Analysis of Active Site Residues in *Escherichia coli* Chorismate Mutase by Site-Directed Mutagenesis

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The X-ray crystal structures of three proteins that catalyze the Claisen rearrangement of chorismate (**1**) to prephenate (**2**) have been solved as complexes with the *endo*-oxabicyclic transition state analogue **3** (Scheme 1). Analysis of the structures of the chorismate mutase from *Bacillus subtilis*,[1,2] the N-terminal 109 amino acid catalytic fragment of the bifunctional *Escherichia coli* chorismate mutase-prephenate dehydratase ("P protein"),[3,4] and the catalytic antibody 1F7[5] indicate that the active sites of both enzyme and antibody mutases are complementary to the conformationally restricted transition state analogue **3**. In the case of the enzymes, the structures also reveal a number of groups that could function in the formation or stabilization of a polar transition state generated by the heterolysis of the O7−C5 bond.[1−5] These structures, taken together with earlier studies[6−13] and recent mutational analyses of active site residues,[14] provide a unique opportunity to identify common mechanistic features associated with this novel biological transformation. We describe here the generation and characterization of 13 active site mutants of the *E. coli* monofunctional mutase (EcCM, Figure 1) and compare the properties of these mutants with the analogous mutants of the *B. subtilis* enzyme (BsCM).

In order to facilitate isolation of the wild-type (wt) and mutant enzymes, six histidines were added directly to the carboxy terminus of the protein by PCR amplification[15] of the EcCM gene encoded on the plasmid pJS42.[16] The resulting histidine-tagged enzyme was cloned into pAED4,[17] an IPTG-inducible T7 expression vector, to afford plasmid pDRL1. The specific activity of the histidine-tagged EcCM was comparable to that of the nontagged mutase as determined by *in vitro* expression and quantitation using [^{35}S]methionine. A series of active site mutants (Table 1) was generated using the method of Kunkel,[18] and the resulting proteins were expressed in *E. coli* strain BL21[19] and purified on nickel-chelating resin[20] (Novagen). The yields of purified mutases were highly dependent on the nature of the mutation, ranging from 25 μg/L of culture for Arg28Ala to 6 mg/L for Lys39Ala. Circular dichroism (CD) was used to assess the secondary structure of mutant proteins (except for Glu52Gln, Arg28Ala, Arg28Lys, which were not sufficiently pure). The CD spectra[21] of all mutants were superimposable with the wt spectrum, exhibiting a strong minimum at 220 nm with much smaller minima at 208 and 212 nm. A small shoulder at ∼210 nm was observed in the CD spectra of the Lys 39 mutants, raising the possibility that, while still largely helical, these three mutants may deviate from the precise structure of wt EcCM. Enzyme activity assays were conducted spectrophotometrically[22] by monitoring the conversion of chorismate to prephenate at pH 7.5 (Table 1).[23]

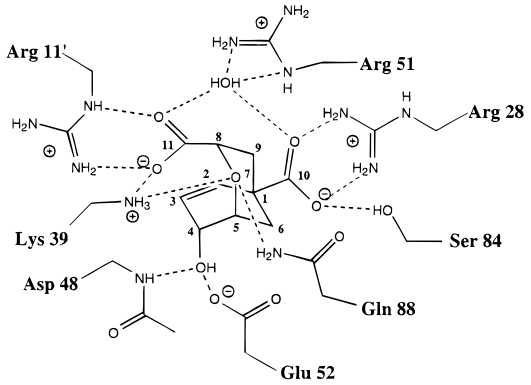
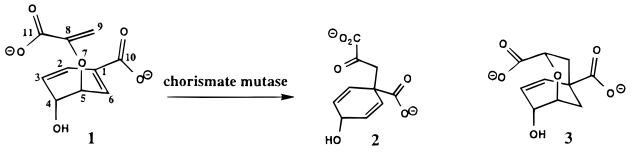


Figure 1. Active site of EcCM bound to transition state analogue **3**.

Scheme 1



The degree to which hydrogen bonding and electrostatic interactions between active site residues and the C10 and C11 carboxylates of chorismate contribute to catalysis is evidenced by an analysis of the Arg 11 and Arg 28 mutants. The role of Arg 11 in hydrogen bonding to the C11 carboxylate is especially important as indicated by the 10^3-fold and 10^4-fold decrease in k_{cat}/K_m for the Arg11Lys and Arg11Ala mutants, respectively, compared with wild-type mutase. These effects are similar to those seen for mutants of the corresponding active site residue, Arg 7, in the *B. subtilis* enzyme[14] and underscore the importance of the bidentate C11 carboxylate−arginine side chain interaction in orienting the substrate in the energetically less favored but reactive pseudodiaxial conformation. In addition to an orientational role, Arg 11 may also exert an electronic effect on the reaction. Electron-withdrawing groups at C2 of allyl vinyl ether, corresponding to C8 of chorismate, have been shown experimentally[24] and in theoretical models[25] to accelerate the nonen-

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(15) PCR was executed with the primers below (start and stop codons are boldfaced, histidine tag is underlined) using a thermal cycle of 94 °C, 30 s; 55 °C, 30 s; 74 °C, 1 min; 25 cycles; 1.5 mM $MgCl_2$): 5′ CTTGACCTGCAT**ATG**ACATCGGAAAACCCGTTA 3′ (introducing a *Nde*I site upstream of the start codon); 5′ CAGGTCAAGAAGCTT**TTA**GTGGTGGTGGTGGTGGTGGAGGATCGATCCGAGAAA 3′ (introducing a *Hin*dIII site downstream of the stop codon).
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(21) CD spectra were taken at 25 °C in 10 mM Tris pH 7.8, 10% glycerol, 100 mM NaCl at a protein concentration of 30 μg/mL.
(22) The assays were conducted as described in the preceding paper.
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Table 1. Kinetic Constants of EcCM Mutants[a]

mutant	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_i (μM)
wt	296 ± 19	72[b]	240000	3.66 ± 0.17
R11A	>2000		26 ± 4.1	
R11K	>2000		230 ± 10	
R28A	>2000		170 ± 12	
R28K	>2000		230 ± 6.4	
K39A	>2000		4.3 ± 0.57	
K39Q	>2000		7.3 ± 0.44	
K39R	>2000		1.9 ± 0.26	
E52A	4580 ± 450	0.49 ± 0.032	110	218 ± 23
E52D	1440 ± 160	3.1 ± 0.20	2200	78.4[c] ± 4.7
E52Q	1080 ± 87	24 ± 1.0	23000	26.8 ± 1.7
Q88A	>2000		12 ± 0.74	
Q88E	>2000		361 ± 7.4	
Q88E (pH 4.9)	141 ± 31	43 ± 4.5	300000	7.3 ± 0.58
wt (pH 4.9)	147 ± 26	31 ± 2.7	210000	
Q88K	>2000		12 ± 2.0	

[a] The concentration of chorismate used in the assays was limited by the absorbance range of the spectrophotometer; as a result K_m and k_{cat} for mutants with a K_m higher than roughly 2−4 mM could not be determined. Concentrations of mutant proteins were determined by comparison with known quantities of wild-type EcCM (determined by its activity assuming a k_{cat} of 72 s^{-1})[4] on an SDS−PAGE gel stained with Commassie Blue. K_i values for **3** were determined by monitoring the reaction at a variety of inhibitor concentrations.[15] [b] Reference 4. [c] Velocity vs [**3**] curve for E52D did not fit standard competitive inhibition equations at concentrations of **3** ≥100 μM. At low concentrations of **3**, K_i was determined to be 78 μM.

zymatic Claisen rearrangement of allyl vinyl ether; strong hydrogen bonds made to C11 in chorismate may promote the rearrangement in a similar manner. The results of the Arg28Ala and Arg28Lys mutants, both exhibiting k_{cat}/K_m values approximately 10^3 times lower than wild-type, also imply a significant role played by hydrogen bonds made to the C10 carboxylate. This interaction in the *E. coli* enzyme may be more important than that in the *B. subtilis* enzyme, in which the analogous Arg116Lys mutant exhibits only a 74-fold decrease in k_{cat}/K_m and relatively little change in k_{cat}. These results are consistent with differences in the three-dimensional structures of the two enzymes;[1,3] in the case of BsCM the C10 carboxylate of **3** is largely solvent exposed, whereas the transition state analogue is buried within the EcCM active site.

The ϵ-amino group of Lys 39 is within hydrogen-bonding distance of both the ether oxygen (O7) of **3** and an oxygen of the C11 carboxylate group. The extremely low activities of the Lys39Ala, Lys39Gln, and Lys39Arg mutants ((3.3×10^4)-fold to (1.3×10^5)-fold drop in k_{cat}/K_m) clearly identify Lys 39 as a critical active site residue. Although the CD spectra of the Lys 39 mutants differ slightly from that of the wt protein, it is likely that this residue plays a major catalytic role in the enzyme. Mutations of the corresponding residue Arg 90 in BsCM, which also bridges O7 and the C11 carboxylate, result in similar decreases in k_{cat}/K_m.[14] The importance of interactions between EcCM and O7 is further reinforced by the analysis of mutations to Gln 88, which is also proposed to hydrogen bond to O7. The Gln88Ala and Gln88Lys mutants manifest a (2×10^4)-fold decrease in the value of k_{cat}/K_m. Given the large effects of mutations in Lys 39, Arg 90, and Gln 88, it is tempting to speculate that these residues, in addition to providing an orientational role in catalysis, stabilize developing negative charge on the enolpyruvyl moiety in a polar transition state. Interestingly, the activity of the Gln88Glu mutant, 700-fold lower than wt at pH 7.5, is rescued almost 10^3-fold by lowering the pH to 4.9. This dramatic increase in activity presumably results from the protonation of Glu 88, reestablishing the hydrogen bond to O7, and provides convincing evidence that hydrogen bonding to the enolpyruvyl oxygen of chorismate is critical to both the k_{cat} and the K_m of mutase.

The side chain of Glu 52 is in close proximity to the C4 hydroxyl of **3** and could potentially act as a general acid or general base catalyst[9] or stabilize charge developing near C4 in a polar transition state. The values of k_{cat} for the Glu52Gln, Glu52Asp, and Glu52Ala mutants are, respectively, 3.0-, 23-, and 150-fold lower than that of wild-type mutase. The high activity of the Glu52Gln mutant argues against a role in which the carboxylate group of Glu 52 stabilizes developing positive charge on C4 as well as against mechanisms invoking Glu 52 as a general acid or base. In contrast to the other active site residues, the activities of mutants of Glu 52 do not parallel those of the corresponding residue (Glu 78) in the *B. subtilis* enzyme.[14] In the latter case the order of activity is Glu 78 > Asp 78 ≫ Gln 78 ≥ Ala 78, and thus a carboxylate in the vicinity of the C4 hydroxyl is likely to be important for catalysis. One possible explanation for these differences may be that the carboxylate group of Glu 78 in BsCM is essential for positioning of both the C4 hydroxyl and the side chain of the critical residue Arg 90.[14] In the case of the *E. coli* enzyme, the Glu 52 side chain makes no obvious interactions with other critical active site residues. In addition, modeling suggests that the loss of the Glu 52−C4 hydroxyl hydrogen bond may lead to an inverted and nonproductive binding mode[26] of chorismate, resulting in an apparent decrease in k_{cat}. Thus the role of Glu 52 may be largely orientational in EcCM.

The mutagenesis data of the chorismate mutases from *E. coli* and *B. subtilis* argue against general acid or base catalysis in the former enzyme and against nucleophilic catalysis in the latter. These results instead point to the common importance of hydrogen bonds between mutases and the enolpyruvyl oxygen and C11 carboxylate of chorismate as most critical to the catalytic efficiencies of these enzymes. Hydrogen bonds made to the C4 hydroxyl likely play an orientational role in addition to the possibility of playing a more complex role in BsCM. Other residues appear to contribute equally to the binding of the substrate and transition state. The ability to efficiently express both enzymes *in vitro* will allow us to further probe the mechanisms of these novel enzymes using unnatural amino acid mutagenesis.[27−29] For example, the electronic versus orientational effects of the interactions made to O7 are being examined by replacing Lys 39 and Gln 88 in EcCM with amino acids which have varying abilities to serve as hydrogen bond donors such as 5-fluorolysine, 5,5-difluorolysine, 4-fluoroglutamine, and thioglutamine.

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(26) Inverting the bound molecule of **3** in the active site such that the C10 and C11 carboxylates exchange places results in the loss of the two hydrogen bonds to O7 (and presumably the loss of turnover), but also in the possible formation of two new hydrogen bonds: the C4 hydroxyl may hydrogen bond with the side chain N of Gln 88 and with the hydroxyl of Ser 84. The C11 and C10 carboxylates exchange hydrogen-bonding partners in this orientation but maintain the number and geometry of these hydrogen bonds. Perhaps in the Glu52Asp and Glu52Ala mutants, the loss of the Glu 52−C4 hydroxyl hydrogen bond makes the inverted binding mode more attractive relative to the normal binding mode, leading to increased nonproductive binding.

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