (Trp), *arom-8* (DH[8, 20, and 19^r]) have been tested for complementation and none has been observed in the combinations: DH(8 + 20 and 8 + 19^r). Hence in each case, all of the mutations affecting one isoenzyme appear to be alleles; only one gene is known to be involved in the specification of each isoenzyme.

If the alternative hypothesis is considered, namely that more than one gene (in this example two) is involved in the specification of each isoenzyme, and if two simplifying assumptions are made, (i) that both polypeptide chains are involved in specification of the allosteric and catalytic functions and (ii) that mutation leading to each phenotype is equally likely in each gene of a given pair, then the probability of obtaining the above result is 1/64 for DAHP synthase (Tyr), 1/32 for DAHP synthase (Phe), and 1/4 for DAHP synthase (Trp). From these considerations it is most probable that DAHP synthase (Phe) and DAHP synthase (Tyr) are each composed of a single unique species of polypeptide chain; DAHP synthase (Tyr) encoded in *arom-6* and DAHP synthase (Phe) in *arom-7*. However, insufficient data are available to be reasonably confident that *arom-8* is the only structural gene for DAHP synthase (Trp).

SUMMARY

The three isoenzymes of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (DAHP synthase) are each inhibitable by one of the aromatic amino acids phenylalanine, tyrosine, and tryptophan.—DAHP synthase (Tyr) is simultaneously rendered insensitive to allosteric inhibition and less stable in the absence of phosphoenolpyruvate by a revertible mutation, DH24^p. DAHP synthase (Phe) is similarly affected by another pleiotropic mutation, DH33^p. It is inferred that DH24^p alters a structural gene for DAHP synthase (Tyr) and that

this gene codes for a polypeptide concerned with the formation of both the catalytic and inhibitor sites. Similarly, DH33^p would appear to alter a structural gene for DAHP synthase (Phe) coding for a polypeptide involved in the formation of both the catalytic and inhibitor sites of this isoenzyme.—As complementation tests suggest that two activity-negative mutants, four allosteric inhibition-negative mutants, and the pleiotropic mutant (DH24^p) are all alleles of the gene *arom-6*, it is likely that *arom-6* is the only structural gene for DAHP synthase (Tyr). Negative results from complementation tests between two activity, three allosteric, and the pleiotropic mutation (DH33^p), each affecting DAHP synthase (Phe), suggest that another gene, *arom-7*, is the only structural gene for this isoenzyme. The case for a single structural gene for DAHP synthase (Trp) is weaker, as only two activity and one allosteric mutant have been grouped as alleles of *arom-8*, a third gene, by complementation tests.

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