BBA 65797

THE EXOGENOUS ENVIRONMENT AS A FACTOR IN THE CONTROL OF THE 3-DEOXY-D-*ARABINO*-HEPTULOSONATE 7-PHOSPHATE SYNTHASE OF *NEUROSPORA CRASSA*

COLIN H. DOY AND DOROTHY M. HALSALL

Research School of Biological Sciences, Australian National University, Canberra, A.C.T. (Australia)

(Received April 1st, 1968)

SUMMARY

(1) The 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase activity (7 phospho-2-keto-3-deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating), EC 4.1.2.15) (DAHP synthase) found in wild type *Neurospora crassa* 74A grown for 60-66 h on minimal medium or in the presence of all possible combinations of aromatic amino acids, is the basal repressed level.

(2) Growth of an aromatic auxotroph on limiting aromatic amino acids results in at least a 10-fold derepression of the phenylalanine *plus* tyrosine inhibited portions of DAHP synthase.

(3) Addition of the aromatic amino acids to the growth medium is capable of inhibiting DAHP synthesis completely, or nearly completely.

(4) Methods have been developed for measuring DAHP or DAH in samples containing tryptophan and non-ionic inhibitors of the colorimetric assay.

INTRODUCTION

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (7-phospho-2-keto-3 deoxy-D-*arabino*-heptonate D-erythrose-4-phosphate-lyase (pyruvate-phosphorylating EC 4.1.2.15) (DAHP synthase) is the first specific enzyme of the aromatic pathway in microorganisms[1]. In *Neurospora crassa* the pre-formed function is controlled by the substrates erythrose 4-phosphate and phosphoenolpyruvate (activators) and the aromatic amino acids phenylalanine, tyrosine and tryptophan (deactivating modifiers)[1-3]. The present paper is concerned with the interaction of DAHP synthase and aromatic amino acids added to the exogenous environment.

At the time of this work, mutants of DAHP synthase were unknown, suggesting the possibility that the enzyme consisted of more than one kind of polypeptide and that

Abbreviations: DAH, 3-deoxy-D-*arabino*-heptulosonate; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate.

mutation was required in more than one of the corresponding structural genes if all activity was to be lost. An assessment was made of the feasibility of using allosteric end product inhibition and repression as the basis for selection of possible mutants of DAHP synthase differing from wild type with regard to active sites inhibited by different allosteric ligands. The penicillin selection procedure cannot be used for *N. crassa* and auxotroph mutant selection depends on non-germination of the required mutant conidia during a period of filtration enrichment[4]. Non-germination and non-growth would in this example depend on complete or nearly complete inhibition of DAHP synthesis by the three aromatic amino acids added to the medium. This is demonstrated for the more rigorous conditions of DAHP synthesis under conditions favouring germination and growth. The isolation of mutants and their properties will be described separately[5] (but see also refs. 16 and 18).

Preliminary experiments had suggested that the level of DAHP synthase found in wild type growing on minimal medium was likely to represent a base level for possible repression by aromatic amino acids added to the exogenous environment[6]. This is confirmed, but derepression to at least 10 times this activity is demonstrated indicating that repression–derepression is a factor in control of this enzymic function and therefore of aromatic biosynthesis.

MATERIALS AND METHODS

Strains

N. crassa wild type 74A and the mutants used in this work were obtained from Professor N. GILES.

TABLE I

DEREPRESSION OF DAHP SYNTHASE WHEN COMMON AROMATIC PATH MUTANTS ARE GROWN ON LIMITING AROMATIC AMINO ACIDS

All strains were grown on VOGEL minimal medium[7] supplemented with phenylalanine ($5 \cdot 10^{-4}$ M) tyrosine ($5 \cdot 10^{-4}$ M), tryptophan (10^{-4} M), 4-aminobenzoate ($3.65 \cdot 10^{-6}$ M) and harvested at 66 h (see also MATERIALS AND METHODS). Cultures grow at different rates and therefore are not strictly comparable with regard to growth phase, all except wild type were past exponential phase. The enzymic deficiencies of mutants are: *arom-1* dehydroshikimate reductase, *arom-2* dehydroquinate synthase, *arom-3* chorismate synthase, *arom-4* 3-enolpyruvylshikimate synthase, *arom-5* shikimate kinase; E polarity mutants lack all the above except chorismate synthase, but also lack dehydroquinase (see GILES *et al.*[11]).

Genome	*Strain*	*DAHP synthase*	
		Relative activity	*% inhibited by Phe (10^{-4} M) + Tyr (10^{-4} M)*
Wild type	74-A	1.0	84
arom-1	81-A	2.2	86
arom-2	81-4A	4.8	81
arom-3	87-5A	7.0	94
arom-4	49-7A	7.7	95
arom-5	75-A	7.4	95
E polarity	34-2A	6.1	90

Media

VOGEL medium[7] was used for growth of wild type. This medium was supplemented with phenylalanine, tyrosine, tryptophan and 4-aminobenzoate as required (see Table I, Figs. 1 and 2). All media were sterilised by autoclaving at 10 lb/20 min.

Culture and harvesting

The medium was inoculated from a conidial suspension. Mycelium was grown at 25° in 1 l medium in 2-l flasks on a New Brunswick gyrotory shaker.

Harvesting was by Buchner filtration, the mycelium being washed with water, pressed dry and deep frozen. Supernatant samples were taken prior to washing. Where supernatant samples were required during culture, they were removed aseptically.

Wet weight determination

Following harvesting, the mycelium was squeezed in several thicknesses of filter paper and tissue, with at least three changes until no more moisture could be removed.

This treatment gave reproducible wet weights, the dry weights being 20.8% of the wet weight.

Dry weight determinations

The mycelium was dried to wet weight and then placed at 80° for 24 h. The dry weights were checked following a further 12 h at 80°.

Dry weight estimations

Where a portion of the mycelium was required for preparation of extracts, the dry weight was estimated as follows. The wet weight of the total sample was taken, the required portion was removed and the wet weight retaken. This mycelium was dried and the estimated dry weight calculated on the basis of the proportion of the wet weight which was used to determine dry weight.

Preparation of mycelial extract

Frozen mycelium was ground by hand using cleaned glass powder and 2 ml/g of 0.1 M KH_2PO_4–NaOH buffer (pH 6.4). The preparation was centrifuged for 20 min at 20 000 $\times$ *g* and the supernatant retained. All preparation and centrifugation was at approx. 4°, the extract being prepared freshly as required.

Estimation of DAHP synthase in mycelial extracts

Freshly prepared extracts were dialysed against 1 l, 0.05 M KH_2PO_4–NaOH buffer (pH 6.4) for 1.5 h at approx. 4° with two further changes of buffer. DAHP synthase was estimated essentially as described previously using cyclohexanone extraction[3,8]. After preliminary analysis the volume of extract added by microsyringe was selected to give an $A_{549\ m\mu}$ of 0.8–1.0. Each assay consisted of two total activity controls, a zero time blank, and estimates in the presence of phenylalanine, tyrosine and phenylalanine *plus* tyrosine respectively, each inhibitor at 10^{-4} M.

Estimation of DAH–DAHP in supernatant samples

Dowex 1 X-8 AG resin (chloride form) was converted to the acetate form with 5 × the theoretical requirement of 1 M acetic acid. The acetate form was shown experimentally to be more effective in absorbing DAH–DAHP.

Small columns containing resin (5 g) were used to treat samples (10 ml) of supernatant, interfering substances were eluted in water (100 ml) and the DAH–DAHP eluted with 1 M HCl (10 ml). DAH–DAHP was effectively eluted in the first 8 ml. Samples of the eluate (0.1 ml) were then tested for DAH–DAHP as in the DAHP synthase assay.

In samples where the supernatant contained a high concentration of tryptophan, a column of Dowex 50 (2 g) was used to remove tryptophan prior to the Dowex 1 X-8 AG acetate resin column. This was necessary as tryptophan is known to interfere with the chemistry of the assay system. The effective removal of tryptophan was checked by bioassay using the tryptophan auxotroph *Aerobacter aerogenes* NC-3.

Estimation of preformed DAH–DAHP in mycelial extracts

Samples of extract (1 ml) were passed through miniature columns prepared in Pasteur pipettes. The lower part of the pipette was packed with Dowex 1 X-8 AG acetate resin (0.5 g) separated from the upper part which was packed with Dowex 50 (0.5 g) resin by a section of glass wool (approx. 2.5 cm). Following the application of the mycelial extract, interfering substances were eluted in water (10 ml). The Pasteur pipette was cut above the glass wool and the DAH–DAHP eluted from the lower resin in 1 M HCl (1 ml). The DAH–DAHP content of the extract was estimated as in the DAHP synthase assay.

Protein estimation

Protein was estimated by the method of LOWRY *et al.*[9] using crystalline bovine serum albumin as standard.

DAH(P) accumulation by washed mycelial suspensions

Mycelium was harvested from six individual 2-l flasks (1 l culture) after growth on limiting aromatic amino acids (25°, 48 h, see Figs. 1 and 2) by filtration without suction. Each sample was washed with VOGEL minimal medium[7] (500 ml, 25°) and resuspended in a further 500 ml. Accumulation of DAH(P) was followed (see above) for 4 h before the addition of inhibitors (see Fig. 3) and for a further 5 h.

RESULTS

Wild type

Wild type *N. crassa* 74A was grown for 60-66 h in VOGEL minimal medium[7] and supplements of single, all possible pairs and the three aromatic amino acids (each 10^{-3} M). No significant variation of DAHP synthase occurred either as total activity or as portions inhibited by a specific amino acid. For example, the portion inhibited by phenylalanine was not repressed by phenylalanine.

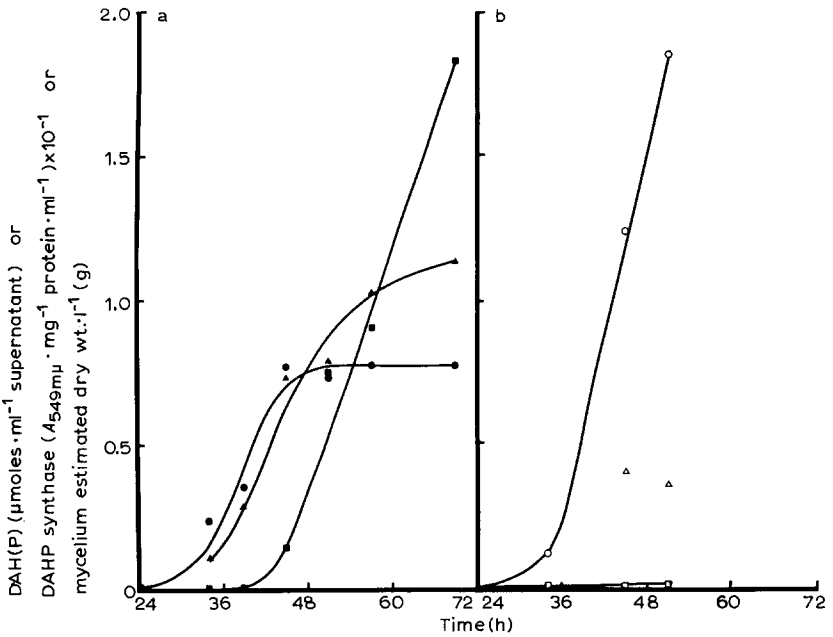

Fig. 1. (a) Filled symbols: *arom-2* (81-4A) grown on VOGEL minimal medium supplemented with phenylalanine ($2 \cdot 10^{-4}$ M), tyrosine ($2 \cdot 10^{-4}$ M), tryptophan ($4 \cdot 10^{-5}$ M) and 4-aminobenzoate (10^{-6} M), this medium limits growth. (b) Open symbols: supplements, phenylalanine (10^{-3} M), tyrosine (10^{-3} M), tryptophan ($4 \cdot 10^{-5}$ M) and 4-aminobenzoate (10^{-6} M); this medium does not limit growth. (●, ○) Growth, g estimated dry wt.·l^{-1}; (▲, △) DAHP synthase, $A_{549\ m\mu} \cdot mg^{-1}$ · protein ml^{-1}; (■, □) DAH(P) μmoles·ml^{-1} supernatant. See also MATERIALS AND METHODS.

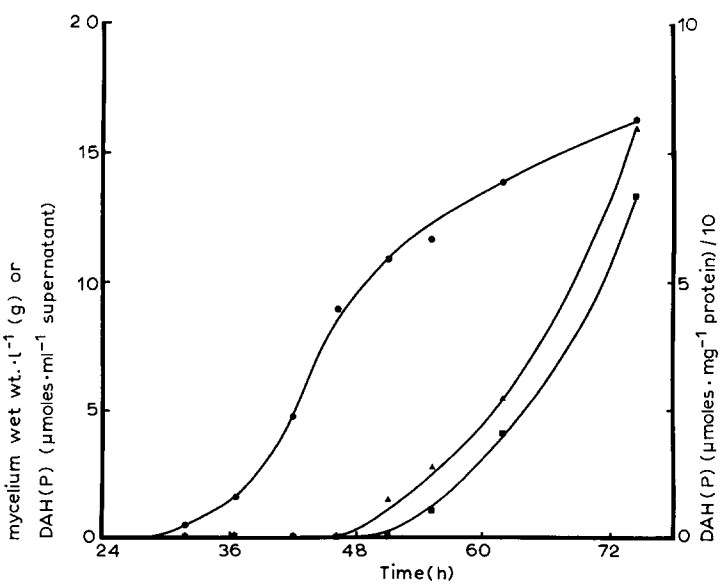

Fig. 2. *Arom-2* (81-4A) grown with limiting aromatic amino acid supplements (see Fig. 1). (●) Growth, g wet wt.·l^{-1}, (▲) intramycelial DAH(P) (μmoles·mg^{-1} protein)/10, (■) DAH(P) μmoles·ml^{-1} supernatant. See also MATERIALS AND METHODS.

Common path auxotrophs

At the time of this work, separate structural genes associated with five of the six common path enzymic functions subsequent to DAHP synthase were known[10,11]. An example of each genotype, *plus* a non-complementing (polarity) mutant lacking the five common path aggregate functions[11], was tested for DAHP synthase after growth on limiting aromatic amino acids. Compared to wild-type grown in the same manner, the DAHP synthase of the mutants was derepressed from 2 to 7.7 fold depending on the strain (Table I). There was a tendency for an increase in the proportion inhibited by phenylalanine *plus* tyrosine in parallel with the extent of derepression (Table I). This demonstrates derepression and moreover a preferential derepression of the phenylalanine and tyrosine inhibited DAHP synthase, presumably at the expense of tryptophan inhibited activity[1,3,12].

The relationship between DAHP synthase, DAHP synthesis and the level of aromatic amino acids added for growth of an arom-2 allele

Control by allosteric end product inhibition and repression was investigated further with an *arom-2* allele lacking dehydroquinate synthase and therefore capable of accumulating DAHP or the dephosphorylated derivative DAH. These compounds are equivalent in the chemical assay portion of the DAHP synthase assay. For chemical assay, interfering substances such as tryptophan and sucrose must be removed (see MATERIALS AND METHODS).

Arom-2 strain 81-4A was grown in two environments, having different levels of aromatic amino acid supplement, one growth limiting, the other not growth limiting. The results of these experiments are presented in Figs. 1 and 2, and Table II. In Fig. 1, growth, the appearance of DAH(P) in the medium and the DAHP synthase activity of mycelial extracts are compared for the two environments. DAH(P) begins to appear in the medium before the end of growth on the limiting aromatic amino acid supplement but only traces appear with the non-limiting supplement. Progressive derepression of DAHP synthase occurs during growth on limiting supplement. When this is analysed in terms of inhibition by phenylalanine and tyrosine it is evident that the portion of activity inhibited by a mixture of these amino acids increases with derepression (Table II). In Fig. 2 DAH(P) synthesis is examined in more detail for growth on the

TABLE II

DEREPRESSION OF DAHP SYNTHASE AND CHANGE OF INHIBITION CHARACTERISTICS WHEN *AROM-2* (81-4A) IS GROWN ON LIMITING AROMATIC AMINO ACIDS

Time harvested (h)	*DAHP synthase*	
	Specific activity ($A_{549\,m\mu} \cdot mg^{-1}$ *protein* $\cdot ml^{-1}$)	*Inhibition by Phe* (10^{-4} *M*) + *Tyr* (10^{-4} *M*)
34	1.16	86
39	2.92	89
45	7.4	91
51	7.95	92
57	10.25	97
69	11.31	90

limiting supplement. In the previous experiments the extra-mycelial DAH(P) was taken as the index of synthesis, but in this experiment the intra-mycelial concentration was determined also. As anticipated these compounds can be detected in the mycelium before excretion into the medium. The results confirm that DAH(P) synthesis is switched on before the end of growth.

Inhibition by aromatic amino acids of DAH(P) excretion by a non-growing suspension of 81-4A

The above experiments show that aromatic amino acids added exogenously control DAH(P) synthesis by repression and presumably a combination of repression and allosteric inhibition. Allosteric inhibition is classically demonstrated for bacteria by taking a non-growing suspension of fully derepressed cells and following the rate of synthesis (more strictly excretion) of the product in question both in the absence and presence of the supposed allosteric inhibitor(s) added exogenously. This was done for 81-4A in the presence of all the possible combinations of aromatic amino acids (Fig. 3).

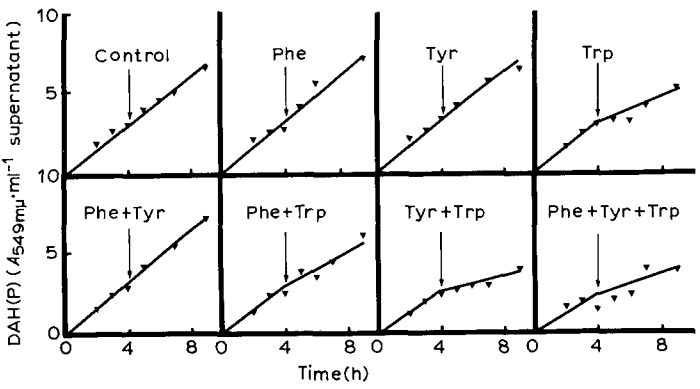

Fig. 3. Accumulation of DAH(P) by *arom-2* (81-4A) harvested at 48 h from limiting aromatic amino acid supplements (see Fig. 1) and resuspended in minimal medium. Aromatic amino acids were subsequently added at the times indicated (arrow).

The properties of dialysed extracts of these mycelia indicated that at least 92% of the total DAHP synthase was inhibited by phenylalanine *plus* tyrosine. In contrast the only significant inhibitor of DAH(P) synthesis by whole cells was tryptophan or combinations including tryptophan (Fig. 3). The small differences in degree of inhibition (either stimulation or interference) for the various combinations are presumably the result of interactions concerned with permeability or accessibility to the enzyme. Depending on the degree of derepression of the tryptophan inhibited DAHP synthase the limits of tryptophan inhibition are 1–8% and from previous experience there is also a small non-inhibited portion[3]. However, the synthesis of DAH(P) given in Fig. 3 is at least 50% inhibited by tryptophan, indicating that under the experimental conditions the functional enzyme is mainly or entirely these minor components. It is not known if this conclusion applies also to the data of Figs. 1 and 2 gained with growing mycelium.

DISCUSSION

These experiments show that the level of DAHP synthase found in wild-type cannot be repressed significantly further by the addition of aromatic amino acids to the exogenous environment. In contrast with *E. coli*[13], the components of activity are not further selectively repressed by the corresponding inhibiting allosteric end product.

If the condition of wild type growth in minimal medium is taken as "normal" the *N. crassa* DAHP synthase is well controlled by endogenous aromatic amino acid synthesis. In this, *N. crassa* resembles *Saccharomyces cerevisiae*[14] which suggests that natural selection has geared these organisms to control by endogenous synthesis rather than an exogenous supply. This conclusion is supported also by the lifting of control over DAH(P) synthesis before the end of growth limited by aromatic amino acids.

That repression is a factor in endogenous control is demonstrated by derepression under conditions favouring starvation of aromatic amino acids. Further, this derepression favours the phenylalanine *plus* tyrosine inhibited portion of DAHP synthase, indicating that this component or components can vary independently from the tryptophan inhibited activity. This was the first indication of the sub-unit character of *N. crassa* DAHP synthase since confirmed by differential stability[3], recognition of isoenzymes[1,15,17] and biochemical genetics[5,16]. Attempts to differentially derepress or repress the phenylalanine and tyrosine inhibited portions have failed (C. H. DOY, unpublished results) but are not unequivocal proof that this cannot be done. Other experience suggests the possibility that these portions of activity interact physically and may therefore be naturally interdependent[2,3,15].

The observation that DAH(P) synthesis was negligible for at least 50 h, when 81-4A was grown in the presence of excess aromatic amino acids, demonstrated the feasibility of looking for mutants of DAHP synthase based on selective feedback inhibition–repression and filtration enrichment. As a consequence, at least three structural genes have been recognised for this function[5,16].

The experiments with non-growing mycelium (Fig. 3) indicate that the bulk of DAHP synthase is not functioning. The simplest explanation is that the internal environment is such that the enzyme is saturated with phenylalanine and tyrosine. It may be that growth was limited by tryptophan (Figs. 1 and 2) rather than other end products. If so, derepression of the phenylalanine and tyrosine inhibited activity (Fig. 1, Table II) occurs in the presence of phenylalanine and tyrosine. This would mean that these end products alone are insufficient for the repression of the portions of DAHP synthase that they inhibit. The details of control by repression–derepression (or by degradation) have not yet been investigated as conclusive results are difficult to obtain with the methods described and are best obtained with a chemostat.

ACKNOWLEDGEMENT

S. PAUL is thanked for her technical assistance.

REFERENCES

1 C. H. DOY, *Rev. Pure Appl. Chem.*, 18 (1968) 41.
2 C. H. DOY, *Biochem. Biophys. Res. Commun.*, 28 (1967) 851.
3 C. H. DOY, *Biochim. Biophys. Acta*, 159 (1968) 352.

4 D. G. CATCHESIDE, *J. Gen. Microbiol.*, 11 (1954) 34.
5 D. M. HALSALL AND C. H. DOY, in preparation.
6 C. H. DOY, *Neurospora Newsletter, No. 10*, (1966) 8.
7 H. J. VOGEL, *Microbial Genet. Bull.*, 13 (1956) 42.
8 C. H. DOY AND K. D. BROWN, *Biochim. Biophys. Acta*, 104 (1965) 377.
9 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
10 N. H. GILES, *Natl. Cancer Inst. Monograph*, 18 (1965) 341.
11 N. H. GILES, M. E. CASE, C. W. H. PARTRIDGE AND S. I. AHMED, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 1453.
12 C. H. DOY, *Biochem. Biophys. Res. Commun.*, 26 (1967) 187.
13 K. D. BROWN AND C. H. DOY, *Biochim. Biophys. Acta*, 118 (1966) 157.
14 C. H. DOY, *Biochim. Biophys. Acta*, 151 (1968) 293.
15 C. H. DOY, in preparation.
16 D. M. HALSALL AND C. H. DOY, *Neurospora Newsletter No. 12*, (1967).
17 C. H. DOY, *Proc. Australian Biochem. Soc.*, (1968) 69.
18 D. M. HALSALL AND C. H. DOY, *Proc. Australian Biochem. Soc.*, (1968) 70.