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Purification and properties of the 3-deoxy-D-*arabino*-heptulosonate-7-phosphate synthase (phenylalanine-inhibitable) of *Saccharomyces cerevisiae*

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The phenylalanine-inhibitable 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (dHpl*P*) synthase from *Saccharomyces cerevisiae* has been purified to apparent homogeneity by a 1250-fold enrichment of the enzyme activity present in wild-type crude extracts, employing an overproducing strain. The estimated molecular mass of 42 kDa corresponds to the calculated molecular mass of 42.13 kDa deduced from the previously determined primary sequence. Gel filtration indicates that the active enzyme is a monomer. The enzyme is an Fe protein and is inactivated by EDTA in a reaction which is reversible by several bivalent metal ions. The Michaelis constant of the enzyme is 18 μM for phospho*enol*pyruvate (*P*-pyruvate) and 130 μM for erythrose 4-phosphate (Ery4*P*) and the rate constant was calculated as 10 s^{-1}. Inhibition by phenylalanine is competitive with respect to erythrose 4-phosphate and non-competitive to phospho*enol*pyruvate, with a K_i of 10 μM.

The first step in the biosynthesis of aromatic compounds in microorganisms and plants is the condensation of phospho*enol*pyruvate and erythrose 4-phosphate to give 3-deoxy-D-*arabino*-heptulosonate 7-phosphate. This reaction is catalysed by dHpl*P* synthase and is closely analogous to an aldol condensation, although the precise reaction mechanism is still unclear [1]. The reverse reaction has not been observed.

It has been established that two isoenzymes of dHpl*P* synthase exist in *Saccaromyces cerevisiae*, one being feedback-inhibited by phenylalanine and encoded by the gene *ARO3*, and the other one inhibited by tyrosine, coded for by the gene *ARO4* [2]. Other microorganisms such as *Escherichia coli* and *Neurospora crassa* even possess three dHpl*P* synthases, each one regulated by one of the three aromatic amino acids.

Extensive work has been done to analyse the *ARO3* gene at the molecular level. In our laboratory, Teshiba et al. [3] isolated single *aro3* and double *aro3 aro4* mutant strains and cloned the gene *ARO3*. The gene is regulated under the system of the general control of amino acid biosynthesis in *S. cerevisiae*, which upon starvation of a single amino acid coordinately increases mRNA and, hence, enzyme levels of more than 30 genes of a number of amino acid biosynthetic pathways [3 – 5]. The DNA sequence of the *ARO3* gene has been determined and regulation of gene expression at the transcriptional level has been studied in detail [6, 7].

Here, we describe the purification and characterization of the *S. cerevisiae* phenylalanine-inhibitable dHpl*P* synthase. The yeast strain we constructed harbours the cloned *ARO3* gene on a high-copy-number plasmid, thus overexpressing the enzyme. Furthermore, the strain is defective in the *ARO4* gene and expresses no more active tyrosine-inhibitable dHpl*P* synthase. This strategy represents a simple method to enrich one out of two isoenzymes, which otherwise could not easily be separated. The pure enzyme is a monomer and is a metalloenzyme with Fe as a cofactor. Since little is known about the regulation of the *ARO3*-derived enzyme, we have established some of its kinetic and inhibitory parameters and demonstrate that inhibition by phenylalanine is competitive with one of its substrates, erythrose 4-phosphate and is non-competitive with regard to phospho*enol*pyruvate.

MATERIALS AND METHODS

Materials

Erythrose 4-phosphate (as sodium salt), phospho*enol*-pyruvate and phenylmethylsulfonyl fluoride were purchased from Sigma (St. Louis, MO). Amino acids and dithiothreitol were of the purest grade available from Fluka (Buchs, Switzerland). Ethylamino-Sepharose was synthesized by the method of Jenissen and Heilmeyer [8], using Sepharose CL4B from Pharmacia (Uppsala, Sweden) as matrix. All other chemicals were from either Fluka (Buchs, Switzerland), Merck (Darmstadt, FRG) or Sigma (St. Louis, MO).

Strains, media, plasmids and growth conditions

The yeast strains used are derivatives of the *S. cerevisiae* laboratory strains X2180-1A (*MATa gal2 SUC2 mal CUP1*) and X2180-1B (*MATα gal2 SUC2 mal CUP1*) obtained from T. Manney (Manhattan, KA) and appear in Table 1 with their genotype. Strain RH1326 was transformed with the *ARO3* gene on the multicopy plasmid pME543 [3], which is a derivative of pJDB207 [9]. Transformation was carried out by the spheroplast method of Hinnen et al. [10] with the modifications suggested by Hsiao and Carbon [11]. MV minimal medium for the cultivation of yeast was described before [4].

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Abbreviations. dHpl*P*, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; Ery4*P*, erythrose 4-phosphate; *P*-pyruvate, phospho*enol*pyruvate.

Enzymes. 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase (EC 4.1.2.15).

For the protein purification, cells were grown at 30°C in 5-l Erlenmeyer flasks with indentations on a rotary shaker. Growth was followed turbidometrically in a Pye Unicam spectrophotometer at 546 nm. $A_{546} = 1$ corresponded to approximately 1.5×10^7 cells/ml culture. Cells were harvested in mid-log phase at a density of $A_{546} = 4$. For a typical purification procedure, 25 g wet cell paste was used.

Enzyme assays and protein gel electrophoresis

Enzyme activities are specified in International Units (1 U = appearance of 1 µmol product/min or disappearance of 1 µmol substrate/min). Specific enzyme activities are given as mU/mg protein.

dHpl*P* synthase was determined with a stop assay (A) and a kinetic assay (B). (A) For determination of dHpl*P* synthase in crude extracts, exponentially growing cells were broken by three passages through a French pressure cell at 4000 N/cm^2 and centrifuged at $40000 \times g$ for 10 min (4°C). The crude extracts were adjusted to 1 mM $MgSO_4$ and nucleic acids were digested with DNase (10 µg/ml) and RNase A (10 µg/ml) for 1 h on ice. dHpl*P* synthase was assayed as described by Gollub et al. [12] except that the coloured product formed after boiling was directly determined at 549 nm instead of following extraction with cyclohexanone. For stability of the colour, the reaction mixture was kept at 55°C until measurement. Method A was also used to follow dHpl*P* synthase activity during the purification procedure. (B) Kinetic data were obtained in an assay based on absorbance difference of phospho*enol*pyruvate and dHpl*P* at 232 nm, described by Schoner and Herrmann [13]. *P*-Pyruvate and Ery4P in 50 mM potassium phosphate buffer, pH 6.8 (500 µl), were prewarmed to 30°C. The reaction was started by the addition of 2 µl purified enzyme. The decrease in absorbance at 232 nm was followed with time at 30°C in a Kontron Uvikon 820 spectrophotometer (Kontron AG, Zürich, Switzerland). Protein content of the cells was measured by the method of Herbert et al. [14]. For rapid determination of protein concentration the method of Bradford [15] was used. The standard in both methods was bovine serum albumin.

Gel electrophoresis of proteins

SDS/polyacrylamide gels were run according to Lämmli [16]. For monitoring protein purification the Mini-Protean II gel system (Bio-Rad, Richmond, CA) was used. The proteins were stained with Coomassie blue.

Column chromatography of proteins

For the hydrophobic interaction chromatography a 1.5 cm × 30 cm ethylamino-Sepharose column with a volume of 50 ml was used. Capacity of the material was 20 mg protein/ml; columns were packed by gravity.

Anion-exchange chromatography was carried out on a MonoQ™ HR5/5 column on an FPLC™ system from Pharmacia (Uppsala, Sweden) with a capacity of 20–50 mg total protein with 5 mg protein in a single peak.

Gel filtration of proteins

Gel filtration of dHpl*P* synthase was performed with a 1.5 cm × 60 cm column with a volume of 110 ml. The Sephacryl S-300 was equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM *P*-pyruvate and 1 mM dithiothreitol. 100–500 µg pure protein was layered onto the column in a maximum volume of 500 µl and eluted with the same buffer. 1-ml fractions were collected at a flow rate of 0.5 ml/min. For the calibration curve bovine serum albumin (65 kDa), ovalbumin (45 kDa) and chymotrypsin (25 kDa) were used.

Table 1. *Overexpression of the phenylalanine-inhibitable dHpl*P *synthase of* S. cerevisiae

Phenylalanine-inhibitable dHPl*P* synthase activities were determined in the presence of 1 mM L-tyrosine to inhibit the *ARO4* gene product

Strain	Genotype	*ARO3*-encoded dHpl*P* synthase activity
		mU/mg
X2180-1A	*ARO3, ARO4*	11
RH558-1	*ARO3, ARO4, gcd2-1*	60
RH1326	*aro3-2, aro4-1, gcd2-1 leu2-2*	–
RH1326 [pME543]	*[ARO3], aro4-1, gcd2-1 leu2-2*	1250

Atomic absorption spectroscopy

Metal concentrations were measured using an Instrument Laboratory atomic absorption spectrometer model Video 12.

RESULTS

*Purification of the phenylalanine-inhibitable dHpl*P *synthase*

The Phe-inhibitable dHpl*P* synthase of *S. cerevisiae* was purified from the *aro3 aro4* double mutant strain RH1326 harbouring the *ARO3* gene on the high-copy-number plasmid pME543 [3]. In addition, this strain carried a *gcd2-1* mutation resulting in increased enzyme levels derived from genes regulated under the general control system of amino acid biosynthesis [4, 5]. Together, expression of the dHpl*P* synthase (Phe) activity of the transformed strain RH1326 was increased 115-fold compared to the wild-type strain X2180-1A (Table 1). Moreover, due to the *aro4* mutation enzyme activity from the tyrosine-inhibitable isoenzyme was completely absent.

All steps in the purification procedure described below were carried out at 4°C. The protease inhibitor phenylmethylsulfonyl fluoride as well as dithiothreitol were added to each buffer to give final concentrations of 100 µM and 1 mM, respectively. The dHpl*P* synthase (Phe-inhibitable) was purified to apparent homogeneity in a four-step procedure.

Step 1: preparation of cell-free extract. Cells were cultivated to $A_{546} = 3-4$. Wet cells were washed twice with 100 mM potassium phosphate, pH 7.0, and suspended in the same buffer (1.5 ml/g cells). Crude extracts were prepared as described for the dHpl*P* synthase stop assay in Materials and Methods, yielding supernatant one.

Step 2: ammonium sulfate precipitation. The potassium phosphate concentration of supernatant one was increased to 0.5 M (pH 8.0) by adding 13 ml 2 M potassium phosphate, pH 8.0, to 40 ml supernatant one. The solution was then adjusted to 40% saturation with ammonium sulfate at pH 7.0. After 15 min of stirring at 4°C, the precipitate was removed

Table 2. *Purification steps of the* ARO3 *gene product dHplP synthase (Phe-inhibitable) from the overproducing strain RH1326 (pME543)*

Step	Protein	Specific dHpl*P* synthase activity	Purifi-cation	Recovery
	mg	U/mg	-fold	%
Crude extract				
RH1326 (pME543)	960	1.25	1	100
Ammonium sulfate	204	2.5	2	42
Ethylamino sepharose	43	11.25	9	40
MonoQ™	24	13.8	11	28

by centrifugation at 25000 g for 20 min, yielding supernatant two.

Step 3: hydrophobic-interaction chromatography. Supernatant two was loaded on an ethylamino-Sepharose CL4B column previously equilibrated with 0.5 M potassium phosphate buffer, pH 7.0, at 40% saturation with ammonium sulfate and containing 1 mM dithiothreitol but no phenylmethylsulfonyl fluoride. After washing with two column volumes of the same buffer, adsorbing proteins were eluted with a linear gradient of decreasing potassium phosphate concentration from equilibration buffer to H_2O. 5-ml fractions were collected at a flow rate of 1 ml/min.

Step 4: anion-exchange chromatography. The pooled fractions of step 3 were dialysed overnight against 20 mM Tris/HCl, pH 8.4, (buffer A) and loaded onto a MonoQ™ column previously equilibrated with the dialysis buffer. After extensive washing with the same buffer, proteins were eluted with a linear gradient formed by mixing buffer A and buffer B (buffer A containing 1 M sodium chloride) at 0.3 ml/min. The *ARO3* dHpl*P* synthase was eluted from the column faster than the rest of the protein. The peak fractions were collected, pooled and stored at −70°C after freezing in liquid nitrogen at a concentration of 1 mg/ml in a buffer containing 100 mM Tris/HCl, pH 7.0, 100 mM potassium chloride, 1 mM dithiothreitol and 100 μM phenylmethylsulfonyl fluoride. At a concentration of 100 μg/ml the enzyme remained stable for at least 2 h at 25°C and 2 days at 4°C, whereas at 10 μg/ml more than 50% of the activity was lost after 1 h at 25°C.

A purification protocol is summarized in Table 2. The dHpl*P* synthase was purified 11-fold with a 28% yield of recovery. Including the 115-fold overproduction due to the high copy number of the plasmid and the *gcd2-1* mutation of strain RH1326 this corresponds to a 1250-fold enrichment of the chromosomally encoded dHpl*P* synthase (Phe) of *S. cerevisiae* strain X2180-1A. An SDS/polyacrylamide gel (Fig. 1) showed a strong band at 42 kDa on a Coomassie-blue-stained gel after the MonoQ™ column, which corresponded to the molecular mass previously deduced from the DNA sequence [6].

Apparent native molecular mass

The subunit molecular mass was determined by gel filtration on a Sephacryl S-300 column. The enzyme activity was eluted as a single peak in a volume corresponding to a molecular mass of 53 kDa, as estimated by comparison with standard markers (data not shown). Compared with the results described above, these data suggest that the active enzyme is a monomer.

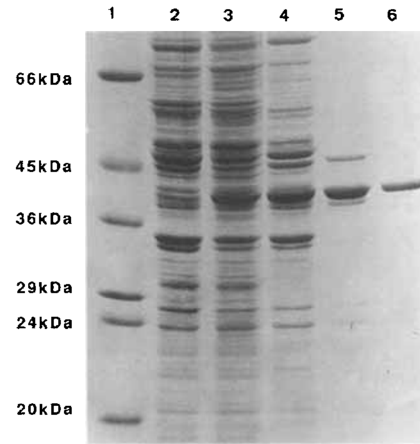

Fig. 1. *Purification steps of the phenylalanine-sensitive dHplP synthase analysed by SDS/polyacrylamide gel electrophoresis.* A 13% SDS/polyacrylamide gel was stained with Coomassie blue. (1) Marker proteins with the indicated molecular mass; (2) crude extract of the wild-type strain X2180-1A (50 μg); (3) crude extract of RH1326 overexpressing the *ARO3* gene 115-fold (30 μg); (4) supernatant of ammonium sulfate precipitation (20 μg); (5) ethylamino-Sepharose pool (10 μg); (6) MonoQ™ pool (4 μg)

Table 3. *Metal-ion dependence of dHplP synthase (Phe-inhibitable)* Phenylalanine-inhibitable dHpl*P* synthase activities were determined in the presence of 1 mM L-tyrosine to inhibit the *ARO4* gene product

Addition to purified enzyme	dHpl*P* synthase activity
	%
None	100
EDTA	11
EDTA at 0°C	66
Co^{2+}	112
Mn^{2+}	95
Zn^{2+}	82
Fe^{2+}	98
EDTA + Co^{2+}	104
EDTA + Mn^{2+}	118
EDTA + Zn^{2+}	91
EDTA + Fe^{2+}	95
EDTA + *P*-pyruvate	16
P-pyruvate + EDTA	60
EDTA + Ery4P	10
Ery4P + EDTA	8

pH and metal ion dependence of enzyme activity

Takahashi and Chan [17] reported for the Phe-inhibitable dHpl*P* synthase in protamine-sulfate-treated and dialysed crude extracts a flat pH profile with a broad maximum ranging from pH 6.5 to pH 8.0. Our pure enzyme shows a narrower pH optimum for activity around pH 6.8.

In order to test whether the enzyme needs a metal ion as cofactor, in a first approach the effect of EDTA on the activity of the purified Phe-inhibitable dHpl*P* synthase was tested as follows. The pure enzyme was incubated with EDTA (final concentration 1.3 mM) in 50 mM potassium phosphate buffer (pH 6.8) for 5 min at room temperature and assayed. Enzyme

Table 4. *Steady-state kinetics of dHplP synthases from various organisms*
The given values of the yeast dHpl*P* synthase (Phe) are averages of four independent measurements (SD < ± 25%).
The kinetic data for the *E. coli* [13, 25] and for the *N. crassa* [26] dHpl*P* synthases were described earlier. Not determined, nd

Organism	Enzyme	*k*	$K_{m,app}$ with		K_i	Quaternary structure
			P-pyruvate	Ery4*P*		
		s^{-1}	μM			
S. cerevisiae	dHpl*P* synthase (Phe)	10	18	130	10	monomer
E. coli	dHpl*P* synthase (Phe)	nd	80	900	75	tetramer
	dHpl*P* synthase (Tyr)	121	5.8	96.5	82	dimer
N. crassa	dHpl*P* synthase (Trp)	nd	12	2.7	nd	tetramer

activity was largely inhibited by the addition of EDTA (Table 3). When the enzyme/EDTA mixture was kept at 0 °C, however, before being assayed, only partial inactivation of the enzyme was found. In a second set of experiments, after inactivation of the enzyme by EDTA, metal ions were added as sulfates and the mixture was again incubated for 5 min at 25 °C before activity was measured. Metal ions such as Co^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2+} were able to restore enzyme activity, whereas ions such as Ca^{2+}, Mg^{2+}, Cu^{2+} and K^{+} were unable to regenerate activity. None of the metal ions, however, was able to increase the activity of the pure enzyme when added alone (Table 3). One of the two substrates, *P*-pyruvate, protected the protein at 2 mM, partially from inactivation by EDTA, but was not able to restore activity when added after EDTA. Ery4*P* had no impact on enzyme activity in the presence of EDTA.

Atomic absorption spectrometry suggests that the metal ion which is present in the enzyme *in vivo* is iron which was found in a concentration of 0.6 mol/mol protein. No cobalt and manganese was found and the amount of zinc was corresponding to the assay buffer.

Substrate kinetics

Kinetic analyses with the pure enzyme preparation were performed to determine the steady-state kinetic parameters of the Phe-inhibitable dHpl*P* synthase. In each case, the initial velocity (*v*) was determined as a function of the concentration of one substrate at a fixed saturating level of the other substrate. The decrease of *P*-pyruvate was followed spectrophotometrically at 232 nm as described in the kinetic assay (B) in Materials and Methods.

First, *P*-pyruvate concentrations were kept constant at a saturating level of 600 μM and initial velocities of the reaction in varying amounts of Ery4*P* were measured. Then, analogous data were obtained with *P*-pyruvate as the varied substrate at a fixed concentration of 800 μM Ery4*P*. On the basis of these data, an apparent K_m for Ery4*P* of 130 μM and an apparent K_m for *P*-pyruvate of 18 μM was determined. With the apparent V_{max} value and the known enzyme concentration $[e_0]$ the rate constant was calculated as 10 s^{-1}. The Lineweaver-Burk plots of initial velocities measured at several fixed *P*-pyruvate and varying Ery4*P* concentrations have a common intercept in the second quadrant and thus the kinetic data suggest a sequential reaction mechanism. Product-inhibition studies, however, to determine the order of substrate binding are yet to be carried out.

The kinetic parameters are listed in Table 4 together with the corresponding values of the Phe-, Tyr- and Trp-sensitive dHpl*P* synthases from other organisms and are discussed below.

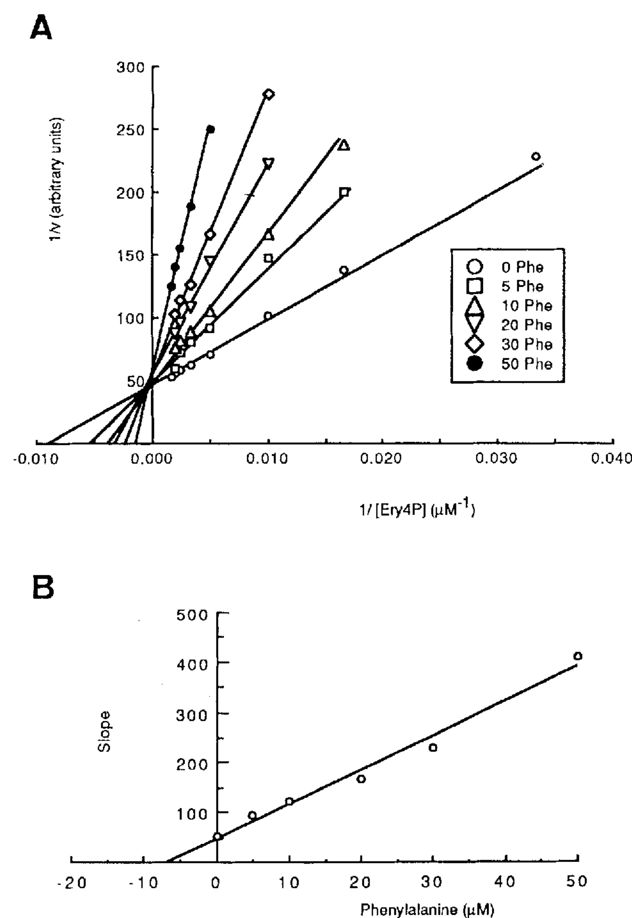

Fig. 2. *Inhibition by phenylalanine.* (A) Double-reciprocal plots of initial velocity (*v*) against Ery4*P* concentrations at 400 μM *P*-pyruvate and several fixed phenylalanine concentrations. The values are means of four independent measurements (SD < ± 25%). (B) Variation of the slopes (arbitrary units) as a function of phenylalanine concentrations

Inhibition by phenylalanine

An inhibition study was conducted to determine (a) the inhibition constant K_i of phenylalanine and (b) the type of inhibition exerted to phenylalanine on the pure dHpl*P*

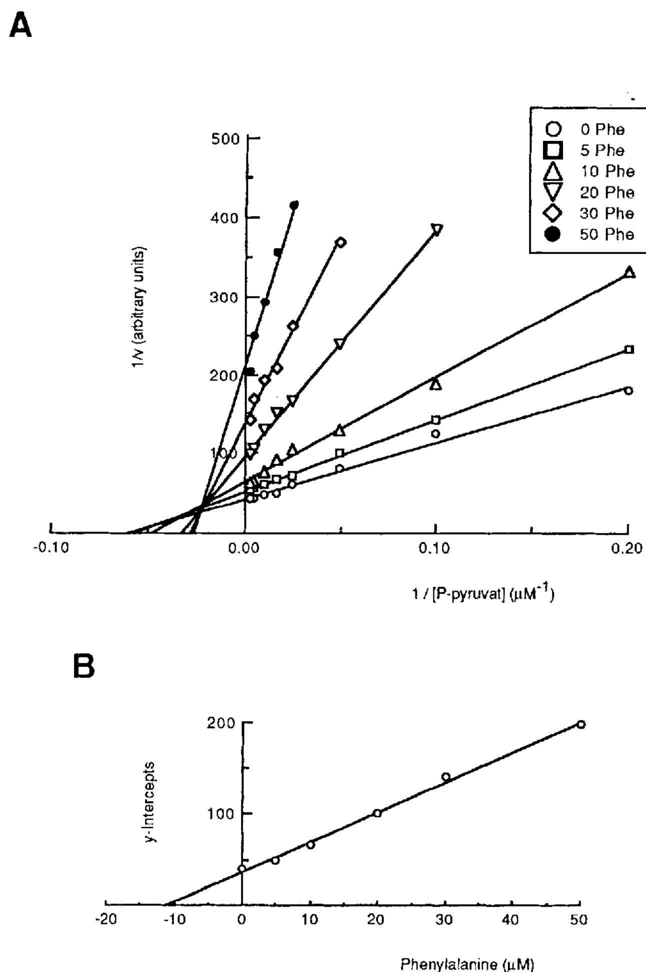

Fig. 3. *Inhibition by phenylalanine.* (A) Double-reciprocal plots of initial velocities (v) are given as a function of P-pyruvate. The Ery4P concentration was 400 µM. The values are means of four independent measurements (SD < ± 25%). (B) Secondary plot of the intercepts (arbitrary units) against the phenylalanine concentrations

synthase. Fig. 2A shows the Lineweaver-Burk plots of the initial velocities of the reaction against varying concentrations of Ery4P in the presence of 0, 5, 10, 20, 30 and 50 µM phenylalanine. P-Pyruvate concentrations were kept at 400 µM. The lines intersect on the y-axis, which suggests that phenylalanine is a competitive inhibitor of the reaction with respect to Ery4P. The inhibition constant was determined by plotting the slopes of the lines of Fig. 2A versus inhibitor concentration (Fig. 2B). The intersect of the graph with the x-axis is K_i, and the K_i for phenylalanine was 7 µM. The inverse arrangement was also measured. The Ery4P concentration was kept constant at 400 µM and initial velocity was determined at 0, 5, 10, 20, 30 and 50 µM phenylalanine in varying P-pyruvate concentrations. In the Lineweaver-Burk plot (Fig. 3A) the corresponding lines cross in the second quadrant, indicating non-competitive inhibition of phenylalanine with respect to P-pyruvate. The inhibition constant K_i was also obtained from a secondary plot; the y-axial intercepts were plotted versus the inhibitor concentration and K_i was determined as 12 µM (Fig. 3B).

The results from Figs 2 and 3 thus correspond to each other, suggesting that phenylalanine acts as competitor for the binding of Ery4P to the enzyme and hence inhibition is non-competitive with respect to P-pyruvate with an average K_i of 10 µM.

Neither L-tyrosine nor L-tryptophan, at concentrations up to 1 mM, inhibit the pure enzyme (data not shown).

DISCUSSION

The reaction catalysed by dHpl$P$ synthase, the condensation of P-pyruvate derived from glycolysis and Ery4P from the pentose-phosphate pathway, is the first step in aromatic compound biosynthesis. The flow of carbon through the shikimate pathway is controlled by modulation of the activity of dHpl$P$ synthase. The Phe-inhibitable dHpl$P$ synthase of *S. cerevisiae* is encoded by the gene *ARO3*. Control of the gene at the level of transcription has been analysed before [6, 7].

In this paper we have described the purification and characterization of the phenylalanine-inhibitable dHpl$P$ synthase of *S. cerevisiae*. The problem to separate the two dHpl$P$ synthase isoenzymes of yeast was circumvented using current gene technology. A strain was constructed overexpressing the *ARO3* gene product and being simultaneously defective in the *ARO4* gene. As was described for the *TRP1* and *TRP3* gene products [18, 19], proteolytic degradation in the early steps of the purification was overcome by addition of protease inhibitors and by maintaining high salt concentrations. Moreover, ethylamino-Sepharose presumably removed a major portion of the numerous protease activities present in cells of *S. cerevisiae*.

A 1250-fold enrichment was necessary to obtain the pure protein. Hence, the dHpl$P$ synthase corresponds to approximately 0.1% of the total cellular protein. This is in agreement with an estimation of the *ARO3* mRNA level in the cell, which, based on the codon usage index according to Bennetzen and Hall [20], amounts to roughly 0.05% of the total mRNA.

A striking difference between the dHpl$P$ synthases, compared in Table 4, is their quaternary structure. Even within *E. coli*, where similarity between the tyrosine- and the phenylalanine-inhibitable dHpl$P$ synthases is 70% (unpublished data, calculated according to primary data of [21] and [22]), the number of subunits of the active enzyme is different. The Phe-inhibitable dHpl$P$ synthase is a tetramer, whereas the Tyr-inhibitable enzyme is a dimer. According to gel-filtration studies, however, the yeast Phe-inhibitable dHpl$P$ synthase activity eluted in a volume corresponding to a molecular mass of 53 kDa, pointing to a monomeric structure of the active enzyme.

Atomic absorption spectroscopy suggests that the Phe-sensitive dHpl$P$ synthase of *S. cerevisiae* is a Fe metalloenzyme. The enzyme can be inactivated by EDTA and restored to full activity by several bivalent metal ions. Similar results were found for the Phe-inhibitable *E. coli* enzyme [23–25]. We observed only partial inactivation by EDTA in the presence of P-pyruvate or at low temperatures, similar to the inactivation of the Phe-sensitive dHpl$P$ synthase from *E. coli* [23–25] or the Trp-sensitive dHpl$P$ synthase from *N. crassa*, both of which were unaffected at low temperatures [26].

The kinetic data of the Phe-sensitive yeast dHpl$P$ synthase suggest a sequential reaction mechanism as it was also proposed for the tyrosine-inhibitable *E. coli* dHpl$P$ synthase [13]. The kinetic parameters were compared with those obtained for the two available dHpl$P$ synthase isoenzymes from *E. coli* and the Trp-sensitive enzyme from *N. crassa* (Table 4). The available apparent K_m for P-pyruvate are similar in all dHpl$P$

synthases of Table 4. For Ery4*P*, however, the reported values vary between 2.7 μM and 900 μM. A reason for this finding may be that Ery4*P* forms dimers in solution, but the enzyme distinguishes between monomers and dimers [26], so that the concentration of this substrate available to the enzyme could be overestimated. The reported inhibition constants for all the dHpl*P* synthases listed in Table 4 are in the same order of magnitude. For the yeast enzyme, inhibition was competitive with respect to Ery4*P* and non-competitive with respect to *P*-pyruvate. The *N. crassa* enzyme showed the same pattern of inhibition, but only qualitative results were obtained, as the inhibition by Trp was not hyperbolic and the intercept or slope replots were curving upward [26]. The present knowledge suggests that the interplay of dHpl*P* synthase subunits and the regulatory behaviour seems to be different in various organisms though the high degree of similarity might correspond to similarities in the catalytic behaviour.

In summary, we have reported a fast purification method for the yeast phenylalanine-inhibitable dHpl*P* synthase from an overproducing strain using a combination of molecular genetics and biochemistry and have determined some of its steady-state kinetic parameters. Once the *ARO4* gene is cloned and available for overexpression, we expect a similar approach to be useful also for enrichment of the tyrosine-inhibitable isoenzyme; it will then be interesting to compare the kinetic and inhibitory properties of the two enzymes.

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