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Selective Stabilization of the Chorismate Mutase Transition State by a Positively Charged Hydrogen Bond Donor

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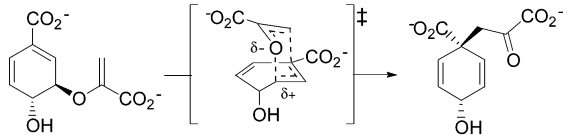
Chorismate mutase (CM) catalyzes the Claisen rearrangement of (−)-chorismate (**1**) to prephenate (**3**), the first committed step in the biosynthesis of phenylalanine and tyrosine.[1] Despite extensive experimental and theoretical work, its mechanism of action remains controversial.



1 **2** **3**

The enzymatic reaction, like its uncatalyzed counterpart, proceeds via a chairlike[2] pericyclic transition state (**2**) in which C−O cleavage precedes C−C bond formation.[3] Electrostatic stabilization of this highly polarized species by a positively charged residue, either an arginine or a lysine, positioned next to the ether oxygen of the breaking C−O bond has been suggested by crystallographic and mutagenesis studies of the structurally diverse *Bacillus subtilis* (BsCM),[4−6] *Escherichia coli* (EcCM),[7,8] and yeast (ScCM)[9] mutases. This hypothesis has found further support in QM/MM calculations.[10] However, more recent computational studies indicate that the cationic group stabilizes chorismate in the ground and transition state to similar extents.[11] It has even been argued that preferential transition state binding is unimportant for chorismate mutase activity.[12] Instead, the efficiency of the enzyme has been attributed mainly to conformational restriction of the substrate in such a way as to confine the reactive centers to contact distances.[12,13]

The critical cationic residue in BsCM is Arg90 (Figure 1). Even conservative substitution of this amino acid with a positively charged lysine leads to substantial reductions in catalytic efficacy.[5,6] However, the mutations that have been investigated to date have not been isosteric with arginine and are typically accompanied by large increases in the K_m value for chorismate, making it difficult to distinguish unambiguously between ground state and transition state effects. To overcome this problem, we have prepared a BsCM variant containing citrulline, an isosteric but neutral arginine analogue, at position 90. This minimal substitution of a charged with a neutral hydrogen bond donor allows the importance of the positive charge in the differential stabilization of the ground and transition states to be assessed directly.

BsCM and the Arg90Cit variant were prepared by a native chemical ligation strategy,[14] exploiting Cys88 to mediate segment condensation. The N-terminal peptide fragment corresponding to residues 1−87 was biosynthesized in *E. coli* strain KA13 as a fusion with the *Mxe* GyrA intein and a chitin-binding domain.[15] KA13 has deletions of both endogenous *E. coli* chorismate mutase genes,[16] so that contamination from chromosomally encoded chorismate mutases can be excluded. The fusion protein was purified on a chitin column and the intein splicing intermediate was captured with

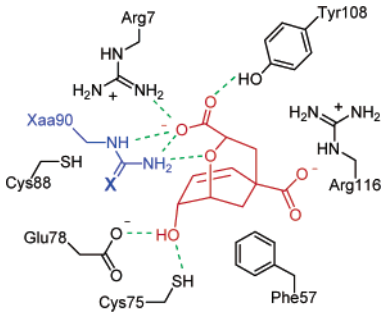


Figure 1. Schematic view of the BsCM active site with bound transition state analogue (compound **4**, red).[4] In the wild-type enzyme the blue residue at position 90 is arginine ($X = NH_2^+$) and in the Arg90Cit variant it is citrulline ($X = O$).

Table 1. Kinetic Parameters of BsCM Variants[a]

enzyme	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)	k_{cat}/k_{uncat}	K_i (μM)
BsCM*	46	98	4.7×10^5	4.0×10^6	1.2
Arg90Cit BsCM*	0.0026	270	9.6	230	6.8

[a] The enzymes were assayed as previously described[6] at 30 °C in 50 mM phosphate buffer (pH 7.5). K_i values were obtained by standard inhibition assays with **4**.[6,17] Errors on all parameters were less than 15%. BsCM* is the semisynthetic *B. subtilis* chorismate mutase containing the D102E mutation. The recombinant BsCM* protein measured under identical conditions gave $k_{cat} = 49 \pm 2\ s^{-1}$ and $K_m = 105 \pm 12\ \mu M$, in good agreement with the published kinetic parameters for wild-type BsCM.[6,18] It was inhibited by **4** with a K_i of $1.0 \pm 0.2\ \mu M$.

2-mercaptoethane sulfonate to give the BsCM(1−87) fragment as a thioester. The C-terminal fragments, corresponding to residues 88−127 with either arginine or citrulline at position 90, were prepared by solid phase peptide synthesis using standard Fmoc protocols. Asp102 in these peptides was replaced by glutamate to avoid problems with aspartimide formation. Control experiments with recombinant D102E BsCM (BsCM*) confirmed that this mutation does not significantly alter the catalytic properties of the enzyme (Table 1). Following cleavage from the solid support, the synthetic BsCM(88−127)* fragments were purified by HPLC and separately coupled with the BsCM(1−87) thioester. Chemical ligations were performed with ca. 1 mM of each peptide in 100 mM Tris-HCl buffer (pH 8) containing 6 M guanidinium chloride and 2.5% thiophenol for 24 h. The ligated polypeptides, BsCM* and Arg90Cit BsCM*, were subsequently folded by 100-fold dilution in 50 mM glycine buffer (pH 8.9) containing 5% 2-propanol and 10% glycerol. After concentration, the folded proteins were purified by ion-exchange chromatography on a MonoQ column. A separate dedicated column was used for the Arg90Cit variant to preclude contamination by another chorismate mutase.

The semisynthetic proteins were analyzed by electrospray ionization mass spectroscopy (ESI-MS) and shown to have the

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expected mass (BsCM*: found 14 502 ± 2 daltons, expected 14 503 daltons; Arg90Cit BsCM*: found 14 503 ± 2 daltons, expected 14 504 daltons). Their circular dichroism spectra are superimposable on that of the recombinant enzyme purified under native conditions, indicating identical overall secondary structure. Like recombinant BsCM, they are homotrimeric in solution, eluting as a single peak with identical retention times from a Superose 12 size-exclusion column. Their trimeric quaternary structure was additionally confirmed by sedimentation velocity ultracentrifugation, which yielded average molecular masses of 41 500 and 43 600 daltons for semisynthetic BsCM* and for the citrulline variant, respectively, in good agreement with the expected mass for a trimer of 43 509 daltons.

Semisynthetic BsCM* is fully active as a catalyst, affording k_{cat} and K_m parameters for the chorismate mutase rearrangement that are indistinguishable from those of its recombinant counterpart (Table 1). The Arg90Cit variant is also active, albeit substantially less so than the wild-type enzyme. Replacement of arginine by citrulline causes a $>10^4$-fold decrease in k_{cat} and a more modest 2.7-fold increase in the K_m value for chorismate (Table 1). The small change in K_m suggests only minor perturbation of the ground-state Michaelis complex, although the nonequality of K_m and the dissociation constant for chorismate for wild-type BsCM[19] precludes accurate quantification of this effect. In contrast, the destabilizing effect of the Arg90Cit mutation on apparent transition state binding can be estimated directly from the equation $\Delta\Delta G = RT \ln[(k_{cat}/K_m)_{mut}/(k_{cat}/K_m)_{WT}]$ to be 6.5 kcal/mol. Effects on catalysis and protein stability of similar magnitude have been observed previously upon removal of a charged hydrogen bond donor or acceptor from a buried salt bridge,[20] supporting the idea that the guanidinium group of Arg90 forms a complementary electrostatic interaction with the developing negative charge at the ether oxygen of chorismate in the transition state.

The ligand binding properties of Arg90Cit BsCM* were further probed with the conformationally constrained inhibitor **4**,[17] which mimics the geometry of the chorismate mutase transition state reasonably well but not its dissociative character. Paralleling the small increase in K_m for chorismate, the inhibition constant value for **4** increases 5.7-fold upon mutation (Table 1), which corresponds to a 1.1 kcal/mol less favorable free energy of binding. The moderate decrease in affinity for the transition state analogue contrasts dramatically with the much larger destabilization of the true transition state. The neutral urea group of citrulline apparently interacts only slightly less well than the positively charged guanidinium group with the neutral ether oxygen of the stable inhibitor (and, by analogy, that of chorismate in the ground state), but it is more than 4 orders of magnitude worse at accommodating the partially anionic ether oxygen in the transition state.

Not surprisingly, even more deleterious effects are observed when either of the partners in the salt bridge is replaced with a non-hydrogen-bonding group. For example, mutation of Arg90 to alanine results in a complete loss of catalytic activity,[5,6] whereas wild-type BsCM is unable to catalyze the analogous Cope rearrangement of carbaprephenate into carbachorismate,[21] in which an apolar methylene group replaces the ether oxygen.

Taken together, these results demonstrate the importance of Arg90 at the active site of BsCM as a positively charged hydrogen bond donor involved in selective stabilization of chorismate in the transition state. It is noteworthy that structurally unrelated AroQ mutases such as EcCM[7] and ScCM[9] have a similarly positioned cation, albeit a lysine rather than an arginine, whereas the relatively inefficient catalytic antibody 1F7 lacks this feature.[22] Efficient catalysis of the chorismate mutase rearrangement evidently requires more than an active site that is simply complementary in shape to the reactive substrate conformer;[12] electrostatic stabilization of the polarized transition state appears to be paramount.

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Supporting Information Available: Experimental details for the synthesis and characterization of BsCM* and Arg90Cit BsCM*. This material is available free of charge via the Internet at http://pubs.acs.org.

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