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COMMUNICATION

# Computationally designed variants of *Escherichia coli* chorismate mutase show altered catalytic activity

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**Computational protein design methods were used to predict five variants of monofunctional *Escherichia coli* chorismate mutase expected to maintain catalytic activity. The variants were tested experimentally and three active site mutants exhibited catalytic activity similar to or greater than the wild-type enzyme. One mutant, Ala32Ser, showed increased catalytic efficiency.**

*Keywords*: enzyme design/chorismate mutase/protein design

## Introduction

The Claisen rearrangement of chorismate to prephenate (Figure 1) is a rare enzyme-catalyzed pericyclic reaction that proceeds through the same mechanism uncatalyzed in solution. Chorismate mutases from various organisms provide rate enhancements of around $10^6$ despite strong dissimilarities in three-dimensional fold (Chook *et al*., 1993; Xue *et al*., 1994; Lee *et al*., 1995). The metabolic importance of chorismate as the key branch point in the shikimate pathway has prompted extensive experimental investigation of the chorismate-prephenate rearrangement since the 1960s (Gibson and Gibson, 1964) and has driven complementation experiments to probe the structural determinants of enzyme catalysis (Woycechowsky and Hilvert, 2004). The concerted, unimolecular nature of the rearrangement and the lack of covalent protein contacts have encouraged numerous theoretical studies of the catalyzed and uncatalyzed reactions. The question of how chorismate mutases achieve rate enhancement has been actively discussed in recent years (Crespo *et al*., 2003; Guimarães *et al*., 2003; Hur and Bruice, 2003; Štrajbl *et al*., 2003; Ranaghan *et al*., 2004).

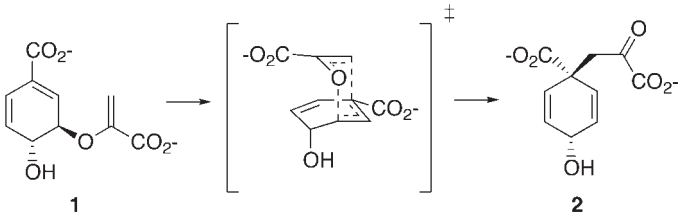


**Fig. 1.** Claisen rearrangement of chorismate (**1**) to prephenate (**2**).

We used computational protein design techniques to identify mutations within the active site of the chorismate mutase domain (EcCM) of *Escherichia coli* chorismate mutase-prephenate dehydratase ('P-protein') (Stewart *et al*., 1990) consistent with catalytic activity. The objective of the study was to evaluate the viability of a rotamer-based approach with an empirical mechanics force field in modeling the active site. We report three active site mutations that permit catalytic activity at or above the level of the wild-type enzyme. One of these appears to have enhanced catalytic efficiency relative to wild-type.

## Materials and methods

An *ab initio* calculated transition-state structure (Wiest and Houk, 1994) was modeled at the position of a transition state analog in the EcCM crystal structure, pdb code 1ecm (Lee *et al*., 1995). Translation (±0.2 Å each axis) and rotation (±5° each axis) of the transition-state structure was allowed and all amino acids except glycine, proline, cysteine and methionine were permitted in the following positions: 28, 32, 35, 39, 46, 47, 48, 51, 52, 55, 81, 84, 85, 88 (chain A); 7, 11, 14, 18 (chain B). Residues from both chains constitute each of the two active sites in this symmetric homodimer. A backbone-dependent side chain rotamer library (Dunbrack and Cohen, 1997) was used with expansion by one standard deviation about $\chi 1$ and $\chi 2$ for all amino acids except arginine and lysine. The energy function used in the ORBIT protein design software (Dahiyat and Mayo, 1997a) was based on the DREIDING force field (Mayo *et al*., 1990) and includes a scaled van der Waals term (Dahiyat and Mayo, 1997b), hydrogen bonding and electrostatic terms (Dahiyat *et al*., 1997) and a surface-area based solvation potential (Street and Mayo, 1998). Transition-state partial atomic charges were obtained as reported previously (Wiest and Houk, 1994). An additional energy penalty was applied to effectively eliminate from consideration all sequences that could not maintain key contacts between the transition-state structure and Arg11 and Arg28. These contacts were previously demonstrated to be necessary for catalysis (Liu *et al*., 1996). The HERO rotamer optimization method was used to obtain the minimum energy amino acid sequence and conformations (Gordon *et al*., 2003). Six mutations were predicted in the optimized structure: Leu7Ile, Ala32Ser, Val35Ile, Asp48Ile, Ile81Leu and Val85Ile. Visual inspection and subsequent calculations indicated that four of the mutations were independent, so they were treated separately.

The gene encoding EcCM residues 1–109 was amplified from genomic DNA (ATCC 700926D) and inserted into the pTYB11 vector from the IMPACT-CN intein fusion system (New England Biolabs). Inverse PCR mutagenesis (Hemsley

*et al*., 1989) was used to construct five variants: Leu7Ile, Ala32Ser, Val35Ile, Asp48Ile and Ile81Leu/Val85Ile. Variant and wild-type proteins were expressed in *E.coli* BL21(DE3) and purified by chitin affinity chromatography. Further purification by gel filtration followed self-cleavage of the affinity tag. Protein characterization followed procedures recently reported for the same construct (Zhang *et al*., 2003). Protein concentration was determined by the Bradford assay using BSA as a standard. Chorismate mutase activity was determined by following the disappearance of chorismate with UV absorbance at 275 nm. Activity assays were conducted at 37°C with 20 nM protein in 50 mM Tris pH 7.8, 2.5 mM EDTA, 20 mM β-mercaptoethanol and 0.01% BSA. Initial velocities were buffer corrected and were determined with <6% depletion of initial substrate concentration. All proteins were initially tested using a substrate concentration range of about 50–500 μM. The wild-type and the Ala32Ser mutant were further assayed with substrate ranges of ~20–2000 μM and a minimum of five trials including two separate protein preparations each. Kinetic parameters were determined by non-linear fitting to the Michaelis–Menten equation.

## Results and discussion

As can be seen in Table I, three of the five variants showed catalytic efficiency similar to or greater than that of the wild-type enzyme. The Ala32Ser mutation results in a slightly more efficient catalyst than wild type owing to both a decrease in $K_M$ and an increase in $k_{cat}$. The fact that substrate binding is enhanced in addition to catalysis is consistent with the observation that factors stabilizing the transition state also contribute to ground-state stabilization in the catalyzed rearrangement (Štrajbl *et al*., 2003). The rate enhancement corresponds to a change in activation energy of <1 kcal/mol, making a detailed structural explanation unwarranted. However, it should be noted that in the predicted structure of this mutant, Ser32 is capable of hydrogen bonding with Gln88 (Figure 2), a residue that makes an essential contact with the ether oxygen of the breaking bond (Liu *et al*., 1996).

Val35Ile shows increased $k_{cat}$ but also increased $K_M$, resulting in $k_{cat}/K_M$ similar to that for wild type. The Leu7Ile mutation also does not have a significant effect on catalytic efficiency. Both mutations create slightly different hydrophobic packing environments near the essential residue Arg11 in the predicted structures. The double mutant Ile81Leu/Val85Ile was predicted to alter packing against the hydrophobic ring face of the reacting molecule. This rearrangement of hydrophobic amino acids does not result in a substantial change in $k_{cat}$. However, the mutations result in increased $K_M$ and reduced catalytic efficiency. The relative insensitivity of the enzyme to changes in some amino acids is not surprising given the recent finding that the reaction is efficiently catalyzed by a protein exhibiting all the characteristics of a molten globule (Vamvaca *et al*., 2004).

The Asp48Ile mutation abolished measurable catalysis. This position makes backbone contacts to the hydroxyl group of the transition-state analog in the EcCM crystal structure. Hydroxyl contacts to a negatively charged residue were suggested to create a favorable electrostatic gradient in the *Bacillus subtilis* enzyme (Kast *et al*., 1996). However, in the *E.coli* structure the Asp48 side chain points away from the active site and is distant from the transition-state analog. Molecular dynamics simulations provide some insight into a possible function of Asp48 that this mutant lacks. Averaged structures from equilibrated systems (see Supplementary data available at *PEDS* Online) show Asp48 hydrogen bonding to Arg11 in both the unliganded and inhibitor-bound enzyme. In simulations of unliganded Asp48Ile mutants, however, Arg11 is dramatically displaced into solution, suggesting that one role of Asp48 may be to stabilize this key active site side chain in a conformation compatible with substrate binding and catalysis.

While the choice of mutations in this experiment was based solely on computational modeling and no sequence alignment information was used in the process, a BLAST search (Altschul *et al*., 1997) using the EcCM sequence as the query showed that sequence variations corresponding to the Ala32Ser, Val35Ile, Val85Ile and Ile81Leu mutations were observed in chorismate mutases from related organisms.

Our design procedure stabilizes a static active site configuration with a bound transition-state structure. Although the substrate of the reaction is not explicitly considered, we expect that modeling interactions using the structure and charges of the transition state should promote some degree of differential stabilization of the transition state relative to substrate. The present study demonstrates that this approach can be used effectively to represent the active site of a natural enzyme. In this case, the predicted mutations were in residues not directly contacting the reacting molecule. The favorable result from

**Table I.** Kinetic parameters of wild-type and mutant EcCM[a]

| | $k_{cat}$ ($min^{-1}$) | $K_M$ (μM) | $k_{cat}/K_M$ ($min^{-1}$ $\mu M^{-1}$) | %WT $k_{cat}/K_M$ |
|---|---|---|---|---|
| WT | 2332 ± 306 | 304 ± 52 | 7.8 ± 1.0 | 100 |
| Ala32Ser | 2708 ± 364 | 220 ± 29 | 12.4 ± 1.0 | 159 |
| Val35Ile[b] | 3046 ± 172 | 365 ± 59 | 8.5 ± 1.1 | 109 |
| Leu7Ile[b] | 2193 ± 291 | 249 ± 54 | 9.1 ± 2.4 | 117 |
| Ile81Leu/Val85Ile[b] | 2004 ± 241 | 669 ± 165 | 3.1 ± 0.6 | 40 |
| Asp48Ile[c] | – | – | – | – |

[a]Reported errors are standard deviations from at least three trials.
[b]Three mutants were assayed with a limited substrate concentration range; see text.
[c]Reaction rates with the Asp48Ile mutant were within error the same as for the uncatalyzed solution reaction.

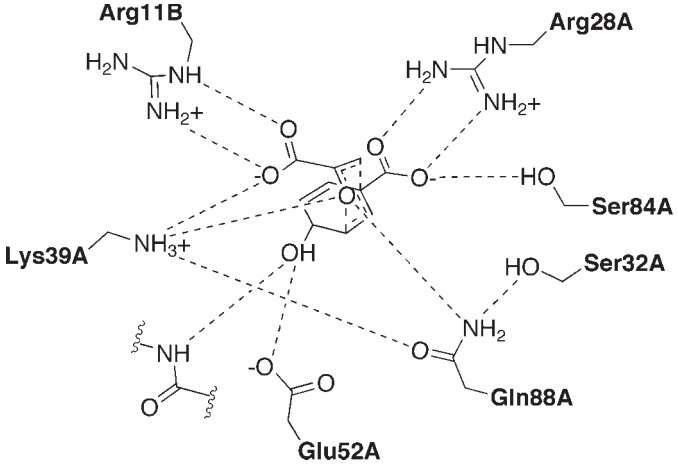


**Fig. 2.** Predicted hydrogen bonding in the Ala32Ser chorismate mutase variant. Interactions in the wild-type crystal structure are the same except for position 32A.

the Ala32Ser mutation suggests that such secondary contacts are important in the enzyme design process. The complete loss of catalytic activity from the Asp48Ile mutation implies that improved treatment of electrostatics and consideration of the unbound enzyme could offer some benefit in future design efforts.

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