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The HARO7 gene of the methylotrophic, thermotolerant yeast Hansenula polymorpha was cloned by functional complementation. HARO7 encodes a multifunctional 280-amino-acid protein with chorismate mutase (EC 5.4.99.5) activity that catalyzes the conversion of chorismate to prephenate, a key step in the biosynthesis of aromatic amino acids. The HARO7 gene product shows strong similarities to primary sequences of known eukaryotic chorismate mutase enzymes. The homologous overexpression and purification of the 32-kDa protein, its kinetic parameters (Km = 319.1 s⁻¹, nM = 1.56, [S]₉₀ = 16.7 mM) as well as its allosteric regulatory properties were determined. Tryptophan acts as heterotropic positive effector; tyrosine is a negative-acting, heterotropic feedback inhibitor of enzyme activity. The influence of temperature on catalytic turnover and the thermal stability of the enzyme were determined and compared to features of the chorismate mutase enzyme of Saccharomyces cerevisiae. Using the Cre-loxP recombination system, we constructed mutant strains carrying a disrupted HARO7 gene that showed tyrosine auxotrophy and severe growth defects. The amount of the 0.9-kb HARO7 mRNA is independent of amino acid starvation conditions but increases twofold in the presence of methanol as the sole carbon source, implying a catabolite repression system acting on HARO7 expression.

Methylotrophic yeasts have gained increasing recognition in basic research as well as in applied biotechnology in the last few years. Most of them are ascomycetes of the genera Hansenula, Pichia, and Candida (38), with Hansenula polymorpha (synonym, Pichia angusta) representing the most prominent member (for a review, see reference 26). Utilization of methanol as sole source of carbon and energy by Hansenula polymorpha is generally accompanied by strong proliferation of microbodies, so-called peroxisomes, and high-level induction of peroxisomal matrix enzymes required for C₁ metabolism (49, 67). The first step in the methanol-utilizing pathway is the oxidation of methanol to formaldehyde and H₂O₂, catalyzed by the so-called peroxisomal catalase (EC 1.11.1.6) encoded by the CAT1 gene, a catalase (EC 1.11.1.6) encoded by the CATI gene, and a formate dehydrogenase activity (EC 1.2.1.2) which is the FMD gene product (9, 31, 33). In the presence of glucose, expression of these genes is subject to a repression system, whereas upon methanol utilization the promoters of these genes are strongly induced (12). The tightly regulated strength of genes involved in methanol metabolism forms the basis for the biotechnological and commercial use of Hansenula polymorpha in recombinant gene expression systems. In recent years, a tractable vector-host system has been developed using either homologous or heterologous metabolic genes as selectable markers in combination with defined mutant strains and taking advantage of the strong promoters of genes that are part of the methanol-utilizing machinery (14, 20). Integration of autonomously replicating plasmids into the chromosomal DNA can be achieved, yielding up to 100 tandemly repeated copies of the transforming DNA that are mitotically stable in the Hansenula polymorpha genome (19, 34). Additionally, Hansenula polymorpha is able to grow at temperatures of up to 48°C, with an optimal growth temperature of 37°C, which is unusual for methylotrophic yeasts (40).

In contrast to the specialized methanol-utilizing pathway of methylotrophic yeasts, biosynthesis of aromatic amino acids is a common feature of most living organisms. Chorismic acid, the end product of the shikimate pathway, is formed in seven invariable enzyme-catalyzed reactions starting with compounds of primary metabolism, erythrose 4-phosphate and phosphoenolpyruvate (27). Conversion of chorismate to anthranilate initiates the biosynthetic branch resulting in l-tryptophan, whereas intramolecular rearrangement of the enolpyruvyl side chain of chorismate to yield prephenate is the initial step in the synthesis of l-tyrosine and l-phenylalanine (68). The latter reaction is unique, as it is the only Claisen rearrangement identified so far in primary metabolism (18). Generally, the conversion of chorismate to prephenate is catalyzed by chorismate mutases (EC 5.4.99.5) which have been identified and characterized in archa, bacteria, fungi, and plants (for a review, see reference 50). Crystallographic data for three natural enzymes have led to a classification based on structural elements as well as primary sequence information. AroH class chorismate mutases are α/β-barrel proteins, as is the trimeric Bacillus subtilis enzyme (6), whereas the AroQ class comprises all-helix bundle polypeptides that are often part of a bifunctional enzyme like the chorismate mutase domain of the Escherichia coli chorismate mutase-prephenate dehydratase activity (8, 39). Eukaryotic chorismate mutases are also classified in the latter class on the basis of conservation.

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of crucial catalytic residues and related tertiary structure (43, 71). Whereas a number of prokaryotic genes encoding chorismate mutase activities have been cloned to date, only a few sequences that originate from eukaryotic organisms and code for chorismate mutase enzymes are available. The best-studied eukaryotic enzyme with respect to structure, allosteric regulation, and mechanism of catalytic turnover is that of the baker’s yeast *Saccharomyces cerevisiae* (42, 56, 57, 65). Recently, additional data for the chorismate mutase enzyme of the filamentous fungus *Aspergillus nidulans* which is encoded by the *aroC* gene, have become available (25). The *A. nidulans* chorismate mutase was found to be similar in catalytic and structural properties to the well-characterized enzyme of *S. cerevisiae*. Nevertheless, different mechanisms for allosteric regulation upon effector binding have been proposed for these two chorismate mutases.

To extend the eukaryotic subclass of AroQ enzymes, we here present the cloning and characterization of the *HARO7* gene coding for a chorismate mutase activity of the methyloptic yeast *H. polymorpha*, an organism closely related to *S. cerevisiae*. *H. polymorpha* disruption strains were constructed by establishing the Cre-loxP recombination system of bacteriophage P1 (63) in this yeast to constitute *HARO7* as a new marker gene to the vector-host expression system of *H. polymorpha* for biotechnological applications (G. Gellissen, G. Braus, R. Fries, S. Georgieva, Heide Mitchell, and A. W. Strasser, German patent application (69)). *H. polymorpha* membranes from Millipore (Eschborn, Germany) were obtained and has been described by Weydemann et al. (69).

**Materials and Methods**

Materials. Chorismic acid as barium salt was purchased from Sigma (St. Louis, Mo.), 5-Fluoroorotic acid was obtained from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). L-Tyrosine for supplementation was obtained as free base (Sam Chemicals A. G.) from Sigma-Aldrich (Munich, Germany) and alternatively from Fluka (Neu-Ulm, Germany) in BioChemika grade (>99%; foreign amino acids, <0.3%). Protein solutions were concentrated by using stirred cells (volumes of 180 and 10 ml) with PM-10 ultrafiltration membranes from Millipore (Eschborn, Germany). The Mini 2D sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) system and Bradford protein assay solution for determination of protein concentrations were from Bio-Rad Laboratories (Hercules, Calif.). Platinum Pf DNA polymerase from Life Technologies GmbH (Karlsruhe, Germany) was used for PCRs. All other chemicals were supplied by Fluka or Sigma-Aldrich.

Strains, media, growth conditions, and transformation procedures. Plasmid DNA was propagated in Escherichia coli DH5α (70). *S. cerevisiae* RH2105 (MATa suc2-Δ9 ura3-52 leu2-3,112 his3-52 A101 LEU2 GAL2) (59) with the genetic background of the laboratory strain X2180-1A (MATa pol2 SUC2 null CUP1) was used as recipient for cloning of a DNA fragment containing the *HARO7* gene of *H. polymorpha* from a genomic library. The uracil-auxotrophic *H. polymorpha* strain RH11 (see below) was obtained from Rhein Biotech (Düsseldorf, Germany) and has been described by Weydemann et al. (69). *H. polymorpha* RH2408 (old FMD promoter: *HARO7 UR43) generally was grown at 37°C as shaken flask (750 ml) culture in YNB supplemented with 5% glycerol as sole carbon source for *FMD* promoter derepression. Cells were harvested at an optical density of 546 nm (OD546) of 7 to 8, washed twice with 50 mM potassium phosphate buffer (pH 7.6), and stored in 1 ml of buffer per g of wet cells at −20°C in the presence of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 1 mM (β-dithiotreitol). For purification, 20 to 30 g of cells was thawed and passed three times through a French pressure cell (30,000 psi). The chorismate mutase enzyme was purified as described by Schmidheini et al. (32), with the modification that for buffer changing or desalting pooled enzymatic activity assays. Native PAGE was performed as described by Anders-Schneider et al. (7) at a final concentration of 10% (w/v) agarose gel (1 mm thick), 8% (w/v) acrylamide gel, and 0.5% ammonium sulfate. Gels were stained with Coomassie blue (20). The purified enzyme was homogeneous as judged by SDS-PAGE and native PAGE analysis.

Enzyme assays and data evaluation. The chorismate mutase enzyme was measured as described previously (54). Enzymatic reactions were carried out at 37°C as briefly described in Table 1. Protein concentrations were determined by using a gradient of 0.1% (w/v) Folin reagent in a 1:1 mixture with 15% (v/v) isopropanol at 595 nm. All assays were performed in triplicate.

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5. **Figure 1.** Purification and characterization of the chorismate mutase from *H. polymorpha*. Purified enzyme was separated by native PAGE and subjected to Coomassie blue staining. The enzyme purity was determined by densitometry.

6. **Figure 2.** Enzymatic activities were measured by using a Cary 1 spectrophotometer. Protein concentrations were determined by using the Bradford protein assay.

7. **Table 2.** Enzymatic reactions were carried out at 37°C as described above. Enzyme activities were measured by using a Cary 1 spectrophotometer. Protein concentrations were determined by using the Bradford protein assay.

8. **Figure 3.** Purification and characterization of the chorismate mutase from *H. polymorpha*. Purified enzyme was separated by native PAGE and subjected to Coomassie blue staining. The enzyme purity was determined by densitometry.

9. **Figure 4.** Enzymatic activities were measured by using a Cary 1 spectrophotometer. Protein concentrations were determined by using the Bradford protein assay.

10. **Table 3.** Enzymatic reactions were carried out at 37°C as described above. Enzyme activities were measured by using a Cary 1 spectrophotometer. Protein concentrations were determined by using the Bradford protein assay.

11. **Figure 5.** Purification and characterization of the chorismate mutase from *H. polymorpha*. Purified enzyme was separated by native PAGE and subjected to Coomassie blue staining. The enzyme purity was determined by densitometry.

12. **Figure 6.** Enzymatic activities were measured by using a Cary 1 spectrophotometer. Protein concentrations were determined by using the Bradford protein assay.

13. **Table 4.** Enzymatic reactions were carried out at 37°C as described above. Enzyme activities were measured by using a Cary 1 spectrophotometer. Protein concentrations were determined by using the Bradford protein assay.
of phenylpyruvate is temperature dependent due to a keto-enol equilibrium, the assay was standardized by keeping the spectrophotometer cell at 30°C. Evaluation of kinetic data was performed as described previously (reference 35 and references therein). Thermal stabilities were determined according to Segel (60), and 3-deoxy-D-arabinoheptulosonic acid (DAHP) synthase activities were measured as described by Teshiba et al. (66).

Sequence alignments. Sequence analyses were performed using the LASERGENE Biocomputing software from DNASTAR (Madison, Wis.). Alignments were created based on the Lipman-Pearson method (41).

Nucleotide sequence accession number. The sequence obtained from plasmid pME1525 for the HARO7 gene has been assigned GenBank accession no. AF240738.

RESULTS

The HARO7 gene of H. polymorpha codes for a chorismate mutase enzyme. The HARO7 gene from the methylophilic yeast H. polymorpha was cloned by functional complementation of an S. cerevisiae aro7A mutant strain. Strains of S. cerevisiae with a deleted ARO7 gene are devoid of endogenous chorismate mutase activity and generally are unable to grow on medium lacking tyrosine or phenylalanine. S. cerevisiae RH2185 (aro7::LEU2 ara3-52) (59) was transformed with genomic DNA of H. polymorpha RB11 (odc1) (69) cloned into the high-copy-number plasmid pRS426 (61). Transformants were selected for viability on minimal medium YNB lacking tyrosine and phenylalanine. One colony appeared after 5 days of growth. A plasmid (pME1524) isolated from this clone was able to complement the auxotrophy of the recipient aro7Δ S. cerevisiae strain. The recipient strain harboring this plasmid grew more slowly than the positive control. Restriction analysis of this plasmid indicated that it contained a genomic DNA insert 5 kb in length. Subcloning of this fragment revealed a 1.7-kb ApaI/Sau3A fragment that was able to complement the Tyr/Phe auxotrophy of S. cerevisiae RH2185 when recloned into pRS426. The recipient strain transformed with this subclone plasmid (pME1525) grew at a rate similar to that of an S. cerevisiae wild-type strain. The DNA insert of pME1525 was subjected to sequence analyses. The genomic sequence is 1,648 bp in length and includes an open reading frame of 843 bp with 281 codons with the capacity to encode a polypeptide with a calculated Mr of 32,067. The 5′ flank region of the genomic fragment spans 342 nucleotides, whereas the 3′ region is 467 bp in length. Conserved splicing motifs described for yeast (51) are not present within the coding region of the identified gene, indicating the absence of any intron sequences. Upon alignment, the deduced amino acid sequence of the gene exhibited high degrees of identity and similarity to genes of known chorismate mutases of other eukaryotic organisms (Fig. 1). The best alignment was to the enzyme of S. cerevisiae, with 54% identity and 70% similarity when conservative changes are taken into account. With the described primary sequence of chorismate mutase from Schizosaccharomyces pombe, 43% identical and 65% similar residues, including conservative replacements, were found. Comparison to chorismate mutase of the filamentous fungus A. nidulans revealed 43% identity and 65% similarity. The plastidic chorismate mutase of Arabidopsis thaliana is less related; the deduced amino acid sequence is 35% identical to the mature plant enzyme and 58% similar when conservative exchanges are included. Because of this strong similarity to described chorismate mutases and the functional complementation of a chorismate mutase-deficient S. cerevisiae strain, the isolated gene from H. polymorpha was named HARO7, in analogy to the homologous ARO7 gene of S. cerevisiae.

Disruption of HARO7 in H. polymorpha results in tyrosine auxotrophy. Gene replacement mutant strains were constructed via homologous integration to characterize the HARO7 gene in more detail (Fig. 2A). Therefore, H. polymorpha RB11 was transformed with a disruption cassette consisting of a loxP-ODC1MX-loxP module flanked by 5′ and 3′ homologous sequences of the HARO7 locus. In this construct, 93% of the HARO7 coding sequence is replaced by the marker cassette expressing orotidine-5′-phosphate decarboxylase, which is encoded by the ODC1 gene (46). Transformants were selected on minimal medium supplemented with tyrosine and phenylalanine but lacking uracil. Auxotrophic mutants were identified by replica plating on minimal medium without supplements. Out of approximately 1,100 Ura+ transformants, five independent, auxotrophic clones were isolated, in line with the low frequency of homologous recombination reported previously for H. polymorpha (16). Retransformation with a DNA fragment comprising the HARO7 coding sequence restored prototrophy of these strains, whereas in negative control experiments no transformants were able to grow. A descendant without the ODC1 expression cassette was obtained from one of these clones (RH2409), taking advantage of the loxP recombination sites in the disruption construct (53). For this purpose, H. polymorpha RH2409 was transformed with the autonomously replicating plasmid pME1690, carrying the cre coding sequence inserted between the inducible FMD promoter and the MOX termination region and the HARO7 coding sequence as a marker gene in addition to the ODC1 gene. Transformants were selected on minimal medium and propagated for 24 h on glycerol-containing medium to derepress expression of the Cre recombinase driven by the FMD promoter. Cured clones in which the ODC1 cassette had been removed by forced homologous recombination between the flanking loxP sites were counterselected on supplemented medium in the presence of 5-fluoroorotic acid (4). One strain (RH2410) isolated by this procedure showed uracil auxotrophy.

The correct genotype of both haro7A mutant strains was confirmed by diagnostic PCR with oligonucleotides specific for HARO7 5′ and 3′ flanking regions (Fig. 2B). Whereas in the wild-type H. polymorpha HARO7 strain RB11 a 1.2-kb fragment was amplified from genomic DNA, insertion of the loxP-ODC1MX-loxP cassette resulted in a 2-kb amplicon. Removal of the ODC1 expression cassette in strain RH2410 was indicated in the PCR approach by amplification of a 0.5-kb DNA fragment. In Southern analysis, the HARO7 promoter-specific probe was hybridized to ApaI/KpnI-digested genomic DNA of H. polymorpha RB11, RH2409, or RH2410 (Fig. 2C). For RB11, a 3.2-kb signal corresponding to the wild-type HARO7 locus was observed. Insertion of the marker module introduced an additional ApaI site in RH2409, thus shortening the hybridizing signal to 0.8 kb. Due to the removal of this ApaI site within the ODC1 gene, a 1.6-kb signal was detected in Southern analysis of RH2410. As expected, the haro7Δ strains showed no growth on solid, nonsupplemented medium but grew on minimal medium supplemented with standard concentrations of tyrosine, phenylalanine, and uracil. No growth was observed on complex medium YEFP even in the presence of tyrosine and phenylalanine or on synthetic complete medium. Surprisingly, both strains grew slowly but reproducibly on minimal medium containing tyrosine as sole amino acid. Therefore, the haro7A mutant strains of H. polymorpha are auxotrophic for the aromatic amino acid tyrosine but bradytrophic for phenylalanine. In liquid cultures of minimal medium, retarded growth was observed only when tyrosine was added at five times the concentration used for supplementation in solid medium. In summary, both mutant strains showed growth defects that depended on the composition of the growth medium, indicating the importance of chorismate mutase activity for

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growth of *H. polymorpha*. This conclusion is supported by the fact that strains retransformed with the HARO7 coding sequence, either as a linear DNA fragment or on a plasmid, did not show any of the growth defects described above and were able to grow on complex medium. HARO7 expression is regulated transcriptionally upon methanol utilization but not upon amino acid starvation. In silico analysis of the flanking 5′ region of HARO7 identified two sequence elements that resemble conserved binding sites for yeast transcription factors. One motif (5′-CACGTG-3′, positions 214 to 218 relative to the translational start codon AUG) matches a binding site for Pho4p (5′-CANNTG-3′) (17), the ultimate effector for phosphate utilization in *S. cerevisiae*. Additionally, an upstream regulatory sequence specific for *H. polymorpha* was identified in the HARO7 promoter region. This sequence element (5′-TTGCCACCGGAA-3′, positions 275 to 281) is similar to the core region of a binding site for Mbp1p (5′-TGCCACCAGAA-3′) within the promoter of the *MOX* gene encoding the peroxisomal methanol oxidase of this methylotrophic yeast (22, 37). A similar motif is found in the promoter of the *H. polymorpha* CAT1 gene, which codes for a peroxisomal catalase (5′-TTGCCACCGGAA-3′) (9). In contrast, no conserved sequence elements directing 3′-end formation were identified in the HARO7 gene fragment of pME1525. The putative Mbp1p-binding element in the HARO7 promoter region implies a transcriptional regulation of HARO7 expression upon methanol utilization. To monitor transcription of the HARO7 gene, steady-state transcript levels were quantified in Northern hybridization analyses. The length of the HARO7-encoded mRNA was determined using an RNA size standard as approximately 0.9 kb (not shown). HARO7 transcription was monitored with respect to availability of dif-

different carbon sources, using expression of the ACT gene of *H. polymorpha* as an internal standard (Fig. 3). To this end, RB11 was cultured in minimal medium containing glycerol as a non-fermentable carbon source or methanol as an inducer of the methanol-utilizing metabolic pathway in *H. polymorpha* (11). Cells were harvested at mid-exponential phase of growth and 4 or 8 h later for total RNA preparation. In addition, glucose was added to identical cultures grown in the presence of glycerol or methanol, respectively, and cultivation was continued for 4 and 8 h prior to RNA preparation. Northern analysis using ACT transcript levels as internal standard revealed different expression patterns of *HARO7* transcription. Transcript levels increased slightly but reproducibly when cells were grown in glycerol-containing medium. Furthermore, methanol as carbon source had a more pronounced effect on *HARO7* transcription, with transcript levels increasing by a factor of 2 compared to glucose-grown cells. These effects induced by the nonoptimal carbon sources were diminished when glucose was added to the medium, implying repression of *HARO7* transcription.

Chorismate mutase is an enzyme in aromatic amino acid biosynthesis. Therefore, transcript levels of *HARO7* mRNA were quantified under conditions of amino acid starvation. *H. polymorpha* RB11 was grown in minimal medium supplemented with the false feedback inhibitor 3-amino-1,2,4-triazole (3-AT) at 1 mM to induce histidine starvation and to derepress the general control system of amino acid biosynthesis (3, 28). Specific DAHP synthase activities (EC 4.1.2.15) determined in crude extracts of RB11 grown in the absence or presence of 3-AT, respectively, were used as a control and showed an increase by a factor of 2 (data not shown), indicating that the general control of amino acid biosynthesis had been induced by the false feedback inhibitor (66). The specific chorismate mutase activity was unaffected by the absence or presence of 3-AT (data not shown). Total RNA was prepared from cultures in mid-log phase (OD$_{546}$ $\approx$ 1.2) and subjected to Northern analysis. Quantification of signal strength revealed no significant increase of *HARO7* transcript levels after the shift to starvation conditions (Fig. 3). We conclude, therefore, that *HARO7* transcription is not triggered by the general control system of amino acid biosynthesis.

Chorismate mutase of *H. polymorpha* is allosterically regulated by tyrosine and tryptophan. The *HARO7* gene product is a key enzyme in the biosynthesis of aromatic amino acids. Chorismate mutase activity has to be regulated stringently to control the flux through the branch point. As no regulation of expression is evident with respect to amino acid availability, we...
were interested in whether certain amino acids might influence catalytic activity. To determine its enzymatic properties, the HARO7 gene product was overproduced and purified. The HARO7 coding sequence was cloned into the expression vector pFPMT121, where it is fused to the promoter of the *H. polymorpha* FMD gene coding for formate dehydrogenase (31) and flanked by the termination region of the MOX gene (37). *H. polymorpha* RB11 was transformed with this expression plasmid (pME1686) and sequentially grown in selective and rich medium to obtain mitotically stable transformants (19). One clone (RH2408) analyzed by Southern hybridization was identified to harbor approximately 50 copies of the expression construct ectopically integrated into the genome of the host strain (data not shown). Cultivation in minimal medium containing glycerol as sole carbon source derepressed the FMD-driven expression of HARO7. The resulting chorismate mutase activity was purified to homogeneity from this overexpressing strain by the purification procedure described in Materials and Methods (Fig. 4A and B). In a gradient PAGE under nonde-naturing conditions, the purified protein displayed an apparent molecular mass of approximately 70 kDa (Fig. 4A). This indicates that the native enzyme consists of two protomers combined to form a dimeric quaternary structure. Kinetic stop assays for determination of catalytic parameters were performed at 37°C, which is the optimal temperature for growth of *H. polymorpha* (Fig. 4C; see below). In the absence of effectors, the enzyme showed positive cooperativity toward its substrate chorismate, resulting in a sigmoid substrate saturation curve. An \([S]_{1/2}\) value of 16.7 mM and a maximal turnover rate of 319.1 s\(^{-1}\) per active site were determined. The calculated Hill coefficient, \(n_{H}\), of 1.56 clearly supports positive cooperativity. Additionally, the regulatory properties of the enzyme were determined by kinetic assays in the presence of allosteric effectors. Tryptophan at 10 \(\mu\)M acts as strong heterotropic positive effector of enzymatic activity due to increased affinity of the enzyme for its substrate. A loss of cooperativity was observed (\(n_{H} = 0.97\)), resulting in Michaelis-Menten-type kinetics with a \(K_{m}\) of 1.6 mM and a maximum turnover value of 303.8 s\(^{-1}\). In contrast, tyrosine inhibits chorismate mutase activity; the turnover rate decreased to 89.3 s\(^{-1}\), and an \([S]_{1/2}\) value of 12.0 mM concentration of this heterotropic effector. An \(n_{H}\) of 1.32 indicates retention of cooperativity.

In summary, the HARO7-encoded chorismate mutase enzyme of *H. polymorpha* is strictly regulated in its activity. Whereas HARO7 transcription is constitutive with respect to amino acid starvation and derepressed in the presence of methanol, catalytic turnover is triggered in an allosteric manner by homotropic and heterotropic effectors specific for the biosynthetic pathway of aromatic amino acids.

*Unliganded H. polymorpha chorismate mutase shows a higher optimal temperature for catalytic turnover than its S. cerevisiae counterpart despite a lower thermal stability.* Kinetic stop assays with the unliganded enzyme were carried out to characterize the temperature profile of catalytic activity of the HARO7-encoded chorismate mutase, and purified yeast chorismate mutase from *S. cerevisiae* was subjected to identical assays for comparison (Fig. 5A). For the enzyme derived from the thermotolerant yeast *H. polymorpha*, maximum enzymatic activity was achieved at a temperature of 48°C. In comparison, the *S. cerevisiae* enzyme shows a decrease in catalytic turnover at temperatures higher than 38°C. With respect to the different maxima of catalytic turnover at elevated temperatures, we were interested in the stability of both enzymes upon incubation at different temperatures. To determine the rate constants in the decrease of catalytic activity due to irreversible denaturation, aliquots of both enzymes were preincubated for different time periods at the specified temperatures before residual chorismate activity was determined in stop assays at low temperature with 2 mM substrate and no effectors present (Fig. 5B). Both enzymes displayed thermal stability at 37°C. After preincubation at 50°C, a decrease in catalytic activity was determined for the *H. polymorpha* chorismate mutase, with a calculated half-life (\(t_{1/2}\)) at this temperature of approximately 8 min. At 55°C, the \(t_{1/2}\) decreased to 2 min; in preincubation experiments at 60°C, catalytic activity displayed a significant decrease over the recorded time period, with a \(t_{1/2}\) of nearly 1 min. For the *S. cerevisiae* chorismate mutase, \(t_{1/2}\) of 7 and 2.74 min were determined at 55 and 60°C, respectively. After preincubation at 65°C, also for this enzyme there was a sharp drop in catalytic activity, with a deduced \(t_{1/2}\) of 13 s. In conclusion, the chorismate mutase of *H. polymorpha* displays a lower thermal stability in comparison to its *S. cerevisiae* homologue, as deduced from the higher rate constants of inactivation at the temperatures used.

**DISCUSSION**

Chorismic acid, the formerly called elusive branch-point compound (21), is an intermediate of several metabolic pathways, like biosynthesis of ubiquinone and other quinones, 4-aminobenzoate, or aromatic amino acids, in which it is the last common compound of a branched biosynthetic cascade. Conversion of chorismate to prephenate, finally resulting in tyrosine and phenylalanine, is an unusual chemical reaction in
primary metabolism that is accelerated by chorismate mutase enzymes up to a factor of $10^6$. In addition, eukaryotic chorismate mutases, especially, have been established as model enzymes for allosteric regulation of catalytic turnover.

We have cloned the *H. polymorpha HARO7* gene coding for the chorismate mutase activity of this methylotrophic yeast. This newly identified enzyme extends the number of described sequences that constitute chorismate mutases. The *H. polymorpha* chorismate mutase is highly similar to the enzyme of the related yeast *S. cerevisiae*, placing the *HARO7* gene product into the AroQ class of chorismate mutases. Additionally, from alignment of the published primary sequences of chorismate mutases a consolidated consensus sequence can be deduced, indicating invariant residues for catalytic activity as well as for regulatory properties. For the yeast enzyme derived from *S. cerevisiae*, several residues have been identified and characterized in detail with respect to enzymatic function (23, 58, 59). Almost all of these specific amino acids are conserved in the *H. polymorpha* enzyme, except for the effector-binding residue at position 143. In *S. cerevisiae*, this position corresponds to Thr$^{145}$, whereas a methionine is found at this position in the *H. polymorpha* enzyme. Nevertheless, overall alignment with other enzymes reveals that this particular position is variable in primary sequence. In contrast, a highly conserved region is present within the primary structures, spanning from Cys$^{146}$ to Phe$^{167}$ in the *S. cerevisiae* enzyme. Based on crystal structures of the *S. cerevisiae* enzyme, this protein segment constitutes a helix (helix 8) that is part of the active site as well as of the regulatory site at the dimer interface and that contributes to the strong hydrophobic interaction between the monomers. The general importance of this secondary structure element accounts for its strictly conserved primary sequence. In the global alignment of cloned eukaryotic chorismate mutases, the *H. polymorpha* enzyme has a unique C-terminal extension. Molecular modeling studies based on the crystal structures determined for the *S. cerevisiae* enzyme imply an additional turn in the C-terminal helix (not shown), but functionality of this extension with respect to catalytic or regulatory properties remains to be elucidated.

Using a *loxP-ODC1MX-loxP* cassette, we were able to con-
struct an *H. polymorpha* strain disrupted in its HARO7 locus (RH2409). Retransformation of HARO7, either as linear DNA fragment or plasmid bound, restored growth of the disruptant on the complex medium YEPD. This clearly supports the idea that the observed growth defect of strain RH2409 is linked to its haro7Δ genotype and not to a background mutation. Surprisingly, the mutant strain showed auxotrophy for tyrosine but not for phenylalanine, with no residual chorismate mutase activity detectable in crude extracts of the disruption strain. One explanation, that the HARO7 gene might encode a bifunctional enzyme like a chorismate mutase-prephenate dehydrogenase activity (T protein), was ruled out because the HARO7 gene was not able to complement a tyr1 mutant strain of *S. cerevisiae* which lacks prephenate dehydrogenase activity (45).

We conclude, therefore, that the HARO7-encoded activity is the only chorismate mutase enzyme in *H. polymorpha* and that no other redundant catalytic activity is encoded by a homologous gene. This is in agreement with results determined by Bode and Birnbaum, who found no evidence for the occurrence of isoenzymic chorismate mutases in yeasts, among them *H. polymorpha* (2). The reasons for the unexpected Phe° phenotype remain obscure, but we speculate that spontaneous, noncatalytic rearrangement of chorismate to prephenate is sufficient in *H. polymorpha* to feed the tyrosine-specific branch, implying a higher affinity of prephenate to the dehydrogenase activity than to the dehydratase enzyme. Alternatively, *H. polymorpha* might be able to synthesize phenylalanine via additional routes or from exogenous tyrosine, but both possibilities are unlikely since no catalytic activities sufficient for such pathways have been described so far. By transient expression of the Cre recombinase, we were able to rescue the genetic marker in the haro7 disruption strain RH2409 due to excision of the ODC1 expression construct. The resulting strain, RH2410, was identical in growth phenotypes to its progenitor but in addition required uracil. With respect to biotechnological applications, this strain is a new suitable recipient in the vector-host system of *H. polymorpha*, as it is able to harbor two expression plasmids carrying different metabolic marker genes. Furthermore, we have demonstrated for the first time that the Cre-loxP recombination system can be applied in *H. polymorpha*, providing an efficient tool for repeated marker rescue following gene disruptions.

Taking advantage of the vector-host system of *H. polymorpha*, we were able to overexpress the HARO7 gene in a homologous way and to purify the encoded chorismate mutase to homogeneity in order to characterize its enzymatic properties in detail. Catalytic activity of a given enzyme is generally linked to temperature. We have shown that the *H. polymorpha* chorismate mutase reaches maximal turnover at a temperature 10°C higher than that of the related yeast *S. cerevisiae*. In experiments like these, elevated turnover based on increased enzyme-substrate collisions is superimposed by irreversible denaturation of the enzyme. We therefore addressed the question of thermal stability of both enzymes. Surprisingly, the *H. polymorpha* enzymes displayed higher rate constants of inactivation upon incubation at elevated temperatures in compar-
son to its S. cerevisiae counterpart. We therefore conclude that the higher optimum temperature for the former is based mainly on increased turnover at the catalytic site. In its allosteric regulation of catalytic activity the H. polymorpha chorismate mutase fits well in the theory of concerted transition as proposed by Monod et al. in 1965 (47). The substrate chorismate acts as homotropic, positive effector that shifts the equilibrium between tense and relaxed state to the more active relaxed state, indicated by a sigmoid curvature of initial velocities as determined in saturation assays as well as by n_H of 1.56. Additionally, this value for n_H indicates that the enzyme contains at least two binding sites for the substrate. Furthermore, regulation of catalytic turnover is achieved by heterotropic effects of two aromatic amino acids. Tyrosine, one end product of the chorismate mutase-specific branch, reduces catalytic efficiency by a factor of 2.6 (k_cat/K_m = 7.44 versus 19.1 mM^{-1} s^{-1}), whereas tryptophan, the final product of the opposite branch, increases the k_cat/K_m by a factor of 9.8 to 188.1 mM^{-1} s^{-1} and abolishes cooperativity. This activating effect of tryptophan is based on increased affinity for the substrate, indicated by the K_m of 1.6 mM, and accounts for an allosteric K system. The overall modulation range of catalytic efficiency via both allosteric effectors is given by a factor of 25. Altering catalytic efficiency is one mode of regulation for a given enzyme. An additional and more general way to tune the flux through a metabolic pathway is based on altered expression levels of a gene product. We have shown for the HARO7 gene that transcription is not increased upon the environmental signal of amino acid starvation, as induced by the false feedback inhibitor 3-AT. This is not unusual for chorismate mutase-encoding genes, as neither ARO7 from S. cerevisiae nor aroC from A. nidulans is the target of a cross-pathway control system acting on amino acid starvation in fungi (35, 55). In contrast to this constitutive expression pattern, HARO7 transcription is induced twofold upon methanol utilization, a specific metabolic feature of H. polymorpha, whereas glycerol as nonoptimal carbon source slightly derepresses HARO7 transcription. Both effects are abolished in the presence of glucose, which accounts for a repression system acting on HARO7 transcription. This is the first example of transcriptional regulation of an eukaryotic chorismate mutase-encoding gene. As methanol utilization is accompanied by drastically increased expression of enzymes specific for this pathway, this mode of chorismate mutase expression might reflect the general need for larger amino acid pools in the yeast cell. We have identified a putative binding site for the MOX-binding factor (Mbf1p) in the HARO7 promoter region. This sequence elements differs from the characterized upstream activating sequence found in the MOX promoter by one transversion and one insertion. Nevertheless, this conserved motif is a promising candidate for a positive, cis-acting element triggering HARO7 transcription.

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REFERENCES


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