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APER

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Regulative fine-tuning of the two novel DAHP isoenzymes aroFp and aroGp of the filamentous fungus *Aspergillus nidulans*

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Abstract Two novel genes, *aroF* and *aroG*, from the filamentous fungus *Aspergillus nidulans* were isolated and the regulative fine-tuning between the encoded, differentially regulated 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthases was analyzed. A wide range of DAHP synthase isoenzymes of various organisms are known, but only a few have been characterized further. DAHP synthases (EC 4.1.2.15) catalyze the first committed step of the shikimate pathway, which is a putative target for anti-weed drugs. The reaction is the condensation of erythrose-4-phosphate (E4P) and phosphoenolpyruvate (PEP) to yield DAHP. The two purified DAHP synthases showed different affinities for the substrates: 175 μM for PEP and 341 μM for E4P for the aroFp isoenzyme and weaker affinities of 239 μM (PEP) and 475 μM (E4P) for the aroGp isoenzyme. The enzymes are differentially regulated by tyrosine (aroFp) and phenylalanine (aroGp). The calculated k_{cat} values are 7.0 s^{-1} for the tyrosine-inhibitable (aroFp) and 5.5 s^{-1} for the phenylalanine inhibitable (aroGp) enzyme. Tyrosine is a competitive inhibitor of the aroFp DAHP synthase in its reaction with PEP. Phenylalanine is a competitive inhibitor of the isoenzyme aroGp in its reaction with E4P. Both enzymes are inhibited by the chelating agent EDTA, which indicates a metal ion as cofactor.

Keywords *Aspergillus nidulans* · Protein purification · Enzyme kinetics · Regulation · 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase

Abbreviations *DAHP* 3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase · *PEP* phosphoenolpyruvate · *E4P* erythrose-4-phosphate

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Introduction

The enzyme 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHP synthase) catalyzes the first step of the aromatic amino acid biosynthetic pathway, i.e., the condensation of phosphoenolpyruvate (PEP) and erythrose-4-phosphate (E4P) to DAHP and inorganic phosphate (Haslam 1993). A number of DAHP synthase isoforms, distinguished according to their regulatory properties, exist in microorganisms and plants (Byng et al. 1982; Byng and Jensen 1983). The only known DAHP synthases from filamentous fungi are the three DAHP synthase isoforms of *Neurospora crassa*. Each is regulated by one of the following three aromatic amino acids: phenylalanine, tyrosine, and tryptophan (Hoffman et al. 1972; Nimmo and Coggins 1981a, b). Only one of the three isoforms, the tryptophan-inhibitable protein, has been analyzed further.

Although all DAHP synthases catalyze the same reaction, they differ in size and mode of competition. On average, there is a size spectrum of 350 amino acids for *Escherichia coli* enzymes, 370 amino acids for the yeast *Saccharomyces cerevisiae* enzymes, and 540 amino acids for the plant DAHP synthases. Catalytic analyses have given insight into the mode of action of specific inhibitors. In yeast, tyrosine acts as a competitive inhibitor of DAHP synthase in its reaction with PEP and as a noncompetitive inhibitor of the enzyme in its reaction with E4P. Conversely, phenylalanine is a competitive inhibitor of DAHP synthase in its reaction with E4P and a noncompetitive inhibitor of the enzyme in its reaction with PEP (Paravicini et al. 1989; Schnappauf et al. 1998). Three isoforms of DAHP synthase exist in *E. coli*, each regulated by one of the three aromatic amino acids. Tyrosine reacts with the synthase as in yeast, whereas phenylalanine competitively inhibits interaction of the enzyme with both PEP and E4P (Schoner and Herrmann 1976; Staub and Denes 1969).

The activity of all characterized DAHP synthases depends on bivalent metal ions. Various bivalent metal ions are able to restore enzyme activity either partially or fully

after complexing of the native metal with the chelating agent EDTA (Stephens and Bauerle, 1991).

The DAHP synthases accelerate the input reaction of the shikimate pathway, which means that their activities must be strictly regulated. In filamentous fungi, a regulatory network acts on gene expression, the so-called cross pathway control (Paluh et al. 1988). The final effector of this system is a transcriptional activator protein that binds to conserved sequence elements in the promoter regions of target genes. Under conditions of amino acid starvation, the transcription of these target genes is induced to counteract the starvation. The cross pathway control network regulates all characterized genes coding for DAHP synthases of fungal origin.

To date, no DAHP-synthase-encoding gene of the filamentous fungus *Aspergillus nidulans* has been identified. Here we present a detailed characterization of the *aroF* and *aroG* gene products, two proteins that regulate the flux into the shikimate pathway in *A. nidulans*.

Materials and methods

Strains, media, libraries, growth conditions

Saccharomyces cerevisiae strain RH2424 (*MATa, can1–100, GAL, aro3*::*HIS3, aro4*::*LEU2, ura3–1*) was used as recipient to clone the *aroF* and *aroG* genes by complementation of the auxotrophy, employing an inducible *A. nidulans* cDNA expression library (Hoffmann et al. 2000). The genomic regions were determined using the chromosome-specific recombinant cosmid-library of *A. nidulans* constructed by Brody et al. (1991). Transformation of *S. cerevisiae* was carried out by the LiOAc method (Ito et al. 1983). Minimal vitamins medium for the cultivation of yeast was described earlier (Miozzari et al. 1978). All chemicals were supplied by FLUKA or Sigma-Aldrich (Steinheim, Germany).

RNA preparation and analysis

Total RNA was prepared from vegetatively growing *A. nidulans* cultures using TRIzol reagent (Life Technologies, Rockville, Md.) following the suppliers' instructions. Transcript levels were analyzed by Northern hybridization (Alwine et al. 1977) using a Bio-Imaging Analyzer (Fuji Photo Film, Tokyo, Japan).

Purification of *A. nidulans* DAHP synthases

For overexpression, a derivative of plasmid p426MET25 (Mumberg et al. 1994) was used in *S. cerevisiae* strain RH2424. The plasmid-carrying yeast cells were grown at 30 °C in 10-l rotatory fermentors under aeration. Cells were harvested in mid-exponential phase at an OD_{546} of 3–4. For a typical purification procedure, 15 g of cells (wet wt.) were used.

The DAHP synthases from *A. nidulans* were purified according to the protocol of Schnappauf et al. (1998) with the following modifications: the anion-exchange chromatography was carried out with 10 mM potassium phosphate (pH 7.6) buffer instead of 10 mM Tris-HCl buffer. DAHP synthases were detected by SDS-PAGE (Laemmli 1970) and enzyme assays. Protein solutions were concentrated in stirred cells with PM-10 ultra filtration membranes from Millipore (Eschborn, Germany). Protein concentrations were determined using the Bradford (1976) assay.

Western blot analysis

DAHP synthases of *A. nidulans* were immunologically detected with a polyclonal rabbit antibody against the tyrosine-inhibitable DAHP synthase of *S. cerevisiae*. A second antibody with horseradish peroxidase activity was detected using the ECL-method (Tesfaigzi et al. 1994).

Enzyme assays

The DAHP synthase activities were determined with the stop assay described by Takahashi and Chan (1971) with the modifications described by Schnappauf et al. (1998). Enzyme activities are specified in International Units (1 U=appearance of 1 µmol product/min). Specific enzyme activities are given as mU (mg protein)$^{-1}$.

Determination of kinetic parameters

Initial velocity data were determined by varying the concentration of one substrate at various fixed concentrations of the second substrate (Bisswanger, 1979). Double-reciprocal plots of the initial velocity vs concentrations of the varied substrate showed a set of intersecting lines. A plot of the interceptions of these lines with the ordinate vs the reciprocal of the concentrations of the fixed substrate gave a straight line. The Michaelis constants were determined from the value of these lines on the abscissa, corresponding to the reciprocal of the absolute value. The reciprocals of the apparent maximal velocities $1/V_{max}$ were on the ordinate. Rate constants k were determined from these values, and the known enzyme concentration e_o was determined according to $k=V_{max}/e_o$. Inhibitory constants were determined by varying the concentrations of a single substrate under various fixed concentrations of the inhibitor. The second substrate was kept at a constant concentration. The data were transformed to double-reciprocal plots. The K_i value was determined from secondary plots of the reciprocal intersections of the lines with the ordinate (noncompetitive inhibition) or the slopes of the lines (competitive inhibition) against inhibitor concentration. The intersection with the abscissa provided the value for K_i.

Results

Isolation of the *aroF* and *aroG* genes of *Aspergillus nidulans*

The genes *aroF* and *aroG* of *A. nidulans* encode a phenylalanine- and a tyrosine-inhibitable DAHP synthase, respectively. Both genes were isolated by functional complementation of the auxotrophic *S. cerevisiae* strain (RH2424) in which the *ARO3* and *ARO4* genes encoding the two DAHP synthases were deleted. This yeast strain is devoid of endogenous DAHP synthase activity and therefore not able to grow on minimal medium without supplementation of the three aromatic amino acids phenylalanine, tyrosine, and tryptophan. *S. cerevisiae* strain RH2424 was transformed with an *A. nidulans* cDNA expression library under the control of the yeast *GAL1* promoter (Hoffmann et al. 2000). Transformants were selected on medium without aromatic amino acids containing 2% galactose as sole carbon source to induce the *GAL1* promoter and thus expression of the putative DAHP-synthase-encoding cDNAs. Sixty clones whose growth phenotype was rescued by the expression of a plasmid-encoded *A. nidulans* cDNA were

Table 1 Protein sequence alignment of the DAHP synthases from *Aspergillus nidulans*, *Saccharomyces cerevisiae* and *Escherichia coli*. The data show the percentages of the sum of sequence identity and the rate of high similarity. The calculated values are based on the alignment method ClustalW (weight matrix: BLOSUM; window size: 5; gap penalty: 3) and were carried out at the IBCP, Lyon (France); http://pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html

ClustalW (%)	*A. nidulans*		*S. cerevisiae*			*E. coli*	
	aroFp	aroGp	Aro3p	Aro4p	AroF	AroG	AroH
A. nidulans							
aroFp	100	74.3	74.0	75.9	68.9	68.0	65.2
aroGp	74.3	100	77.9	73.7	66.9	69.0	62.3
S. cerevisiae							
Aro3p	73.4	77.7	100	76.4	69.8	70.4	62.8
Aro4p	75.9	73.7	76.4	100	66.5	69.7	64.2
E. coli							
AroF	68.9	66.3	69.8	66.5	100	70.8	66.5
AroG	68.3	69.0	70.4	69.3	69.8	100	74.9
AroH	65.2	62.3	62.8	64.2	66.5	74.9	100

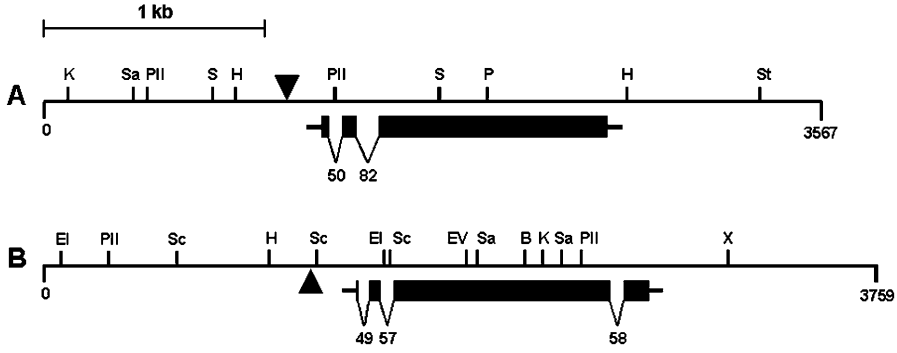

Fig. 1 Structure of the *aroF* (**A**) and *aroG* (**B**) genes of *Aspergillus nidulans*. The chromosomal loci of the two genes are shown. The GeneBank accession numbers for these sequences are AF283008 for the *aroF* gene and AF283009 for the *aroG* gene. The *black boxes* show the ORFs, which are interrupted by introns, two in *aroF* and three in *aroG*. The ▼ indicates the putative CpcAp/Gcn4p binding sites. *B Bam*HI, *EI Eco*RI, *EV Eco*RV, *H Hin*dIII, *K Kpn*I, *P Pvu*I, *PII Pvu*II, *S Sph*I, *Sa Sal*I, *Sc Sac*I, *St Stu*I, *X Xba*I. *Numbers* indicate base pairs

isolated. Restriction digest analysis of the recovered plasmids revealed that two different cDNAs were able to complement the yeast auxotrophy. One cDNA of each group was subcloned into pME1513 for overexpression and DAHP synthase activity measurements in strain RH2424. Specific enzyme tests from crude extracts revealed that the two cDNAs encode DAHP synthases, one inhibited by tyrosine and the other by phenylalanine. No inhibition by tryptophan was detected. Even careful rescreening of the cDNA library never revealed any gene encoding a tryptophan-dependent DAHP synthase. This suggests that no tryptophan-inhibitable DAHP synthase exists, although it cannot be completely excluded that a third gene exists which is not significantly expressed under hyphal growth conditions. In accordance with the putative homologous genes of *E. coli*, the genes of *A. nidulans* were named *aroF* and *aroG*. Both cDNAs were analyzed by DNA sequencing. *aroF* has an ORF of 1089 bp encoding 362 codons with the capacity to express a polypeptide with a deduced molecular mass of 39.3 kDa. The ORF of *aroG* is 1125 bp long, encoding 374 codons and

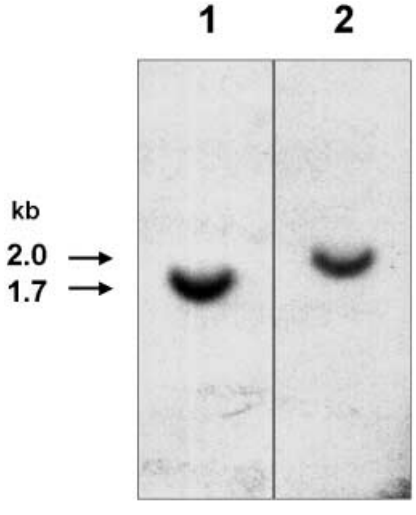

Fig. 2 Southern hybridization analysis of genomic DNA prepared from *A. nidulans* strain FGSC A234 (*yA2, pabaA1, veA1*). The Southern hybridization was carried out as described in Liu et al. (1992). The genomic DNA was digested with *Hin*dIII (*1*) or *Hin*dIII/*Xba*I (*2*), separated on an agarose gel, blotted onto nitrocellulose and probed with ^{32}P-labeled full-length *aroF* cDNA (*1*) and *aroG* cDNA (*2*)

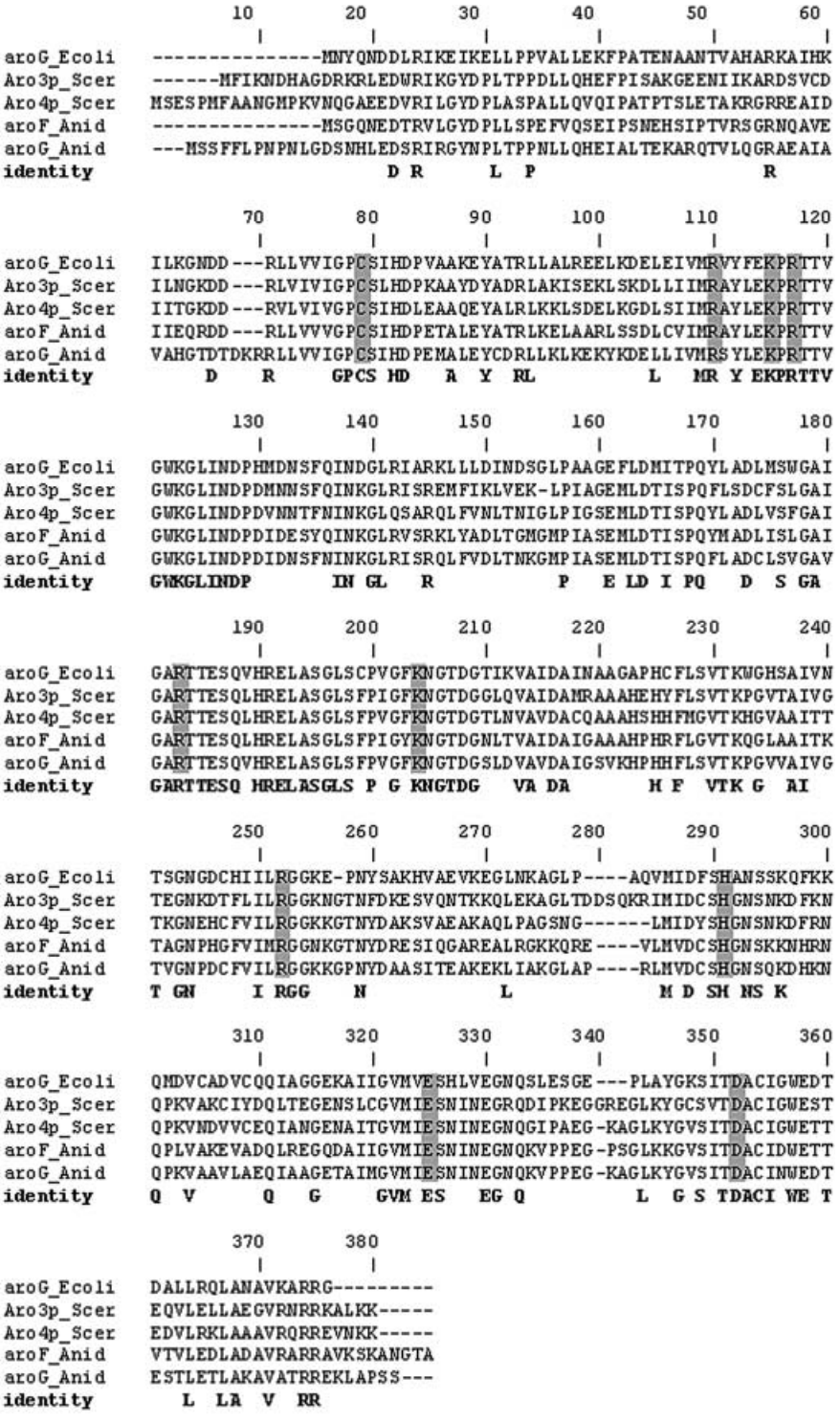

Fig. 3 Sequence alignment of the *aroG*-encoded DAHP synthase (phenylalanine-inhibitable) of *E. coli* with the *A. nidulans* and *Saccharomyces cerevisiae* DAHP synthases. The residues shaded in *light gray* indicate the metal- and PEP-binding sites of the phenylalanine-inhibitable DAHP synthase of *Escherichia coli*, the crystal structure of which was recently published (Shumilin et al. 1999). The *bottom row* shows amino acids that are identical in all five sequences in this alignment

resulting in a protein with a deduced molecular mass of 40.5 kDa. The deduced amino acid sequences show significant stretches of amino acid residues identical to those of known DAHP synthases, suggesting homologies between the encoding genes. The sum of residues identical and similar to those of the *S. cerevisiae* and *E. coli* enzymes varies between 62.3 and 77.7% (Table 1). The genomic loci of the two genes were isolated from a chromosome-wise ordered cosmid library by colony hybridization of the cDNAs and shown to be on chromosome V (*aroF*) and chromosome VIII (*aroG*) (Fig. 1). In a Southern hybridization experiment with the full-length cDNAs as probes, there was no detectable cross-hybridization between the two genes (Fig. 2). No additional band was visible that could have been evidence for a third DAHP synthase of *A. nidulans*. The *aroF* ORF contains two introns,

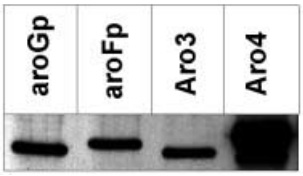

Fig. 4 Western blot analysis of the two DAHP synthases from *A. nidulans*. Crude extracts of yeast strain RH2424 harboring different 2-μm plasmids were analyzed with a polyclonal rabbit antibody raised against the Aro4p of *S. cerevisiae*, showing structural similarities of aroFp and aroGp to that enzyme. Each lane was loaded with 0.8 μg protein

50 and 82 bp long; three introns interrupt the *aroG* coding sequence with lengths of 49, 56, and 57 bp.

Structural similarities between the DAHP synthases of *S. cerevisiae*, *E. coli*, and *A. nidulans*

The phenylalanine-inhibitable *E. coli* enzyme was the first DAHP synthase whose crystal structure has been determined (Shumilin et al. 1999) and thus it can be used for structure studies of other DAHP synthases. A deduced amino acid sequence alignment between the *aroF*- and *aroG*-encoded DAHP synthases of *A. nidulans*, the Aro3p and Aro4p enzymes of *S. cerevisiae*, and the phenylalanine-inhibitable DAHP synthase of *E. coli* revealed a high degree of identity between these enzymes (Fig. 3). Furthermore, the crucial residues involved in metal- and PEP-binding in the *E. coli* enzyme are highly conserved in both *A. nidulans* enzymes. This suggests that the enzymes from all three organisms may have similar three-dimensional structures. Using the purified yeast Aro4 protein, we had previously raised polyclonal antibodies against the *S. cerevisiae* enzyme. By Western blot analysis, we could show that this antibody also specifically recognizes the yeast Aro3 protein and both *A. nidulans* DAHP synthases aroFp and aroGp, when overexpressed in yeast (Fig. 4). This corroborates the results of the theoretical alignment studies, that there are strong structural similarities between the DAHP synthases of these organisms.

The *aroF* and *aroG* genes are transcriptionally regulated by amino acid starvation

Putative CpcAp-/Gcn4p-binding sites were found in the *aroF* and *aroG* promoter regions (Dutton et al. 1997). The upstream positions of these sequence elements are –161 bp (5′-TTGAGTCTG-3′) and –220 bp (5′-GGATGACTCC-3′), respectively, relative to the start codon. The presence of these binding sites suggests that *aroF* and *aroG* are regulated by the cross pathway control of amino acid biosynthesis by CpcA, the protein encoded by the *A. nidulans* homologue of the yeast *GCN4* gene. In fungi, amino acid starvation induces the expression of a genetic network including numerous amino acid biosynthesis

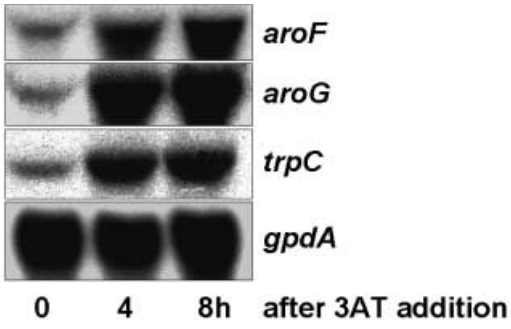

Fig. 5 Northern hybridization analysis of total RNAs prepared from *A. nidulans* strain FGSC A234 (*yA2*, *pabaA1*, *veA1*). The autoradiograph shows the various time points after shifting to 3-amino-1,2,4-triazole (*3AT*)-containing medium. Each lane was loaded with 20 μg total RNA. The RNA was labeled with probes specific for *aroF*, *aroG*, *trpC* or *gpdA*

genes. The central transcriptional activators Gcn4p of yeast, *cpcA* of *Aspergillus niger* (Wanke et al. 1997), and *cpc-1* of *N. crassa* (Paluh et al. 1988) have been characterized and shown to bind to conserved target sequences within regulated promoters. As there are putative binding sites for these transcription factors in the promoter regions of *aroF* and *aroG*, the effect of amino acid starvation on the expression level of these genes was tested. *A. nidulans* strain FGCS A234 (*yA2*, *pabaA1*, *veA1*) was cultivated in liquid minimal medium for 20 h before the mycelia were transferred to fresh medium containing 3-amino-1,2,4-triazole, which acts as a false feedback inhibitor in the histidine biosynthesis pathway and therefore mimics amino acid starvation by depletion of the histidine pool (Hilton et al. 1965). Mycelia were harvested after different time points, and total RNA was prepared and subjected to Northern hybridization analysis using the *aroF* and *aroG* cDNAs as probes (Fig. 5). As controls, the expression of *gpdA* (Punt et al. 1988), a gene encoding a glycolytic enzyme (glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12) that is constitutively expressed, and *trpC*, a tryp-

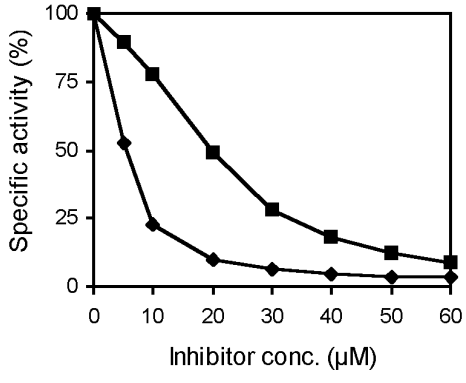

Fig. 6 Inhibition of the DAHP synthases from *A. nidulans* with their specific inhibitors tyrosine for aroF (■) and phenylalanine for aroG (◆). There were no cross-inhibition effects detectable within the range shown. In addition, no inhibition by tryptophan was detected

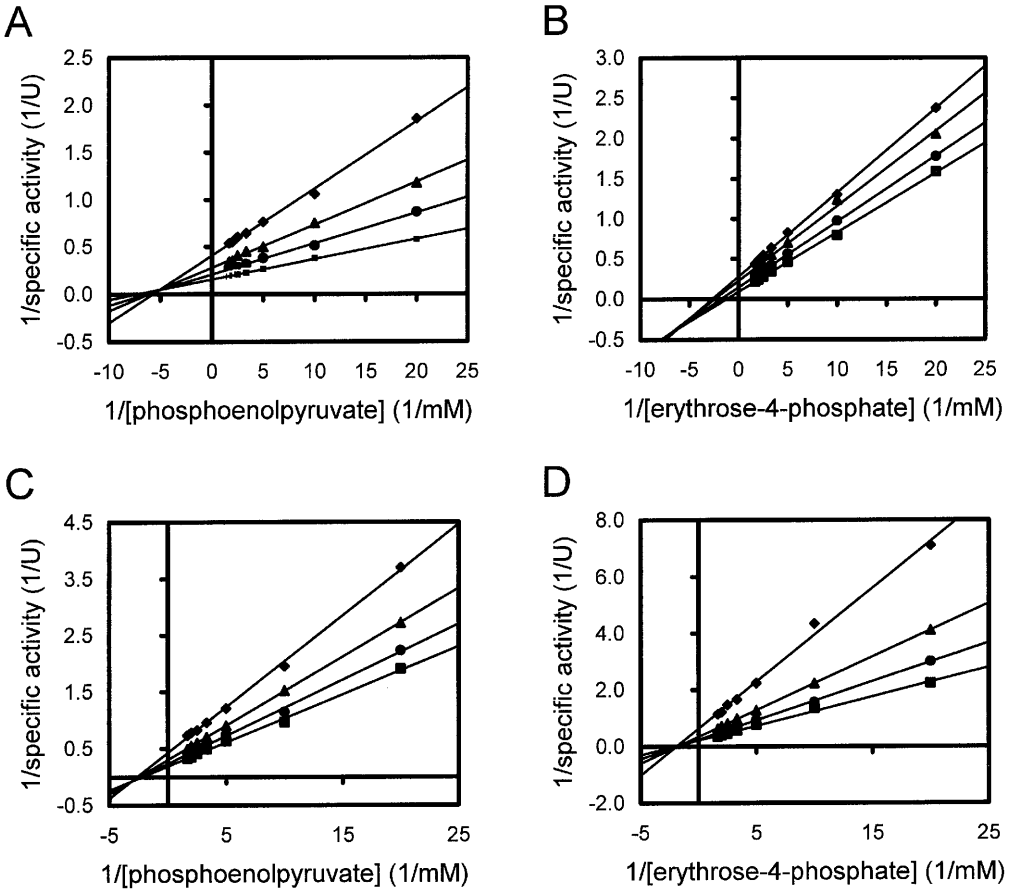

Fig. 7A–D Substrate saturations of the tyrosine- (**A**, **B**) and phenylalanine-inhibitable (**C**, **D**) DAHP synthases of *A. nidulans*. **A**, **C** Lineweaver-Burk plot with various phosphoenolpyruvate concentrations and fixed erythrose-4-phosphate concentrations of ◆ 0.1 mM, ▲ 0.2 mM, ● 0.3 mM and ■ 0.4 mM. **B**, **D** Lineweaver-Burk plot with various erythrose-4-phosphate concentrations and fixed phosphoenolpyruvate concentrations of ◆ 0.05 mM, ▲ 0.1 mM, ● 0.15 mM and ■ 0.2 mM

tophan biosynthesis gene reported to be induced upon amino acid starvation by the cross pathway control (Eckert et al. 1999), were tested. Genes *aroF* and *aroG* were induced by a factor of 3.5 when 3-amino-1,2,4-triazole was added to the culture. The induction was kinetically and quantitatively similar to the induction of the *trpC* gene (Yelton et al. 1983). This result suggests that *aroF* and *aroG* are targets of the cross pathway control network, which is activated by the environmental signal of amino acid starvation.

Feedback regulation of the *aroF*- and *aroG*-encoded DAHP synthases by tyrosine and phenylalanine, respectively

In order to characterize the enzymatic properties of aroFp and aroGp, the respective genes were overexpressed in the yeast strain RH2424 under the control of the yeast *MET25* promoter, and the proteins were purified as described in Materials and methods. The proteins were enriched 60-fold and purified to 95% homogeneity to analyze the enzyme activities and inhibition patterns of the two DAHP synthases of *A. nidulans* at various inhibitor concentrations (Fig. 6). This inhibition study revealed a specifically different behavior of the isoenzymes towards increasing inhibitor concentrations. aroGp, which is inhibited by phenylalanine, is more sensitive to its inhibitor than the isoenzyme aroFp is to its inhibitor tyrosine. At an inhibitor concentration of 10 μM, the difference between the enzyme activities was 55% in favor of the tyrosine-inhibited enzyme aroFp. This is in remarkable contrast to *S. cerevisiae*, where the sensitivities of the enzymes to the corresponding inhibitors are reversed (Schnappauf et al.

Table 2 Kinetic parameters of the DAHP synthases from *A. nidulans* and *S. cerevisiae*. The K_i values refer to the specific inhibitors of each DAHP synthase

	A. nidulans		*S. cerevisiae*	
	aroFp	aroGp	Aro3p[a]	Aro4p[b]
K_m (PEP) (μM)	175	239	18	125
K_m (E4P) (μM)	341	475	130	500
K_i (μM)	8.4	1.2	10	0.9
k_{cat} (s^{-1})	7.0	5.5	7.0	6.0

[a]Paravicini et al. (1989)
[b]Schnappauf et al. (1998)

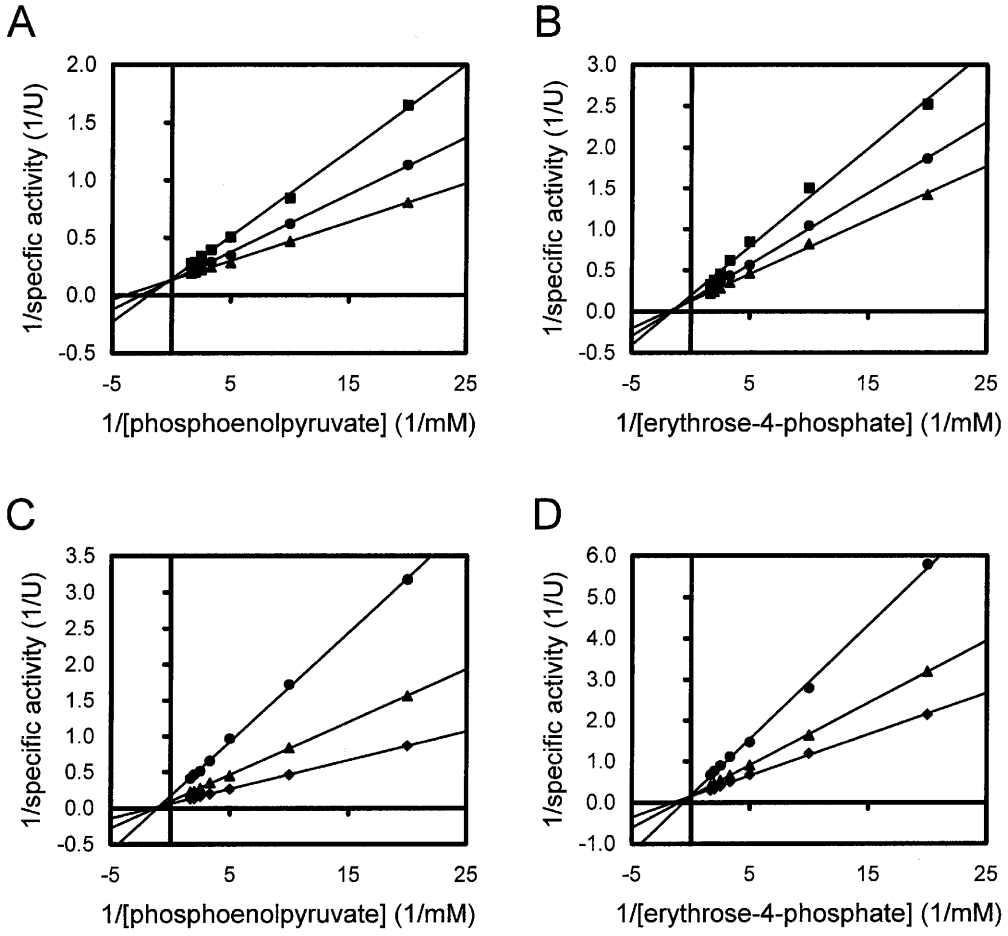

Fig. 8A–D Inhibition studies of the tyrosine- (**A**, **B**) and phenylalanine-inhibitable (**C**, **D**) DAHP synthases of *A. nidulans*. **A**, **B** Lineweaver-Burk plot with 0.5 mM substrate concentrations and fixed tyrosine concentrations of ▲ 5 μM, ● 10 μM and ■ 15 μM. **C, D** Lineweaver-Burk plot with 0.5 mM substrate concentrations and fixed phenylalanine concentrations of ◆ 1 μM, ▲ 2.5 μM and ● 5 μM

1998). No cross-inhibition effects were detected. In addition, tryptophan, at concentrations up to 60 μM, had no effect on either enzymatic activity (not shown). This suggests that the regulation pattern of the DAHP synthase isoenzymes could have developed in evolution after separation between yeast and filamentous fungi.

Kinetic properties of the aroFp and aroGp

The kinetic parameters of the purified enzymes were measured. The reaction catalyzed by DAHP synthases is a two-substrate reaction. To obtain the steady-state kinetics, the concentration of one substrate was varied and the enzyme activity at various fixed concentrations of the other substrate was measured (Fig. 7A–D).

The two enzymes have a common intersection in the double-reciprocal plots (Lineweaver-Burk plots) of initial velocity plotted against the varying substrates. The kinetic parameters were obtained by secondary plots in which the reciprocal substrate concentrations were plotted against the intercepts of the *y*-axis (Table 2). Both K_m values (one for PEP and the other for E4P) of the aroFp enzyme are lower than those of the isoenzyme aroGp. This implies that the affinity of both substrates for aroFp is higher than for the aroGp protein. In addition, for both enzymes the affinity of PEP is higher than that of the other substrate E4P. With the apparent V_{max} values and the known enzyme concentration e_0, the turnover number k_{cat} of both enzymes was calculated per monomer to 7.0 s^{-1} for the protein aroFp and 5.5 s^{-1} for the protein aroGp, which means that the tyrosine-inhibitable DAHP synthase is slightly more effective than the isoenzyme.

Inhibition studies of the DAHP synthases of *A. nidulans*

We had previously shown that in yeast tyrosine and phenylalanine compete with PEP and E4P, respectively, for binding to DAHP synthase (Schnappauf et al. 1998). To verify this competition for the DAHP synthases of *A. nidulans*, enzyme tests with various concentrations of one substrate and a fixed concentration (0.5 mM) of the other were carried out. For inhibition, various fixed inhibitor concentrations of tyrosine (for aroFp) and phenylalanine (for aroGp), were used (Fig. 8A–D). In all of the Line-

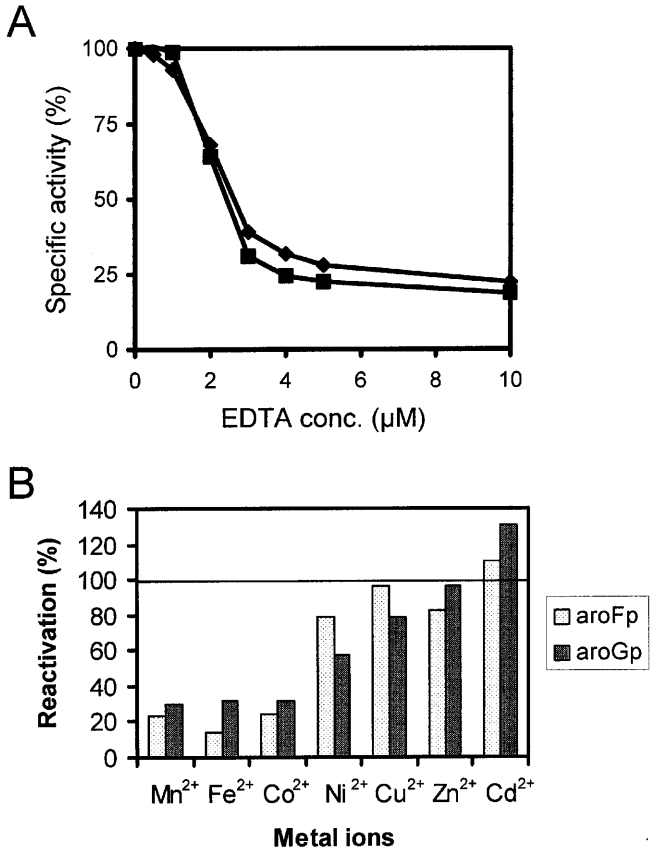

Fig. 9 Inhibition by EDTA. **A** Inhibition patterns of the DAHP synthases aroFp (■) and aroGp (◆) from *A. nidulans* with varying EDTA concentrations. Between 1 and 3 μM EDTA there is 75% loss of activity. Even with concentrations up to 1.5 mM it is not possible to increase this activity loss. **B** Reactivation of the DAHP synthase activities of *A. nidulans* with various bivalent metal ions after EDTA inactivation. The values reflect the activities in percentage compared to the EDTA- and metal-untreated enzyme

weaver-Burk plots, there is a common intersection of the lines representing different inhibitor concentrations. The intersection with the ordinate is at various PEP concentrations (tyrosine-inhibitable enzyme aroFp, Fig. 8A) and at various concentrations of erythrose-4-phosphate (phenylalanine-inhibitable enzyme aroGp, Fig. 8D). Thus, phenylalanine competitively inhibits the interaction of E4P with the aroGp isoenzyme, and tyrosine competitively inhibits interaction of PEP with the aroFp DAHP synthase.

Furthermore, phenylalanine and tyrosine are non-competitive inhibitors of PEP and E4P, respectively, in the reaction of these substrates with the synthase. The K_i values are 8.4 μM for the tyrosine-inhibitable DAHP synthase and 1.2 μM for the phenylalanine-inhibitable isoenzyme. Therefore, phenylalanine has an inhibitory effect on its respective enzyme that is increased by one order of magnitude over the inhibitory effect of tyrosine on its corresponding enzyme.

Both DAHP synthases of *A. nidulans* are metalloproteins

For other DAHP synthases, it has been shown that enzyme activity decreases when the concentration of the metal-chelating agent EDTA increases and that it is possible to destroy enzyme activity completely at high EDTA concentrations (Schnappauf et al. 1998). Therefore, it was of interest to determine whether the *A. nidulans* enzymes are also dependent on a bivalent metal; enzyme assays with increasing EDTA concentrations were carried out (Fig. 9A). At concentrations between 1 and 4 μM, the enzymes lost 75% of their activity. Higher concentrations up to 1.5 mM had no further significant effect on enzyme activity.

Since the enzymes were inhibitable by EDTA, it was of interest to determine whether activity could be restored by the addition of bivalent metals. Several bivalent metals of the first row of transition elements as well as cadmium ions were tested. In the test system, the enzyme samples were incubated with 1.50 mM EDTA at room temperature. After 5 min incubation, 1.75 mM of several Me^{2+} ions (one per test) were added and incubation was carried out for another 15 min at room temperature. After inhibition and reactivation of the proteins, enzymes were assayed to determine the rates of re-activation (Fig. 9B). Enzyme activities of both enzymes were restorable up to at least 80% by the addition of bivalent copper and zinc ions. Cadmium ions activated both *A. nidulans* DAHP synthases. Manganese, iron, and cobalt ions restored enzyme activities only up to 30%.

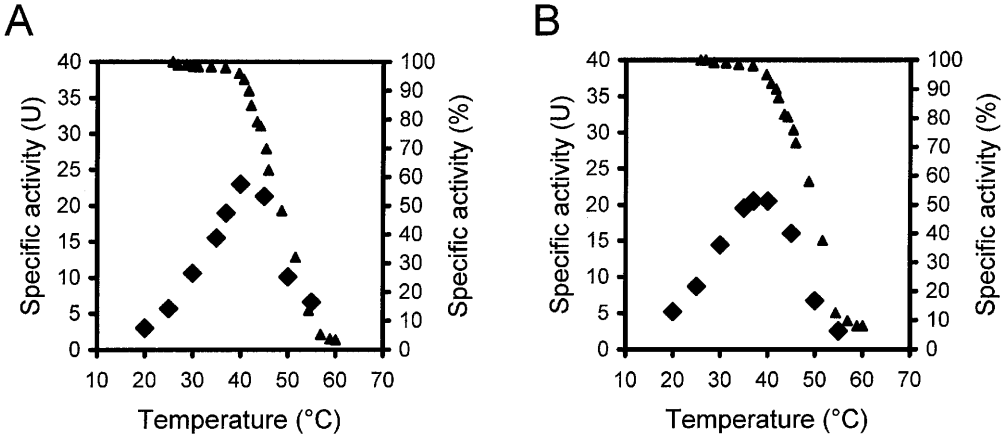

Fig. 10 Temperature effect and heat inactivation of the aroF (**A**) and aroG (**B**) proteins of *A. nidulans*. The enzymatic assays were carried out with substrate concentrations of 0.5 mM of each substrate. The *left y*-axis shows the specific activity after 5 min reaction time (◆) given in units according to the test temperature. The *right y*-axis show the inactivation of the enzymes (▲) after incubating 5 min at a constant temperature. The values are given in percentage according to the enzyme treated at 37 °C

The pH dependence of DAHP synthases activity

The two DAHP synthase isoenzymes from *A. nidulans* show different pH optima. Enzyme activity starts at a pH of about 5.0–5.5, has an optimum between 6.5 and 7.5, and remains nearly stable up to pH 8.0. The tyrosine-inhibitable aroFp had an activity optimum at a pH 6.8; the phenylalnine-inhibitable isoenzyme had an optimum at pH 7.5.

Temperature effects on the *A. nidulans* DAHP synthases

The enzyme activity and stability of both DAHP synthases were tested at various temperatures. First, activities were measured at a range of temperatures using the enzyme assay described in Materials and methods. In a second approach and to investigate the stability properties of both enzymes, the proteins were pre-incubated for 5 min at different fixed temperatures between 25 °C and 60 °C. After incubation, the enzymes were assayed at 37 °C to measure the remaining activities (Fig. 10 A,B). Both enzymes are stable up to a temperature of about 40 °C. Above this temperature the activity sharply decreased. At a temperature of about 55 °C, only 5–10% of activity remained.

Discussion

Two novel genes, *aroF* and *aroG*, encoding DAHP synthases of the filamentous fungus *A. nidulans* were isolated and the gene products were characterized. In contrast to the filamentous fungus *N. crassa*, no gene for a tryptophan inhibitable DAHP synthase could be recovered from a cDNA library, which was constructed from normally growing hyphae. Both genes are part of the cross pathway control of *A. nidulans*. The activation by a transcriptional activator similar to Gcn4p of yeast with a factor of 3.5 for both genes is significant but weak compared to the reported genes so far (Paluh et al. 1988). This is similar in the yeast *S. cerevisiae*, where both enzymes are regulated by the general control. This means that the first step of the aromatic amino acid biosynthesis is a important step and has to be regulated precisely in eukaryotes.

As the DAHP synthases are Me^{2+} enzymes, it was not surprising that EDTA had an inhibitory effect on both *A. nidulans* isoenzymes. More surprising was the fact that even an EDTA concentration up to 1.5 mM did not completely abolish enzyme activity. In contrast, the *S. cerevisiae* enzymes are already inactive under these conditions. This suggests that complete removal of the metal ion from the enzyme is not possible under the conditions tested or that in contrast to the known DAHP synthases, the *A. nidulans* enzyme activities are not completely dependent on a bivalent metal. Reactivation was possible by copper and zinc ions, which indicates that these two metals could be cofactors of the native *A. nidulans* DAHP synthases in vivo. Cadmium even had an activating effect on the EDTA-inhibited enzymes of *A. nidulans*, but the biological implication of that is questionable. In general, the metal binding sites of DAHP synthases seem to be variable to bind more than one kind of metal ion as there are reports of different metal ions which can reactivate activity in different microorganisms (Paravicini et al. 1989; Schnappauf et al. 1998; Stephens and Bauerle 1991).

Although DAHP synthases share common properties concerning their catalytic behavior and presumably their overall fold they show distinct differences according to regulation. They differ mostly in efficiency and inhibitor affinity. DAHP synthases of *A. nidulans* or *S. cerevisiae* with average k_{cat} values of 6 s^{-1} are less efficient than *E. coli* DAHP synthases with k_{cat} values of 21 s^{-1} (tryptophan-inhibitable) and 121 s^{-1} (tyrosine-inhibitable). In contrast to *S. cerevisiae* enzymes, the tyrosine-inhibitable isoenzyme of *A. nidulans* is more efficient. In addition the affinity of both substrates is higher to the tyrosine-regulated enzyme in *A. nidulans*. This is different than in yeast where the phenylalanine-regulated isoenzyme has a higher affinity to both substrates. Therefore, we conclude that the common ancestor of the DAHP synthases isoenzymes had a different regulation pattern.

Both characterized *A. nidulans* DAHP synthases are inhibited by an end product of the biosynthetic pathway. In the filamentous fungus *A. nidulans*, the phenylalanine-regulated enzyme is strongly inhibited with a K_i of 1.2 μM, whereas in *S. cerevisiae* phenylalanine inhibits the corresponding isoenzyme with a ten times lower magnitude of strength (K_i of 10 μM). The tyrosine-inhibitable isoenzyme of *A. nidulans* is regulated with the same magnitude of concentration (K_i of 8.4 μM). This is ten times weaker than the regulation of the tyrosine-isoenzyme in the yeast *S. cerevisiae* (K_i value of 0.9 μM). This means that in contrast to yeast, *A. nidulans* could regulate the flux into the shikimate pathway mainly by phenylalanine (Fell 1992). Compared to the *E. coli* DAHP synthases, where the K_i values are much higher (8-fold) (Schoner and Herrmann 1976; Simpson and Davidson 1976) fungal DAHP synthases seem to be more carefully regulated on the enzymatic level.

Only two differently regulated isoenzymes were found in the filamentous fungus *A. nidulans*, in contrast to *N. crassa*, where three isoforms are present. Compared to the yeast *S. cerevisiae*, phenylalanine seems to play the more important role in regulation of the flux into the shikimate pathway in *A. nidulans*. This suggests that the evolution of the fine-tuning of the DAHP synthase enzyme regulation has developed after the taxonomic splitting of unicellular yeasts and filamentous fungi. It will be intriguing to determine whether phenylalanine or tyrosine is the more important pillar of regulation in *N. crassa*.

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