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* 


Chorismate Mutase of A. nidulans

ultrafiltration membranes from Millipore (Eschborn, Germany). The Mini 2D SDS-polyacrylamide gel electrophoresis system and the Bradford protein assay solution for determination of protein concentrations originated from Bio-Rad. Vent polymerase (BIOLABS, Schwalbach, Germany) was used for polymerase chain reactions. All other chemicals were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany).

**Strains, Media,** **cDNA Library, Plasmids, Growth Conditions**—The _S. cerevisiae_ strain RH2185 (MATa, sec2-9, ural3-52, leu2-3, leu2-112, his3-521, ar0::LEU2, GAL2) (10) with the genetic background of the laboratory strain X2180-1A (MATa, gal2, SUC2, mal, CUP1) was used as a recipient for cloning of _A. nidulans_ DNA out of an inducible expression library. The expression library was constructed after mRNA isolation from _A. nidulans_ FGSC A234 (6, pabaA1, veA1) using the Superscript® cDNA Synthesis Kit from Life Technologies, Inc. (Gaithersburg, MD). cDNAs were ligated as Sau3AI fragments in the shuttle vector pRS316-GAL1 (18) and propagated in _Escherichia coli_. Yeast transformation was carried out as described in Ref. 19. Transformation of _A. nidulans_ was carried out according to Pun and van den Hengel (20). For overexpression, a derivative of plasmid p426MET25 (21) was used in the _S. cerevisiae_ strain RH2192 (MATa, pra1-1, pbr1-1, prc1-1, cps1-3, ural3A5, leu2-3, 112, his3, ar0::LEU2) which is a derivative of the protease-deficient strain _S. bayanus_ xBASY-86 (22). The _A. nidulans_ strain G1100 (aroC1246, riboA1, adg14, A2D) was described earlier (23) and was used for cloning of _A. nidulans_ DNA out of an inducible expression library. The native molecular medium for the cultivation of yeast was described earlier (24) and minimal medium for the cultivation of _A. nidulans_ strains was prepared according to Kafer (25).

**Site-directed Mutagenesis**—A polymerase chain reaction-based method was used for site-directed mutagenesis of _aroC_ (26). The polymerase chain reaction-generated fragments were sequenced (27) to confirm the presence of the mutations and to rule out second-site mutations. Erase chain reaction-generated fragments were sequenced (27) to confirm according to Käfer (25).

**RNA Preparation and Analysis**—Total RNA was prepared from vegetatively growing _A. nidulans_ cultures using the TRIzol™ reagent from Life Technologies, Inc. following the supplier’s instructions. Transcript levels were analyzed by Northern hybridization (28) using a Bio-Imaging Analyzer from Fuji Photo Film Co. Ltd. (Tokyo, Japan). Transcript length was determined using the 0.16–1.77 kb RNA ladder from Life Technologies, Inc.

**Overexpression and Purification of A. nidulans Chorismate Mutase**—Plasmid-carrying yeast strains were grown at 30 °C in 10-liter rotary fermentors under aeration. Cells were harvested in mid-log phase at an OD600 of 4–6, washed twice with 50 ml potassium phosphate buffer, pH 7.6, and stored in 1 ml of buffer/g wet cells at −20 °C in the presence of 0.1 M sodium azide and 0.1 M sodium dodecyl sulfate ( SDS) in 0.2 M EDTA, and 1 ml of di-thiothreitol. For purification, 80–110 g of cells were thawed and run three times through a French Pressure Cell (18,000 p.s.i.). Cell debris was sedimented by centrifugation at 30,000 × _g_ for 20 min.

The chorismate mutase was purified according to the protocol of Schmidlin et al. (14) with the following modifications: in all steps 10 mM potassium phosphate buffer, pH 7.6, was used as solvent, ammonium sulfate precipitation was carried out at 47% saturation, phenylmethylsulfonyl fluoride ( PMSF) was added to the equilibration buffer for the ethylamino-Sepharose column, dialysis was used to desalt protein extracts, and a second run on a Mono Q column at pH 5.8 in 10 mM potassium phosphate buffer was performed. Chorismate mutase was detected by SDS-polyacrylamide gel electrophoresis (99) and enzymatic activity assays. Measurements of protein concentrations were performed using the Bradford assay (30).

**Enzyme Assays**—Chorismate mutase activity was measured as described previously (9) with some modifications. The enzymatic reaction, prephenate, is converted to phenylpyruvate photometrically, 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 enzymatic reactions at 30 °C and equilibrating the spectrophotometer cell to the same temperature. Reaction volumes of 250 μl containing 100 mM potassium phosphate buffer, pH 7.4, 5 mM MgCl2; 0.1 mM potassium chloride, 50 μM tyrosine and 5 μM tryptophan, chorismate mutase enzyme, and chorismate in a range from 0.25 to 13 mM were used. The reaction was started by adding the mixture of all ingredients to the prewarmed chorismate solution. The reaction was stopped by adding 250 μl of 1 M HCl. After an incubation time of 10 min, 4 ml of 1 N NaOH were added and extinction at 320 nm was measured against H2O. To exclude absorbance caused by the uncatalyzed rearrangement of chorismate, blanks of increasing chorismate concentrations without enzyme were prepared and absorbance was measured. These blank absorbances were subtracted from the absorbance values measured for the enzymatic activities. A calibration curve was drawn using different known phenylpyruvate concentrations that were treated the same way as the enzyme reactions. The molecular extinction coefficient at 30 °C was determined as 13095 m ^ 2 cm ^ −1. For determination of the pH optimum, a universal buffer solution with a pH range of 2.5–12 containing 30 mM citric acid, 30 mM KH2PO4, 20 mM diethybarbituric acid and different amounts of NaOH was used.

The collected data were transformed to international units ( μmol min ^ −1) per mg of enzyme. The maximum velocity _V_{max}_, the Hill-coefficient _nH_, and the substrate concentration at half-maximal velocity _S_{0.5}_, or _K_{s}_, were determined using a computer program which applied the Quasi-Newton method (Davidson-Fletcher-Powell algorithm) to fit optimal curves to the data (31). To draw substrate saturation curves, the data were fitted either to the Michaelis-Menten equation ( _v_ = _V_{max}_, S _/K_{m}_, + S), or to the Hill equation ( _v_ = _V_{max}_, S ( _S_{0.5}_, + S) ^ −1), where _S_{0.5}_, _S_{0.5},_, Edadie-Hofstee plots ( _v_ = _S_{0.5}_, − _v_ ) and _v_ were drawn to decide which equation a set of kinetic data had to be applied. Enzyme kinetics without cooperativity result in a linear curve, whereas even small degrees of increased cooperativity result in S-shaped curves. Hill plots (log ( _v_ − _v_ _/v_{max}_, _S_{0.5}_, + S _/K_{m}_,) versus _log_ ( _S_ ) were used to calculate Hill coefficients. The resulting _V_{max}_, values were transformed to catalytic constants ( _k_{cat}_, _V_{max}_, _M_{p}, E_{m}_, − 60 s ^ −1), substrate turnover per active site). The inhibitor constant _K_, for tyrosine was determined according to Dixon by plotting _v_ versus _inhibitor concentration (33).

**Determination of the Native Molecular Weight**—The native molecular weight of the chorismate mutase was estimated by gel filtration on a Superdex 200 prep grade column using 50 mM potassium phosphate, 150 mM NaCl, pH 7.6, as elution buffer. The amount of the column was determined with blue dextran and a calibration plot was defined using a gel filtration chromatography standard from Bio-Rad containing thyroglobulin, bovine γ-globulin, chicken ovalbumin, equine myoglobin, and vitamin B-12. In addition, the molecular weight was determined independently by sedimentation equilibrium at 50,000 rpm (16 °C) and calculation of the sedimentation coefficient and the molecular mass. Three different concentrations of the enzyme in 20 mM potassium phosphate buffer, pH 7.6, were used and all ultracentrifugal analyses were performed on a Beckmann XLA. To confirm the results obtained by gel filtration and analytical ultracentrifugation, the molecular weight was estimated by native polyacrylamide gel electrophoresis using a gradient from 4 to 20% polyacrylamide (34).

**Determination of the Isoelectric Point**—The isoelectric point of the chorismate mutase enzyme was determined 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™ ampholyte ranging from pH 3.5 to 9.5 in a concentration of 0.5%. The column was applied to the Rotofor 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, pH 7.6. Chorismate mutase was detected by enzyme assays as well as by SDS-polyacrylamide gel electrophoresis.

**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**—Immunochemical 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 (version 3.0 d; Astar (Madison, Wisconsin). Alignments were created based on the Lipman-Pearson method (37). For homology modeling, the predicted 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 bank (12) by ProMod3 (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.
The chorismate mutase of *A. nidulans* shows homology to the *S. cerevisiae* enzyme. The alignment shows a comparison of the deduced amino acid sequence of *A. nidulans* chorismate mutase (AnCM) with that of *S. cerevisiae* (ScCM). Identical residues are indicated by vertical bars, conservative replacements by colons, and neutral changes by periods.

**RESULTS**

Isolation of the *aroC* Gene from *A. nidulans*—The *aroC* gene from *A. nidulans* was cloned by functional complementation of a *S. cerevisiae* *aro7A* 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 tyrosine or phenylalanine. Yeast strain RH2185 (*aro7-LEU2, aro3-52*) was transformed with *A. nidulans* cDNA expressed from the *GAL1* promoter (18) and transformants were selected by growth on medium lacking tyrosine and phenylalanine. A total of 80 colonies were obtained, from which plasmids were isolated and analyzed. Southern blot analysis of this region using the cDNA of pME1498 as a probe, revealed that the *aroC* gene does not contain any chorismate mutase.

The genomic region encoding the cDNA insert of pME1498 was isolated and analyzed. Southern blot analysis of this region, using the cDNA of pME1498 as a probe, revealed that the corresponding gene exists as a single copy in the *A. nidulans* genome (data not shown). A sublibrary of *A. nidulans* genomic HindIII fragments of 4–6 kb in size was screened by colony hybridization with the *aroC* cDNA probe and resulted in a 5-kb fragment which was subjected to DNA sequence analyses (Fig. 2A). The genomic fragment contains the same open reading frame as the cDNA, flanked by a 1.2-kb 5’-region and a 2.7-kb 3’-region, and is interrupted by two short intron sequences. Intron I is located 70 bp downstream of the translational start site and is 113 bp in length, while intron II is located 77 bp upstream of the stop codon UAG and 49 bp in size. Both show the conserved 5’ splicing, internal, and 3’ splicing sequences described for *A. nidulans* introns (42). Sequence analysis for upstream regulatory sequences in the promoter region of *aroC* indicated a putative STUA-binding site and a Gcn4p response element in reverse orientation by the open triangle. A *aroC* mutant strain auxotrophic for phenylalanine and tyrosine. For 90% of the isolated transformants were selected on medium lacking tyrosine and phenylalanine. Yeast strains with a plasmid from *A. nidulans* (strain G1100) (43) for ectopic integration (Fig. 2B). The homology goes up to 67% when conservative changes are taken into account.

The genomic region encoding the cDNA insert of pME1498 was isolated and analyzed. The alignment shows a comparison of the deduced amino acid sequence of *A. nidulans* chorismate mutase (AnCM) with that of *S. cerevisiae* (ScCM). Identical residues are indicated by vertical bars, conservative replacements by colons, and neutral changes by periods.
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 Is Regulated by Tyrosine and Tryptophan—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_{cat} was calculated.
Chorismate Mutase of A. nidulans

Kinetic parameters of chorismate mutase enzyme from A. nidulans

<table>
<thead>
<tr>
<th>Inhibited (50 μM tyrosine)</th>
<th>Unliganded</th>
<th>Activated (5 μM tryptophan)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$k_{cat}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td>$s^{-1}$</td>
<td>$s^{-1}$</td>
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<tr>
<td>$[S]_{0.5}$</td>
<td>$[S]_{0.5}$</td>
<td>$[S]_{0.5}$</td>
</tr>
<tr>
<td>82.5</td>
<td>6.4</td>
<td>12.9</td>
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The inhibitory concentration of tyrosine (50 μM) and the corresponding $K_m$ were determined 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.

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. 3A). Without any effector bound, chorismate mutase activity reaches its maximum at a pH of 5.9.

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 mM 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 mM with a turnover rate of 82.5 s$^{-1}$. The Hill coefficient of 1.69 indicates the type of mixed inhibition. 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 nm was determined (data not shown). Using a calculated molecular mass of 30.0 kDa for one single chorismate mutase polypeptide, the specific protein was determined to be at an acidic pH of 4.7 (data not shown). Using a calculated molecular mass of 65 kDa for one single chorismate mutase polypeptide, the specific protein was determined to be at an acidic pH of 4.7 (data not shown).

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 loop220s (Thr226) is important for proper signal transduction from the effector binding sites to the catalytic centers of the homodimer (14). Exchange of that residue was changed to threonine and isoleucine, respectively, in A. nidulans (Fig. 1B). By site-directed mutagenesis, this residue was changed to threonine and isoleucine, respectively, in the aroC gene product. Both alleles (aroC$^{D226T}$, aroC$^{D226I}$) were able to complement the yeast aro7Δ deletion indicating that they are expressed properly in the recipient strain. Aro7p 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.

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 (loop220s).

| Hill coefficient | 1.69 |

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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.

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 ARO7T226D 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<sup>D233I</sup> 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<sup>D233I</sup> 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<sup>D233T</sup> protein, 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 (aro7::LEU2) harboring different 2-μm expression plasmids. Lane 3 contains crude extract from yeast strain RH2191 carrying one chromosomal copy of the ARO7 gene. Proteins cross-reacting with polyclonal antiseraum 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 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.

**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 aroC gene of A. nidulans is the chorismate mutase enzyme of this filamentous fungus. According to its high sequence similarity to the monofunctional chorismate mutase of S. cerevisiae the 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 aroC gene product is tightly regulated in its activity. The substrate, chorismate, serves as homotopic, 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 heterotopic 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 A. nidulans chorismate mutase since allosteric enzymes are often multimeric proteins.

pH dependence of catalytic activity of the chorismate mutase from 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 A. nidulans enzyme at different pH values resemble that of yeast chorismate mutase. For the 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 S. cerevisiae enzyme. In the yeast protein the active site residue Glu246 has been identified to be important in restricting enzyme activity to acidic conditions (10).

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 (Arg75, Arg76, Asn139, Ser142, and Thr145) and allosteric signal transduction (Glu23, Asp24, Phe28, and Tyr234), as well as active site residues (Arg16, Arg157, Lys168, Glu198, and Thr226) are conserved in the chorismate mutase of A. nidulans (Fig. 1B). Additionally, 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, Thr226, of the yeast enzyme is not conserved in its A. nidulans counterpart, as this residue has been characterized as the molecular switch in transmitting the signal for T to 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 S. cerevisiae and 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 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 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.

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 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 aroC transcript levels which is consistent with data obtained for S. cerevisiae (55). Sequence analysis for upstream regulatory sequences indicated a putative STUA-binding site 560 nucleotides upstream of the translational start codon of aroC. This sequence element matches the described consensus for STUA response elements (42). As a filamentous fungus A. nidulans has developed additional regulatory networks that constitute differentiation processes. Preliminary transcript level analyses indicate that 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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