

HAR07 Encodes Chorismate Mutase of the Methylophilic Yeast *Hansenula polymorpha* and Is Derepressed upon Methanol Utilization

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The *HAR07* gene of the methylophilic, thermotolerant yeast *Hansenula polymorpha* was cloned by functional complementation. *HAR07* encodes a monofunctional 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 *HAR07* gene product shows strong similarities to primary sequences of known eukaryotic chorismate mutase enzymes. After homologous overexpression and purification of the 32-kDa protein, its kinetic parameters ($k_{\text{cat}} = 319.1 \text{ s}^{-1}$, $n_H = 1.56$, $[S]_{0.5} = 16.7 \text{ mM}$) 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 *HAR07* gene that showed tyrosine auxotrophy and severe growth defects. The amount of the 0.9-kb *HAR07* 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 *HAR07* expression.

Methylophilic 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 *H. 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 *MOX*-encoded methanol oxidase (EC 1.1.3.13) (37). Additional important gene products involved in methanol assimilation are a dihydroxyacetone synthase (EC 2.2.1.3) encoded by the *DAS* gene, a catalase (EC 1.11.1.6) encoded by the *CAT1* 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 *H. 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 *H. polymorpha* genome (19, 34). Additionally, *H. polymorpha* is able to grow at temperatures of up to 48°C, with an optimal growth temperature of 37°C, which is unusual for methylophilic yeasts (40).

In contrast to the specialized methanol-utilizing pathway of methylophilic 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 enol-pyruvyl 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 archaea, 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 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 been made available (35). 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 methylotrophic yeast *H. polymorpha*, an organism closely related to *S. cerevisiae*. *haro7Δ* 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. Pries, S. Krappmann, and A. W. Strasser, German patent application 19919124.7, April 1999). Transcriptional expression patterns of *HARO7* were monitored with respect to different environmental stimuli like amino acid starvation conditions or alternative carbon sources, indicating that *HARO7* expression is the target of a catabolite repression system but not of the general control of amino acid biosynthesis. Additionally, the enzyme was overexpressed and purified to homogeneity, taking advantage of an *H. polymorpha* expression system. Kinetic assays as well as regulatory analyses of the chorismate mutase indicated that catalytic activity is tightly regulated in an allosterical manner and that this enzyme of *H. polymorpha* has a higher optimal temperature for catalytic turnover than its counterpart from *S. cerevisiae* despite lower thermal stability.

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 (SigmaUltra, >99%) from Sigma-Aldrich Chemie GmbH (Steinheim, 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 *Pfx* 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* DH5 α (70). *S. cerevisiae* RH2185 (*MATa suc2-Δ9 ura3-52 leu2-3 leu2-112 his4-519 aro7::LEU2 GAL2*) (59) with the genetic background of the laboratory strain X2180-1A (*MATa gal2 SUC2 mal 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 RB11 (*odc1*) was obtained from Rhein Biotech GmbH (Düsseldorf, Germany) and has been described by Weydemann et al. (69). *H. polymorpha* RH2408 (*odc1 FMD promoter::HARO7 URA3*) was used for homologous overexpression and purification of the *HARO7* gene product. Mutant strains RH2409 (*odc1 haro7::loxP-ODC1MX-loxP*) and RH2410 (*odc1 haro7::loxP*) are derivatives of *H. polymorpha* RB11 carrying a disrupted *HARO7* gene. *E. coli* cells were grown in LB medium (44) supplemented with ampicillin (100 $\mu\text{g} \cdot \text{ml}^{-1}$) at 37°C. Complex medium for growth of yeasts was YEPD (1% yeast extract, 2% peptone, 2% glucose). Selective medium contained 0.14% yeast nitrogen base (without amino acids and ammonium sulfate) and 0.5% ammonium sulfate. Carbon sources were either 2% glucose, 1% glycerol, or 0.7%

methanol. Supplements were added according to Guthrie and Fink (25). In contrast to *S. cerevisiae*, which was cultivated at 30°C, *H. polymorpha* strains were propagated at 37°C, which is the optimal temperature for growth of this yeast. Transformation of *E. coli* was performed as described by Inoue et al. (32). *S. cerevisiae* was transformed by a modification of the protocol of Elble (13). For transformation of *H. polymorpha*, an electroporation procedure was used (15).

Isolation and analyses of nucleic acids. For isolation of plasmid DNA from bacterial strains, a plasmid purification system from Qiagen (Hilden, Germany) was used. Genomic DNA from yeasts was isolated according to Hoffmann and Winston (30) and analyzed by Southern blotting (62) or diagnostic PCR (52) using oligonucleotides OLSK57 (5'-CAATGCCAGCAATATGGAGACG-3') and RP1 (5'-GAACTAGAATTCGAGAATAATTAAG-3'). Total RNA from *H. polymorpha* cultures was prepared according to Cross and Tinkenberg (7), and transcript levels were determined by Northern hybridization (48) using a Bio-Imaging analyzer from Fuji Photo Film Co. Ltd. (Tokyo, Japan). Transcript length was determined using a 0.16- to 1.77-kb RNA ladder from Life Technologies, Inc. Sequencing reactions were carried out using a BigDye sequencing kit (28) and analyzed on an ABI PRISM 310 genetic analyzer (PE Biosystems, Foster City, Calif.).

Cloning techniques; construction of genomic library and plasmids. Standard techniques for cloning and manipulation of recombinant DNA were applied (44). For identification of the *HARO7* gene, a genomic library was constructed by ligating partially *Sau3A*-digested DNA of *H. polymorpha* RB11 in the *Bam*HI restriction site of shuttle vector pRS426 (61). The ligation products were transformed in *E. coli* to yield a library pool of approximately 100,000 independent clones from which plasmid DNA was isolated. Plasmid pME1524 contains a 5-kb genomic *Sau3A* fragment containing the *HARO7* gene of *H. polymorpha* RB11 in pRS426. Subcloning generated plasmid pME1525, which carries a 1.8-kb *Apa*I/*Hind*III fragment, originating from pME1524, in pRS426. For working purposes, plasmid pME1526, which carries the 1.8-kb *Apa*I/*Hind*III fragment of pME1524 in the bacterial plasmid pBluescript II KS (Stratagene (La Jolla, Calif.)), was constructed. Overexpression of the *HARO7* gene product in RB11 was achieved using plasmid pME1686. This plasmid carries the entire open reading frame of *HARO7* amplified from pME1525 using oligonucleotides OLSK50 (5'-TATAGAATTTCATGGACTTTATGAGCC-3', *Eco*RI site underlined) and RP1 in a PCR; the resulting *Eco*RI fragment was cloned in the expression vector pFPMT121 (Rhein Biotech) to yield an *FMD promoter::HARO7::MOX* terminator expression cassette in a plasmid autonomously replicating in *H. polymorpha*. For characterization of a *haro7Δ* mutant strain, a disruption cassette was constructed. To this end, the 5' flanking sequence of *HARO7* was amplified from pME1524 using OLSK34 (5'-ATATAGATCTACA AAACTAAACAGG-3', *Bgl*II site underlined) as reverse primer and cloned as a 2-kb *Sall*/*Bgl*II fragment. The 3' region flanking *HARO7* was cloned as a 2.5-kb *Bgl*II/*Not*I fragment amplified in a PCR with OLSK35 (5'-ATATAGATCTGATGCGACGCAGAAAAGC-3', *Bgl*II site underlined) as forward primer using a partial *Bgl*II genomic sublibrary of RB11 cloned in pBluescript II KS (*Bam*HI) as template. Both flanking regions were cloned in pBluescript II KS (*Sall*/*Not*I) to yield vector pME1687. A *loxP-ODC1MX-loxP* cassette as a 1.6-kb *Bam*HI fragment was cloned in the *Bgl*II site of this disruption vector. This cassette was constructed from plasmid pUG-ODC1 and is a derivative of the *loxP-kanMX-loxP* module (24) where the kanamycin resistance gene was replaced by the *ODC1* gene of *H. polymorpha*. The resulting vector from which a 6-kb disruption cassette was released by *Kpn*I digestion is pME1688. For forcing recombination between the *loxP* sites in strain RH2409, we used plasmid pME1690, which carries the coding sequence of the *Cre* recombinase (64) as a PCR fragment fused to the *FMD* promoter as well as the *HARO7* gene as an *Eco*21/*Ssp*I fragment cloned in the *Sma*I site of pFPMT121. Plasmid pME1689 carries part of the coding region (exon III) of the *ACT* gene from *H. polymorpha* DL1-L (W. K. Hong, H. A. Kang, J. H. Shon, E. S. Choi, and S. K. Rhee, unpublished data [GenBank accession no. AF085278]) as a *Bam*HI/*Sca*I fragment.

Overexpression and purification of *H. polymorpha* chorismate mutase. *H. polymorpha* RH2408 (*odc1 FMD promoter::HARO7 URA3*) generally was grown at 37°C as shake flask (750 ml) culture in YNB supplemented with 3% glycerol as sole carbon source for *FMD* promoter derepression. Cells were harvested at an optical density at 546 nm (OD_{546}) 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 DL-dithiothreitol). For purification, 20 to 30 g of cells was thawed and passed three times through a French pressure cell (18,000 lb/in²). Cell debris was sedimented by centrifugation at 30,000 \times g for 20 min. The chorismate mutase enzyme was purified as described by Schmidheini et al. (54), with the modification that for buffer changing or desalting pooled fractions after the first anion-exchange run, gel filtration on a Superdex 75pg column was applied. Chorismate mutase was detected by SDS-PAGE (36) and by enzymatic activity assays. Native PAGE was performed as described by Anderson et al. (1), using a gradient of 4 to 15% polyacrylamide. Protein concentrations were measured by the Bradford assay (5).

Enzyme assays and data evaluation. Chorismate mutase activity was measured as described previously (54). Enzymatic reactions were carried out at 37°C; as effector concentrations in substrate saturation assays, 100 μM tyrosine and 10 μM tryptophan were chosen. Enzymatic activity was measured spectrophotometrically, determining the concentration of phenylpyruvate. Since absorbance

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 LASER-GENE 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. AF204738.

RESULTS

The *HARO7* gene of *H. polymorpha* codes for a chorismate mutase enzyme. The *HARO7* gene from the methylotrophic yeast *H. polymorpha* was cloned by functional complementation of an *S. cerevisiae aro7Δ* 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 ura3-52*) (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 M_r of 32,067. The 5' flanking 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 63% 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 1,000 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 *S. cerevisiae URA3* 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 *haro7Δ* 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, a *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 YEPD 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 *haro7Δ* 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

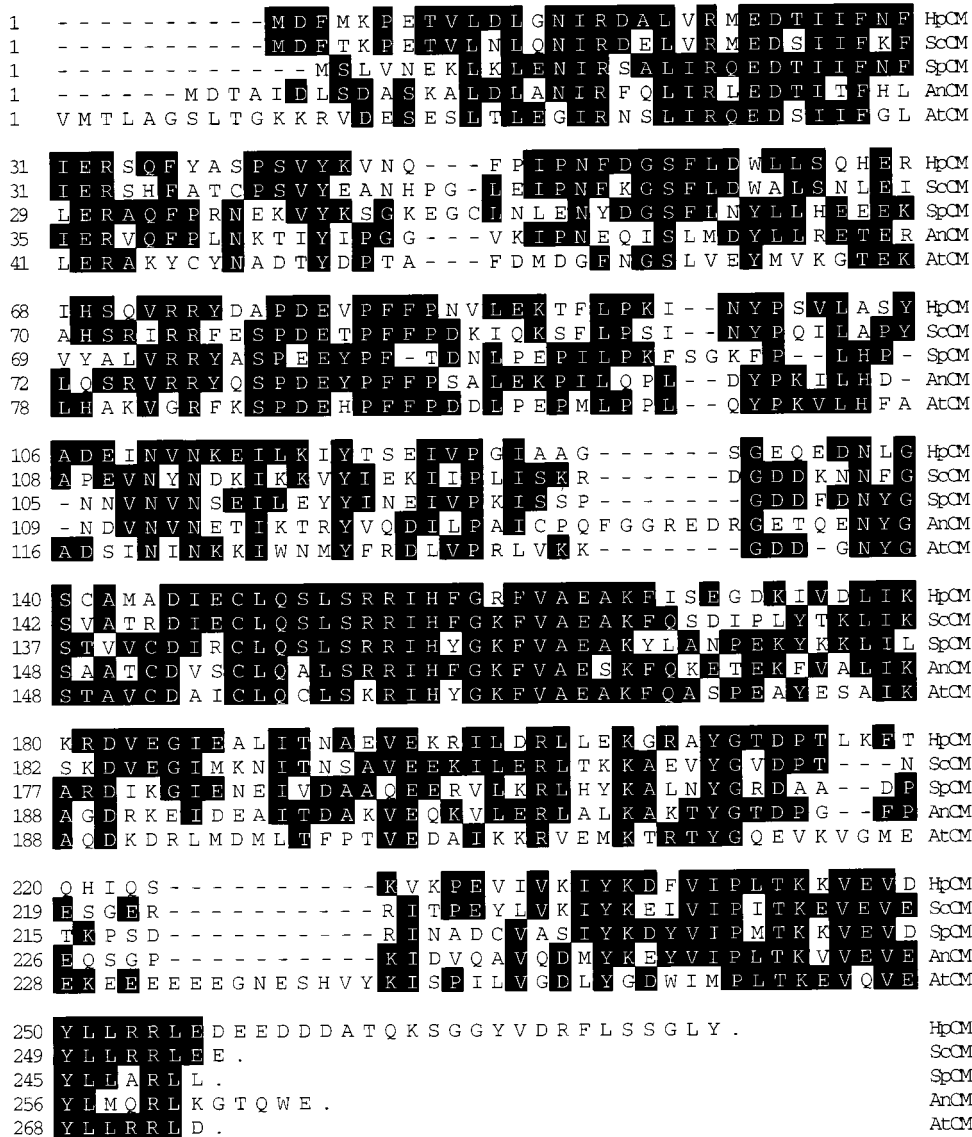


FIG. 1. Multiple sequence alignment of deduced eukaryotic chorismate mutases including the amino acid sequence of *H. polymorpha* chorismate mutase. Sources of sequences: HpCM, *H. polymorpha* (accession no. AF204738); ScCM, *S. cerevisiae* (accession no. M24517) (54); SpCM, *Schizosaccharomyces pombe* (accession no. Z98529) (K. Oliver, D. Harris, B. G. Barrell, M. A. Rajandream, and V. Wood, unpublished data); AnCM, *A. nidulans* (accession no. AF133241) (35); AtCM, *Arabidopsis thaliana*, mature plastidic enzyme (accession no. Z26519) (10). Conserved residues are boxed in black.

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 -140 to -135 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', po-

sitions -275 to -264) is similar to the core region of a binding site for Mbp1p (5'-TTGCACCGCAA-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'-TCGCACCGCAA-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-

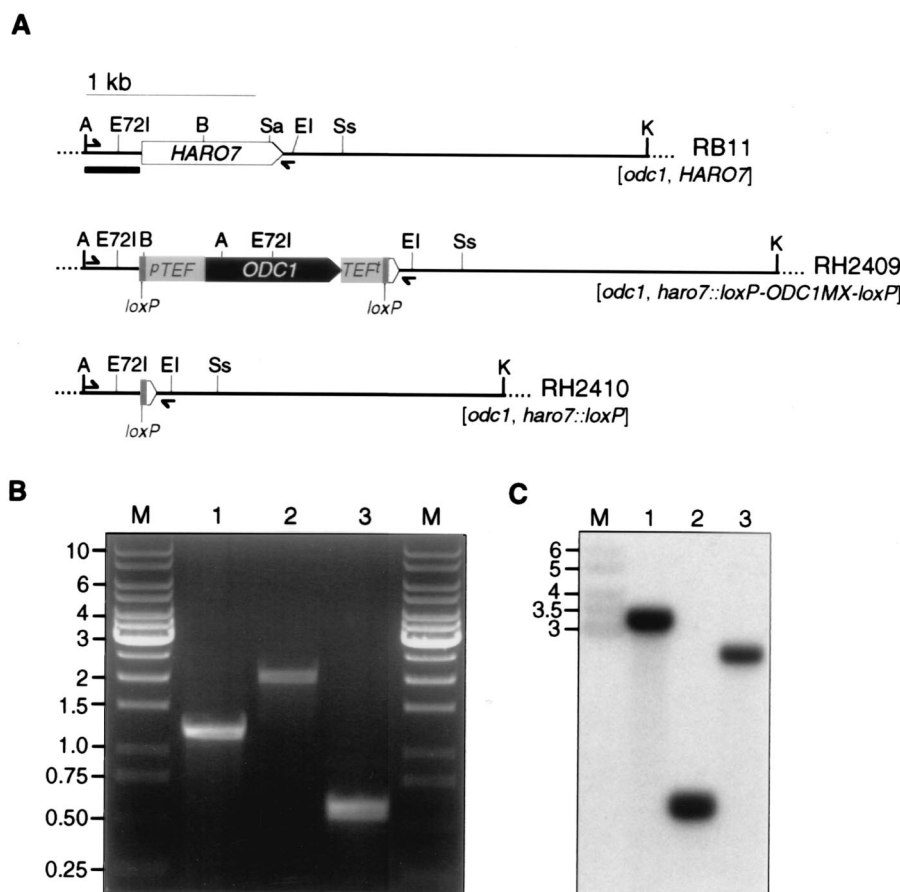


FIG. 2. Construction of *haro7* Δ mutant strains of *H. polymorpha* using the Cre-*loxP* recombination system. (A) Physical maps of the *HARO7*, *haro7::loxP-ODC1MX-loxP*, and *haro7::loxP* loci. Coding sequences of *HARO7* and *ODC1* are schematically drawn as tipped boxes, promoter and termination sequences of the *TEF2* gene from *Ashbya gossypii* are shown as light gray boxes, and *loxP* sites are marked in dark gray. Primer positions chosen for diagnostic PCR are indicated by half arrows. The horizontal bar represents the *HARO7* promoter-specific probe used in Southern analysis. A, *Apa*I; B, *Bgl*II; E, *Eco*RI; E72I, *Eco*72I; K, *Kpn*I; Sa, *Sa*I; Ss, *Ssp*I. (B) Diagnostic PCR on genomic DNA of wild-type *H. polymorpha* *HARO7* strain RB11 (lane 1) and *haro7* Δ mutant strains RH2409 (lane 2) and RH2410 (lane 3) with *HARO7*-specific 5' and 3' oligonucleotides. M, marker DNA fragments of the indicated size in kilobases. (C) Southern hybridization of a specific *HARO7* promoter probe on *Apa*I/*Kpn*I-digested genomic DNA of strains RB11 (lane 1), RH2409 (lane 2), and RH2410 (lane 3). M, nonspecifically hybridizing DNA marker fragments with the indicated length in kilobases.

ferent 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 a key 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 supple-

mented 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

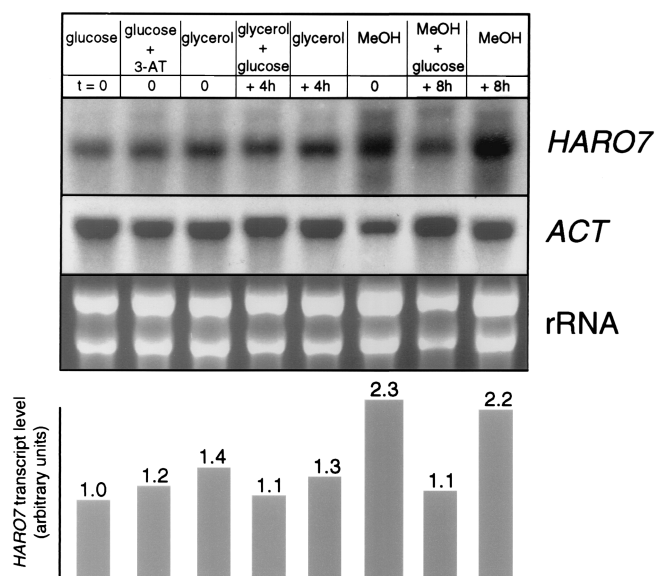


FIG. 3. Expression pattern of *HARO7* under amino acid starvation conditions or in the presence of different carbon sources. *H. polymorpha* RB11 was grown in minimal medium containing glucose (lane 1), glucose and 1 mM 3-AT (lane 2), glycerol (lane 3), or methanol (MeOH; lane 6) to mid-log phase ($OD_{546} \approx 1.2$, $t = 0$), and total RNA was prepared. Additionally, glucose was added to cultures of RB11 grown in glycerol and methanol medium, respectively, and incubation was continued for 4 or 8 h (lanes 4 and 7) before RNA preparation. As a control, RNA was prepared from cultures after prolonged cultivation in the absence of additional glucose (lanes 5 and 8). For Northern analysis, 20 μ g of total RNA was loaded in each lane and probed successively with probes specific for *HARO7* and *ACT* of *H. polymorpha*. Ethidium bromide-stained total RNA is included as a control. Quantified steady-state levels of *HARO7* transcripts are shown in the histogram after standardization with respect to *ACT* transcript levels. The values are averages of two independent experiments with a standard deviation not exceeding 20%.

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 media 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 non-denaturing 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 $[S]_{0.5}$ 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 μ 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 $[S]_{0.5}$ value of 12.0 mM was calculated at a 100 μ M 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, $t_{1/2}$ s 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

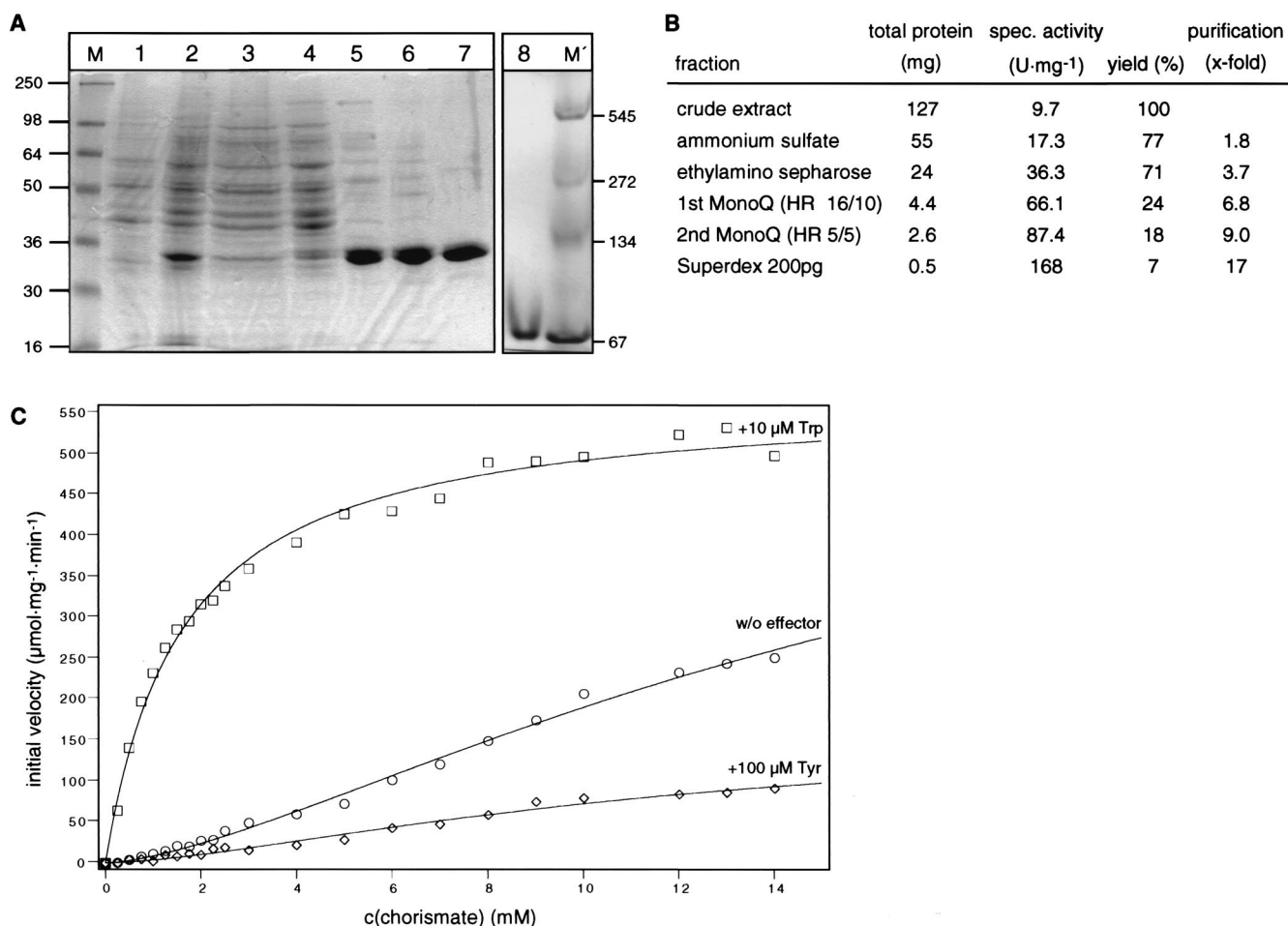


FIG. 4. Purification of chorismate mutase of *H. polymorpha* and enzymatic properties. (A) SDS-polyacrylamide gel of the purification of chorismate mutase of *H. polymorpha* (left) and native PAGE (right). Lanes: M, marker proteins with the indicated molecular masses in kilodaltons; 1, crude extract of RB11; 2, crude extract of RH2409; 3, supernatant of ammonium sulfate precipitation; 4, ethylamino-Sepharose pool; 5, first MonoQ pool; 6, second MonoQ pool; 7, Superdex 200pg pool; 8, purified Haro7p in a nondenaturing polyacrylamide gel (gradient 4 to 15%); M', marker proteins with the indicated native molecular masses in kilodaltons. A total of 5 μ g protein was loaded on each lane. (B) Purification protocol for *H. polymorpha* chorismate mutase. Enzyme assays were performed in the presence of 500 μ M tryptophan and 1 mM chorismate for 10 min. (C) Substrate saturation plot of enzyme assays. Purified *H. polymorpha* chorismate mutase was assayed with 10 μ M tryptophan, without (w/o) effector, or in the presence of 100 μ M tyrosine, as indicated. Catalytic turnover was carried out for 1 min, and data were fitted to functions describing cooperative or Michaelis-Menten-type saturation (solid lines). Specific activities are mean values of at least five independent measurements with a standard deviation not exceeding 20%.

primary metabolism that is accelerated by chorismate mutase enzymes up to a factor of 10⁶. 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

Thr¹⁴⁵, 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 Cys¹⁴⁸ to Phe¹⁶⁷ in the *H. polymorpha* 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-

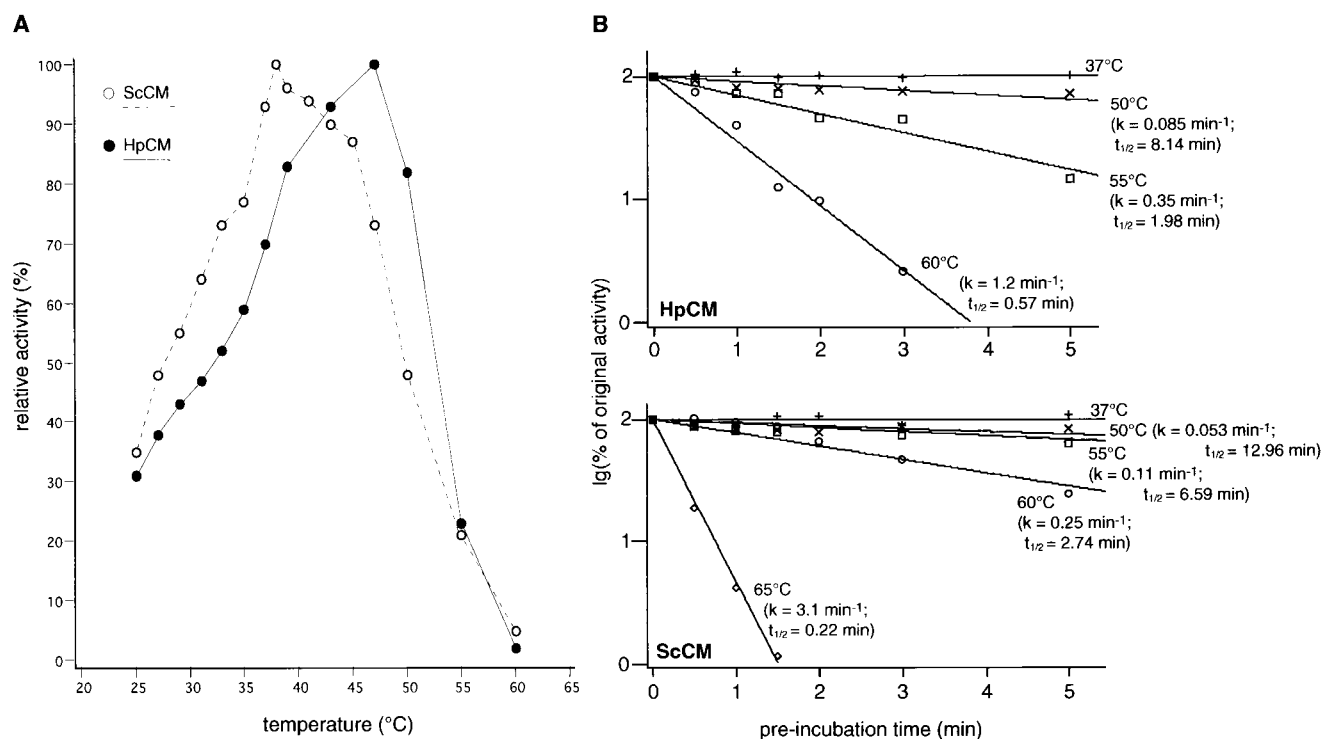


FIG. 5. Temperature profiles and thermal stabilities of chorismate mutases from *H. polymorpha* and *S. cerevisiae*. (A) Determination of optimum temperatures for catalytic turnover. Catalytic activities were quantified with purified chorismate mutase enzymes of *S. cerevisiae* (ScCM) and *H. polymorpha* (HpCM) in the absence of effectors. Enzyme samples in buffer were preincubated for 5 min at the indicated temperature before chorismate was added to a final concentration of 1 mM to start catalytic turnover. Reactions were stopped after 10 min. Each measurement was performed twice with a standard deviation not exceeding 20%; 100% activity equals 13.1 U ($\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$) for ScCM and 5.6 U for HpCM, respectively. (B) Analysis of rate constants for thermal inactivation. Enzyme samples of purified chorismate mutase of *H. polymorpha* (HpCM) and *S. cerevisiae* (ScCM) were preincubated in the absence of effectors for different time periods at the indicated temperature and chilled on ice, and residual activities were determined in stop assays at 37°C (HpCM) and 30°C (ScCM), with 2 mM substrate concentration and 2 min of catalytic turnover after 1 min of incubation at the assay temperature; 100% of original activity equals 49.8 U for ScCM and 15.4 U for HpCM, respectively, and each measurement was performed twice with a standard deviation not exceeding 20%.

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 *haro7* Δ 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 compari-

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 an 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 $19.1 \text{ mM}^{-1} \cdot \text{s}^{-1}$), whereas tryptophan, the final product of the opposite branch, increases the k_{cat}/K_m by a factor of 9.8 to $188.1 \text{ mM}^{-1} \cdot \text{s}^{-1}$ 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 a 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 element 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.

ACKNOWLEDGMENTS

This work was supported by Rhein Biotech GmbH and by grants from the Deutsche Forschungsgemeinschaft, the Fonds der Chemischen Industrie, the Volkswagen-Stiftung, and the Niedersächsischen Vorab der Volkswagen-Stiftung, Forschungsstelle für nachwachsende Rohstoffe.

We thank Markus Hartmann for determination of DAHP synthase activities, Silke Busch for critical reading of the manuscript, and all other members of the laboratory for helpful discussions.

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