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STUDIES CONCERNING THE BIOCHEMICAL GENETICS OF ACTIVITY AND FEEDBACK INHIBITION MUTANTS OF *SCHIZOSACCHAROMYCES POMBE* 3-DEOXY-D-*arabino*-HEPTULOSONATE-7-PHOSPHATE SYNTHASE

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SUMMARY

1. When wild-type extracts of *Schizosaccharomyces pombe* were fractionated with Sephadex G-100 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) activity eluted in one peak, whereas with dialyzed extracts two activity peaks appeared. The same was observed with extracts of *aro*2-C mutants. No difference was observed in the elution patterns of the undialyzed and dialyzed extracts of the *aro*1-C strains. Therefore we conclude that DAHP synthase (phenylalanine) can be present in two molecular forms.

2. Feedback inhibition negative mutants were selected and are described.

3. Complementation tests and genetic linkage studies with feedback inhibition-negative mutants are in agreement with the assumption that the allosteric and catalytic mutations are located on the same polypeptide, and that therefore the DAHP synthase is a homomultimeric enzyme.

INTRODUCTION

In the accompanying paper [1] it is shown that the first reaction of the common aromatic amino acid biosynthesis in *Schizosaccharomyces pombe* is catalyzed by two different isoenzymes. One is the 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (7-phospho-2-keto-3-deoxy-D-*arabino*-heptonate -D-erythrose-4-phosphate lyase (puruvate-phosphorylating), EC 4.1.2.15) (DAHP synthase) (tyrosine) which is inhibited by tyrosine, the other is the DAHP synthase (phenylalanine) which is inhibited by phenylalanine. It seems that two DAHP synthase isoenzymes are characteristic for yeast species, as two isoenzymes are also found in *Saccharomyces cerevisiae* [2]. Little is known about the properties of these two isoenzymes. In this paper we describe some properties of the two DAHP synthase isoenzymes of *S. pombe*.

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

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MATERIALS AND METHODS

Media and culture conditions

All media and abbreviations used are described in the accompanying papers[1]. Incubation was at 30 °C.

Strains

Mutants of the type *aro*1-C (defective in DAHP synthase (phenylalanine)) and *aro*2-C (defective in DAHP synthase (tyrosine)) and *aro*1-C *aro*2-C double mutants have been obtained previously and are characterized in the accompanying paper. The isolation of feedback inhibition negative mutants, as well as of *aro*2-C *aro*1-I *aro*1-C triple mutants and of the diploid strains is described below.

Isolation of feedback inhibition negative mutants

*Aro*1-C and *aro*2-C mutants were plated out at a high density (about $6 \cdot 10^6$ cells per plate) on minimal agar (1 mM tyrosine) and minimal agar (1 mM phenylalanine) respectively. On the lawn of non growing cells some colonies appeared. These were reisolated on the same supplemented minimal medium. Extracts were prepared from the selected isolates and were assayed for DAHP synthase activity and inhibition. Feedback inhibition negative mutants derived from *aro*1-C strains are designated as *aro*1-C *aro*2-1 mutants, and those derived from *aro*2-C strains, as *aro*2-C *aro*1-1. The letter I indicates that the second mutation affects the function of feedback inhibition; the locus is known from the genetic tests described in this paper.

Induction of aro2-C aro1-I aro1-C mutants

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis of *aro*2-C *aro*1-I strains was carried out according to Megnet [3]. The treated cells were plated on malt extract agar (0.5 mM tyrosine + 0.5 mM phenylalanine + 0.5 mM tryptophan + 10 μM 4-aminobenzoate). The resulting colonies were printed on minimal agar, minimal agar (1 mM phenylalanine), minimal agar (1 mM tyrosine), minimal agar (1 mM tryptophan) and minimal agar (1 mM phenylalanine + 1 mM tyrosine + 1 mM tryptophan + 10 μM 4-aminobenzoate). The colonies which grew only on the minimal agar with the four supplements were tested for DAHP synthase activity. If they showed no activity it was assumed that they were triple mutants.

Construction of diploid strains

For complementation tests diploid strains were obtained according to the method developed by Leupold [4].

Growth experiments

These experiments were carried out as described in the accompanying paper [1].

Random spore analysis

The procedure was already described by Leupold [4].

Culturing of cells and preparation of crude extracts

Cells were grown, harvested and disrupted by two methods as described in

the accompanying paper [1]. Cells were lyophilized and disrupted in a Brown homogenizer when the extracts were used for fractionation on Sephadex columns. For all other experiments, fresh cells were disrupted with the sonifier. When required crude extracts were dialyzed for 12–15 h against 0.1 M phosphate buffer (pH 7.0).

DAHP synthase assay

The test is described in the accompanying paper [1].

Protein test

Protein was determined according to the method of Lowry et al. [5].

Gel filtration

Sephadex G-100 gels were prepared and packed as described in "Sephadex-Gelfiltration in theory and practice" (Pharmacia Fine Chemicals, Uppsala, Sweden).

RESULTS

Activity negative mutants

For an initial characterization of the DAHP synthase isoenzymes, we studied the effect of pH and ionic strength of the buffer on DAHP synthase activity and its inhibition by tyrosine and phenylalanine. For a separate analysis of the two isoenzymes, we used extracts of an *aro*1-C and an *aro*2-C mutant, defective in DAHP synthase (phenylalanine) and in DAHP synthase (tyrosine), respectively. The results (Table I and Table II) show that activities and their inhibition by tyrosine and phenylalanine vary greatly with pH and buffer changes.

TABLE I

DEPENDENCE ON pH OF DAHP SYNTHASE ACTIVITY AND INHIBITION

Cells of mutant strains *aro*1-10C and *aro*2-25C were disrupted with the sonifier in 0.05 M phosphate buffer (pH 7.0). 5 μl of the crude extract, conveniently diluted, were given to the test.

pH	Extract of *aro*1-10C		Extract of *aro*2-25C	
	Enzymic activity	Inhibition by tyrosine (%)	Enzymic activity	Inhibition by phenylalanine (%)
5.0	27	84	3.8	85
5.5	36.1		10.2	
6.0	42	71	11.8	87
6.5	42.5		12.5	
7.0	42	53	10.5	49
7.5	41		9.0	

When dialyzed and undialyzed wild-type extracts are fractioned with Sephadex G-100, two different elution patterns of enzyme activity are observed: in the undialyzed extract there is only one activity peak, whereas in the dialyzed extract two activity peaks appear (Fig. 1a). The peak eluting in the earlier fractions will be referred to as high apparent molecular weight form. To determine if both enzymes

TABLE II

DEPENDENCE ON BUFFER CONCENTRATION OF DAHP SYNTHASE ACTIVITY AND INHIBITION

The mutant strains used and the procedure followed were the same as described for Table I.

Buffer molarity (M)	Extract of *aro*1-10C		Extract of *aro*2-25C	
	Enzymic activity	Inhibition by tyrosine (%)	Enzymic activity	Inhibition by phenylalanine (%)
0.025	41	15	10.2	32
0.05	41	34	10.4	50
0.1	40.5	57	9.4	62
0.2	32	66	7.7	72

participate in the high molecular weight form, we examined dialyzed and undialyzed extracts of *aro*1-C and *aro*2-C mutants by gel filtration on Sephadex G-100. In all six tested *aro*1-C mutants there is no difference in the elution patterns of the undialyzed and dialyzed extracts (Fig. 1b). On the other hand, in all four tested *aro*2-C mu-

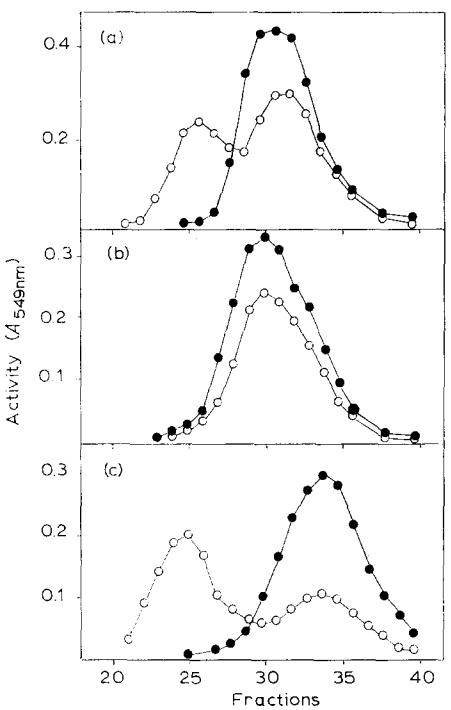

Fig. 1. Elution patterns of dialyzed and undialyzed extracts of the wild-type 972 and of *aro*1-C and *aro*2-C mutants. Undialyzed and dialyzed extracts were layered on a Sephadex G-100 column (2 cm × 58 cm). The following sample volumes were applied on the column: wild-type 972 (1a), 0.8 ml (30 mg protein/ml); *aro*1-10C (1b), 0.8 ml (25 mg protein per ml) and *aro*2-25C (1c), 1.6 ml (25 mg protein per ml). Elution was with 0.1 M buffer (pH 7.0) at a rate of 17 ml/h. Fractions of 2 ml were collected. The protein front elutes in fraction 24. ●, activity in undialyzed extracts; ○, activity in dialyzed extracts.

tants two peaks with DAHP synthase activity are found with dialyzed extracts, while in the undialyzed extracts only one peak appears (Fig. 1c). These results show that of the two isoenzymes, most probably only DAHP synthase (phenylalanine) takes part in the formation of the high molecular weight form. Preliminary results were obtained by Sephadex G-100 filtration according to the method described in ref. 6. The column (2 cm × 58 cm) was calibrated with proteins of known molecular weights: cytochrome *c* (12 500), bovin albumin (134 000 and 67 000), egg albumin (45 000) and bovin γ-globulin (160 000). A molecular weight of about 125 000 and 45 000 for the two forms of the DAHP synthase (phenylalanine) and 75 000 for DAHP synthase (tyrosine) was calculated.

In the course of the experiments, some differences in stability and in inhibition by phenylalanine were uncovered in the two forms of DAHP synthase (phenylalanine). Therefore we wanted to find out whether the high molecular weight form had any function in the mechanism of feedback inhibition. In analogy to *Neurospora crassa*, where a change in the molecular weight represents a part of the inhibitory mechanism [7], it could be possible that the amino acids present in the undialyzed extract inhibit the formation of an aggregate. Our experiments indicated that this is not the case in *S. pombe*. Crude extracts dialyzed and fractionated in the presence of phenylalanine, tyrosine and tryptophan on a preequilibrated Sephadex G-100 column showed the same elution pattern as crude extracts dialyzed and fractionated in buffer not containing aromatic amino acids. (The concentration of phenylalanine, tyrosine and tryptophan in the buffer that was used to dialyze, pre-equilibrate the column and fractionate the extracts was 10 μM. The aromatic amino acids in the eluting buffer were diluted in subsequent analysis of fractions so giving negligible inhibition.)

Feedback inhibition-negative mutants

The activity of 91 mutants, obtained from *aro*1-C strains on the basis of their growth on minimal agar (1 mM tyrosine), was tested. Eight mutants were found to be feedback inhibition negative (Table III). The enzyme activity in these mutants is remarkably lower than in the wild-type or in *aro*1-C mutants. Preliminary results indi-

TABLE III

DAHP SYNTHASE ACTIVITY AND INHIBITION OF *aro*1-C *aro*2-I MUTANTS

Cells were suspended in 0.1 M phosphate buffer (pH 6.5), disrupted with the sonifier and tested for activity and inhibition as described in the accompanying paper [1]. Two *aro*1-C strains and wild-type 972 served as a control.

Strains	Enzymic activity	Inhibition by tyrosine (%)
972	45	45
*aro*1-97C	40	58
*aro*1-37C	41	55
*aro*1-97C *aro*2-4I	10	−6
*aro*1-10C *aro*2-71I	5	2
*aro*1-34C *aro*2-80I	15	13
*aro*1-97C *aro*2-92I	30	9
*aro*1-97C *aro*2-37I	7	22

cate that the activity of these feedback inhibition-negative mutants is altered relative to temperature sensitivity, Co^{2+} stimulation and pH dependence in comparison to the non mutated enzyme. 30 mutants showed no activity at all. We assume (see discussion) that these are also feedback inhibition negative, but that as a result of the mutation, their enzyme activity in crude extracts is very labile. *Aro*2-C *aro*1-I mutants were selected on the basis of their growth on minimal agar (1 mM phenylalanine). 64 clones were tested for activity and inhibition by phenylalanine. Table IV shows the results obtained

TABLE IV

DAHP SYNTHASE ACTIVITY AND INHIBITION OF *aro*2-C *aro*1-I MUTANTS

The procedure to disrupt cells and test the extracts was the same as described for Table III.

Strains	Enzymic activity	Inhibition by phenylalanine (%)
972	45	22
*aro*2-5C	10	61
*aro*2-2C	9	65
*aro*2-2C *aro*1-14I	12	0
*aro*2-2C *aro*1-16I	10	−6
*aro*2-6C *aro*1-25I	11	4
*aro*2-6C *aro*1-26I	10	4
*aro*2-6C *aro*1-27I	9	0
*aro*2-5C *aro*1-54I	8	6
*aro*2-5C *aro*1-55I	9	5
*aro*2-5C *aro*1-56I	10	5
*aro*2-1C *aro*1-65I	11	−1
*aro*2-1C *aro*1-66I	9	−6
*aro*2-2C *aro*1-70I	9	0

with ten mutants which are clearly feedback inhibition negative. In many other mutants not presented here inhibition was reduced, as compared with *aro*2-C mutants. In all cases activity was similar to the non mutated enzyme of *aro*2-C strains. Mutants growing on media containing phenylalanine or tyrosine, and which are not feedback negative, are probably permease defective, or revertants, or not completely feedback negative. The growth of *aro*1-C *aro*2-I and *aro*2-C *aro*1-I mutants was tested quantitatively in liquid media and compared to the *aro*-C mutants they were derived from. These results confirmed the results obtained on solid media, and the assumption that the 30 mutants already mentioned, which show no detectable activity in crude extracts, actually possess activity in vivo.

In the analysis of feedback inhibition-negative mutants we believed to find another way to study a possible relation between the high and low molecular weight forms of DAHP synthase (phenylalanine) and feedback inhibition. Thus crude extracts of five *aro*2-C *aro*1-I mutants were examined on a Sephadex G-100 column, with and without previous dialysis of the extracts. The results shed no light on the problem. The mutants presented either both peaks (corresponding to the high and low molecular weight forms), or only the low molecular weight form peak, or even an intermediate form.

Linkage relationships

As the DAHP synthase is an allosteric enzyme [8], it seemed interesting to determine whether the allosteric and the catalytic sites are located on the same polypeptide chain or not, i.e. if the enzyme is homo- or heteromultimeric. Two different genetic methods have been applied for resolving this problem. When a feedback inhibition negative mutant is crossed to an *aro*1-C *aro*2-C double mutant, 25% of the spore progeny will show the phenotype of the *aro*-C mutant from which the feedback inhibition-negative mutant has been derived, if the allosteric and catalytic sites are coded for by two unlinked genes. If the two sites are linked, however, 50% of the progeny will present the double mutant and 50% the feedback inhibition-negative phenotype. This was in fact observed in all crosses. Ten *aro*2-C *aro*1-I and ten *aro*1-C *aro*2-I mutants were crossed to double mutants, and 500–1000 colonies of each cross were analyzed. Thus it seems that the allosteric and catalytic sites are coded for by the same gene or at least by two very closely linked genes.

In order to differentiate between these two possibilities, complementation tests are carried out [9]. As there is no selective medium for feedback inhibition negative mutants, more complex procedures had to be applied.

We have indications (see above) that many *aro*1-C *aro*2-I mutants show no detectable activity because of the greater instability of their DAHP synthase in vitro. We crossed three of these mutants to *aro*1-C *aro*2-C double mutants. If complementation were present, diploid strains of constitution $\frac{aro1\text{-}C\ aro2\text{-}I\ +}{aro1\text{-}C\ +\ aro2\text{-}C}$ should show DAHP synthase activity when crude extracts are tested. However, in the diploids obtained from our crosses we detected no DAHP synthase activity at all, as in the *aro*1-C *aro*2-I parent. Therefore it seems that there is no complementation between the allosteric and catalytic sites of DAHP synthase (tyrosine).

When an *aro*2-C *aro*1-I mutant is mutated further, so that both catalytic sites are inactive (thus leading to an *aro*1-C *aro*2-C double mutant phenotype), and crossed to an *aro*2-C mutant, complementation can be manifested in vivo if present. Diploid strains of constitution $\frac{aro2\text{-}C\ aro1\text{-}I\ aro1\text{-}C}{aro2\text{-}C\ +\ +}$ should show an *aro*2-C *aro*1-I phenotype if the inactive allosteric site of the *aro*2-C *aro*1-I *aro*-C triple mutant complements with the active catalytic site of the *aro*2-C mutant. Crosses containing diploid zygotes can be plated out on minimal agar (1 mM phenylalanine + Magdala red 20 mg/l) and growth of feedback inhibition-negative colonies would be a consequence of complementation. In our experiments, however, we observed no complementation. The lack of complementation between the catalytically defective mutants with the corresponding inhibition negative mutants seems to indicate that each of the isoenzymes is coded only by one gene, i.e. that both the DAHP synthase (phenylalanine) and the DAHP synthase (tyrosine) are most probably homomultimeric enzymes.

DISCUSSION

By analyzing *aro*1-C and *aro*2-C mutants we have shown that the DAHP synthase (phenylalanine) can be present in a high and low molecular weight form. The

high molecular weight form appears when crude extracts are dialyzed. For the DAHP synthase (tyrosine) we found only one form. The object of many of our experiments was to find a relationship between feedback inhibition and the two forms of DAHP synthase (phenylalanine) as it is known to exist in *N. crassa* [7]. However, all our attempts failed to achieve this.

The presence of phenylalanine, tyrosine or tryptophan in *aro*2-C extracts did not prevent the formation of the high molecular weight form, and the results of molecular sieving in the case of feedback inhibition-negative mutants indicate any clear relationship between the two forms. Nevertheless it is not excluded that feedback inhibition might be related somehow to a change in the molecular organization, but this relationship is by no means as simple and clear as in *N. crassa* [7]. Also the possibility of an association of the low molecular weight form with other enzyme activities involved in the biosynthesis of the aromatic amino acids [10] will have to be taken into account in further investigations.

Feedback inhibition-negative mutants derived from *aro*2-C strains (*aro*2-C *aro*1-I) had the same specific activity as their ancestors. This was not the case for *aro*1-C *aro*2-I mutants. Compared with the *aro*1-C mutants they showed a very low specific activity. It is possible that as Halsall and Catcheside [11] already pointed out for the DAHP synthase (tyrosine) and DAHP synthase (phenylalanine) in *N. crassa* the single mutation alters not only the tyrosine inhibition site but also affects the catalytic site or enzyme stability and therefore exhibits a pleiotropic effect. It is conceivable that in the 30 "*aro*1-C *aro*2-I" mutants which fail to show DAHP synthase activity in vitro, the pleiotropic effect of the induced mutation is extremely strong.

The results of our complementation tests and of our linkage studies are in agreement with the assumption that the allosteric and catalytic mutations are located on the same polypeptide, and that therefore the DAHP synthase is a homomultimeric enzyme, as it is known for the three DAHP synthases in *N. crassa* [12].

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