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GENES AND ISOENZYMES CONTROLLING THE FIRST STEP IN THE AROMATIC AMINO ACID BIOSYNTHESIS IN *SCHIZOSACCHAROMYCES POMBE*

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SUMMARY

1. Crude extracts of *Schizosaccharomyces pombe* wild-type were assayed in order to characterize the first enzyme of the aromatic amino acid pathway, 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). Optimal conditions for activity have been found with a 0.1 M phosphate buffer (pH 6.5). Co^{2+} and Mn^{2+} stimulate activity whereas EDTA inhibits strongly. Inhibition by EDTA is reversed by Co^{2+}. The enzyme activity is feedback inhibited by tyrosine and phenylalanine but not by tryptophan.

2. On analyzing the inhibition pattern in the activity peak obtained by Sephadex G-100 filtration, two overlapping regions with different degrees of inhibition by phenylalanine and tyrosine were found.

3. DAHP synthase mutants were obtained and characterized. *aro*2-C mutants show a reduced growth on minimal medium containing phenylalanine (1 mM) and *aro*1-C mutants grow poorly on minimal medium supplemented with tyrosine. In vitro the DAHP synthase activity of *aro*2-C mutants is inhibited to a greater extent by phenylalanine than in the wild-type, and *aro*1-C mutants are more inhibited by tyrosine compared to the wild-type.

4. Linkage relationships assign all *aro*1-C mutants to one locus, and all *aro*2-C mutants to another locus. The two genes are unlinked.

5. Double mutants obtained from *aro*1-C × *aro*2-C crosses require tyrosine, phenylalanine, tryptophan and 4-aminobenzoate for growth. They do not grow on minimal medium or minimal medium supplemented with only one or with any combination of two aromatic amino acids plus 4-aminobenzoate. In vitro they show no DAHP synthase activity.

6. These results show that in *S. pombe* the first step in the biosynthesis of aromatic amino acids is catalyzed by two isoenzymes.

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

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INTRODUCTION

The common pathway for the biosynthesis of aromatic amino acids has been extensively studied in many microorganisms, and has been found to be the same [1]. The first reaction is catalyzed by 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), which plays an important role in the regulation of the pathway [1]. The biochemical organization of this enzymatic activity shows marked differences in the organisms studied. For example, in *Neurospora crassa* and *Escherichia coli* three distinct isoenzymes of DAHP synthase have been described, which are feedback inhibited by tyrosine, phenylalanine and tryptophan, respectively. In *Bacillus subtilis* the enzyme is a single protein [1] whereas in *Saccharomyces cerevisiae* two isoenzymes seem to be present [2, 3].

The work reported in this publication was initiated in order to study the control of DAHP synthase activity in *Schizosaccharomyces pombe* and to determine whether two isoenzymes are present, as is the case in *S. cerevisiae*, the other yeast species studied.

MATERIALS AND METHODS

Strains

The wild-type strains 972 (heterothallic−) and 975 (heterothallic+) came from the collection of Professor U. Leupold (Institut for General Microbiology, Altenbergrain 21, Berne, Switzerland). Mutants were derived from these strains by a procedure described below.

Media and culture conditions

Yeast extract medium, malt extract medium and minimal medium as well as the agar concentration needed to solidify these media (yeast extract agar, malt extract agar and minimal agar) have been described previously [4].

When media were supplemented, the nature and concentration of the added substance is indicated in brackets. Incubation was at 30 °C.

Chemicals

Inorganic chemicals were of P. A. grade. Organic chemicals were of the highest grade commercially available (supplied by Merck AG, Darmstadt, Germany and Fluka AG, Buchs, Switzerland). D-Erythrose 4-phosphate (Ery-4-*P*) (barium salt of the diethylacetal) and phosphoenolpyruvate (*P*-enolpyruvate) (monosodium salt) were supplied by Boehringer, Mannheim.

Induction and isolation of DAHP synthase isoenzyme mutants

For selecting mutants defective in DAHP synthase activity, it was assumed that in *S. pombe* two or more DAHP synthase isoenzymes were present, each being inhibited specifically by one aromatic amino acid. If two isoenzymes are assumed in the wild-type, the growth in minimal medium of a mutant defective in one of the isoenzymes could be blocked by supplementing the medium with the amino acid which inhibits the activity of the remaining isoenzyme unless biosynthetic channeling occurs

[2]. If three isoenzymes were present and again no biosynthetic channeling occurs, a mutant with only two functional proteins could grow on minimal medium, but not on minimal medium supplemented with the two inhibiting amino acids [5].

*Aro*2-C mutants were obtained according to the procedure described by Megnet [6] with the exception that 0.1 M acetate buffer was used and that the final concentration of *N*-methyl-*N'*-nitro'-*N*-nitrosoguanidine was 3 mg/ml. For enrichment [6], the treated cultures were first washed, resuspended in yeast extract (10 g glucose per l), incubated with aeration at 30 °C for 3 h and washed again. The cells were then suspended in minimal medium (1.5 mM phenylalanine + 1.5 mM tyrosine), 2 deoxy-glucose was added to reach a final concentration of 0.4 mg/ml and incubation was continued for 15 h. Suspensions were diluted and plated out on minimal agar. The resulting colonies were printed on minimal agar and on minimal agar (1.5 mM phenylalanine + 1.5 mM tyrosine + 2 μM 4-aminobenzoate) (selection medium). The mutants showed better growth on minimal medium than on the selection medium.

*Aro*1-C mutants were obtained without this enrichment procedure. The mutagenized cell suspension was directly diluted and plated on yeast extract agar. The colonies were printed on minimal agar and minimal agar (1 mM tyrosine). The mutants showed reduced growth on minimal agar (1 mM tyrosine) but wild-type growth on minimal agar alone.

The mutants of the type *aro*1-C and *aro*2-C are called activity-negative mutants. The letter C indicates that the catalytic site has been altered.

Growth experiments

Cells grown in yeast extract or supplemented yeast extract were washed twice with minimal medium and starved by incubation in minimal medium for 6 h at 30 °C. Tubes containing 5 ml medium were inoculated with the desired cultures and incubated on a reciprocating shaker. Growth was followed on a Lumetron Colorimeter "Photovolt" at a wavelength of 530 nm.

Random spore analysis

The procedure was previously described by Leupold [7]. To obtain double mutants, spores of the *aro*1-C × *aro*2-C crosses were plated on malt extract agar (0.5 mM tyrosine + 0.5 mM phenylalanine + 0.5 mM tryptophan + 10 μM 4-aminobenzoate) and the colonies were printed on minimal agar, minimal agar (1 mM tyrosine), minimal agar (1 mM phenylalanine) and minimal agar (1 mM tryptophan).

Culturing of cells and preparation of crude extracts

Cells were grown in yeast extract, except double mutants which were grown in supplemented yeast extract (0.5 mM tyrosine + 0.5 mM phenylalanine + 0.5 mM tryptophan + 10 μM 4-aminobenzoate). Towards the end of log phase cultures were centrifuged at 0 °C and the cells were broken by two different methods. The enzymic activities obtained from both were comparable.

Following one method, the cells were lyophilized and subsequently treated twice in a Brown Homogenizer (Model MSK), each time for 30 s. Cold phosphate buffer (pH 7.0), 0.05 or 0.1 M was added, and the suspension centrifuged at 48 000 × g for 30 min. Alternatively, cells from 200 ml yeast extract cultures were

centrifuged, washed, suspended in 10 ml 0.1 M phosphate buffer (pH 7.0) and sonicated in a 5.5 cm × 2.5 cm glass vial using a B-12 Sonifier (Branson Sonic Power Company) for two 30-s periods. The cell debris were spun down at 48 000 × *g* for 30 min.

DAHP synthase assay

The enzyme activity was assayed by a modification of the method described by Gollub et al. [8]. The standard reaction mixture consisted of 31 nmoles Ery-4-*P*, 31 nmoles *P*-enolpyruvate, 4 μmoles phosphate buffer (pH 6.5), 0.1 μmole $CoCl_2$, if required tyrosine or phenylalanine, enzyme and water, up to a final volume of 40 μl. The concentration of tyrosine and phenylalanine was always 1 or 0.5 mM, because these concentrations are within the range of maximal inhibition.

Incubation was for 10 min at 37 °C. The reaction was stopped with 50 μl trichloroacetic acid (10%); 100 μl $NaIO_4$ (0.025 M in 0.075 M H_2SO_4) was added and the mixture incubated further for 30 min at 37 °C. The excess of $NaIO_4$ was removed by the addition of 100 μl sodium *m*-arsenite (2% in 0.5 M HCl). As soon as the yellow colour disappeared, 500 μl thiobarbituric acid (0.3%) were added to the assay, which was then boiled for 8 min. The pink solution was mixed with 1 ml cyclohexanon by shaking on a Vortex. The two layers were separated in a small laboratory centrifuge. The test tube was warmed to 37 °C and the upper organic layer removed. Its absorbance was measured at the same temperature on a Unicam SP 18.000 ultraviolet spectrophotometer at 549 nm.

In the described assay the formation of DAHP is linear up to an absorbance of $A_{549\,nm} = 1.0$.

As there was no synthetic DAHP available, a direct correlation between the measured absorption and the DAHP concentration in the test was not possible. Therefore enzymic activity is stated as absorption at 549 nm found after the ten minute incubation period at 37 °C per mg protein.

Protein test

Proteins were determined according to the method of Lowry et al. [9].

Gel filtration

Sephadex gels were prepared and packed as described in "Sephadex-Gel Filtration in Theory and Practice" (Pharmacia Fine Chemicals, Uppsala, Sweden).

RESULTS

Some properties of DAHP synthase in crude extracts

Experiments were carried out in order to find good assay conditions for *S. pombe* DAHP synthase in crude extracts. The optimal conditions for activity and inhibition were determined using a 0.1 M phosphate buffer (pH 6.5). Assays were always carried out at a protein concentration in the range of linearity with activity.

Table I shows the stimulation of DAHP synthase activity by Co^{2+} and Mn^{2+}. EDTA causes a partial inhibition, which is reversed on addition of excess Co^{2+}.

The enzyme in crude extracts is inhibited specifically by the L-isomers of tyrosine and phenylalanine (Table II). This inhibition by the two amino acids is additive to a

TABLE I

INFLUENCE OF SOME BIVALENT IONS AND EDTA ON DAHP SYNTHASE ACTIVITY IN WILD-TYPE CRUDE EXTRACTS

Cells were disrupted with the Brown homogenizer and suspended in 0.05 M phosphate buffer (pH 7.0). The concentrations of the substances in the test are given in parentheses. The value of the enzymic activity corresponding to 100% activity is also given in parentheses.

Added substance	% activity
–	100 (22)
$CoCl_2$ (2.5 mM)	178
$MnCl_2$ (2.5 mM)	162
EDTA (2.5 mM)	55
$CoCl_2$ (2.5 mM) + EDTA (1.2 mM)	166

certain degree (see also Table III). Cobalt has no influence on the extent of the inhibition. At the high concentration (1 mM) used D- and L-tryptophan interfere in the assay of DAHP and at lower concentrations (0.05 mM) tryptophan does not (in presence or absence of Co^{2+}) inhibit DAHP synthase activity. The percentage inhibition of the activity by tyrosine remained constant during the incubation period of 10 min at 37 °C necessary for testing enzymatic activity, while the percentage inhibition by phenylalanine decreased. The addition of cobalt did not stabilize this inhibition.

Separation of DAHP synthase isoenzymes

The experiments with crude extracts suggested the existence of two isoenzymes, one responsible for the inhibition by phenylalanine and the other responsible for the inhibition by tyrosine. Therefore we tried to separate the two activities in crude extracts by gel filtration on Sephadex G-100 and G-200, by chromatography on DEAE-cellulose and DEAE-Sephadex, as well as fractionation with $(NH_4)_2SO_4$. All these

TABLE II

INHIBITION OF DAHP SYNTHASE WILD-TYPE CRUDE EXTRACTS BY AROMATIC AMINO ACIDS

Cells were disrupted with the Brown homogenizer and suspended in 0.05 M phosphate buffer (pH 7.0). The amino acid concentration in the assay was 1 mM. In the controls the amino acids were added after the first 10-min incubation period.

Added amino acid	% inhibition when added before incubation	% inhibition when added after incubation (control)
L-Phenylalanine	25	1
L-Tyrosine	45	−1
L-Phenylalanine + L-Tyrosine	59	1
L-Tryptophan	45	48
D-Phenylalanine	2	0
D-Tyrosine	3	2
D-Tryptophan	46	45

attempts proved unsuccessful. Consequently we tried to further differentiate the DAHP synthase activity peak obtained with molecular sieves by analyzing its inhibition pattern. The results are shown in Fig. 1. Two partially overlapping peaks of inhibited activities can be distinguished within the total activity peak. These results could be

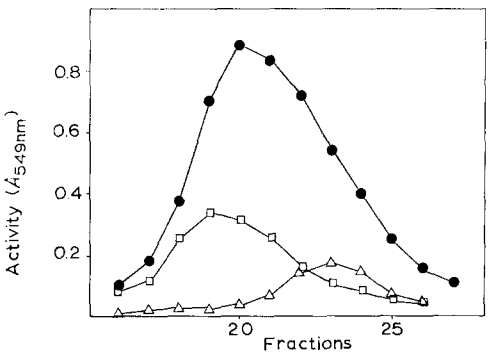

Fig. 1. Gel filtration of crude extracts of wild-type 972: DAHP synthase activity and inhibition. 0.2 ml crude extract (40 mg protein per ml) were layered on a Sephadex G-100 column (1 cm × 24 cm) and eluted with 0.05 M phosphate buffer. The rate of elution was 25 ml/h and the fraction size 0.5 ml. The protein front elutes in fraction 17. ●, total activity; □, activity inhibited by tyrosine; △, activity inhibited by phenylalanine.

due to the presence of, at least, two DAHP synthase isoenzymes, one being inhibited by phenylalanine (DAHP synthase (phenylalanine)), the other by tyrosine (DAHP synthase (tyrosine)).

DAHP synthase isoenzyme mutants

Genetic and physiological data. Six *aro*1-C and four *aro*2-C mutants were obtained as already described in Materials and Methods. *Aro*2-C mutants could be selected on minimal medium containing both phenylalanine and tyrosine or tryptophan, because they grew less on these media than on minimal agar alone.

According to their growth on solid media, and to their linkage relationships, mutants can be classified in two groups: *aro*1-C mutants, which grow on minimal agar and show reduced growth on minimal agar (1 mM tyrosine) and *aro*2-C mutants, which also grow on minimal agar, but show reduced growth on minimal agar (1 mM phenylalanine). Growth rates of the single mutants as well as of the double mutants obtained from *aro*1-C × *aro*2-C crosses were tested quantitatively in liquid media, and compared to the wild-type. The amino acids had no effect on growth of the wild-type, in contrast to the mutants. The growth of *aro*1-C and *aro*2-C mutants was not blocked completely in a medium containing a very high concentration (1 mM) of the inhibitory amino acid.

The generation time of 3 h in minimal medium was the same for the mutants and the wild-type. The generation time for *aro*1-C mutants in minimal medium (1 mM tyrosine) was 6.25 h, and for *aro*2-C mutants in minimal medium (1 mM phenylalanine) it was 7.25 h. Double mutants do not grow in minimal medium or minimal medium supplemented with one, or any combination of two amino acids plus 4-aminobenzoate. They do grow, however, after a 48-h lag, in minimal medium (1 mM

tyrosine + 1 mM phenylalanine + 1 mM tryptophan + 10 μM 4-aminobenzoate). If there is no linkage between the two structural genes for DAHP synthase isoenzymes, 50% of the spore progeny of an *aro*1-C × *aro*2-C cross should be of parental type (i.e. *aro*1-C and *aro*2-C) and 50% should be recombinant (wild-type and double mutant). This was in fact observed in all crosses. The double mutants generally constituted less than the expected 25% of the colonies. This can be ascribed to reduced spore germination.

Biochemical data. In crude extracts of *aro*1-C, *aro*2-C and double mutants, activity and inhibition of DAHP synthase were assayed. Table III summarizes the

TABLE III

INHIBITION OF DAHP SYNTHASE ACTIVITY IN CRUDE EXTRACTS OF WILD-TYPE AND MUTANT STRAINS

Cells were disrupted with the Brown homogenizer (except double mutants which were sonicated) and suspended in 0.05 M phosphate buffer (pH 7.0). The amino acid concentration in the assay was 0.5 mM. Enzymic activity is expressed as percentage of that shown by the wild-type. Inhibition (%) is given relative to that shown by the particular strain with no amino acid present assuming the figure in brackets in the first column to represent 100%.

Strain	Enzymic activity (%) (actual values in parentheses)	Inhibition by tyrosine (%)	Inhibition by phenylalanine (%)	Inhibition by phenylalanine + tyrosine (%)
972	100 (41.8)	46	20	60
*aro*1-8C	94 (39.4)	62	5	—
*aro*1-10C	98 (41)	57	5	—
*aro*1-34C	85 (35.5)	58	4	—
*aro*1-35C	90 (37.7)	56	7	—
*aro*1-37C	96 (40)	59	6	—
*aro*1-97C	91 (38)	61	3	62
*aro*2-1C	28 (11.7)	15	74	—
*aro*2-2C	21 (8.8)	10	60	65
*aro*2-4C	23 (9.6)	8	70	—
*aro*2-25C	27 (11.3)	13	68	—
Double mutant *aro*2-4Ch$^+$ × *aro*1-10Ch$^-$	<5	—	—	—

results. Data with the wild-type are shown for comparison. The inhibition of DAHP synthase activity in *aro*1-C and *aro*2-C mutants by tyrosine and phenylalanine respectively is greater than that found in the wild-type. In *aro*1-C an additional weak inhibition by phenylalanine is observed, and in *aro*2-C mutants tyrosine also has a weak inhibitory effect. This weak inhibition could be due to a cross-inhibition of the two amino acids. This phenomenon could also explain the partial additivity of inhibition observed when crude extracts were incubated with both amino acids together. *Aro*1-C mutants did not show a significant decrease in the specific activity as compared to the wild-type, whereas in *aro*2-C mutants the specific activity is about 1/4 of the wild-type. Double mutants do not show any DAHP synthase activity at all. This, together

with the results of growth and genetic experiments, seem to confirm the existence of only two isoenzymes in *S. pombe*.

DISCUSSION

Activity and inhibition of the DAHP synthase were tested in crude extracts of the wild-type, *aro*1-C, *aro*2-C and *aro*1-C *aro*2-C double mutants. The results show that in *S. pombe* at least two DAHP synthase isoenzymes are present. One of these isoenzymes, the DAHP synthase (tyrosine) is feedback inhibited by L-tyrosine, the other one, DAHP synthase (phenylalanine) by L-phenylalanine. Growth experiments with mutants of constitution *aro*1-C (defective in DAHP synthase (phenylalanine)), *aro*2-C (defective in DAHP synthase (tyrosine)) and double mutants (lacking both enzymatic activities) lead to the conclusion that in *S. pombe* the first step in the biosynthesis of aromatic amino acids is catalyzed only by these two isoenzymes.

Two DAHP synthases might characterize yeast species, as two isoenzymes are found in *S. cerevisiae* as well. In this organism, both isoenzyme activities are of the same order, and the DAHP synthase (tyrosine) can be completely inhibited in vivo and in vitro [2] while in *S. pombe* the activity of the phenylalanine-sensitive isoenzyme amounts only to about 1/4 of the total DAHP synthase activity, and neither of the enzymes is completely feedback inhibited.

It cannot be decided to what extent the two isoenzymes are feedback inhibitable in vivo. Neither can quantitative inhibition of the two isoenzymes be calculated from growth experiments with *aro*-C mutants as, on one hand, the growth inhibiting amino acid both inhibits and feeds the mutant (the inhibiting amino acid does not have to be synthesized any more), and on the other hand it is not known up to which level DAHP synthase activity is growth limiting. However, it should be pointed out that growth experiments with the mutants show that even at very high amino acid concentrations (1 mM) the two isoenzymes are not completely inhibited.

Tryptophan does not act as a feedback inhibitor of DAHP synthase as is the case, e.g. in *N. crassa*, where tryptophan inhibits a third DAHP synthase isoenzyme [5]. It is reasonable to assume that in *S. pombe*, the enzyme activity which is not inhibited by tyrosine or phenylalanine might be responsible for the synthesis of tryptophan.

In *N. crassa* the gene for DAHP synthase (tyrosine) is also responsible for the synthesis of 4-aminobenzoate [5]. In *S. pombe* all *aro*1-C and *aro*2-C mutants grow without 4-aminobenzoate in the medium, which would indicate that 4-aminobenzoate is synthesized. However it is not clear why double mutants require a 48-h lag period for growth on a medium supplemented with the aromatic amino acids and 4-aminobenzoate.

S. pombe DAHP synthase shows other similarities with the enzyme of *N. crassa* and *S. cerevisiae*, e.g. in its positive response to Co^{2+} and Mn^{2+}.

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