Mutagenesis Study of Active Site Residues in Chorismate Mutase from *Bacillus subtilis*

Sharon T. Cload, David R. Liu, Richard M. Pastor, and Peter G. Schultz*

Howard Hughes Medical Institute, Department of Chemistry University of California, Berkeley, California 94720

Received September 13, 1995

Chorismate mutase catalyses the [3,3] Claisen rearrangement of chorismate (**1**) to prephenate (**2**), the committed step in the biosynthesis of tyrosine and phenylalanine in bacteria, fungi, and higher plants[1] (Scheme 1). Despite more than two decades of studies on this novel biological rearrangement,[2−15] the mechanism of the enzyme-catalyzed reaction remains unclear. Substrate labeling[5,7] and kinetic isotope effect studies[6] demonstrated that both the uncatalyzed and catalyzed reactions proceed through a chairlike transition state in which the C5−O7 bond cleavage precedes C9−C1 bond formation. Mechanistic proposals which have been suggested previously include protonation or deprotonation of the C4 hydroxyl by an active site general acid or base, respectively, nucleophilic catalysis by attack of an active site nucleophile at C5, and simple conformational restriction of the substrate.[9,14] Recently, X-ray crystal structures of monofunctional chorismate mutases from *Bacillus subtilis* (BsCM)[16,17] *Escherichia coli* (EcCM),[18] and catalytic antibody 1F7[19] bound to the *endo*-oxabicyclic transition state analogue **3**[11] were solved (Figure 1). Analysis of the active site structures has led to a general mechanistic hypothesis that the enzymes and antibody stabilize the chairlike transition state geometry via a series of electrostatic and hydrogen-bonding interactions,[17−20] which is consistent with the earlier finding that the rearrangement of chorismate and related compounds is more facile in hydrogen-bonding solvents.[8,10] In addition, it has been speculated that enzyme active site residues may stabilize the developing charge on the enol ether oxygen and the cyclohexadiene ring in the polar transition state.[6,10] The availability of the structures combined with the ability to mutate specific residues provides an opportunity to test these mechanistic hypotheses and identify key catalytic residues. We have generated and characterized a series of 16 mutants of the *B. subtilis* enzyme.

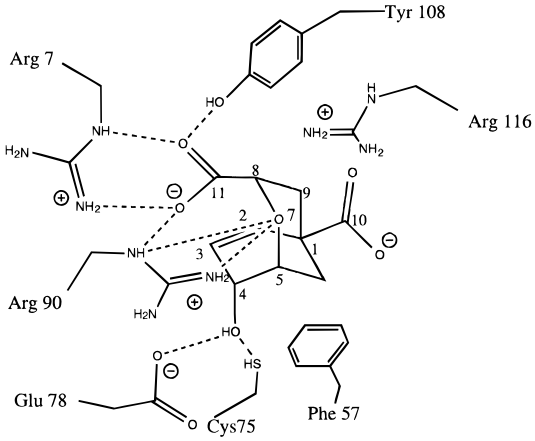
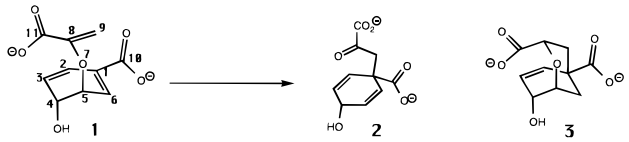


Figure 1. Schematic diagram of transition state analogue **3** bound in the BsCM active site.

Scheme 1



The gene encoding BsCM was subcloned from plasmid pBSCM2[21] into the phagemid pAED4[22] under the control of the T7 RNA polymerase promoter. Six histidines were added to the carboxy terminus using PCR[23] to afford plasmid pCM6XH. Mutant genes were constructed using the method of Kunkel,[24] and the resulting proteins were expressed in *E. coli* strain BL21 which carries the gene for T7 RNA polymerase behind the *lac*UV5 promoter.[25] Mutant proteins were purified to homogeneity (as determined by SDS−PAGE and Coomassie staining) in a single step using IMAC affinity chromatography on Ni(II)-chelating resin (Novagen).[26,27] The structural integrity of the mutants was assayed by circular dichroism (CD) spectroscopy. The CD spectra of all mutants between 200 and 260 nm were superimposable with that of the wild-type (wt) protein, suggesting that no significant structural changes were caused by the mutations. Activities of the mutants were determined by monitoring the disappearance of chorismate spectrophotometrically at 274 or 304 nm ($\Delta\epsilon_{274} = 2340\ M^{-1}\ cm^{-1}$; $\Delta\epsilon_{304} = 808\ M^{-1}\ cm^{-1}$) in 50 mM potassium phosphate pH 7.5 at 30 °C.[28] Table 1 lists the kinetic parameters (k_{cat},

(1) Weiss, U.; Edwards, J. M. *The Biosynthesis of Aromatic Amino Compounds*; Wiley: New York, 1980; pp 134−184.
(2) Andrews, P. R.; Smith, G. D.; Young, I. G. *Biochemistry* **1973**, *12*, 3492−3498.
(3) Andrews, P. R.; Cain, E. N.; Rizzardo, E.; Smith, G. D. *Biochemistry* **1977**, *16*, 4848−4852.
(4) Gorisch, H. *Biochemistry* **1978**, *17*, 3700−3705.
(5) Sogo, S. G.; Widlanski, T. S.; Hoare, J. H.; Grimshaw, C. E.; Berchtold, G. A.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 2701−2703.
(6) Addadi, L.; Jaffe, E. K.; Knowles, J. R. *Biochemistry* **1983**, *22*, 4494−4501.
(7) Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1985**, *107*, 5306−5308.
(8) Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5008−5013.
(9) Guilford, W. J.; Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1987**, *109*, 5013−5019.
(10) Gajewski, J. J.; Jurayj, J.; Kimbrough, D. R.; Gande, M. E.; Ganem, B.; Carpenter, B. K. *J. Am. Chem. Soc.* **1987**, *109*, 1170−1186.
(11) Bartlett, P. A.; Johnson, C. R. *J. Am. Chem. Soc.* **1985**, *107*, 7792−7793.
(12) Bartlett, P. A.; Nakagawa, Y.; Johnson, C. R.; Riech, S.; Luis, A. *J. Org. Chem.* **1988**, *53*, 3195−3210.
(13) Delany, J. J.; Padykula, R. E.; Berchtold, G. A. *J. Am. Chem. Soc.* **1992**, *114*, 1394−1397.
(14) Gray, J. V.; Knowles, J. R. *Biochemistry* **1994**, *33*, 9953−9959.
(15) Gray, J. V.; Eren, D.; Knowles, J. R. *Biochemistry* **1990**, *29*, 8872−8878.
(16) Chook, Y.-M.; Ke, H.; Lipscomb, W. H. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 8600−8603.
(17) Chook, Y.-M.; Gray, J. V.; Ke, H.; Lipscomb, W. N. *J. Mol. Biol.* **1994**, *240*, 476−500.
(18) Lee, A. Y.; Karplus, P. A.; Ganem, B.; Clardy, J. *J. Am. Chem. Soc.* **1995**, *117*, 3627−3628.
(19) Haynes, M. R.; Stura, E. A.; Hilvert, D.; Wilson, I. A. *Science* **1994**, *263*, 646−652.
(20) Lee, A. Y.; Stewart, J. D.; Clardy, J.; Ganem, B. *Chem. Biol.* **1995**, *2*, 195−203.
(21) Gray, J. V.; Golinelli-Pimpaneau, B.; Knowles, J. R. *Biochemistry* **1990**, *29*, 376−383.
(22) Doering, D. S. Thesis, Massachusetts Institute of Technology, Cambridge, MA, 1992.
(23) The N-terminal PCR primer, 5′ CTTGACCTGCATATGATGATT CGCGGAATTCGCGGA 3′, contained an *Nde*I restriction site and a start codon; the C-terminal primer, 5′ CAGGTCAAGAAGCTTTTA$(GTG)_6$-CAATTCAGTATTTTTTGTCAA 3′, contained six histidine codons, a stop codon; and a *Hin*dIII restriction site.
(24) Kunkel *Methods Enzymol.* **1987**, *154*, 367−382.
(25) Grodberg, J.; Dunn, J. J. *J. Bacteriol.* **1988**, *170*, 1245.
(26) Hochuli, E.; Dobeli, H.; Schracher, A. *J. Chromatogr.* **1987**, *411*, 177−184.
(27) Protein concentrations were determined spectrophotometrically (280 nM) using extinction coefficients calculated using the equation $\epsilon 280$ = ntyr*1450 $M^{-1}\ cm^{-1}$ + ntryp*5800 $M^{-1}\ cm^{-1}$; Kuramitsu, S.; Hiromi, K.; Hayashi, H.; Morino, Y.; Kagaminyama, H. *Biochemistry* **1990**, *29*, 5469.
(28) Chorismic acid was isolated from *Klebsiella pneumoniae* strain 62-1 by the method of Gibson: Gibson, F. *Biochem. Prep.* **1968**, *12*, 94.

0002-7863/96/1518-1787$12.00/0

Table 1. Kinetic Constants of *B. subtilis* Chorismate Mutase Mutants

mutant	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	K_i (μM)
wild type	69.5 ± 1.8	87 ± 5	$8.00 \times 10^5 \pm 5.3 \times 10^4$	1.7 ± 0.1
wild type[a]	50	100	1.00×10^6	
R90K			31 ± 1	ND[b]
R90A			<1	ND
R7K			717 ± 11	ND
R7A			1 ± 0.1	ND
Y108F	95.5 ± 7.8	624 ± 84	$1.53 \times 10^5 \pm 2.4 \times 10^4$	7.2 ± 0.7
Y108A			$3.39 \times 10^3 \pm 60$	ND
R116K	16.3 ± 0.7	1505 ± 99	$1.08 \times 10^4 \pm 8.6 \times 10^2$	15.7 ± 1.3
R116A			$1.42 \times 10^3 \pm 65$	ND
C75S	100.4 ± 3.5	382 ± 31	$2.63 \times 10^5 \pm 2.3 \times 10^4$	7.9 ± 0.4
C75A	95.4 ± 8.0	1048 ± 156	$9.11 \times 10^4 \pm 1.5 \times 10^4$	23.5 ± 2.0
C75D	58.0 ± 3.5	817 ± 87	$7.1 \times 10^4 \pm 8.6 \times 10^3$	11.5 ± 1.1
C75D/E78A			$1.66 \times 10^3 \pm 30$	ND
E78D	35.7 ± 2.4	1297 ± 139	$2.75 \times 10^4 \pm 3.5 \times 10^3$	43.6 ± 3.4
E78Q			75 ± 2	ND
E78A			33 ± 1	ND
F57W	29.6 ± 1.4	1071 ± 90	$2.76 \times 10^4 \pm 2.7 \times 10^3$	21.1

[a] BsCM without polyhistidine tag.[21] [b] ND = not determined. Parameters were determined by fitting initial rate data to the Michaelis−Menten equation. In cases in which saturation was not observed at concentrations of chorismate ≥1.5 mM, the k_{cat}/K_m values were determined by a linear fit of initial velocity versus chorismate concentration. Inhibition constants were measured at 30 °C in 50 mM potassium phosphate pH 7.5 at [chorismate] = K_m with various concentrations of **3**.[12] K_i values were determined by fitting the data to the equation $v = (k_{cat}[E_o][S])/([S] + K_m(1 + [I]/K_i))$ in which $K_m = [S]$.

K_m, k_{cat}/K_m, K_i) for the wt and mutant enzymes. The poly-his tag does not significantly affect the catalytic properties of the wt enzyme.

Hydrogen bonds between the C11 carboxylate group of chorismate and Arg 7 have been proposed to play an important role in positioning the enolpyruvyl side chain in the chairlike transition state geometry.[16,17,20] Consistent with this notion, removal of this bidentate hydrogen-bonding interaction in the Arg7Ala mutant leads to an approximately 10^6-fold reduction in k_{cat}/K_m. Substitution of Arg 7 with lysine leads to a 10^3-fold decrease in k_{cat}/K_m, indicating that the ϵ-amino group of lysine interacts with the C11 carboxylate less effectively. The crystal structure also suggests that the guanidinium group of Arg 90 hydrogen bonds to one of the oxygens of the C11 carboxylate group as well as the ether oxygen (O7) of **3**.[16,17] Substitution of Arg 90 with lysine results in a 2.6×10^4 reduction in k_{cat}/K_m relative to wt enzyme, and the Arg90Ala mutant has no measurable activity. The large decrease in activity associated with these mutations indicates that this residue also plays a critical catalytic role. Hydrogen bonds made by Arg 90 may stabilize the negative charge which develops O7 in the transition state as well as stabilize the required chairlike transition state conformation. An analogous bridging residue, Lys 39, is present in the *E. coli* enzyme ($k_{cat}/K_m = 2.4 \times 10^5$ M^{-1} s^{-1}),[29] but in the antibody 1F7, which has significantly lower activity compared to the enzymes ($k_{cat}/K_m = 23.5$ M^{-1} s^{-1}), the corresponding arginine is not positioned well to interact with O7.[19]

The X-ray crystal structure indicates that Tyr 108, Arg 116, Phe 57, and Cys 75 are all proximal to **3** in the active site.[16−18] Tyr 108 is within hydrogen-bonding distance of the C11 carboxylate, and Arg 116 is a potential hydrogen-bonding partner for the C10 carboxylate, although this region is not well-defined crystallographically. The side chain thiol group and backbone amide of Cys 75 are situated within hydrogen-bonding distance of the C4 hydroxyl group of **3**, and Phe 57 lies under C5. Substitutions at each of these positions, Tyr108Phe, Arg116Lys, Phe57Trp, Cys75Ser, Cys75Ala, and Cys75Asp, have little effect on k_{cat} but lead to 4- to 17-fold increases in K_m. Thus it appears that none of these residues plays a significant catalytic role, either orientational (Arg 116 or Tyr 108), as an active site nucleophile (Cys 75), or in stabilizing developing positive charge on the cyclohexadienyl ring system (Phe 57). For mutations at all four sites, the increase in K_m for chorismate correlates well with the increase in K_i for TS‡ analogue **3**, consistent with the fact that k_{cat} changes little.[30] These residues appear to contribute equally to the binding energies of substrate and transition state, indicating that substrate is bound in a pseudodiaxial chairlike geometry.

Reaction mechanisms involving both general acid and general base catalysis at the C4 hydroxyl group of chorismate have been proposed.[9] Active site groups may also stabilize the positive charge that develops in the vicinity of C4 in a dipolar transition state.[14,19] Although 4-deshydroxychorismate is a substrate for the *E. coli* chorismate mutase-prephenate dehydrogenase enzyme, the rate acceleration (k_{cat}/k_{uncat}) for this is 10^2 compared to 10^6 for chorismate.[31] This suggests that while the C4 hydroxyl is not required for catalysis, interactions between this moiety and the enzyme may contribute significantly to the rate acceleration. The carboxylate side chain of Glu 78 is positioned 2.90 Å from the C4 hydroxyl group of **3** and also lies 3.2 Å from NH1 and ϵ-N of Arg 90. Both the Glu78Gln and Glu78Ala mutants have significantly reduced activities (>10^4-fold) compared with wt enzyme, while the Glu78Asp mutant manifests only a 2-fold reduction in k_{cat} with a 15-fold increase in K_m. The activity of the Glu78Ala mutant is rescued 50-fold by replacing C75 with aspartate in the double mutant Glu78Ala/Cys75Asp. Again, the increases in K_m and K_i for Glu78Asp correlate well with the relatively unperturbed k_{cat},[30] suggesting that Glu 78 contributes equally to the binding energy of substrate and transition state. These results indicate that a carboxyl group in the vicinity of the C4 hydroxyl is required, most likely to orient both chorismate and the critical bidentate hydrogen-bonding guanidinium group of Arg 90. Because analogue **3** does not mimic the charge distribution expected in a transition state involving heterolysis of the C5−O7 bond or ionization of the C4 hydroxyl, it is difficult to determine to what degree this residue (and to a lesser degree Asp 78 and Asp 75) could also play an electrostatic role in catalysis. Mutagenesis using unnatural amino acids,[32−34] as well as mutational studies of the corresponding *E. coli* chorismate mutase, should help dissect out electronic versus conformational effects involving the O7 and C4 hydroxyl group of chorismate.

Acknowledgment. This work was supported by NIH Grant (R01GM49220). S.T.C. is supported by a National Institutes of Health postdoctoral fellowship (GM15958); D.R.L. is supported by a Howard Hughes predoctoral fellowship (HHMI-M940) and was formerly supported by NDSEG fellowship DAAH04-94-G-0296. We thank Jeremy Knowles for providing us with pBSCM2 and Whitney Smith for the generous gift of transition state analogue **3**. We also thank Helle Ulrich for helpful discussions.

JA953152G

(29) Stewart, J.; Wilson, D. B.; Ganem, B. *J. Am. Chem. Soc.* **1990**, *112*, 4582−4584.

(30) It has been suggested that the k_{cat} value for BsCM may be limited by the dissociation of prephenate, although there is some discrepancy in the reported prephenate dissociation rates: 60 s^{-1} at 25 °C (Gray, J. V.; Eren, D.; Knowles, J. R. *Biochemistry* **1990**, *29*, 8872−8878) and 270 s^{-1} at 25 °C (Rajagopalan, J. S.; Taylor, K. M.; Jaffe, E. K. *Biochemistry* **1993**, *32*, 3965).

(31) Pawlak, J. L.; Padykula, R. E.; Kronis, J. D.; Aleksejczyk, R. A.; Berchtold, G. A. *J. Am. Chem. Soc.* **1989**, *111*, 3374−3381.

(32) Cornish, V. W.; Mendel, D.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 621−633.

(33) Ellman, J.; Mendel, D.; Anthony-Cahill, S.; Noren, C. J.; Schultz, P. G. *Methods Enzymol.* **1991**, *202*, 301−337.

(34) Noren, C. J.; Anthony-Cahill, S. J.; Griffith, M. C.; Schultz, P. G. *Science* **1989**, *244*, 182−188.