

Tyrosine and Tryptophan Act through the Same Binding Site at the Dimer Interface of Yeast Chorismate Mutase*

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Tyrosine and tryptophan are the regulators of the dimeric yeast chorismate mutase. Biochemical studies reveal two binding sites per molecule for both effectors, tyrosine or tryptophan. A single binding site is built up by helix 8 and helices 4 and 5 of two different subunits. The binding sites have been analyzed in the active enzyme by site directed mutagenesis of critical codons of the coding gene, *ARO7*. Gly-141 and Ser-142, which both reside on helix 8, are involved in the binding of tyrosine or tryptophan presumably by interacting specifically with the amino- and carboxylate-groups of these amino acid effectors. Interaction with Thr-145 of helix 8 is required for a strong tyrosine binding to the allosteric site. Replacement of Arg-75, which connects helices 4 and 5 or of Arg-76, which is part of helix 5 by alanine residues, resulted in unregulated enzymes. These two residues are bonded to the carboxylate group and phenolic hydroxyl group of tyrosine, respectively, but do not interact with tryptophan by hydrogen bonding in the crystal structures. Phenylalanine, which has low binding affinity slightly activated the chorismate mutase. A T145V mutant chorismate mutase, however, showed increased activation by phenylalanine. Our results support a mechanism by which tyrosine contracts the allosteric site by interacting with its phenolic hydroxyl group. Tryptophan works in an inverse way by opening the allosteric site through the steric size of its side chain.

Chorismate is the last common precursor of the amino acids tyrosine, phenylalanine, and tryptophan. Chorismate mutase (EC 5.4.99.5) catalyzes the intramolecular rearrangement of chorismate to prephenate, which is the first committed step in the biosynthesis of phenylalanine and tyrosine (1) (for a review, see Ref. 2). This reaction is the only example of a pericyclic reaction in primary metabolism and has been studied in detail (for reviews see Refs. 3 and 4). The enzyme competes for a common substrate with anthranilate synthase (EC 4.1.3.27), which catalyzes the first step of the tryptophan-specific branch. The chorismate mutase of *Saccharomyces cerevisiae* is a key regulatory element in the aromatic amino acid biosynthesis pathway and is activated by tryptophan and inhibited by tyrosine (5, 6). This antagonism plays an important role in main-

taining a balanced biosynthesis of aromatic amino acids in the cell. The yeast chorismate mutase holoenzyme is composed of two 30-kDa subunits encoded by the *ARO7* gene (6) and serves as simple model for an allosteric enzyme. Phenylalanine is not an inhibitor of yeast chorismate mutase. This is in contrast to the homologous plastidic chorismate mutase from *Arabidopsis thaliana*, which is inhibited by both amino acids, tyrosine and phenylalanine (7). Besides the heterotropic ligand induced effects, yeast chorismate mutase exhibits homotropic cooperative interactions for substrate binding (6). Both kinetic properties of the enzyme can be rationalized in terms of a transition from a conformation having a low affinity for chorismate, the T (tense) state, to a conformation with high affinity for the substrate, the R (relaxed) state (8). According to this two-state model, homotropic and heterotropic interactions proceed by the same transition. The influence of the allosteric regulators is best explained by local conformational changes induced by their competitive binding to the regulatory sites, therefore stabilizing either of the two states or inducing a conformational change of the overall enzyme structure.

The crystal structures of a mutant enzyme (T226I), which is locked in the activated state (resolution of 2.2 Å) and of a tyrosine-bound wild-type enzyme (resolution of 2.8 Å) have been described (6, 9–11). The crystallographic structure determined by x-ray diffraction of the mutant enzyme showed that two tryptophan molecules were copurified and bound at the dimer interface. The three-dimensional structure of the wild-type enzyme cocrystallized with tyrosine showed two tyrosine molecules at those sites where tryptophan binds to the mutant enzyme (Fig. 1, A and B) (11). In addition two crystal structures of chorismate mutase in complex with a transition state analogue and with either the allosteric activator tryptophan (3.0 Å resolution) or the allosteric inhibitor tyrosine (2.8 Å) bound to the regulatory site were determined (12). Superposition of these two structures showed significant differences between their regulatory sites, whereas their catalytic domains superimposed closely. These results suggest that the regulatory and the catalytic domains could acquire different conformations independently of each other. In all crystallographic structures of yeast chorismate mutase characterized so far both effectors bind at a cleft, which is formed by helices 4 and 5 of one subunit and helix 8 of the other subunit (Fig. 1, A and B).

To examine the model suggested by the structural studies we applied site-directed mutagenesis of *ARO7* to investigate the effects of various amino acid replacements at the putative tryptophan and tyrosine binding sites. The wild-type and mutant enzymes were overexpressed in yeast using recombinant DNA technology and then purified for binding and activity assays. We found that tryptophan and tyrosine regulate the yeast chorismate mutase through binding to common residues at the dimer interface of the enzyme.

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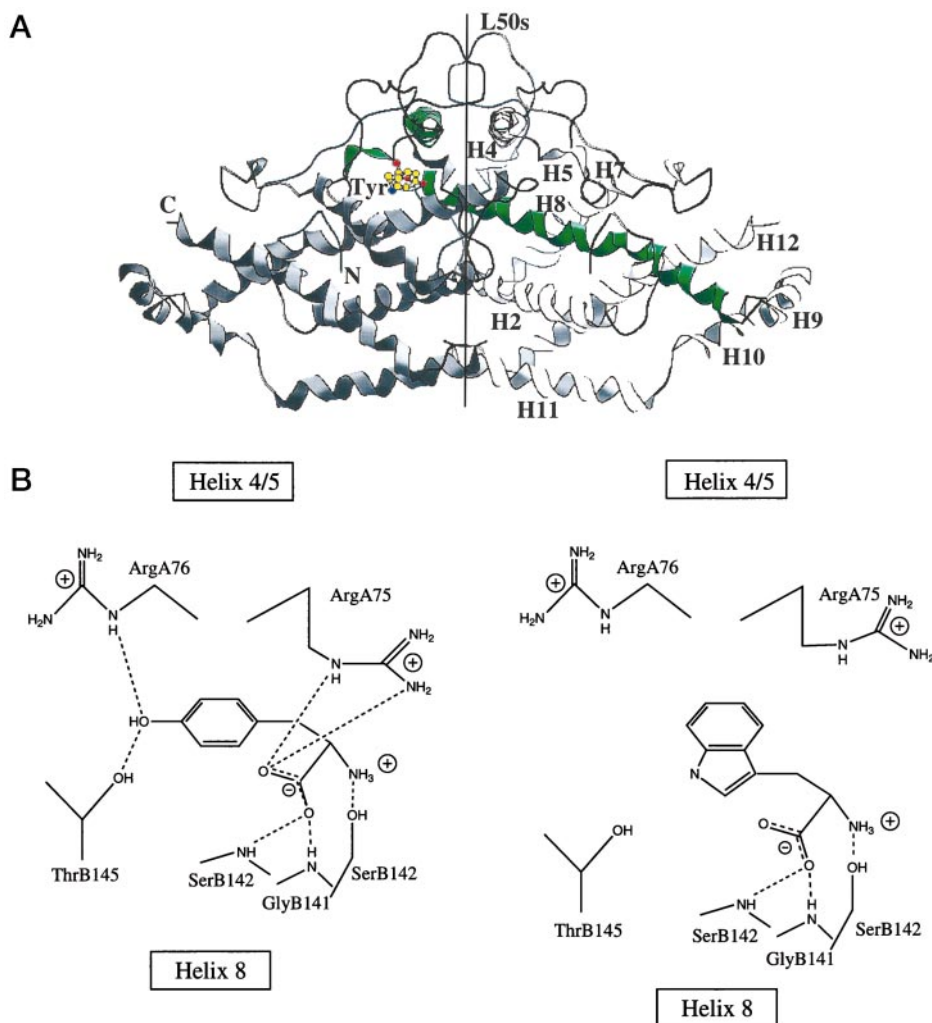


FIG. 1. A, model of the tyrosine-bound yeast chorismate mutase dimer as deduced from the x-ray diffraction data (11). The N and C termini are labeled as *N* and *C*, respectively. One of the two tyrosine molecules bound at the dimer interface is shown in a ball and stick model (C atoms, yellow; N atoms, blue; O atoms, red). The helices 4, 5, and 8 of the other subunit which form the binding site are drawn in green. The numbering of the helices is indicated at the *right subunit*. B, schematic showing the proposed binding of tyrosine and tryptophan to the interface of yeast chorismate mutase in more detail (9, 11). Residues are part of the different subunits of the dimeric enzymes, which are indicated by A and B, respectively. Possible interactions with the effectors are indicated as dotted lines.

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 (13) and by coupling of the ligand, ethylamino-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 Amicon (Danvers, MA). 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 supplied by FLUKA (Buchs, Switzerland) or Sigma.

Strains, Media, Plasmids, Growth Conditions—The *S. cerevisiae* strain RH1242 (*MAT α* , *aro7*, *leu2-2*) with the genetic background of the laboratory strain X2180-1B (*MAT α* , *gal2*, *SUC2*, *mal*, *CUP1*) was used for expression of the chorismate mutase enzymes. Derivatives of plasmid pME605 (10) were used as overexpression system. Yeast transformation was carried out by the LiAc method (14). MV minimal medium for the cultivation of yeasts was described earlier (15).

Site-directed Mutagenesis—A polymerase chain reaction-based method was used for site-directed mutagenesis of *ARO7* (16). Polymerase chain reaction-generated DNA fragments were sequenced (17) to confirm the presence of the mutation and to rule out any second-site mutations.

Purification of Yeast Chorismate Mutase—Yeast cells were grown at 30 °C in 10-liter rotatory fermentors under aeration. Cells were harvested in mid-log phase at an A_{546} of 4–6, washed twice with 50 mM potassium phosphate buffer, and stored in 1 ml of buffer/g of wet cells at –20 °C in the presence of protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, and 1 mM DL-dithiothreitol). 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 \times g for 20 min.

The enzyme was purified as described previously (6) with the following modifications. Phenylmethylsulfonyl fluoride was added to the equilibration buffer for the ethylamino-Sepharose column, dialysis was used to desalt protein extracts, and chorismate mutase was applied to a second MonoQ column (HR 5/5).

Chorismate mutase was detected by SDS-polyacrylamide gel electrophoresis (18) and by enzyme assays. Measurements of protein concentrations were performed using the Bradford assay (19).

Enzyme Assays—Chorismate mutase activity was measured as described previously (5) with some modifications. The enzymatic conversion is stopped after 1 min by addition of HCl, and the product of the enzymatic reaction, prephenate, is converted to phenylpyruvate through a chemical reaction. Enzymatic activity is 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 enzymatic reactions at 30 °C and equilibrating the spectrophotometer cell to the same temperature. Reaction volumes of 250 μ l containing 100 mM Tris, pH 7.6, 2 mM EDTA, 20 mM dithiothreitol, optionally 0.1 mM tyrosine, 0.1 mM phenylalanine or 0.01 mM tryptophan, chorismate mutase enzyme, and chorismate in a range from 0.25 to 10 mM were used. The reaction was started by adding the mix of all ingredients to the prewarmed chorismate solution. Reaction was stopped by adding 250 μ l of 1 M HCl. After an incubation time of 10 min, 4 ml of 1 M NaOH were added, and A_{320} was measured against H_2O . 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 optical densities measured for enzyme activities. A calibration curve was drawn using different known phenylpyruvate concentrations that were treated the same way as the enzyme reactions. The molar extinction coefficient at 30 °C was determined as 13095 $M^{-1} cm^{-1}$.

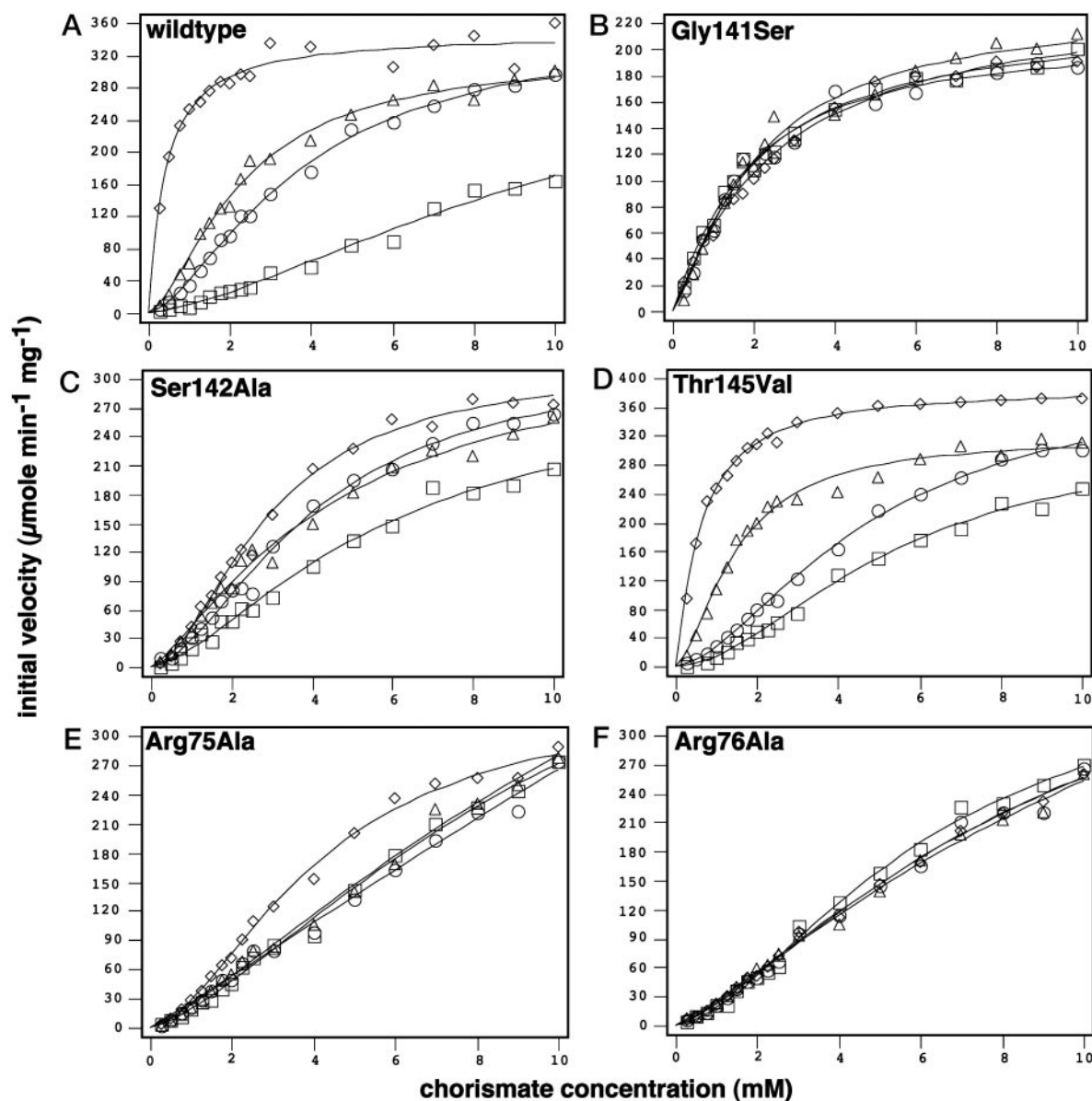


Fig. 2. Substrate saturation plots of wild-type and mutant chorismate mutases. The enzymes were assayed with $10 \mu\text{M}$ tryptophan (\diamond) without effectors (\circ) or in the presence of $100 \mu\text{M}$ tyrosine (\square) or $100 \mu\text{M}$ phenylalanine (\triangle). The data were fitted to functions describing cooperative or Michaelis-Menten-type saturation.

The collected data were transformed to international units ($\mu\text{mol}/\text{min}$) per mg of enzyme. The maximum velocity V_{max} , the Hill-coefficient n_{H} , and the substrate concentration at half maximal velocity $[S]_{0.5}$ or K_m were determined using a computer program which was applying the Quasi-Newton method (Davidon-Fletcher-Powell algorithm) to fit optimal curves to the data (20). To draw substrate saturation curves, the data were fitted either to the Michaelis-Menten equation ($v = V_{\text{max}} [S] / (K_m + [S])$) or to the Hill equation ($v = V_{\text{max}} [S]^n / ([S]^n + S'^{-1})$), where $S'^{(1/n)} = S_{0.5}$. Eadie-Hofstee plots ($v [S]^{-1}$ versus v) were drawn to decide to 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 cooperativity result in concave curvatures of the kinetic data (20). Hill plots ($\log(v(V_{\text{max}} - v)^{-1})$ versus $\log [S]$) were used to calculate Hill coefficients. The resulting V_{max} values were transformed to catalytic constants ($k_{\text{cat}} = V_{\text{max}} M_r E_0^{-1} (60 \text{ s})^{-1}$; substrate turnover per enzyme dimer). The numerical values turned out to be identical due to the use of 60,000 as molecular weight for chorismate mutase.

Binding Assays—The binding of tyrosine, phenylalanine, and tryptophan to chorismate mutase was assayed with an equilibrium dialysis apparatus with $200 \mu\text{l}$ of half cell volumes and Spectra/Por membrane discs with a molecular cut-off of 12,000–14,000 Da (Spectrum, Houston, Texas). Protein samples were dialyzed overnight in 20 mM Tris-HCl to remove glycerol and adjusted to a concentration of $25 \mu\text{M}$ in a final volume of $100 \mu\text{l}$. Ligand concentrations were $100 \mu\text{M}$ for tyrosine, 50

μM for tryptophan, and 100 – $400 \mu\text{M}$ for phenylalanine with a concentration of approximately 100,000 cpm/ $100 \mu\text{l}$ of L-[U- ^{14}C]tyrosine, L-[side chain- ^{14}C]tryptophan, and L-[U- ^{14}C]phenylalanine. The specific molar activity of ligand solution was calculated from its concentration corrected to the added ^{14}C -labeled compound and from its activity determined by counting unequilibrated samples. $100 \mu\text{l}$ of protein and ligand solution were allowed to equilibrate for 3 h at 30°C . Samples from each compartment were subjected to scintillation counting, and the final protein concentrations of the protein-containing half-cell were determined. No membrane leakage for the protein was observed. Additionally, control experiments without protein were performed, in which equilibria generally were achieved after 3 h. Free ligand concentrations were determined from the activity in the enzyme-free compartment, bound ligand concentrations from the activity difference between the two compartments. All data obtained were standardized to the G141S mutant enzyme which showed no detectable effector binding activity.

RESULTS

Allosteric Regulation of Yeast Chorismate Mutase

Fig. 2A shows typical substrate saturation plots of the wild-type chorismate mutase. The enzyme shows cooperativity with respect to the substrate. The sigmoidal substrate saturation

TABLE I
Kinetic parameters of wild-type and mutant chorismate mutases

Values for k_{cat} , K_m , $S_{0.5}$ 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.

Protein	Inhibited (100 μM tyrosine)			Unliganded			Activated					
							(10 μM tryptophan)			(100 μM phenylalanine)		
	k_{cat}	K_m , $S_{0.5}$	n_H	k_{cat}	K_m , $S_{0.5}$	n_H	k_{cat}	K_m , $S_{0.5}$	n_H	k_{cat}	K_m , $S_{0.5}$	n_H
Wild-type	s^{-1}	mM		s^{-1}	mM		s^{-1}	mM		s^{-1}	mM	
R75A	387	11.8 ^a	1.4	360	3.8	1.6	348	0.4	1.0 ^b	320	2.3	1.6
R76A	— ^c	—	—	—	—	—	344	4.2	1.7	—	—	—
G141S	387	6.2 ^a	1.6	486	9.3 ^a	1.3	512	10.0 ^a	1.3	653	14.8 ^a	1.2
S142A	228	2.0	1.1 ^b	207	1.7	1.3 ^b	240	2.5	1.1 ^b	234	2.1	1.2 ^b
T145V	291	5.6	1.6	323	4.0	1.5	313	2.9	1.8	344	4.6	1.4
	318	5.3	1.8	402	4.8	1.6	387	0.6	1.2	319	1.5	1.7

^a Values had uncertainty intervals of more than 10% according to the fitting procedure.

^b Hyperbolic saturation was indicated by linearity of the Eadie-Hofstee plots.

^c Dash indicates the flatness of the substrate saturation curves did not allow any reliable calculation of the data.

curve is markedly depressed upon addition of the inhibitor tyrosine and $S_{0.5}$ is shifted to a higher value (Table I). The enzyme is strongly activated by the addition of tryptophan with a K_m of 0.4 mM. The saturation by the substrate is noncooperative, and the enzyme follows hyperbolic substrate kinetics. The principal heterotropic effect of the regulatory amino acids to the catalytic center is a change in substrate affinity since similar values for k_{cat} were determined. We performed equilibrium dialysis experiments choosing effector concentrations of 100 and 50 μM for tyrosine and tryptophan, respectively, in the unequilibrated samples. This corresponds to a ratio of protein to ligand of 1:4 and 1:2, respectively. Assuming two binding sites per enzyme dimer for each effector, we calculated the fractional saturation of each mutant chorismate mutase under these conditions (Fig. 3). Values of 77 and 83% saturation for the binding of tyrosine and tryptophan, respectively, to yeast chorismate mutase were measured.

Phenylalanine was also tested for an effect on chorismate mutase activity and activated the enzyme *in vitro* as indicated by a reduction of the $S_{0.5}$ value. Binding of phenylalanine to the enzyme at concentrations as high as 400 μM could not be detected, presumably because it is too weak to be detected by the binding assay. Hence the tyrosine hydroxyl group, which lacks the phenylalanine molecule is important for strong binding as well as inhibition of yeast chorismate mutase.

X-ray data suggested that two tyrosine or two tryptophan molecules bind at the same sites at the interface of the dimeric yeast chorismate mutase. A schematical drawing of the computer graphics model derived from the crystals is shown in Fig. 1B (9, 11). According to this model tyrosine and tryptophan are similarly orientated at the interface of the enzyme. The carboxylate groups of both amino acid effectors interact with the backbone of the enzyme at Gly-141 and Ser-142. The amino groups bind to Ser-142. The side chain of Arg-75 interacts with the carboxylate group of tyrosine but not with tryptophan. The side chain of tyrosine interacts with both subunits by hydrogen bonding to Arg-76 and Thr-145.

We mutated these putative effector binding sites by site-directed mutagenesis of the *ARO7* gene. All mutant *aro7* alleles complemented the tyrosine and phenylalanine auxotrophy of an *aro7* minus strain. The mutant enzymes, which were named R75A, R76A, G141S, S142A, and T145V according to their replacements, were purified to homogeneity. We investigated the effects of the amino acid replacements on regulation of the enzymes and on binding of tyrosine and tryptophan, respectively. The curves for the substrate saturation experiments are shown in Fig. 2. The numerical values for k_{cat} , K_m or $S_{0.5}$ and n_H are given in Table I.

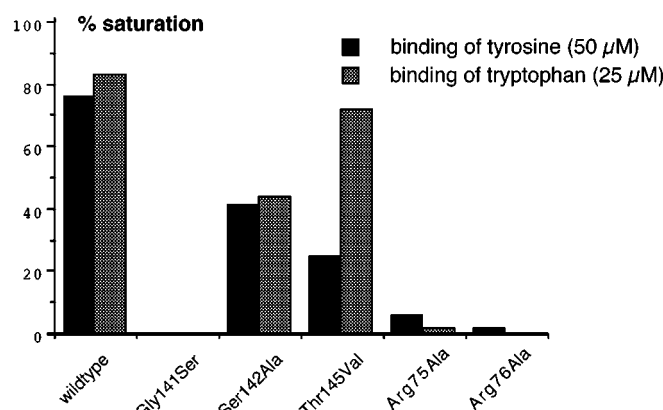


FIG. 3. Effector binding of wild-type and mutant chorismate mutases. Binding of tryptophan and tyrosine at fixed free ligand concentrations was determined by equilibrium dialysis as described under "Experimental Procedures." One hundred percent saturation corresponds to two ligand molecules bound per enzyme dimer, whereas zero percent saturation corresponds to the data obtained for the G141S mutant enzyme. Each experiment was performed at least three times. The standard deviation did not exceed 25% except for the R75A and the R76A mutant enzymes for which values were close to the detection limit.

Amino Acid Residues of Helix 8 Are Part of the Allosteric Site of Yeast Chorismate Mutase

Gly-141 Interacts with the Carboxylate Groups of Tyrosine and Tryptophan, Respectively—X-ray data suggested that the carboxylate groups of both effectors, tyrosine and tryptophan, are located in the co-crystals close to Gly-141 of helix 8 and interact with the main chain NH group of Gly-141. If Gly-141 is part of the allosteric site, computer graphics modeling suggested that a serine at position Gly-141 should prevent binding of both amino acid effectors. Correspondingly, we found that all characteristic homotropic and heterotropic effects exhibited by the wild-type chorismate mutase were eliminated when Gly-141 is substituted by serine (Fig. 2B). The enzyme is unresponsive to tyrosine, tryptophan, and phenylalanine and has characteristics of a Michaelis-Menten enzyme. The K_m value is reduced compared with the unliganded wild-type chorismate mutase. Amino acid binding experiments indicated that the enzyme is unable to bind any of the effectors (Fig. 3). These results suggest that the enzyme is not subject to heterotropic effects, because the amino acid effectors are unable to bind to the enzyme and demonstrate that the position of tyrosine and tryptophan in the co-crystals corresponds to the allosteric site of yeast chorismate mutase. Tyrosine and tryptophan share a

common site for their allosteric binding at the interface of the dimeric yeast chorismate mutase, where they act as inhibitor and activator, respectively.

Ser-142 Interacts with Both Amino Acid Effectors by Bonding with Its Side Chain—The x-ray structure suggests that the carboxylate groups of both effectors, tyrosine and tryptophan, are bonded to the backbone NH group of Ser-142. Furthermore, the side chain hydroxyl group of Ser-142 seems to be hydrogen bonded to the amino groups of both effectors. Replacement of Ser-142 with an alanine should remove the interactions of the serine side chain with the regulatory amino acids. The substrate saturation curves are given in Fig. 2C. The enzyme shows heterotropic response toward tyrosine and tryptophan, but the changes in substrate affinity upon the addition of tyrosine and tryptophan are not as pronounced as those for wild-type chorismate mutase (Table I). This is due to a reduced binding for both effectors, tyrosine and tryptophan (Fig. 3). Interaction with the serine hydroxyl group is therefore equally important for binding of the inhibitor as well as the activator to yeast chorismate mutase. It is not essential for the mechanistic process of action of both amino acid effectors on enzyme activity. The curve for the enzyme in the presence of phenylalanine shows no significant difference to unliganded chorismate mutase.

Thr-145 Is Involved in Tyrosine Inhibition—In the x-ray structures, Thr-145 of helix 8 is hydrogen bonded to the phenolic hydroxyl-group of the inhibitor tyrosine, yet it does not interact with the bound tryptophan. We replaced Thr-145 by a valine to prevent interaction with tyrosine. A valine at this position should make the allosteric binding pocket more hydrophobic, and might even allow enhanced binding of phenylalanine in a conformation that is used by tyrosine in the wild-type enzyme. The capability of this mutant enzyme to respond to tyrosine is reduced to a narrower range of regulation, which is mirrored by a moderate increase of the $S_{0.5}$ value (Fig. 2D; Table I). Thr-145 is obviously important for the strong binding of tyrosine to the wild-type enzyme, since T45V shows only approximately 35% of the binding capacity of the wild-type enzyme (Fig. 3). Activation of T145V chorismate mutase by phenylalanine is enhanced compared with the wild-type enzyme. Nevertheless binding of phenylalanine to the enzyme was too weak to be detected with the binding assay. Activation of the T145V mutant enzyme by tryptophan is the same as of the wild-type enzyme and binding of tryptophan is preserved (Fig. 3). These data indicate that Thr-145 is neither involved in tryptophan binding nor involved in the mechanism of activation. Yet, it is important for the binding of tyrosine to the allosteric site and the inhibition of yeast chorismate mutase.

Amino Acid Residues of Helices 4 and 5 within the Second Monomer form the Other Part of the Yeast Chorismate Mutase Allosteric Site

Arg-75 and Arg-76 are required for inhibition by tyrosine and activation by tryptophan. Arg-76 is part of helix 5 and Arg-75 is part of the interconnection between helices 4 and 5. These elements were assumed to constitute the second part of the effector binding site opposite to helix 8 of the other monomer. In the x-ray structure of the co-crystals the side chain of Arg-75 interacts with the carboxylate group of tyrosine and Arg-76 is hydrogen-bonded to the hydroxyl group of the inhibitor. Binding of tryptophan does not result in ionic contacts or hydrogen bonding according to the co-crystals. When Arg-75 and Arg-76 are replaced by an alanine, the two enzymes show a dramatically reduced response toward any effector amino acid (Fig. 2, E and F). The R75A mutant enzyme displays only residual activation by tryptophan, although the binding assay shows no

tryptophan binding (Fig. 3). The flatness of the curves for R75A chorismate mutase did not allow any reliable calculation of the catalytic parameters, only the kinetic parameters for the tryptophan-activated enzyme could be evaluated. The enzyme still showed homotropic cooperative interactions toward the substrate. Substrate affinity is largely reduced, and even for the tryptophan-activated enzyme it is still lower than for the wild-type enzyme without effectors.

The R76A mutant enzyme is totally unresponsive toward tyrosine, tryptophan, and phenylalanine. Sigmoidal curvature was determined for all curves. The $S_{0.5}$ values are increased compared with wild-type chorismate mutase (Table I). No significant binding of the effector amino acids could be detected (Fig. 3).

Residues Arg-75 and Arg-76 seem to be required for the binding of both, tyrosine and tryptophan and play an essential role in the allosteric process of regulation of yeast chorismate mutase.

DISCUSSION

Long range effects play an important role in the biological properties of allosterically regulated enzymes. An allosteric domain includes a regulatory site for binding of small effector molecules. The signal "binding of an effector" has to be transduced to the catalytic site and has to change the structure of this site in a manner which is reflected by the change in catalytic parameters. The simple organization of yeast chorismate mutase composed of only two subunits with exclusively helical structural elements and no β -sheet elements, offers an attractive model system for the study of allosteric regulation of proteins. The crystallographic structures of activated and inhibited yeast chorismate mutase offer an ideal guide for the design of site-directed mutagenesis studies on the allosteric control of the enzyme.

Crystal structure studies indicated that the two binding sites of the allosteric domain are located at the interface of the dimeric enzyme and that each binding site is defined by specific amino acid residues of both monomers. To corroborate these structural studies on a molecular level, we have replaced five amino acid residues located adjacent to two tyrosine or tryptophan molecules in the corresponding crystal structures.

All replacements had an effect on the regulation of yeast chorismate mutase. Replacements of Gly-141, Arg-75, and Arg-76 resulted in unregulated enzymes that are not able to bind any of the amino acid effectors. Replacement of Ser-142 resulted in an enzyme with reduced binding capacity for the effectors. These results lead to the conclusion that regulation of the activity of yeast chorismate mutase by tyrosine and tryptophan, the two allosteric regulators, is achieved by binding to the same sites.

In contrast, Thr-145 interacts specifically with the inhibitor tyrosine, and changes in this residue do not affect activation of yeast chorismate mutase by tryptophan. Thr-145 presumably interacts with the phenolic hydroxyl group of tyrosine and contributes to part of the binding of the inhibitor to the allosteric site. We therefore conclude that inhibition of chorismate mutase involves amino acid residues, which are not of functional importance for activation. In the homologous plastidic chorismate mutase from *A. thaliana*, a valine is located at a position corresponding to Thr-145 in yeast chorismate mutase (7). The plant enzyme is inhibited by tyrosine and by phenylalanine. A T145V replacement in yeast chorismate mutase leads to an enhanced response toward phenylalanine, but the effect is still activation.

The residues which are part of the regulatory binding site reside on three different helices, helices 4 and 5 of one monomer and helix 8 of the other monomer. The location of the

regulatory binding site at the dimer interface of yeast chorismate and the involvement of functional residues from both subunits indicates that dimer formation is required for effector binding and allosteric regulation. The involvement of the same subunit interfaces in the action of tyrosine and tryptophan is consistent with the proposed mechanism of heterotropic interaction, which implies that tyrosine and tryptophan act in reverse ways by either contracting or opening the helix bundle of the allosteric domain.

Tyrosine interacts with all residues described here by hydrogen bonding and inhibits the enzyme by pulling the two subunits together through hydrogen-bonds to each subunit. Therefore the enzyme subunits in the T state structure might be constrained by strong bonds that propagate and change the structure needed for substrate binding at the catalytic site. The tyrosine hydroxyl group is specifically required for inhibition of the chorismate mutase because phenylalanine lacking only this group is not an inhibitor of the enzyme. The hydroxyl group interacts with two amino acid residues located in both monomers. The hydrogen bond to Arg-76 is especially important since disruption of this interaction abolishes tyrosine inhibition. Hydrogen bonding of Thr-145 to the tyrosine hydroxyl group is important for the strong binding of tyrosine to the allosteric site. A T145V mutation which disrupts this interaction still results in the transmission of a reduced signal "binding of tyrosine" to the active site. Interaction of Arg-76 with the tyrosine hydroxyl group presumably triggers this remaining intramolecular signal transduction activity between the allosteric and catalytic site.

Activation of yeast chorismate mutase is achieved because tryptophan pushes the two subunits apart by the steric size of its side chain. Arg-75 and Arg-76 do not have polar contacts to the tryptophan molecule in the crystal structure. However, replacements of Arg-75 as well as Arg-76 to alanine resulted in enzymes that are not activated by tryptophan, because these enzymes are unable to bind tryptophan, and demonstrate the importance of helices 4 and 5 in the process of allosteric activation.

Comparison of the T and R state crystal structures shows that the side chain of Arg-75 changes conformation resulting in an interaction with the carboxylate group of tyrosine but not with tryptophan. Arg-76 interacts by hydrogen bonding with the phenolic group of tyrosine, but not with tryptophan. In the activated structure Arg-76 is very close to the bound tryptophan, suggesting strong van der Waals interactions between protein and effector, which might trigger activation by tryptophan.

If activation is caused by an increase in the distance between residues of both subunits, the distance in wild-type yeast chorismate mutase in the absence of effectors could represent a third structure between that found for the T and R state structures. Tyrosine and tryptophan might directly influence the proportion of the T and R states by the involvement of an "induced fit" promoted by effector binding. Another possibility is the existence a preexisting T-R equilibrium. Tyrosine and tryptophan would simply act by stabilizing either of the two states suitable for binding and therefore shifting the equilibrium. No mutant enzyme of the allosteric site could be found which was still binding tyrosine or tryptophan but no more responding toward the effectors. One might speculate that binding of the effectors to the enzyme might just be appropriate if the enzyme is able to exist in the activated and inhibited structures. Binding of tyrosine or tryptophan would trigger an allosteric transition, which then results in optimal binding of the effectors.

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