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Modulation of the Allosteric Equilibrium of Yeast Chorismate Mutase by Variation of a Single Amino Acid Residue

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Chorismate mutase (EC 5.4.99.5) from the yeast *Saccharomyces cerevisiae* is an allosteric enzyme which can be locked in its active R (relaxed) state by a single threonine-to-isoleucine exchange at position 226. Seven new replacements of residue 226 reveal that this position is able to direct the enzyme's allosteric equilibrium, without interfering with the catalytic constant or the affinity for the activator.

Chorismate is the last common precursor of the amino acids tyrosine, phenylalanine, and tryptophan and an important intermediate in the biosynthesis of other aromatic compounds in fungi, bacteria, and plants (9). The enzyme chorismate mutase (EC 5.4.99.5) catalyzes the conversion of chorismate to prephenate, which is the first committed step towards formation of phenylalanine and tyrosine. Two properties of this enzyme deserve particular attention. First, the rearrangement of chorismate is a rare example of an enzymatically catalyzed pericyclic process, proceeding via a transition state of chair-like geometry (18). Distinct types of chorismate mutase with no similarity in sequence and differing in regulation have been identified in various organisms (1, 5, 6, 17), and catalytic antibodies with chorismate mutase activity have been constructed (11, 12). It is suggested that proteins capable of arranging the substrate molecule in a transition-state-like conformation have appeared independently several times during evolution (10). The second aspect of interest refers to the enzyme's strategic position in the aromatic amino acid biosynthetic pathway (3). Chorismate mutase competes for a common substrate with its counterpart anthranilate synthase (EC 4.1.3.27)—catalyzing the first step of the tryptophan-specific branch—and is therefore an important point of metabolic regulation in most organisms. Our studies focus on the allosteric regulation of the chorismate mutase from *Saccharomyces cerevisiae*. This enzyme is a monofunctional homodimer of two 30-kDa subunits encoded by the gene *ARO7* (15). Its crystal structure has only recently been solved (19) and found to be profoundly different from the only other known structure, the monofunctional enzyme from *Bacillus subtilis* (4). Yeast chorismate mutase is feedback inhibited by tyrosine and activated by tryptophan and therefore appears to be similar to chorismate mutases from other fungi (2) and from plant chloroplasts (7). The mutation of residue 226 from threonine to isoleucine was shown to lock the enzyme in its active allosteric R state (15). In order to determine the side chain characteristics required at this position for proper regulation of the enzyme, we have changed

amino acid 226 of yeast chorismate mutase to glycine and alanine (small), aspartate and arginine (charged), serine (hydrophilic, similar to the wild-type threonine), cysteine (sulfur containing), proline (backbone constrained), and threonine and isoleucine (wild type and R-state locking).

Codon 226 of the *ARO7* gene was altered to code for one of the eight desired amino acids by using a PCR-based mutagenesis procedure (8). Each of the artificial *ARO7* alleles was integrated into the original locus on chromosome XVI. Remarkably, every one yielded viable transformants, indicating that no serious damage was caused to the enzyme by the amino acid substitutions. However, enzyme assays performed with crude extracts revealed abnormal regulatory responses to tyrosine and tryptophan (data not shown). For a detailed analysis, all engineered chorismate mutases were expressed in yeast cells from high-copy-number plasmids analogous to pME605 (16). Overexpression rates of 40- to 60-fold were obtained, as estimated from activity measurements with crude extracts. The wild type and all eight mutant enzymes were purified as described previously (15). Batches of approximately 40 g of cells yielded 5 mg of pure protein on average, with no significant differences between the various mutant forms in elution behavior on any chromatographic column. Purities of 90 to 98%, as estimated from Coomassie- and silver-stained sodium dodecyl sulfate-polyacrylamide gels, were achieved.

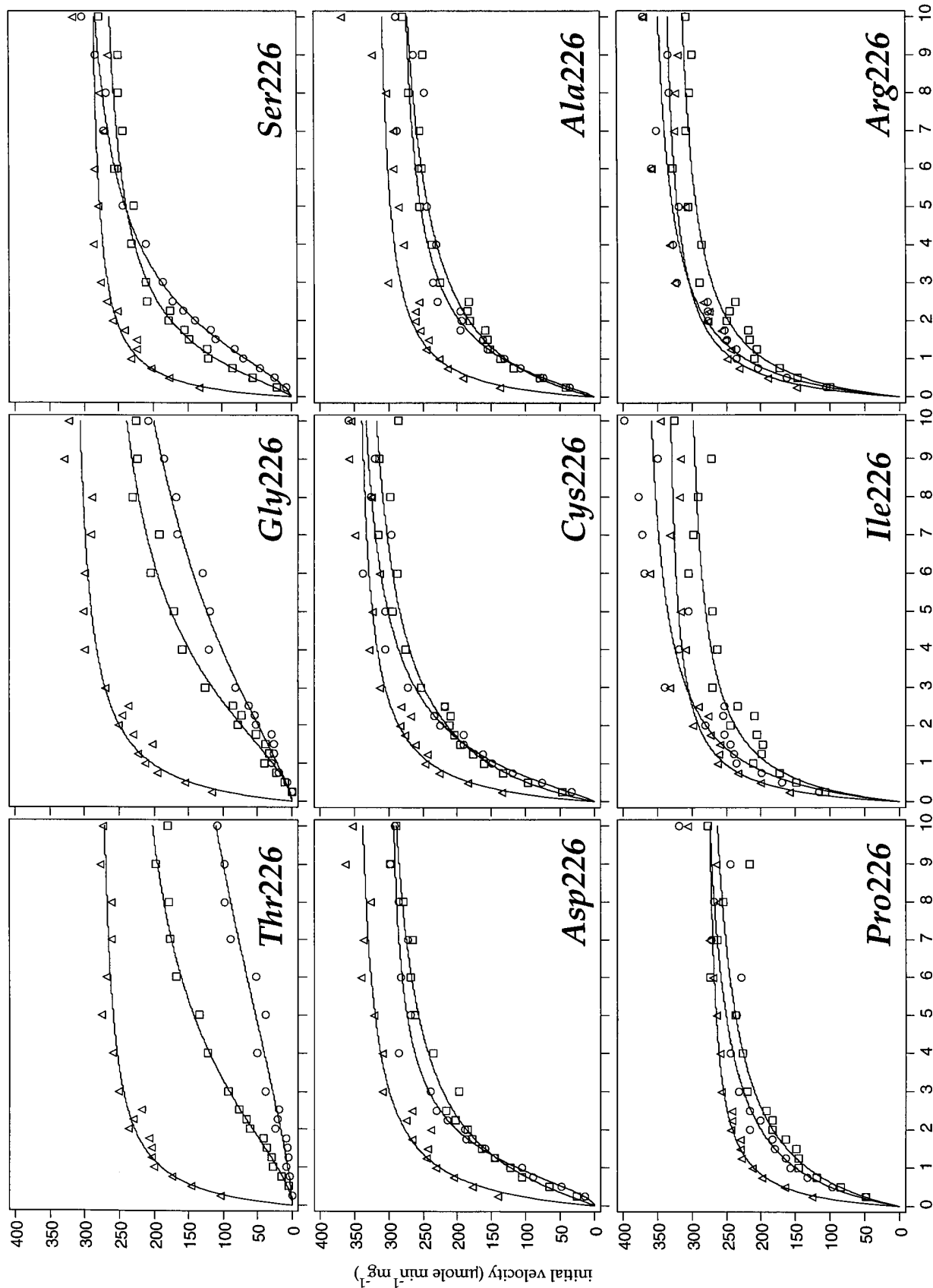
The substrate saturation curves for each mutant enzyme were determined in the absence of effectors, in the presence of the inhibitor tyrosine (100 μ M), and in the presence of the activator tryptophan (10 μ M) (Fig. 1). A modification of the previously described method (15) was applied. All measurements were performed at least four times to reduce errors in initial velocity data to less than 15%. Curvatures of Eadie-Hofstee plots and slopes of Hill plots were used to discriminate between the sigmoid and hyperbolic curves characteristic of cooperative or Michaelis-Menten-type substrate binding. According to these results, data were fitted by a program using the Marquardt algorithm (13) to the equations describing Michaelis-Menten-type hyperbolic saturation or Monod-Wyman-Changeux-type cooperative saturation (14). The resulting V_{\max} values were transformed to catalytic constants (k_{cat}) ($k_{\text{cat}} = V_{\max} M_r E^{-1}$ [60 s^{-1}]; substrate turnover per enzyme dimer). The numerical values for V_{\max} and k_{cat} turned out to be identical because of the use of 60,000 as the molecular weight for chorismate mutase. A summary of k_{cat} (V_{\max}), $S_{0.5}$ (for cooperative enzymes) or K_m , and Hill constants (n_H) is given in Table 1.

The Thr-226 panel in Fig. 1 shows the characteristic diagram

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chorismate concentration (mM)

FIG. 1. Substrate saturation plots of wild-type and mutant chorismate mutases. Purified wild-type (Thr-226) and mutant enzymes were assayed in the presence of 100 μ M tyrosine (circles) or 10 μ M tryptophan (triangles) or were assayed unliganded (squares). Each datum point was measured at least four times, and the collected data were fitted to functions describing either cooperative or Michaelis-Menten-type saturation.

TABLE 1. Kinetic parameters of wild-type and mutant chorismate mutases^a

Amino acid at residue 226	Value for protein in state:								
	Inhibited (100 μ M tyrosine)			Unliganded			Activated (10 μ M tryptophan)		
	k_{cat} (s^{-1})	K_m , $S_{0.5}$ (mM)	n_H	k_{cat} (s^{-1})	K_m , $S_{0.5}$ (mM)	n_H	k_{cat} (s^{-1})	$S_{0.5}$ (mM)	n_H
Thr	312 ^b	14.8 ^b	1.52	251 ^b	4.2 ^b	1.60	284	0.5	1.07
Gly	340 ^b	7.7 ^b	1.42	287 ^b	3.8	1.58	325	0.6	0.92
Ser	311	2.3	1.63	280	1.4	1.32	296	0.3	1.06
Asp	301	1.3	1.66	321	1.5	1.22	357	0.6	0.96
Cys	360	1.5	1.31	361	1.4	1.02	355	0.5	0.87
Ala	290	1.1	1.15	310	1.4	1.05	321	0.4	0.78
Pro	301	1.0	1.09	295	1.2	1.04	283	0.4	1.01
Ile	386	0.8	1.04	318	0.7	0.91	342	0.4	0.88
Arg	372	0.7	1.03	335	0.7	0.94	350	0.5	0.78

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

^b Value has an uncertainty interval of >15% from the fitting procedure (see text).

for wild-type chorismate mutase. The sigmoid saturation curve is markedly depressed upon addition of tyrosine but is dramatically elevated and becomes hyperbolic in the presence of tryptophan. From all three curves, k_{cat} values in the range of 300 s^{-1} were determined. However, the extrapolation of the flat unliganded and inhibited curves resulted in considerable uncertainty intervals. The difference between this value and the previously reported data (15) is due to an additional calibration step in the enzyme assay. The K_m values reflect the strong regulation of the wild-type enzyme: in the absence of effectors it reaches half-maximal activity at approximately 4 mM substrate, whereas in the presence of the activator only 0.5 mM is required. Inhibition by tyrosine shifts the K_m to an estimated 10 to 20 mM (Table 1; Fig. 1).

The mutant enzymes displayed the following properties. (i) All eight mutants were indistinguishable from the wild type when activated by tryptophan, suggesting that they all are able to form a regular R state. (ii) In the unliganded and inhibited states, the k_{cat} values of all enzymes converged in the same range around 300 s^{-1} . Thus, regardless of the residue at position 226, chorismate mutase displays the behavior of a K system according to the Monod-Wyman-Changeux model (14). (iii) In the absence of effectors the K_m of each mutant enzyme was lower than that of the wild type, indicating a shift of the allosteric equilibrium towards the R state. This shift was weakest in Gly-226 and Ser-226 mutants; stronger in Asp-226, Cys-226, Ala-226, and Pro-226 mutants; and complete in Ile-226 and Arg-226 mutants. (iv) Every decrease in K_m in enzymes in the unliganded state was accompanied by an even more prominent one in the presence of tyrosine, leading to a rapid disappearance of feedback inhibition: a significant regulatory effect of the inhibitor was observed only in Gly-226 and Ser-226 mutants. (v) Every decrease in K_m was found to go along with a loss of cooperativity. Formally, hyperbolicity (Hill constants of 1.1 and less) was reached at an $S_{0.5}$ of around 1.4 mM.

In accordance with the Monod-Wyman-Changeux model, it has been shown that both the wild type and the R-state-locked Ile-226 mutant are equally able to bind the activator tryptophan, whereas the ability to bind the inhibitor is lost in the mutant (15). We tested all our enzymes for equilibrium binding of L-[¹⁴C(U)]tyrosine or L-[side chain-3-¹⁴C]tryptophan, respectively, using equilibrium dialysis as described previously

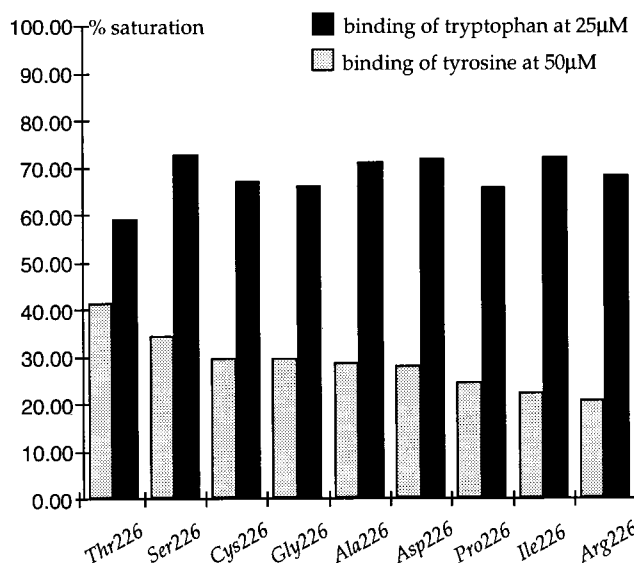


FIG. 2. Relative effector binding by wild-type (Thr-226) and mutant chorismate mutases. Binding of tryptophan and tyrosine at fixed free ligand concentrations was determined by equilibrium dialysis as described in Materials and Methods. One hundred percent saturation corresponds to two ligand molecules bound per enzyme dimer.

(15). Experiments were performed at free effector concentrations that lead to approximately half-maximal saturation of wild-type chorismate mutase, i.e., 50 μ M for tyrosine and 25 μ M for tryptophan. Assuming two binding sites for each effector per dimeric enzyme, we calculated the fractional saturation of each mutant chorismate mutase, as summarized in Fig. 2. All enzymes showed tryptophan saturations of 59 to 73%, indicating that activator binding is not negatively affected by the exchange of amino acid 226. The binding of tyrosine, however, was clearly influenced by the mutagenesis: all mutants showed intermediary binding values between the wild-type level (42%) and those of the constitutively activated Ile-226 (22%) and Arg-226 (21%) mutants.

The mutations at residue 226 have the remarkably distinct effect of interfering only with the allosteric transition of the enzyme, likely without directly affecting any of the allosteric binding sites. There is no obvious correlation of the strength of the effect with any particular property of the introduced amino acid. Since only shifts of the allosteric equilibrium towards the R state were observed, it appears that the wild-type residue, threonine, is required to stabilize the T (tense) state, thereby allowing the enzyme to display its strong response to the effectors. With the three-dimensional structure available, these observations will be of great value for the detailed description of the allosteric conformational change of yeast chorismate mutase.

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