The aroC Gene of Aspergillus nidulans Codes for a Monofunctional, Allosterically Regulated Chorismate Mutase*

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Sven Krappmann, Kerstin Helmstaedt, Thomas Gerstberger, Sabine Eckert, Bernd Hoffmann, Michael Hoppert, Georg Schnappauf, and Gerhard H. Braus‡

From the Institute of Microbiology & Genetics, Georg-August-University, Grisebachstrasse 8, D-37077 Göttingen, Germany

The cDNA and the chromosomal locus of the aroC gene of Aspergillus nidulans were cloned and is the first representative of a filamentous fungal gene encoding chorismate mutase (EC 5.4.99.5), the enzyme at the first branch point of aromatic amino acid biosynthesis. The aroC gene complements the Saccharomyces cerevisiae aro7Δ as well as the A. nidulans aroC mutation. The gene consists of three exons interrupted by two short intron sequences. The expressed mRNA is 0.96 kilobases in length and aroC expression is not regulated on the transcriptional level under amino acid starvation conditions. aroC encodes a monofunctional polypeptide of 268 amino acids. Purification of this 30-kDa enzyme allowed determination of its kinetic parameters (Km = 82 s⁻¹, nH = 1.56, [S]ₜ₁₀₀ = 2.3 mM), varying pH dependence of catalytic activity in different regulatory states, and an acidic pI value of 4.7. Tryptophan acts as a heterotropic activator and tyrosine as a negative acting, heterotropic feedback-inhibitor with a Kᵢ of 2.8 μM. Immunological data, homology modeling, as well as electron microscopy studies, indicate that this chorismate mutase has a dimeric structure like the S. cerevisiae enzyme. Site-directed mutagenesis of a crucial residue in loop220s (Asp233) revealed differences concerning the intramolecular signal transduction for allosteric regulation of enzymatic activity.

Chorismic acid is the last common compound in the biosynthesis of aromatic amino acids. The metabolic branch leading to L-tryptophan is initiated by its conversion to anthranilate catalyzed by the enzyme anthranilate synthase (EC 4.1.3.27), whereas the catalytic step to prephenate finally leads to L-phenylalanine and L-tyrosine (1, 2). The latter reaction is the only known Claisen rearrangement in primary metabolism of living organisms and is catalyzed by a unique enzyme, the chorismate mutase (EC 5.4.99.5) (3). Chorismate mutases are found in archaea, bacteria, fungi, and plants (4). Based on primary sequence information and determination of the crystal structure of three natural enzymes, chorismate mutases are classified into two groups: the chorismate mutase of Bacillus subtilis represents the AroH class and is characterized by its trimeric pseudo α/β-barrel structure (5, 6). In contrast, polypeptides of the AroQ class are all-helix bundle proteins and are often part of a bifunctional enzyme containing a chorismate mutase domain (7). According to Hilvert and co-workers (8), eukaryotic chorismate mutases, which additionally contain regulatory domains, also fall into the latter class despite of the rare primary amino acid sequence similarity with their prokaryotic counterparts. The enzymatic properties of some eukaryotic chorismate mutases have been studied in detail, but only a limited number of the corresponding genes have been cloned yet (4).

The chorismate mutase of the bakers’ yeast Saccharomyces cerevisiae is the most prominent member of the AroQ class and has been characterized in extensive studies (9–11). Its allosteric modulation by tyrosine and tryptophan serves as a model in understanding the regulatory properties of a branch point enzyme. Determination of different crystal structures has given insight into the structural basis for the regulatory processes controlling the flux of chorismate into one of the two branches in the biosynthesis of aromatic amino acids (see Ref. 12, and references therein). In addition, molecular dynamics studies have given hints to understand the mechanism of the enzymatic conversion performed by this enzyme (13). The homodimeric yeast enzyme consists of 2 × 12 helices with the catalytically active domain built up by three helices of each subunit. The loop preceding one of these helices has turned out to be crucial for transmitting the signal of T to R state transition. Conversion of one residue in this loop (T226I) results in a constitutively activated enzyme that is unresponsive to its inhibitor tyrosine (14).

To date, no gene coding for a chorismate mutase of a filamentous fungus has been characterized. Here we present the characterization of the chorismate mutase-encoding gene aroC of Aspergillus nidulans. This filamentous fungus has become a model organism concerning metabolism as well as differentiation in recent decades (15, 16). The aroC gene product was overexpressed in yeast using recombinant DNA technology and then purified for kinetic assays and regulatory analysis. The quaternary structure was determined by computer modeling and compared with the yeast enzyme. Additionally, site-directed mutagenesis was applied to investigate the role of a putatively crucial residue (Asp233) in allosteric transition as this amino acid residue corresponds to residue Thr226 in the yeast chorismate mutase. We found that the newly characterized chorismate mutase shares structural similarities with its yeast homologue, but that the molecular basis of the mechanism for T-R transition is not conserved.

EXPERIMENTAL PROCEDURES

Materials—Chorismic acid as barium salt was purchased from Sigma. Ethylamino-Sepharose was prepared following the protocol for activation of Sepharose CL-4B (17) and by coupling of the ligand ethylamine-HCl to the activated matrix. 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 2 DS-polyacrylamide gel electrophoresis system and the Bradford protein assay solution for determination of protein concentrations originated from Bio-Rad. Venti polymerase (BIOLABS, Schwalbach, Germany) was used for polymerase chain reactions. All other chemicals were obtained by E. Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), 5-methyl-2-thio-2-deoxyuridine, and horseradish peroxidase (HRP) were purchased from Sigma (St. Louis, MO). All other chemicals were obtained from Sigma. 

**Chorismate Mutase of A. nidulans**

The chorismate mutase enzyme was purified using the Bio-Rad Rotofor system according to the supplier’s instructions. A pH gradient in 18 ml of 10 mM potassium phosphate buffer, pH 7.6, was set up by a Bio-Lyte 3/10 ampholyte ranging from pH 3.5 to 9.5 in a concentration of 0.5%. The enzyme was applied to the column and after 4 h the run was completed. The content of the focusing chamber was fractionated and the pH of each fraction was measured. Before detection of chorismate mutase, NaCl was applied to 1 M final concentration and fractions were dialyzed against 10 mM potassium phosphate buffer, pH 7.6. Chorismate mutase was detected by enzyme assays as well as by SDS-polyacrylamide gel electrophoresis using a gradient from 4 to 20% polyacrylamide (34).

**Electron Microscopy**—Negative staining of protein samples was performed as described in Ref. 35 with 4% uranyl acetate solution. Electron microscopic images were taken at a EM 301 transmission electron microscope (Philips, Eindhoven, Netherlands) at an acceleration potential of 80 kV. Magnification was calibrated using a cross-grid replica.

**Western Blot Analysis**—Immunological detection of chorismate mutase proteins was performed using a polyclonal rabbit antibody raised against purified yeast chorismate mutase (10) and a second antibody with horseradish peroxidase activity. Detection was carried out using the ECL method (36).

**Sequence Alignment and Homology Modeling Studies**—All sequence analyses were performed using the LASERGENE Biocomputing software from DNASTAR (Madison, WI). Alignments were created based on the Dayhoff-Pearson method (37). For homology modeling, the deduced primary structure of the A. nidulans chorismate mutase was aligned to the crystallographic data of yeast chorismate mutases as described in the Brookhaven protein data base (12) by ProModII (38) and refined by the SWISS-MODEL service (39, 40). Using the MOLMOL software (41), a three-dimensional structure model could be established by calculation of secondary structures.
Chorismate Mutase of A. nidulans

Results

Isolation of the aroC Gene from A. nidulans—The aroC gene from A. nidulans was cloned by functional complementation of a S. cerevisiae aro7Δ mutant strain (10). Yeast strains with a deleted ARO7 gene do not contain any chorismate mutase activity and therefore were unable to grow on minimal medium lacking tryptophan or phenylalanine. Yeast strain RH2185 (aro7-LEU2, aro3-52) was transformed with A. nidulans cDNA expressed from the GALI promoter (18) and transformants were selected on medium lacking tryptophan and phenylalanine and tyrosine. For 90% of the isolated transformants a complementation of the Phe/Tyr auxotrophy was observed, indicating that we had isolated the aroC gene of A. nidulans described for S. cerevisiae (45). The described phenotype of mutants a complementation of the Phe/Tyr auxotrophy was observed, indicating that we had isolated the aroC gene of A. nidulans described for S. cerevisiae (45). For this purpose, the genomic HindIII fragment was transformed into strain G1100 (aroC1248, riboA1, adG14, yA2) different time points after shifting to medium containing 5-aminoo-1,2,4-triazole (3AT). Each lane was loaded with 20 μg of total RNA and probed successively with probes specific for aroC, trpC, and gpdA. Ethidium bromide-stained total RNA is included as control.

aroC Expression Is Not Regulated Transcriptionally upon Amino Acid Starvation Conditions. A, schematic drawing of a genomic HindIII/AatII DNA fragment containing the aroC gene. The GenBank accession number for this sequence is AF133241. Solid boxes indicate the open reading frame interrupted by two introns. A putative STUA-binding site is indicated by the solid triangle and a Gen4p response element in reverse orientation by the open triangle. A, AaII; B, BstEII; El, EcoRI; EV, EcoRV; H, HindIII; P, PvuII; Sa, SalI; St, Stul; X, XbaI. B, Northern analysis of total RNA prepared from A. nidulans strain FGSC A234 (yA2, pabaA1, veA1) different time points after shifting to medium containing 5-aminoo-1,2,4-triazole (3AT). Ethidium bromide-stained total RNA is included as control.

aroC gene and expression under amino acid starvation conditions. A, schematic drawing of a genomic HindIII/AatII DNA fragment containing the aroC gene. The GenBank accession number for this sequence is AF133241. Solid boxes indicate the open reading frame interrupted by two introns. A putative STUA-binding site is indicated by the solid triangle and a Gen4p response element in reverse orientation by the open triangle. A, AaII; B, BstEII; El, EcoRI; EV, EcoRV; H, HindIII; P, PvuII; Sa, SalI; St, Stul; X, XbaI. B, Northern analysis of total RNA prepared from A. nidulans strain FGSC A234 (yA2, pabaA1, veA1) different time points after shifting to medium containing 5-aminoo-1,2,4-triazole (3AT). Ethidium bromide-stained total RNA is included as control.

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thetic pathway and because of the existence of a putative GCRE in its promoter region we were interested in whether *aroC* expression is affected by amino acid starvation conditions. For that purpose, *A. nidulans* strain FGSC A234 (yA2, pabaA1, veA1) was cultivated in liquid minimal medium for 20 h and mycelia were transferred to fresh medium containing 3-amino-1,2,4-triazole (3AT). This reagent acts as false feedback-inhibitor on the histidine biosynthesis and therefore mimics amino acid starvation by depletion of the histidine pool within the fungus (46). After different time points mycelium was harvested and total RNA was prepared. Following Northern blot the *aroC* transcript levels were determined by probing with the cDNA fragment. Additionally, the levels of the *gpdA* (47) and the *trpC* (48) transcripts were detected with specific probes serving as internal controls (Fig. 2B). *gpdA*, which encodes an enzyme of glycolysis (glyceraldehyde-3-phosphate dehydrogenase, EC 1.2.1.12), is known to be unregulated in its transcription upon 3-amino-1,2,4-triazole addition. In contrast, *trpC*, which codes for a trifunctional enzyme of tryptophan biosynthesis, has been shown to be transcriptionally regulated by amino acid starvation conditions. Quantification of signal strength reveals constant expression of *aroC* after shifting to amino acid starvation conditions. Expression of *gpdA* shows the identical pattern, whereas *trpC* transcription is increased by a factor of 15, 8 h after the onset of the environmental stimulus. Therefore we conclude that transcription of the *aroC* gene is not affected by a regulatory network that acts upon the environmental signal amino acid starvation.

**Chorismate Mutase of *A. nidulans***

The enzyme was purified by overexpression in *S. cerevisiae* strain RH2192 (aro7::LEU2, ura3-52) from a high-copy plasmid carrying the *A. nidulans* *aroC* cDNA fragment driven by the *MET25* yeast promoter. The protein was enriched 64-fold and purified to homogeneity to determine the properties of the *aroC* gene product.

Kinetic stop assays with the unliganded enzyme were performed to reveal the catalytic properties of the *A. nidulans* chorismate mutase (Fig. 3A, Table I). In the absence of effectors the enzyme shows positive cooperativity toward its substrate chorismate leading to a sigmoidal substrate saturation curve. A [S]₀.₅ value of 2.3 mM and a Hill coefficient *n*ₕ of 1.56 were determined and the maximal turnover rate *k*ₘₐₓ was calculated

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**Fig. 3. Characteristics of *A. nidulans* chorismate mutase.**

*A*, substrate saturation plot of enzyme assays. The enzyme was assayed with 5 µM tryptophan (○), without effector (■), or in the presence of 50 µM tyrosine (○). The data were fitted to functions describing cooperative or Michaelis-Menten-type saturation. Specific activities are mean values of five independent measurements with a standard deviation not exceeding 20%. *B*, Dixon plot of enzyme assays in the presence of tyrosine at different concentrations. Specific activities were measured in the presence of 2 mM (○), 3 mM (■), and 4 mM (□) substrate and plotted reciprocally versus tyrosine concentrations. The point of intersection determines the inhibitory constant *K*ᵢ for tyrosine. *C*, pH optima for chorismate mutase activities under different effector conditions at 1 mM chorismate concentration. The optima are given on the right side. *D*, determination of native molecular weight by gel filtration. Calibration of a Superdex 200 column was performed as described under “Experimental Procedures.” Using a void volume of 47.19 ml as determined by blue dextran and a total column volume of 120 ml, the *K*ᵢ of the native chorismate mutase (□) was calculated to be 0.67. This value corresponds to a polypeptide with an apparent molecular mass of 62,187 Da.
Kinetic parameters of chorismate mutase enzyme from A. nidulans

| Inhibited (50 μM tyrosine) | Unliganded | Activated (5 μM tryptophan) |
|---------------------------|------------|-----------------------------|
|                           | $k_{cat}$  | $K_m$ | $S_{0.5}$ | $n_H$ | $k_{cat}$ | $K_m$ | $S_{0.5}$ | $n_H$ |
| $s^{-1}$                  | $\mu M$   | $n_M$ | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ | $s^{-1}$ | $\mu M$ |
| 82.5                      | 6.4        | 1.69  | 82        | 2.3     | 1.56     | 92     | 0.1      | 0.95  |

Table I

Values for $k_{cat}$, $K_m$, and $S_{0.5}$ were defined by fitting initial velocity data to equations describing hyperbolic or cooperative saturation, respectively. Hill coefficients ($n_H$) were calculated from Hill plots by linear regression.

To be 82 s$^{-1}$ per active site. By isoelectric focusing, the pl of the protein was determined to be at an acidic pH of 4.7 (data not shown). The solvent pH also has an influence on the catalytic activity of the enzyme (Fig. 3C). Without any effector bound, chorismate mutase activity reaches its maximum at a pH of 5.9.

Table I

To reveal the regulatory behavior of the enzyme, kinetic assays were performed in the presence of allosteric effectors (Fig. 3A, Table I). Tryptophan at 5 μM concentration has a strong effect on the catalytic rate. Cooperativity is lost ($n_H = 0.95$), leading to a Michaelis-Menten-type kinetic with a $K_m$ of 0.1 μM and the maximal turnover number is increased to 92 s$^{-1}$. In contrast, tyrosine acts as inhibitor of chorismate mutase activity. 50 μM of this amino acid resulted in a $S_{0.5}$ value of 6.4 μM with a turnover rate of 82.5 s$^{-1}$. The Hill coefficient of 1.69 indicates the retained cooperativity. The influence of tyrosine was further examined by kinetic assays in the presence of different amounts of this effector. Evaluation of these data according to Dixon (33) leads to a set of linear curves, one for each chorismate concentration (Fig. 3B). The point of intersection reveals an inhibitory constant $K_i$ of 2.8 μM and further indicates the type of mixed inhibition. In summary, chorismate mutase of A. nidulans is tightly regulated in its catalytic activity by tryptophan and tyrosine, with tryptophan as positive effector having a stronger influence on enzymatic behavior. This is indicated by the fact that alteration of enzyme kinetics is achieved at 10-fold lower concentration (5 μM) compared with the inhibitory concentration of tyrosine (50 μM). The allosteric effectors also show an influence on enzymatic activity with respect to solvent pH (Fig. 3C). Tyrosine shifts the catalytic maximum to a value of 5.4, whereas in the presence of tryptophan maximal catalytic activity is achieved at pH 7.1. In addition, tryptophan broadens the pH range of detectable catalytic activity.

The Chorismate Mutase of A. nidulans Is a Dimer—In order to elucidate the quaternary structure of the aroC gene product, different approaches were carried out. By analytical ultracentrifugation a mean sedimentation constant S of 4.35 ± 0.2 was determined (data not shown). Using a calculated molecular mass of 30.0 kDa for one single chorismate mutase polypeptide, this S value indicates the existence of a homodimeric structure. Gel filtration analysis supports this result (Fig. 3D). The purified protein eluted from a calibrated Superdex 200 column at a defined elution volume corresponding to a $K_d$ value of 0.67. This value matches the estimated $K_d$ for a protein of 62.2 kDa, therefore the chorismate mutase had passed the column as a dimer. Additionally, gradient polyacrylamide gel electrophoresis under non-denaturing conditions indicated an apparent molecular mass of the native enzyme of approximately 65 kDa (data not shown).

To analyze the structure of AROC, electron microscopic images of the purified enzyme were taken at a magnification of 1:3.1 × 10$^6$ (Fig. 4A). The images show the presence of a globular protein, approximately 13 × 7 nm in size. Different projections of the protein show a cleft between two subunits, indicating a structure where two identical subunits are connected by a dimeric interface.

Antibodies against the Yeast Chorismate Mutase Recognize the A. nidulans Enzyme—Given the globular, homodimeric structure of the chorismate mutase from A. nidulans and its similarity in the deduced amino acid sequence to the yeast enzyme, we performed molecular modeling studies based on the homology to known crystal structures. A three-dimensional structure of the A. nidulans enzyme was deduced on the basis of the crystal structures of the yeast chorismate mutases and the secondary structure elements of this newly created structure were calculated. The proposed three-dimensional structure of the enzyme from A. nidulans shows an all-helical structure (Fig. 4B) consisting of 12 helices that resembles that of the yeast protein. Superposition of both structures points out the similarity between them which is highest for the helical elements. Differences between the structures exist in the loops that connect these helices, especially for the loop preceding helix 12 (loop220a).

The modeling studies suggest that similar epitopes exist on the A. nidulans chorismate mutase in comparison to the yeast enzyme. To test this hypothesis, we performed immunoblotting with a polyclonal rabbit antibody raised against purified yeast chorismate mutase. Western blots of cell extracts of yeast strain RH2192 harboring the coding cDNA for aroC or the ARO7 gene of S. cerevisiae, respectively, on a 2-μm overexpression plasmid revealed a high affinity of this antibody to the A. nidulans enzyme (Fig. 4C). Therefore, we conclude that similar epitopes exist on both chorismate mutases and that the structure of the A. nidulans enzyme resembles that of the yeast protein.

A Crucial Region for Allosteric Regulation of the Yeast Enzyme Is Not Conserved in the A. nidulans Chorismate Mutase—Given the strong homology of the aroC gene product to yeast chorismate mutase, we were interested in whether the mechanism of allosteric transition is conserved in these related proteins. For the Aro7p of S. cerevisiae, it has been shown that a single threonine residue in loop220a (Thr226) is important for proper signal transduction from the effector binding sites to the catalytic centers of the homodimer (14). Exchange of that amino acid residue to isoleucine (Aro7p T226I) leads to a constitutively activated enzyme that is locked in the R state. Upon alignment of the primary structures of AROC and Aro7p, an aspartate residue (Asp235) corresponds to that position in the A. nidulans enzyme (Fig. 1B). By site-directed mutagenesis, this residue was changed to threonine and isoleucine, respectively, in the aroC gene product. Both alleles (aroC T226I, aroC D235I) were able to complement the yeast aro7Δ deletion indicating that they are expressed properly in the recipient strain. After overexpression in the yeast aro7Δ mutant strain RH2192, desalted crude extracts were prepared and specific chorismate mutase activities were determined in the absence or presence of effectors, respectively. In addition, the corresponding ARO7 alleles, ARO7WT, ARO7 T226I and ARO7 D235I, were expressed from the same plasmid in the aro7Δ strain and the specific activities were determined in desalted crude extracts under identical conditions (Table II).
Chorismate Mutase of A. nidulans

Catalytic activities were determined in desalted crude extracts of yeast strain RH2192 expressing different chorismate mutase-encoding alleles on a 2-μm overexpression plasmid driven by the MET25 promoter. The values measured for each enzyme were standardized for plasmid copy number by Southern analyses.

| Protein       | Specific activity (nmol·min⁻¹·mg⁻¹) | Range of modulation |
|---------------|-------------------------------------|---------------------|
|               | Unliganded | Inhibited | Activated |               |
| AROC          | 32.5       | 8.4       | 88.5      | 11             |
| AROC<sub>D233I</sub> | 30.3       | 13.4      | 63.0      | 4.7            |
| AROC<sub>D233T</sub> | 41.3       | 22.0      | 64.3      | 2.9            |
| Aro7p         | 4.8        | 1.2       | 40.2      | 34             |
| Aro7<sub>T226I</sub>p | 20.6       | 21.7      | 26.4      | 1.3            |
| Aro7<sub>T226T</sub>p | 3.7        | 3.3       | 11.1      | 3.4            |

* Assay conditions were 50 μg of total protein and 2 mM chorismate, 3-min reaction time, and 100 μM tyrosine or 500 μM tryptophan, respectively. Each value is the mean of three independent measurements with a standard deviation not exceeding 20%.

Generally, the A. nidulans chorismate mutase enzymes showed higher specific activities in these assays than their yeast homologues. For the AROC wild-type enzyme a specific activity of 32.5 units/mg of total protein was measured, which is repressed 3.9-fold to 8.4 units mg⁻¹ in the presence of 100 μM tyrosine, whereas tryptophan at 500 μM concentration leads to a 2.7-fold increase in specific activity to a value of 88.5 units mg⁻¹. In contrast, yeast chorismate mutase activity expressed from the ARO7<sub>T226I</sub> allele was measured to be 3.7 units mg⁻¹.

In its inhibited form the enzyme is slightly repressed in its activity (3.3 units mg⁻¹). In the presence of tryptophan, activity is increased 3-fold to 11.1 units mg⁻¹. The proteins with a substitution to isoleucine clearly differ in their enzymatic properties. The unliganded aroC<sub>D233I</sub> gene product shows a specific activity of 30.3 units mg⁻¹, which is repressed 2.3-fold when liganded by tyrosine (13.4 units mg⁻¹) and increased 2.1-fold to 63.0 units mg⁻¹ by its activator tryptophan. The yeast counterpart Aro7<sub>T226I</sub>p has a specific activity of 20.6 units mg⁻¹ and shows almost no regulatory response to both effectors which is characteristic for this constitutively activated enzyme. Substitution of residue 233 in the A. nidulans enzyme to threonine leads to a chorismate mutase with a reduced regulatory range. The uneffected enzymatic activity of 41.3 units mg⁻¹ is decreased 1.9-fold to 22.0 units mg⁻¹ by tyrosine and increased 1.6-fold to 64.3 units mg⁻¹ by tryptophan. The corresponding wt-Aro7p enzyme shows a specific activity of 4.8 units mg⁻¹ in its unliganded state. This value is decreased 4-fold to 1.2 units mg⁻¹ in the presence of tyrosine, whereas tryptophan leads to a 8.4-fold increase of specific activity to 40.2 units/mg of protein.

In summary, both AROC mutant proteins exhibit a reduced range of regulatory properties in comparison to the wild-type enzyme. In the wild-type enzyme, carrying the charged amino acid aspartate at position 233, modulation of chorismate mutase activity by the heterotropic effectors tyrosine and tryptophan, respectively, is given by a factor of 11. In the protein derived from the aroC<sub>D233I</sub> mutant allele, the substitution to an apolar amino acid residue leads to reduced modulation and enzymatic activity is within a range of 5. The AROC<sub>D233T</sub> gene product was modeled on known three-dimensional structures of yeast chorismate mutase monomers by SWISS-MODEL. In the superimposition the tertiary structure of the yeast protein is represented as gray ribbons, structures of AROC are indicated as a black line. C indicates the C terminus of the protein, N its N terminus. Important helices (H) are indicated as well as loop220s connecting helix 11 and 12. The alignment shows a section of both enzymes compromising the region of loop220s with identical residues in bold. C, a polyclonal rabbit antibody raised against yeast chorismate mutase binds the AROC enzyme with high affinity. The immunoblot shows 15 μg of crude extracts of yeast strain RH2192 (aro7Δ::LEU2) harboring different 2-μm expression plasmids. Lane 3 contains crude extract from yeast strain RH2191 carrying one chromosomal copy of the AROT gene. Proteins cross-reacting with polyclonal antiserum raised against purified yeast chorismate mutase were detected using enhanced chemiluminescence.
protein shows almost no response to the effectors with a narrow window of regulation by a factor of 3. The exchange of the aspartate residue to the polar amino acid threonine therefore seems to disturb the intramolecular signal transduction pathway for the allosteric switch.

Taken together, the data clearly show that the chorismate mutase enzymes of the bakers' yeast and the filamentous fungus \textit{A. nidulans} share regulatory and structural properties. Despite these similarities the intramolecular signal transduction pathway for allosteric transition as proposed for the yeast enzyme seems to be not conserved in the AROC protein.

\textbf{DISCUSSION}

The metabolic pathway of aromatic amino acid biosynthesis is a conserved reaction cascade converting two compounds of primary metabolism to phenylalanine, tyrosine, and tryptophan. The flux of compounds through this pathway has to be strictly regulated as synthesis of aromatic amino acids is an energy-consuming process. One mode of regulation lies in controlling the activity of branch point enzymes within a pathway, either by altering their catalytic properties or via altered enzyme levels within a cell. In the aromatic amino acid biosynthetic pathway, the chorismate mutase enzyme is one major point of attack in regulating the flux of chorismic acid into the tyrosine/phenylalanine-specific branch.

We have demonstrated that the protein specified by the \textit{aroC} gene of \textit{A. nidulans} is the chorismate mutase enzyme of this filamentous fungus. According to its high sequence similarity to the monofunctional chorismate mutase of \textit{S. cerevisiae} the \textit{A. nidulans} enzyme has to be classified as a member of the AroQ class of chorismate mutases. The kinetic properties of this enzyme demonstrate that the \textit{aroC} gene product is tightly regulated in its activity. The substrate, chorismate, serves as homotropic, positive effector as deduced from positive cooperativity in substrate saturation assays. The determined Hill coefficient of 1.56 clearly indicates that the enzyme contains at least two substrate-binding sites. In addition, two aromatic amino acids show heterotropic effects on enzymatic activity. Tyrosine, one end product of the chorismate mutase-specific branch, influences catalytic efficiency negatively, whereas tryptophan, the end product of the opposite branch, strongly increases catalytic turnover. Therefore this chorismate mutase enzyme fits well in the model of allosterism as established by Monod and co-workers (49). In this simple model a given enzyme exists in two (or more) structural states, tense (T-) or relaxed (R-), with different catalytic activities. The equilibrium between these states is changed upon substrate binding to the active site or by binding of inhibitory or activating ligands at distinct allosteric sites. Further reference to allosterism is given by the homodimeric structure of the \textit{A. nidulans} chorismate mutase since allosteric enzymes are often multimeric proteins.

\textbf{pH dependence of catalytic activity of the chorismate mutase from \textit{A. nidulans}} shows three distinct optima. For the unliganded enzyme the pH optimum of 5.9 fits quite well the intracellular pH in filamentous fungi, which is in a range of 5.7 to 6.5 (50). The negative effector tyrosine shifts this optimum only slightly to a value of 5.4, whereas tryptophan alters the range of catalytic activity dramatically: maximum turnover is achieved at the neutral pH of 7.1 and catalytic activity is present over a pH range between 4 and 12. This pH-dependent catalytic behavior is contrary to that known from bacterial chorismate mutases where highest catalytic activities are achieved at alkaline pH (51, 52). On the other hand the catalytic activities of the \textit{A. nidulans} enzyme at different pH values resemble that of yeast chorismate mutase. For the \textit{A. nidulans} chorismate mutase, similar absolute catalytic activities were determined as described for the yeast enzyme (10). Without any effector present, enzymatic activity was measured over 4.5 pH units and tyrosine restricted catalytic activity to a range of 3 pH units (Fig. 3C). One difference concerning pH dependence is the range of detectable catalytic activity in the presence of tryptophan. The heterotropic positive effector broadens the pH range of activity to 8 pH units compared with a value of 6 units as reported for the \textit{S. cerevisiae} enzyme. In the yeast protein the active site residue Glu\textsuperscript{248} has been identified to be important in restricting enzyme activity to acidic conditions (10). Upon alignment, this particular residue is conserved within the primary structure of the \textit{A. nidulans} enzyme (Glu\textsuperscript{253}).

In yeast chorismate mutase, different domains within the dimeric structure have been identified (53). Upon effector binding, the two subunits rotate relative to each other and the allosteric signal is transmitted toward the polypeptide to the catalytic domain. The dimeric structure and all specific amino acids of the yeast enzyme which are important for binding of effectors (Arg\textsuperscript{77}, Arg\textsuperscript{76}, Asn\textsuperscript{139}, Ser\textsuperscript{142} and Thr\textsuperscript{145}) and allosteric signal transduction (Glu\textsuperscript{23}, Asp\textsuperscript{24}, Phe\textsuperscript{25}, and Tyr\textsuperscript{254}), as well as active site residues (Arg\textsuperscript{16}, Arg\textsuperscript{157}, Lys\textsuperscript{168}, Glu\textsuperscript{198}, and Thr\textsuperscript{242}) are conserved in the chorismate mutase of \textit{A. nidulans} (Fig. 1B). Additionally, \textit{in silico} studies showed that AROC can be modeled quite closely onto the tertiary structure of the yeast protein. Therefore, it was surprising that one particular residue, Thr\textsuperscript{226}, of the yeast enzyme is not conserved in its \textit{A. nidulans} counterpart, as this residue had been characterized as the molecular switch in transmitting the signal for \textit{T} to \textit{R} state transition (9). By site-directed mutagenesis we created two mutant AROC enzymes. None of these enzymes turned out to be locked in either allosteric state, but both proteins showed decreased regulatory properties upon effector binding. We conclude that this narrow window of regulatory modulation represents intermediate states between tense and relaxed state. The role of loop220s in transmitting the intramolecular signal from the effector binding sites to the catalytic domains is obviously different in the chorismate mutases of \textit{S. cerevisiae} and \textit{A. nidulans}. Whereas in the yeast protein substitution of one particular residue in loop220s locks the whole enzyme in its activated state, we did not find such a behavior in the AROC mutant enzymes. Taking into account that the \textit{A. nidulans} enzyme resembles its yeast homologue with respect to catalytic and regulatory behavior as well as structural properties this difference is surprising. It implicates that the structure of this loop preceding helix 12, which is part of the catalytic domain, is more flexible in the \textit{A. nidulans} enzyme than in the yeast chorismate mutase. Additionally, we suggest that alternative pathways within the molecule could exist for signal transduction to the active site in contrast to one exclusive via loop220s.

\textbf{Allosterism} is one possible way in regulating enzymatic activity. In living systems additional mechanisms of flux control through a metabolic pathway exist which affect the rate of expression of a given enzyme. For the \textit{aroC} gene product data indicate that its expression is not regulated transcriptionally via the cross-pathway control network (54). Amino acid starvation conditions showed no influence on \textit{aroC} transcript levels which is consistent with data obtained for \textit{S. cerevisiae} (55). Sequence analysis for upstream regulatory sequences indicated a putative STUA-binding site 560 nucleotides upstream of the translational start codon of \textit{aroC}. This sequence element matches the described consensus for STUA response elements (42). As a filamentous fungus \textit{A. nidulans} has developed additional regulatory networks that constitute differentiation processes. Preliminary transcript level analyses indicate that \textit{aroC} expression is down-regulated drastically after the developmental program of asexual conidiation is initiated (not shown).
Future research will have to identify trans factors as well as cis elements responsible for this type of regulation and elucidate whether this is specific for chorismate mutase expression or, in contrast, is a general effect after the developmental program has been established.

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