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Electrostatic Catalysis of the Claisen Rearrangement: Probing the Role of Glu78 in *Bacillus subtilis* Chorismate Mutase by Genetic Selection

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Conversion of (−)-chorismate to prephenate by chorismate mutase (CM) is a rare example of an enzyme-catalyzed sigmatropic rearrangement.[1] Structural studies of CMs from *Bacillus subtilis*[2,3] and *Escherichia coli*[4] (BsCM and EcCM, respectively) indicate that the enzymes exert considerable conformational control over the flexible chorismate molecule.[5] Both proteins also possess cationic and anionic residues oriented for potential electrostatic stabilization of a putative dipolar,[6,7] chairlike[8] transition state in which C−O bond cleavage substantially precedes C−C bond formation[7,9] (Figure 1A). Theoretical studies have examined the importance of such electrostatic interactions,[10] and mutagenesis experiments with BsCM have confirmed that a positive charge close to the ether oxygen of the substrate is essential for activity.[11] We have now used targeted randomizing mutagenesis and selection to probe the role of Glu78. Our results support the notion[3,12] that an electrostatic gradient in the active site is a major factor in CM catalysis.

The chorismate-to-prephenate rearrangement is an essential step in the biosynthesis of phenylalanine and tyrosine in *E. coli*,[1] making direct genetic selection for CM activity possible.[11] To evaluate the role of key residues in the catalytic site of the *aroH*-encoded BsCM,[13] the corresponding codons can be randomized and functional variants of the protein selected for their ability to allow a host strain lacking endogenous CM genes to grow on minimal medium.[11] As shown in Figure 1, the position of Glu78 at the bottom of the binding pocket suggests[3,12] that its carboxylate could stabilize the partial positive charge on the cyclohexadienyl ring of the presumed dipolar transition state. To assess the importance of a negative charge at position 78,

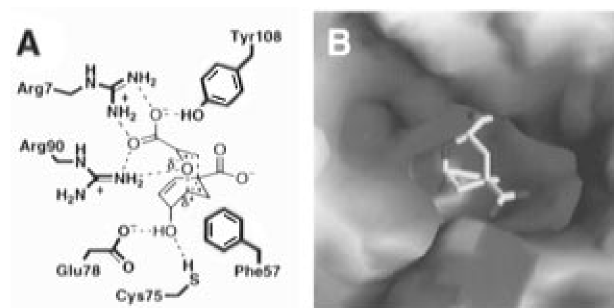

Figure 1. (A) Schematic view of the active site of wild-type BsCM[2,3] with the presumed transition state for the chorismate-to-prephenate rearrangement (thin lines). (B) Electrostatic surface potential (negative, red; positive, blue) of the enzyme calculated with the program GRASP.[21] A transition state analog is complexed at the active site, and the negatively charged Glu78 is visible as the red patch at the bottom of the predominantly positively charged binding pocket.

aroH libraries I (randomized codon 78) and II (simultaneously randomized positions 75 and 78) were constructed.[14]

The libraries were introduced into the host strain KA12/pKIMP-UAUC,[11] and clones able to produce their own phenylalanine and/or tyrosine were analyzed by sequencing.[11] Additionally, randomly chosen control clones grown under nonselective conditions were included.[15] Figure 2A,B compiles all unambiguously identified CM variants (without secondary mutations or wild-type codons) from libraries I and II, respectively. Table 1 summarizes additional *in vivo* and *in vitro* data obtained with a subset of clones.[16] Intracellular concentrations of CM were shown by SDS−polyacrylamide gel electrophoresis to vary by less than a factor of 2 for all mutants except for a clone with an amber stop codon at position 78, where no CM band was visible (data not shown). Strain KA12 (*supE44*) partially suppresses this stop codon with Gln.[17]

When only codon 78 was randomized, all 20 fast-growing clones encoded Glu78 (Figure 2A). The fact that the isosteric but uncharged variant Gln78 is essentially inactive (Table 1) underscores the importance of the negative charge of Glu78. That the carboxylate provides more than a hydrogen bond to chorismate's hydroxyl group is consistent with substrate modification experiments showing that the substrate alcohol is not essential for catalysis by an EcCM.[18] Nevertheless, a low level of activity is obtained when Glu78 is replaced with some residues capable of hydrogen bonding, such as His, Cys, Ser,

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(14) The partially randomized *aroH* libraries I and II were constructed using the polymerase chain reaction (PCR) with oligonucleotides 5′-ATCAAGCGGCCGCACTAGT and 5′-GTATGTACCGGTAACATG-TATGCAG*NN(G/C)*ATGGACGTCACAGGCGGTCTTA (library I) or 5′-GTATGTACCGGTTACT*NN(G/C)*ATGCAG*NN(G/C)*ATGGATGTC-ACAGGCGGTCTTA (library II). Randomized codons are italicized. The gel-purified 197 base pair (bp) PCR products were digested with *Pin*AI (site underlined) and *Hin*dIII. The 144 bp library fragments were ligated into a 3017 bp *Pin*AI-*Hin*dIII fragment of plasmid pKCMT-W previously incubated with *Bsr*GI and gel purified. Detailed procedures and subsequent manipulations are described in ref 11; wild-type *aroH* contamination was eliminated by digestion of the library II plasmid pool with *Aat*II prior to transformation of the selection strain KA12/pKIMP-UAUC.

(15) Sequencing of control clones revealed that codon 78 in library I exhibited a biased (4-fold lower) T-content, probably due to unequal coupling efficiencies during oligonucleotide synthesis. Nevertheless, we believe that the large number of 51 selected active clones surveyed gives a representative picture of permissible alternatives at position 78. The composition of library II was random.

(16) In general, there was good correlation between assays *in vivo* (growth on plates and doubling time determinations) and *in vitro* (enzyme activity). Exceptions can probably be explained by instability of individual variants *in vitro* as a consequence of the location of the alterations at the subunit interface. Comparisons between the assays used are discussed in ref 11.

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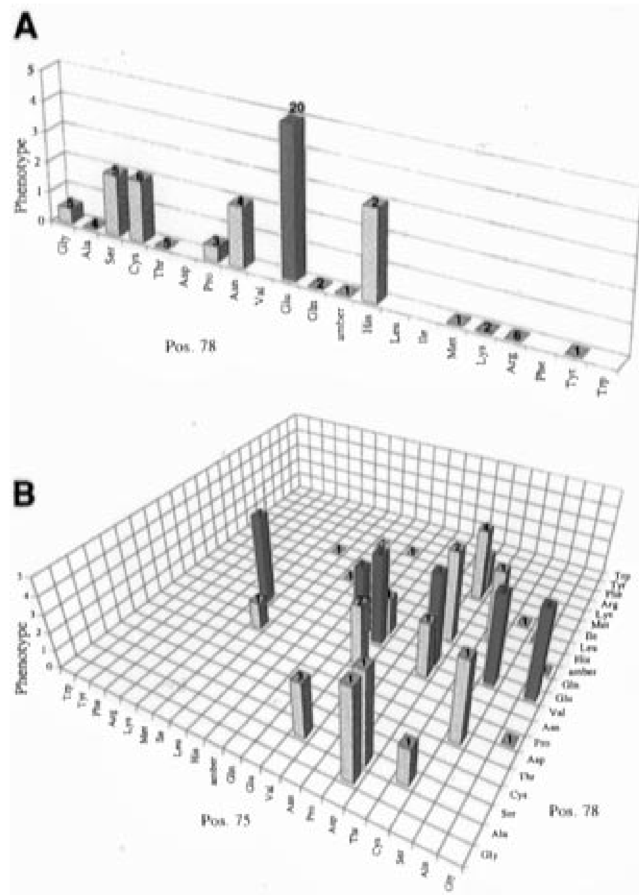

Figure 2. Summary of results from analysis of libraries randomized at position 78 (A) and positions 75 and 78 (B) of BsCM. The vertical axis displays *in vivo* activity (colony size on selective media) of individual clones ranging from 0 (no growth) to 5 (fast, wild-type-like growth).[11] Numbers on the tops of columns indicate the occurrence of that variant. Clones encoding Glu78 are identified by red columns. Amino acids are ordered according to increasing side-chain volume.[22]

or Asn (Table 1), perhaps because of compensatory structural changes at the active site.

The importance of the negative charge is further emphasized by the pattern of residues selected from the combinatorial library II. As visualized in Figure 2B, the only variants with wild-type-like activity *in vivo* contain either Glu at position 78 or Asp at position 75. The relatively high activities *in vivo* and *in vitro* of variants Ser75Glu78 and Gly75Glu78 (Table 1) show that Cys at position 75 is unessential, contrary to recent speculations.[5] A carboxylate group alone is not sufficient for complementation, however; its proper placement appears equally important,[19] given that Asp78 only appears in selected library I clones as the fortuitous double-mutant Asp78Gly81.[16] The Val81-to-Gly81 exchange may cause a surface loop to pack closer against Phe57 and thereby move Asp78 deeper into the active site (Figure 1). Exchange of Cys against the smaller Ser in the active mutant Ser75Asp78[16] may similarly allow better positioning of the aspartyl carboxylate.

Table 1. Characterization of a Subset of Selected Mutants

clone	phenotype *in vivo*[a] on plates	phenotype *in vivo*[a] t_d (h)	specific activity *in vitro*[b] (% of wild type)
wild type[c]	5	2.8	100
vector[c]	0	no growth	<0.05
His78	3	4.5	0.10
Cys78	2	nt	0.05
Ser78	2	7.7	<0.05[d]
Asn78	2	7.9	<0.05
Gly78	0.5	9.0	<0.05[d]
Pro78	0.5	15	<0.05
Gln78	0	9.9	<0.05
amber78	0	nt	<0.05
Ala78	0	17	<0.05
Met78	0	nt	<0.05
Asp78Gly81	5	3.1	<0.05[e]
Ser75Glu78	5	2.5	25[d]
Asp75Glu78	5	nt	21[d]
Gly75Glu78	5	nt	15[d]
Met75Glu78	5	nt	7.2
Val75Glu78	5	nt	1.8
Asp75Ser78	5	3.0	0.32[d]
Asp75Ala78	5	nt	0.23
Ser75Asp78	4.5	3.4	<0.05[e]
Asp75Met78	4	4.7	0.08
Val75Ser78	3	7.4	0.05
Asp75Val78	3	nt	<0.05
Cys75Ser78	2	nt	<0.05
Ile75Asn78	1.5	nt	<0.05

[a] Growth behavior under selective conditions was quantified on agar plates and in liquid cultures as described.[11] Doubling times (t_d) were determined in duplicate with standard deviations of less than 7% between measurements; nt, not tested. [b] *In vitro* CM assays were carried out with 50 μM chorismate and crude protein extracts.[11] Accuracy is within a factor of 2. [c] Data for wild type (plasmid pKCMT-W) and negative control (plasmid pBLS) were from a parallel study.[11] [d] Specific activities were essentially obtained under k_{cat}/K_m conditions, as preliminary measurements in crude extracts indicate $\geq$3-fold increases in K_m relative to wild type (K_m = 85 μM). [e] See footnote 16.

These results, together with other mutagenesis studies,[11] support the idea[3,12] that Glu78 and Arg90 may contribute to the efficiency of BsCM by complementing charge separation in the transition state (Figure 1A). The carboxylate component of this dipolar binding site (Figure 1), which apparently contributes up to 10^3-fold to catalysis, can be relocated from position 78 to 75, illustrating the advantages of combinatorial mutagenesis. Asp at position 75 is virtually superimposable on Glu52 in EcCM,[4,5] suggesting that Glu52 constitutes the relevant negative pole in EcCM and that a dipolar binding site may well be a general feature of efficient CMs. The CM catalytic antibody 1F7,[12,20] which lacks an analogous negative charge, is 10^4 times less active, and engineering of a dipolar binding site into 1F7 may prove profitable for catalysis.

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