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The two 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase isoenzymes from *Saccharomyces cerevisiae* show different kinetic modes of inhibition

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Abstract Activity of the tyrosine-inhibitable 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (EC 4.1.2.15) from *Saccharomyces cerevisiae* that was encoded by the *ARO4* gene cloned on a high-copy-number plasmid was enhanced 64-fold as compared to the wild-type. The enzyme was purified to apparent homogeneity from the strain that harbored this recombinant plasmid. The estimated molecular weight of 42,000 of the enzyme corresponded to the calculated molecular mass of 40 kDa deduced from the DNA sequence. The enzyme could be inactivated by EDTA in a reaction that was reversed by several bivalent metal ions; presumably a metal cofactor is required for enzymatic catalysis. The Michaelis constant of the enzyme was 125 μM for phospho*enol*pyruvate and 500 μM for erythrose 4-phosphate. The rate constant was calculated as 6 s^{-1}, and kinetic data indicated a sequential mechanism of the enzymatic reaction. Tyrosine was a competitive inhibitor with phospho*enol*pyruvate as substrate of the enzyme (K_i of 0.9 μM) and a noncompetitive inhibitor with erythrose 4-phosphate as substrate. This is in contrast to the *ARO3*-encoded isoenzyme, where phenylalanine is a competitive inhibitor with erythrose 4-phosphate as a substrate of the enzyme and a noncompetitive inhibitor with phospho*enol*pyruvate as substrate.

Key words *Saccharomyces cerevisiae* · 3-Deoxy-D-*arabino*-heptulosonate-7-phosphate synthase · Protein purification · Enzyme kinetics

Abbreviation *DAHP* 3-deoxy-D-*arabino*-heptulosonate-7-phosphate

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Introduction

The 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase catalyzes the first step in the aromatic amino acid biosynthetic pathway, which yields the three amino acids tyrosine, tryptophan, and phenylalanine. The enzyme catalyzes a condensation of phospho*enol*pyruvate and erythrose 4-phosphate to produce 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (Haslam 1993). A broad diversity of differentially controlled isoenzyme species has been observed for this enzyme in nature (Byng et al. 1982; Byng and Jensen 1983).

In *Saccharomyces cerevisiae*, two differently regulated DAHP synthases carry out the first regulated step in the aromatic amino acid biosynthetic pathway (Lingens et al. 1967; Teshiba et al. 1986; Paravicini et al. 1989b; Braus 1991; Künzler et al. 1992). The two DAHP synthases are encoded by the genes *ARO3* and *ARO4* and are inhibited by phenylalanine and tyrosine, respectively. Other microorganisms such as *Escherichia coli* and *Neurospora crassa* possess a third DAHP synthase that is regulated by the third end product of the pathway, tryptophan (Hoffmann et al. 1972; Nimmo and Coggins 1981a,b; Byng and Jensen 1983). In *E. coli*, the tryptophan-sensitive enzyme accounts for only a small portion of DAHP synthase activity (Pittard 1996). *Bacillus subtilis* has a single, bifunctional DAHP synthase-chorismate mutase enzyme that mediates the flux of carbon into the shikimate pathway (Huang et al. 1974a,b).

The *ARO3* and *ARO4* genes of yeast have been cloned and encode proteins with similar size and amino acid sequence (Teshiba et al. 1986; Künzler et al. 1992). In addition, there are regions with high similarity to other DAHP synthases, e.g., the three enzymes of *E. coli* (Künzler et al. 1992). The *ARO3* and *ARO4* genes are part of a transcriptional regulatory network. Both genes are regulated by the transcriptional activator Gcn4p (Hinnebusch 1988; Paravicini et al. 1988, 1989a; Künzler et al. 1992). GCN4p functions by derepressing transcription of the genes during amino acid starvation and by maintaining a basal level

of *ARO3* transcription in the presence of amino acids (Paravicini et al. 1989a). Basal expression of *ARO3* is additionally regulated by the global factor ABF1 and by the global protein complex BUF (Künzler et al. 1995).

We have previously purified and characterized the *ARO3*-encoded, phenylalanine-inhibitable DAHP synthase from yeast (Paravicini et al. 1989b). Activity of the enzyme is regulated through competitive feedback inhibition by phenylalanine with erythrose 4-phosphate as substrate and through non-competitive inhibition with phospho*enol*-pyruvate as substrate. In contrast, in *E. coli* the phenylalanine-sensitive enzyme is competitively inhibited with either compound as a substrate (Staub and Denes 1969b), and the tyrosine-sensitive enzyme is competitively feedback-inhibited by tyrosine with phospho*enol*pyruvate as substrate and noncompetitively inhibited with erythrose 4-phosphate as substrate. This prompted us to investigate whether regulation of the tyrosine-inhibitable enzyme of yeast is subject to competitive inhibition with phospho*enol*pyruvate as substrate.

Here we describe the purification and characterization of the *S. cerevisiae* isoenzyme Aro4p, the tyrosine-inhibitable DAHP synthase. The steady-state kinetics of the purified *ARO4*-encoded DAHP synthase have been measured, and the results are characteristic for a sequential enzyme mechanism (Cleland 1967, 1970). The mechanism of feedback was studied. The enzyme was strongly, competitively inhibited by tyrosine with phospho*enol*pyruvate as substrate, and noncompetitively inhibited with erythrose 4-phosphate as substrate. These results are the opposite of those with inhibition of the isoenzyme by phenylalanine.

Materials and methods

Strains, media, plasmids, and growth conditions

The yeast strains used are derivatives of the *S. cerevisiae* laboratory strains X2180-1A (*MAT*α *gal2 SUC2 mal CUP1*) and X2180-1B (*MATa gal2 SUC2 mal CUP1*) obtained from T. Manney (Kansas State University, Manhatten KA, USA). Strain RH1326 (*MATa aro3-2 aro4-1 gcd2-1 leu2-2*) was transformed with the multicopy plasmid pME1201, which is a derivative of pJDB207 (Beggs 1978) carrying the *ARO4* gene on a genomic 1.9-kb *Bcl*I fragment. Cells were transformed by the LiAc method (Ito et al. 1983). Strain RH1316 (*Mata aro3-2*) was used to determine cellular *ARO4* activitiy. MV minimal medium for the cultivation of yeast has been previously described (Miozzari et al. 1978).

For protein purification, 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, 25 g of cells (wet wt.) was used.

Enzyme assays

Enzyme activities are specified in International Units (1 U = appearance of 1 μmol product per min). Specific enzyme activities are given as mU (mg protein)$^{-1}$.

The DAHP synthase activity was determined with a stop assay based on the absorbance at 550 nm of a secondary product of DAHP as described by Takahashi and Chan (1971) with the exception that the pH of the enzymatic reaction was adjusted to pH 6.8 instead of pH 6.5 and that sodium periodate was dissolved in 0.25 instead of 0.125 M H_2SO_4. Assays were performed with substrate concentrations such that absorbance of the product was proportional to the amount of enzyme added and were measured on a Kontron Uvikon 922. Reconstitution of enzymatic activity by Fe^{2+} was measured under a nitrogen atmosphere to prevent oxidation of the metal.

Purification of DAHP synthase (Aro4p)

All steps in the purification procedure were performed at 4 °C, and dithiothreitol and the protease inhibitor phenylmethyl-sulfonylfluoride were added to each buffer to a final concentration of 1 mM and 100 μM, respectively. For the hydrophobic interaction chromatography, a 1.5 × 30-cm ethylamino-Sepharose (Jennissen and Heilmeyer 1975) column with a volume of 50 ml was used. Capacity of the material was 20 mg protein ml^{-1}; columns were packed by gravity.

The *ARO4*-encoded DAHP synthase was purified to apparent homogeneity in a four-step procedure.

Step 1: preparation of crude extracts

Cells were cultivated to an OD_{546} of 3–4. Wet cells were washed twice with 50 mM potassium phosphate (pH 7.6) and were suspended in an equal volume of buffer before addition of phenylmethylsulfonylfluoride and dithiothreitol. Crude extracts were prepared as described previously by using a French press cell (Kradolfer et al. 1977).

Step 2: ammonium sulfate precipitation

The potassium phosphate concentration of the supernatant was increased to 0.5 M (pH 7.6) by adding the appropriate amount of K_2HPO_4. The suspension was then adjusted to 30% saturation of ammonium sulfate, and the pH was corrected to 7.6 when necessary. After 20 min of stirring at 4 °C, the precipitate was removed by centrifugation at 25,000 × *g* for 40 min, yielding ammonium sulfate extract as supernatant.

Step 3: hydrophobic interaction chromatography

The ammonium sulfate extract was loaded onto an ethylamino-CL4B-Sepharose column previously equilibrated with 0.5 M potassium phosphate buffer (pH 7.6) at 30% saturation with ammonium sulfate. After the column was washed with equilibration buffer, bound proteins were eluted with a 1-l linear gradient of equilibration buffer and H_2O. Fractions (10 ml) were collected at a flow rate of 1.5 ml min^{-1}. Fractions containing DAHP synthase activity were pooled and dialyzed against 10 mM Tris-HCl (pH 7.6).

Step 4: anion-exchange chromatography

A Mono Q column (HR 16/10) from Pharmacia (Uppsala, Sweden) was equilibrated with 10 mM Tris-HCl (pH 7.6), and the dialyzed fractions were applied to the column. After washing with the same buffer, proteins were eluted with 380 ml of a linear NaCl gradient (0–250 mM) in equilibration buffer. DAHP synthase containing fractions was collected, concentrated to 4 mg protein ml^{-1}, adjusted to 50% glycerol, and stored at –20 °C.

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 ve-

locity against concentrations of the varied substrate showed a set of intersecting lines. A plot of the interceptions of these lines with the ordinate against 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 $1/V_{max}$ are on the ordinate. Rate constants k were determined from these values, and the known enzyme concentration e_o was determined according to $k = V_{max}/e_0$.

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.

Gel electrophoresis of proteins

SDS/polyacrylamide gels were run according to Lämmli (1970) on the Mini-Protean II gel system (BioRad, Richmond, Calif., USA). For monitoring protein purification, the gel was stained with Coomassie blue.

Results

Purification of the tyrosine-inhibitable DAHP synthase from yeast

The tyrosine-inhibitable DAHP synthase of *S. cerevisiae* was purified from crude extracts of the *aro3 aro4* double mutant strain RH1326, which harbored the *ARO4* gene on the high-copy-number plasmid pME1201. This strain carried a *gcd2-1* mutation resulting in constitutively increased enzyme levels derived from genes regulated under the general control system of amino acid biosynthesis (Miozzari et al. 1978; Hinnebusch 1988). Strains carrying a *gcd2-1* mutation reveal an approximately fourfold derepression of the *ARO4* gene as compared to the regulatory wild-type strain (Künzler et al. 1992). In addition, we determined a 13-fold increased copy number of the *ARO4* gene when it was expressed from the high-copy-number plasmid pJDB207 (Fig. 1A). Activity measurements resulted in a 64-fold higher activity of the enzyme as compared to the activity of a strain with a genomic copy of *ARO4* and an *aro3* mutation. This value corresponds to a theoretical 52-fold overexpression that is the product of a increased transcription of *ARO4* due to a *gcd2-1* mutation and the increased copy number of *ARO4*.

From each purification step, a sample was taken, and the proteins were separated by SDS/PAGE and stained with Coomassie-Blue (Fig. 1B). Lane 5 shows a single protein band with a molecular mass of 42 kDa, which is in agreement with the calculated molecular mass of 40 kDa deduced from the *ARO4* DNA sequence. In 50% glycerol, the enzyme remained stable at –20 °C for at least 4 weeks. Quantitation of a typical purification protocol is summarized in Table 1. The DAHP synthase was purified 7.5-fold with a 34% yield. Including the 64-fold overproduction

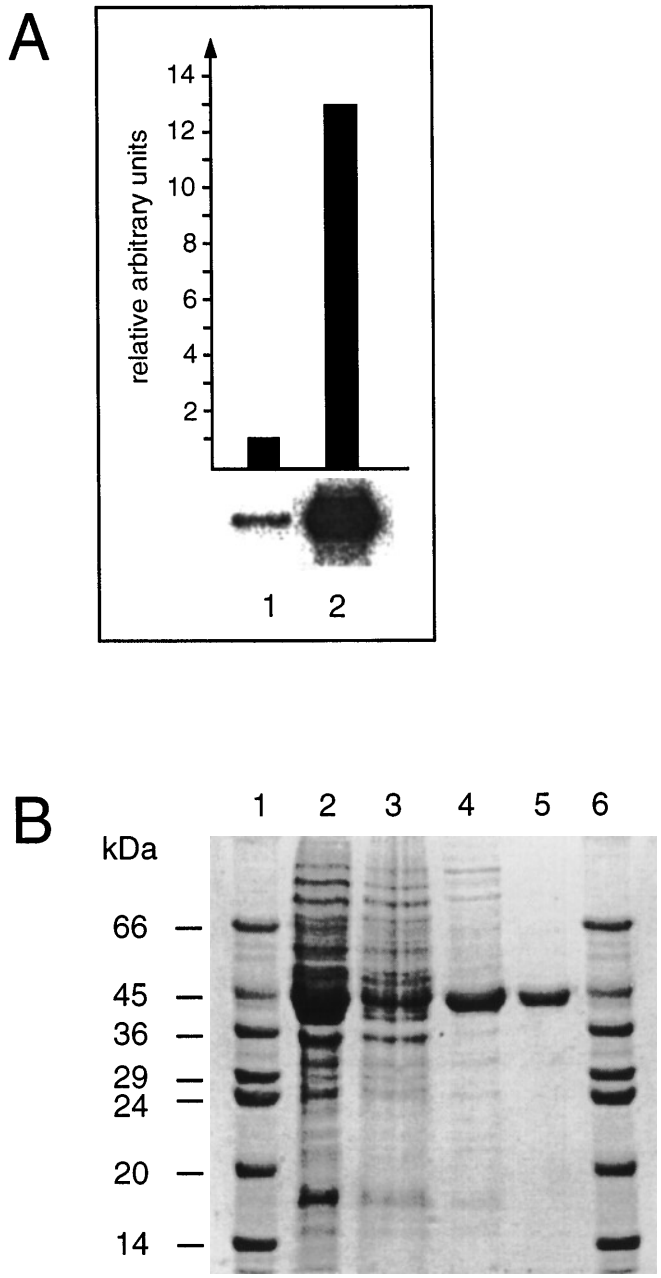

Fig. 1A, B Purification of the tyrosine-inhibitable 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase (ARO4p). **A** *ARO4* gene copy number on the high-copy-number plasmid pJDB207. DNA of yeast strain RH1316 carrying a genomic *ARO4* copy (*lane 1*) and of yeast strain RH1326 expressing the *ARO4* gene from the high-copy-number plasmid pJDB207 (*lane 2*) were hybridized with a radioactively labeled *ARO4* DNA probe. Several Southern blots were analyzed on a phosphoimager and indicated an average 13-fold increased copy number of *ARO4* of strain RH1326 as compared to that of strain RH1316. One Southern blot is depicted. **B** Analysis of the purification of the tyrosine-inhibitable DAHP synthase by SDS/PAGE. *Lanes 1*, *6* marker proteins with the indicated molecular masses in kDa, *2* crude extract of RH1326 overexpressing the *ARO4* gene (22 μg), *3* supernatant of ammonium sulfate precipitation (13 μg), *4* ethylamino-Sepharose pool (6 μg), and *5* Mono Q pool (1 μg). Proteins were stained with Coomassie blue

Table 1 Quantitation of the purification of overexpressed *ARO4*-encoded 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase

Step	Protein (mg)	Specific activity (U/mg protein)	Recovery (%)	Purification (-fold)
Crude extract	708	1.1	100	1.0
Ammonium sulfate	512	1.3	87	1.2
Ethylamino-Sepharose	136	4.38	77	4.0
Mono Q	32	8.24	34	7.5

due to the high copy number of the plasmid and the *gcd2-1* mutation of strain RH1326, this corresponds to a 480-fold enrichment of the chromosomally encoded DAHP synthase.

pH optimum

Activity of the purified tyrosine-inhibitable DAHP synthase increase from an acidic pH to a maximum pH of 6.5–7.0 and remains almost at the same level up to pH 8.0. This is in agreement with results obtained from studies with the partially purified enzyme (Takahashi and Chan 1971).

Substrate kinetics

Using the purified enzyme, it was possible to study the steady-state kinetics of the tyrosine-inhibitable DAHP synthase isoenzyme. All measurements were performed at least three times to reduce errors in initial velocity data to less than 10%. Measurements of enzyme batches from independent purifications reproduced our data. The initial velocity (v) was determined as a function of the concentration of one substrate at a fixed level of the other substrate. The results are shown as double-reciprocal (Lineweaver-Burk) plots. For the double-reciprocal plots of velocity against phospho*enol*pyruvate concentration at a series of fixed erythrose 4-phosphate concentrations, the lines appear to have a common intersection, which is a typical pattern for a sequential mechanism in which all substrates must bind to the enzyme before any product is released (Fig. 2A). The curves obtained by varying the erythrose 4-phosphate concentration at different phospho*enol*pyruvate concentrations also intersect in a common point, which again supported a sequential reaction mechanism (Fig. 3A). Similar results were obtained with the purified DAHP synthases (phenylalanine- and tyrosine-sensitive) of *E. coli*, where the reaction mechanism was ordered and sequential with respect to the substrates, with phospho*enol*pyruvate being the first substrate to bind (Schoner and Herrmann 1976; Simpson and Davidson 1976). Kinetic parameters were determined from secondary plots of the data shown in Figs. 2A and 3A and are shown in Figs. 2B and 3B. K_m values of 125 μM for phospho*enol*pyruvate and 500 μM for erythrose 4-phosphate

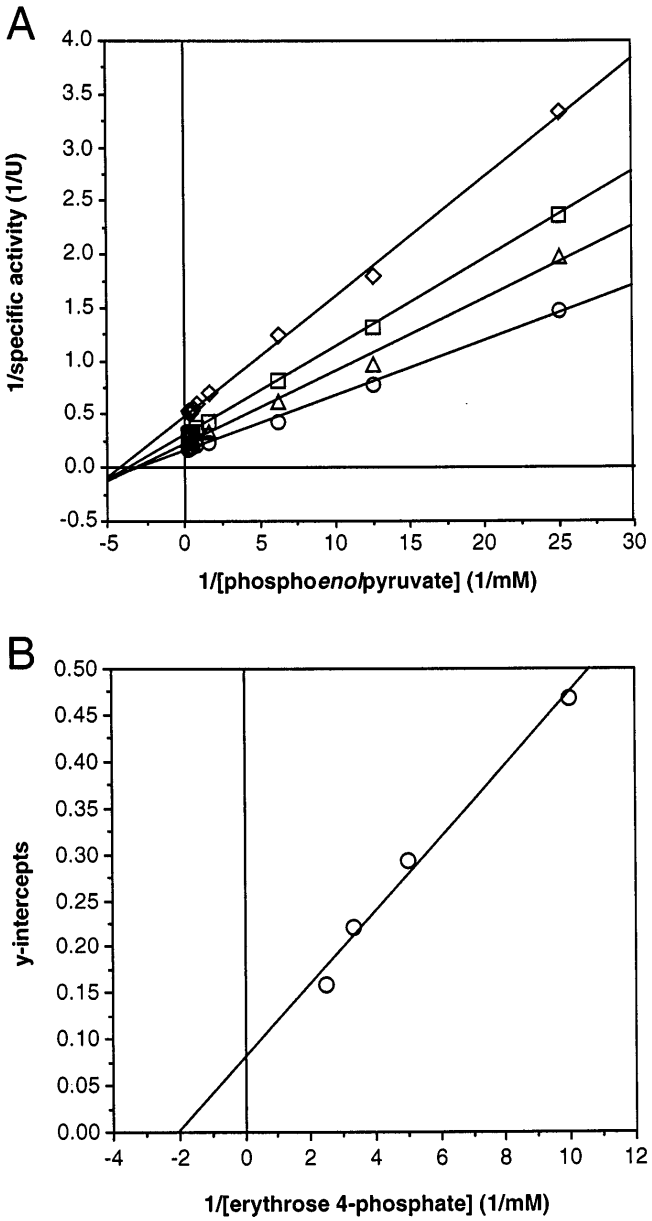

Fig. 2A, B Phospho*enol*pyruvate saturation of tyrosine-sensitive 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase. **A** Double-reciprocal plots of initial velocity (v) plotted against phospho*enol*pyruvate concentration at erythrose 4-phosphate concentrations of ◇ 0.1 mM, □ 0.2 mM, △ 0.3 mM, and ○ 0.4 mM. **B** Reciprocal erythrose 4-phosphate concentrations plotted against intercepts with the y-axis

were obtained. Higher affinity for phospho*enol*pyruvate as compared to erythrose 4-phosphate has also been determined for the respective enzymes of *E. coli*, and it has been suggested that the enzyme-phospho*enol*pyruvate complex might be the native form for both the tyrosine- and the phenylalanine-sensitive DAHP synthases (McCandliss et al. 1978). With the apparent V_{max} values and the known enzyme concentration e_o, the rate constant k of yeast DAHP synthase was calculated to be 6 s^{-1} according to the formula explained in Materials and methods.

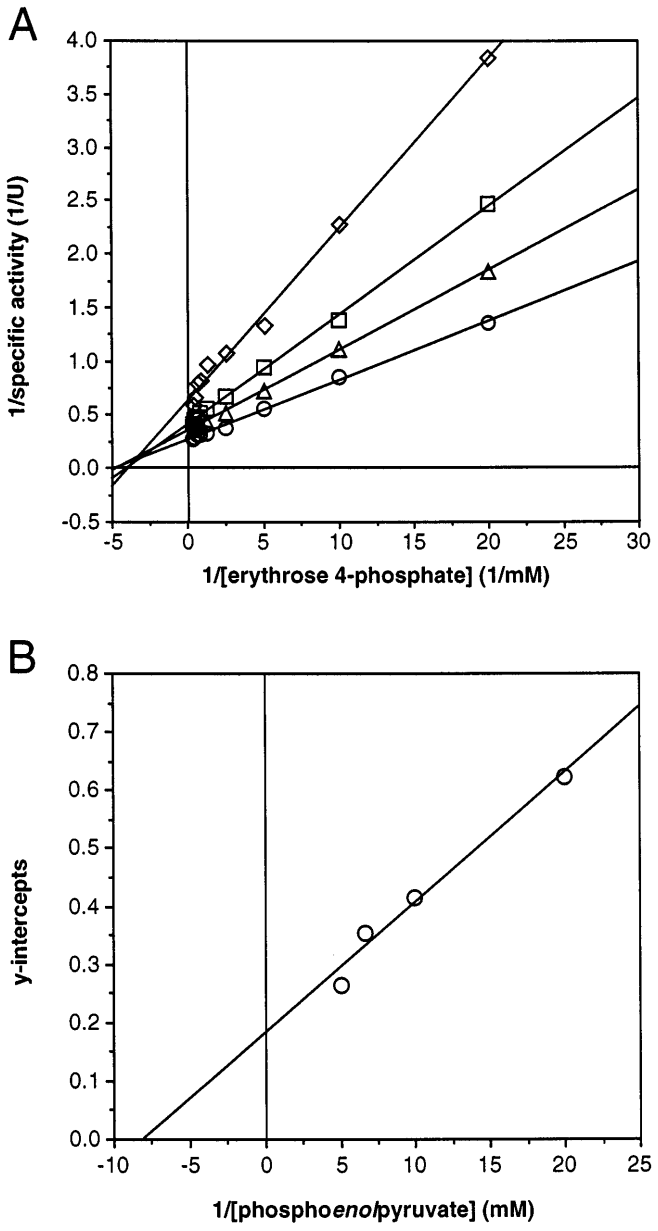

Fig. 3A, B Erythrose 4-phosphate saturation of tyrosine-sensitive 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase. **A** Double-reciprocal plots of initial velocity (*v*) plotted against erythrose 4-phosphate concentration at phospho*enol*pyruvate concentrations of ◇ 0.05 mM, □ 0.1 mM, △ 0.15 mM, and ○ 0.2 mM. **B** Reciprocal phospho*enol*pyruvate concentrations plotted against intercepts with the y-axis

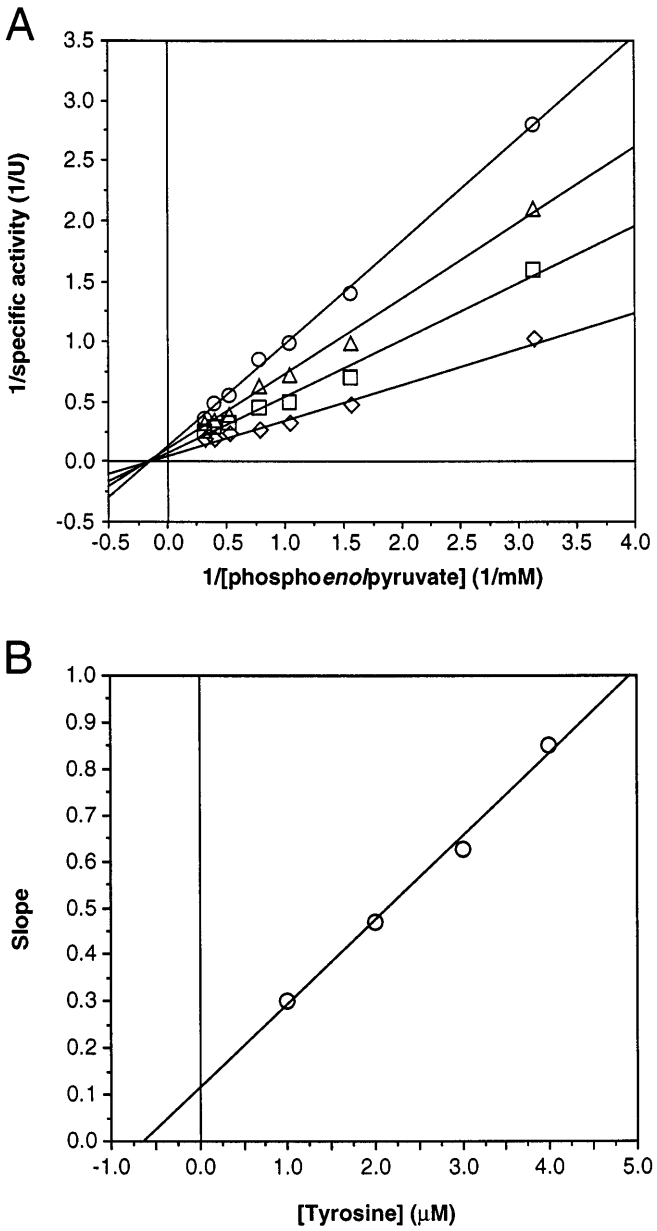

Fig. 4A, B Tyrosine inhibition of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase. **A** Double-reciprocal plots of initial velocity (*v*) plotted against phosphoenolpyruvate concentration at tyrosine concentrations of ◇ 1 μM, □ 2 μM, △ 3 μM, and ○ 4 μM. Erythrose 4-phosphate was kept at a constant concentration of 400 μM. **B** Tyrosine concentrations are plotted against variation of the slopes

Inhibition studies

The effect of tyrosine and of phenylalanine on the enzyme was studied by adding the amino acids at subsaturating levels. Erythrose 4-phosphate concentration was kept constant at 400 μM. The initial velocities of the reaction, which were dependent upon varying amounts of phospho*enol*pyruvate with different concentrations of tyrosine and phenylalanine, were measured. With different fixed tyrosine concentrations, the graphs of the double-reciprocal plots of initial velocity against concentration of the varied substrate have a common intersection on the ordinate for phospho*enol*pyruvate saturation (Fig. 4A). In an analogous experiment, we kept the phospho*enol*pyruvate concentration constant at 400 μM and varied the erythrose 4-phosphate concentration. Values were measured at different fixed tyrosine concentrations. The lines for the Lineweaver-Burk plots of substrate saturations crossed in the second quadrant (Fig. 5A). The results of both experiments suggest that tyrosine competitively inhibits the en-

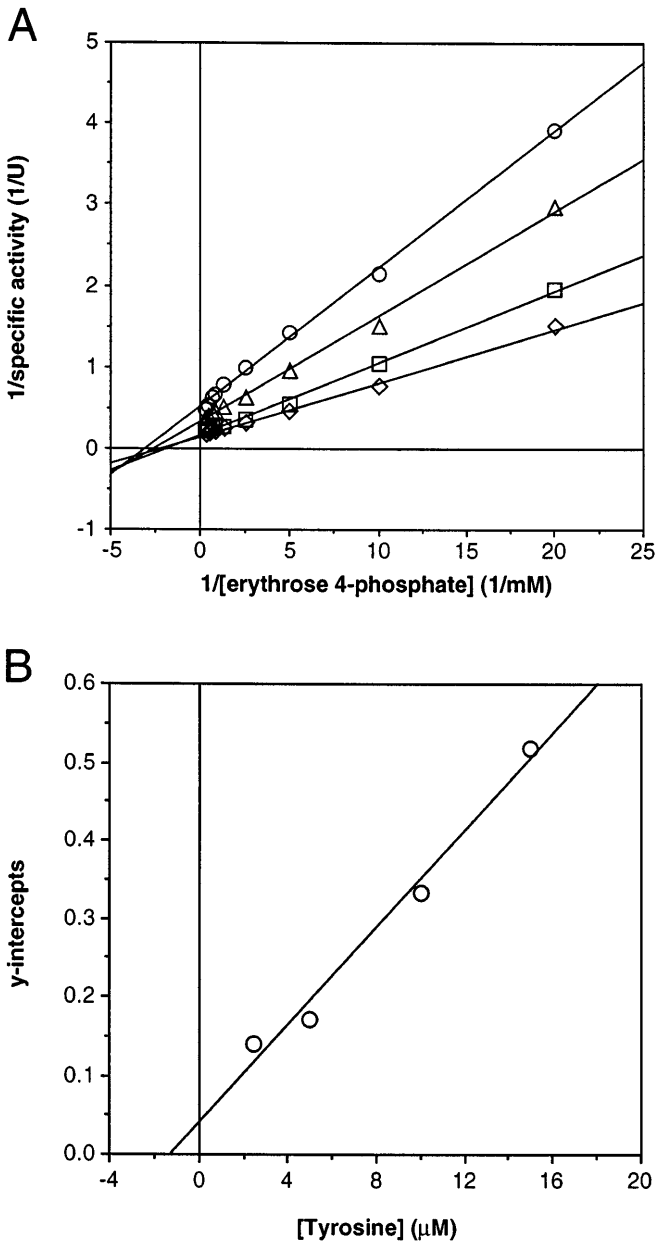

Fig. 5A, B Tyrosine inhibition of 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase. **A** Double-reciprocal plots of initial velocity (v) plotted against erythrose 4-phosphate concentration at tyrosine concentrations of ◇ 2.5 μM, □ 5 μM, △ 10 μM, and ○ 15 μM. Phospho*enol*pyruvate was kept at a constant concentration of 400 μM. **B** Tyrosine concentrations are plotted against intercepts of lines with the y-axis

zyme with phospho*enol*pyruvate as substrate and noncompetitively inhibits it with erythrose 4-phosphate as substrate. Phenylalanine in high concentrations also had an inhibitory effect on the enzyme. This inhibition was also found to be competitive with phospho*enol*pyruvate as substrate and noncompetitive with erythrose 4-phosphate as substrate (data not shown). The inhibition constants were calculated from secondary plots, where the intersections with the abscissa or slopes of the lines were plotted versus the inhibitor concentration (Figs. 4B and 5B). The average K_i values were determined to be 0.9 μM for tyrosine and 270 μM for phenylalanine.

Metal ion dependence of enzyme activity

The *ARO3*-encoded DAHP synthase was reported to be a metalloprotein with Fe^{2+} as cofactor (Paravicini et al. 1989b). When the growth medium of the yeast strain overexpressing Aro4p was supplemented with varying amounts of Fe^{2+} and Zn^{2+}, the same DAHP synthase activity was obtained, suggesting that a metal cofactor is not limiting during growth. However, we observed that the activity of the tyrosine-sensitive DAHP synthase can be completely abolished by the addition of EDTA. To test whether this effect is due to the sequestration of a metal cofactor, the pure enzyme was incubated with 1.5 mM EDTA in 50 mM potassium phosphate buffer (pH 6.8) for 5 min at room temperature. No DAHP synthase activity was detected after this treatment. We then added several metal ions as sulfate salts and tested for their ability to regenerate enzymatic activity after incubation for 15 min at 25 °C. Co^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+} ions were able to restore enzyme activity most effectively. Co^{2+} increased activity to approximately 167% of the control, which was not treated with EDTA. Cd^{2+} and Ni^{2+} addition induced a partial regeneration of the enzyme to an activity of less than 70% of the untreated enzyme. Mn^{2+} and Mg^{2+} were the least effective: enzyme activities were less than 20% of the untreated enzyme. The results indicate that an endogenous, bivalent metal ion was copurified and is essential for catalytic activity. The results also suggest that other bivalent metal ions may be used as cofactor.

Discussion

The reaction catalyzed by DAHP synthase, the condensation of phospho*enol*pyruvate derived from glycolysis and erythrose 4-phosphate from the pentose phosphate pathway, is the initial step in the biosynthetic pathway of aromatic compounds. The amino acid sequence of DAHP synthases is conserved from bacteria to higher eukaryotes (Künzler et al. 1992). Different end products participate in the early-pathway control of the activity of this enzyme. In yeast, the reaction is catalyzed by two isoenzymes, the *ARO3*- and *ARO4*-encoded DAHP synthases. The expression and purification system described here has made it possible to obtain substantial amounts of highly pure *ARO4*-encoded, tyrosine-sensitive DAHP synthase. Purification of the enzyme has enabled us to study many of the properties of this enzyme in detail and to compare them with results obtained with the purified *ARO3*-encoded isoenzyme. Regulation of the isoenzymes differs not only in the nature of the feedback inhibitors, but also in the type of competitive inhibition with each of the two substrates.

Apparent native molecular weight

Sedimentation-velocity centrifugation determined the molecular weight of the major portion of the Aro4p to be 7.2 ± 0.1 S. The molecular mass of this portion was found to be 98 kDa, which corresponds to at least two 42-kDa subunits. However, portions of the enzyme with molecular masses corresponding to monomeric state and higher molecular aggregates were also found.

Although the DAHP synthases of several organsims are very similar in their sequence and mobility on SDS-PAGE, variations among the native molecular weights of the enzymes between different organisms and among the isoenzymes of one organism have been described. The tyrosine- and tryptophan-inhibitable DAHP synthases of *E. coli* have been reported to form a dimer, while phenylalanine-sensitive DAHP synthase has been determined to consist of four subunits (Schoner and Herrmann 1976; Simpson and Davidson 1976; McCandliss et al. 1978; Ray and Bauerle 1991). Native *ARO3*-encoded DAHP synthase of yeast has been reported to be a monomer (Paravicini et al. 1989b).

The three isoenzymes of *N. crassa* have also been shown to exist as oligomeric aggregates (Doy 1970; Nimmo and Coggins 1981a). The apparent molecular weight of the phenylalanine- and tyrosine-sensitive enzymes is lowered in the presence of regulatory amino acids. Dissociation of the enzymes seems to be part of the mechanism of allosteric inhibition.

Tyrosine-sensitive DAHP synthase is a metalloenzyme

Several DAHP synthases from different organisms have been described to be EDTA sensitive and to be stimulated by bivalent metal ions (Staub and Denes 1969a,b; Schweingruber and Wyssling 1974; McCandliss et al. 1978; Nimmo and Coggins 1981b; Whitaker et al. 1981; Paravicini et al. 1989b). Studies on the three isoenzymes from *E. coli* indicate that these enzymes are dependent on iron, although the function of this cofactor remains unclear (McCandliss and Herrmann 1978; Ray and Bauerle 1991; Stephens and Bauerle 1991). Our results indicate that the *ARO4*-encoded DAHP synthase of *S. cerevisiae* requires a functional enzyme-metal complex for enzymatic activity. Several metal ions were able to restore enzymatic activity after treatment with the chelating agent EDTA and might be able to replace the naturally occurring cofactor if available in sufficient amounts.

Allosteric mechanism

The most striking difference between the two DAHP synthases from yeast is the nature of the feedback inhibition with the different substrates. Tyrosine acts as a competitor for the binding of phospho*enol*pyruvate to the tyrosine-inhibitable DAHP synthase, whereas phenylalanine acts as a competitor for the binding of erythrose 4-phosphate to the phenylalanine-inhibitable enzyme. The tyrosine-sensitive enzyme of *E. coli* is similar to the tyrosine-sensitive DAHP synthase of yeast in that it is also inhibited competitively by tyrosine with phospho*enol*pyruvate as substrate and noncompetitively with erythrose 4-phosphate as substrate (Schoner and Herrmann 1976). Yet, phenylalanine inhibits the isoenzyme noncompetitively with each of the substrates (Staub and Denes 1969b). In yeast, phenylalanine shows a cross-reaction with tyrosine on enzyme activity of the *ARO4*-encoded DAHP synthase only at nonphysiologically high concentrations. Therefore, the tyrosine hydroxyl group seems to be responsible for the strong inhibition of the tyrosine-inhibitable DAHP synthase with a K_i of 0.9 μM. Assuming that the inhibition by tyrosine of the *ARO4*-encoded DAHP synthase and by phenylalanine of the *ARO3*-encoded DAHP synthase is caused by binding of each inhibitor to the same site on the enzymes, it is possible that a single amino acid replacement in the allosteric binding site of each of the two yeast isoenzymes could result in different specificities towards the two amino acids tyrosine and phenylalanine. However, it does not explain the different mode of action of the competition of the inhibitory amino acids with the substrates. The nature of the feedback inhibitors does not determine the different patterns since inhibition of *ARO4*-encoded DAHP synthase by phenylalanine is competitve with phospho*enol*pyruvate as substrate and noncompetitve with erythrose 4-phosphate as substrate, as is inhibition by tyrosine. One might assume that the binding sites for the feedback inhibitors of the *ARO3*- and *ARO4*-encoded DAHP synthases might be located at different sites of the enzymes. The kinetic differences could also reflect differences in the ways in which the two amino acid binding sites overlap with the active site, although the amino acids bind at fundamentally the same sites.

In yeast, no tryptophan-regulated enzyme that could indicate a primary pathway flow towards the tyrosine and phenylalanine branch has been detected. A K_i value of 75 μM determined for the phenylalanine-inhibitable DAHP synthase (Paravicini et al. 1989b) as compared to 0.9 μM for the isoenzyme described here indicates a major flux towards phenylalanine in the aromatic amino acid biosynthesis of yeast. Examination of deregulated mutants could yield further insight into the importance of the different regulatory patterns with the different substrates of the two DAHP synthases in yeast. Since the similarities between structural genes of isoenzymes suggest that they are derived from a common ancestor, it is of particular interest to determine whether *ARO3* and *ARO4* hybrid genes encode functional DAHP synthases. We are currently investigating whether the two DAHP synthases can be structurally divided into regulatory and catalytic domains. We will then try to construct DAHP synthases with new regulatory patterns, e.g., unregulated enzymes or enzymes that are regulated by both effectors, tyrosine and phenylalanine.

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