Identification of Active Site Cysteine Residues that Function as General Bases: Diaminopimelate Epimerase

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D,L-*meso*-Diaminopimelate (DAP), a precursor to L-lysine in bacterial lysine biosynthesis, is a diamino acid which is incorporated into the pentapeptide of the Gram-negative peptidoglycan moiety.[1] Diaminopimelate epimerase (EC 5.1.1.7), a member of the pyridoxal phosphate-independent amino acid racemases, catalyzes the interconversion of L,L and D,L-*meso*-diaminopimelate (Figure 1).[2] Mechanistically similar to the well-studied PLP-independent amino acid racemases, especially proline racemase[3] and glutamate racemase,[4,5] DAP epimerase uses two cysteine residues. The thiolate form of one of the cysteines functions as the general base, and the other cysteine thiol functions as the general acid in one direction, while these ionization states, and functions, must be necessarily reversed for the other direction. An early mechanistic study of the *Escherichia coli* diaminopimelate epimerase supported a two-base mechanism.[2] The irreversible inhibition of the *E. coli* enzyme due to the alkylation of Cys73 by an active site-directed inhibitor, 2-(4-amino-4-carboxybutyl)-aziridine-2-carboxylate (aziDAP), suggested that this cysteine residue is present at or near the active site.[6] The three-dimensional structure of the *Haemophilus influenzae* diaminopimelate epimerase has been determined, and the 274-amino acid monomeric enzyme has the two conserved cysteine residues, Cys73 and Cys217, in disulfide linkage at the interface of two structurally superimposable domains.[7] Kinetic and isotopic studies of the reduced, active enzyme suggest these two cysteine residues are the catalytic acid and base.[8] The assignment of the two active-site cysteines responsible for proton abstraction in the L,L → D,L and D,L → L,L directions has been investigated using diasteriomeric 3-fluoro-DAP substrates and cysteine site-directed mutants of *H. influenzae* diaminopimelate epimerase.

The 3-fluoro analogue of L,L-DAP, (2*S*,3*R*,6*S*)-3-fluoro-2,6-diaminopimelic acid (**1**) and the 3-fluoro analogue of D,L-DAP, the 2*R*,3*S*,6*S* isomer (**2**), were synthesized and tested with the *E. coli* DAP epimerase.[9] Although **1** and **2** were competitive inhibitors of wild-type *E. coli* DAP epimerase, (exhibiting K_i values of 4 μM and 25 μM, respectively), the enzyme was also found to rapidly eliminate hydrogen fluoride (HF) from **2**, epimerize **1**, and slowly eliminate HF from **1**. The product formed from this elimination is the enamine (**3**), which spontaneously cyclizes (possibly via hydrolysis to the intermediate 2-keto-6-aminopimelate) to tetrahydrodipicolinic acid (**4**, Figure 2). Monitoring hydrogen fluoride release by ^{19}F NMR, both elimination and epimerization products were identified with wild-type *H. influenzae* DAP epimerase (data not shown). Single mutants, C73A, C73S, C217A, and C217S, and the double mutant, C73S/C217S, were prepared and purified from *E. coli*.[10] Each mutant was incubated with **1** and **2** and monitored continuously for HF elimination (Table 1).

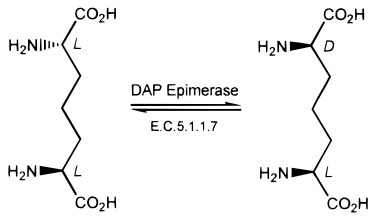

Figure 1. Reaction catalyzed by diaminopimelate epimerase.

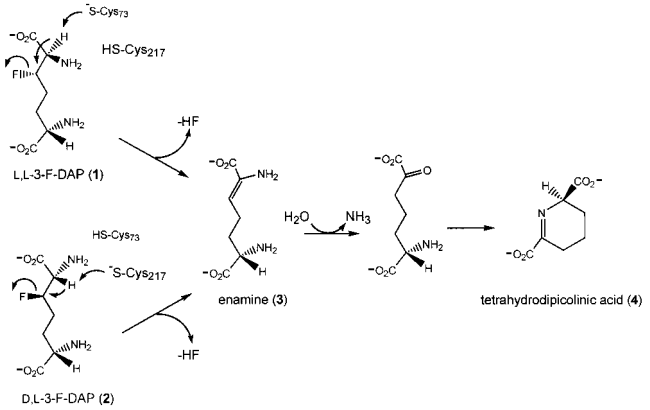

Figure 2. Proposed mechanism of hydrogen fluoride elimination by DAP epimerase. The product after HF elimination of L,L- and D,L-3-fluoro-DAP (**1** and **2**) forms the enamine (**3**), which spontaneously hydrolyzes and cyclizes to generate tetrahydrodipicolinic acid (**4**).

Although the single mutants of diaminopimelate epimerase, C73A and C217A, were found to be inactive as epimerases, these enzymes were able to catalyze the elimination of hydrogen fluoride via abstraction of the C-2 hydrogen. The C73A mutant was able to rapidly catalyze elimination of the D,L-3-fluoro-DAP analogue (**2**) and was essentially unable to catalyze elimination with the L,L-3-fluoro-DAP analogue (**1**, Figure 3a and 3b). This trend was reversed with the C217A mutant, which catalyzed HF elimination from L,L-3-fluoro-DAP but was incapable of catalyzing HF elimination from D,L-3-fluoro-DAP (Figure 3c and 3d). The single mutants, C73S and C217S were able to catalyze both epimerization of DAP and HF elimination of the fluoro-DAP analogues (Table 1). Qualitatively, the C73S mutant was able to

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(10) Mutations at the Cys73 and Cys217 positions of the *H. influenzae* diaminopimelate epimerase (*dapF* gene) were introduced by PCR (Perkin-Elmer). Sequencing of each of the plasmid DNA confirmed successful mutation(s) of the enzyme. Expression and purification of the mutant enzymes were accomplished following the protocol for the wild-type enzyme.[8] Electrospray ionization mass spectrometry confirmed the mass of the mutant epimerases as well as the purity of the samples. Further details of the cloning, expression, and purification of the mutant enzymes will be published separately.

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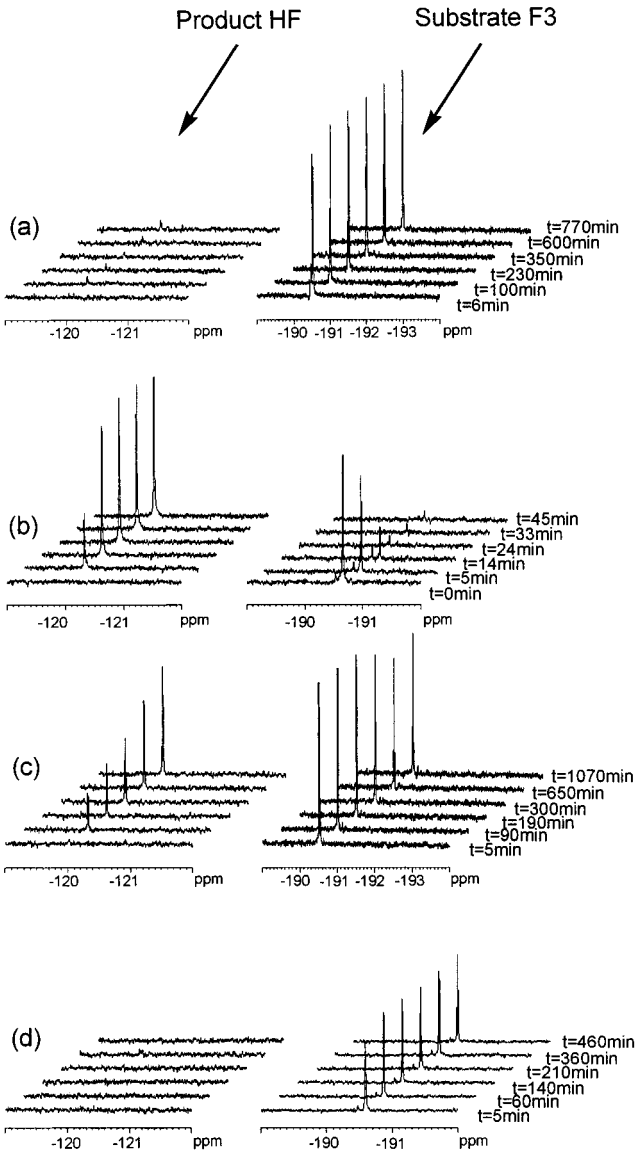

Figure 3. ^{19}F NMR time course of the reaction of the C73A mutant diaminopimelate epimerase with (a) **1** and (b) **2**, and of the C217A mutant with (c) **1** and (d) **2**. Method: The reaction was initiated by the addition of C73A or C217A to **1** (1.8 mM solution, final enzyme concentrations were 10 and 1 μM, respectively) or to **2** (1.4 mM and 2.0 mM solution, respectively; final enzyme concentration was 3 μM), 50 mM potassium phosphate, and D_2O (10%). The final volume was 330 μL. The experiment was carried out at pH 7.8 and 25 °C in a 5 mm symmetrical microtube matched with D_2O (Shigemi). ^{19}F NMR spectra (proton decoupled) were recorded on a Bruker DRX 300 spectrometer. Acquisition time = 1.05 s; number of scans = 128 (4.4 m); spectral width = 62.5 kHz. No window function was applied prior to Fourier transformation and phasing.

catalyze elimination from **2** more readily than from **1**, and the reverse trend was seen with the C217S mutant. Interestingly, the fluoro-epimerization product of **1** was not detected for either of these mutants, suggesting that the cysteine to serine mutation interferes with epimerization of the L,L-3-fluoro-DAP analogue. The alternative explanation would be that HF elimination is faster than epimerization for these mutants (in contrast to the wild-type enzyme), but this appears improbable since the conformational orientation of the fluorine in the active site (unfavorable for elimination) is likely to remain the same.[9a] Epimerization activity of the double mutant, C73S/C217S was not detectable by the coupled enzyme activity assay nor the circular dichroism assay.[8] However, the double mutant was able to slowly eliminate HF from both **1** and **2**.

Table 1. Relative Activity of *H. Influenzae* DAP Epimerase Enzymes

	epimerization (%)[a]	HF elimination[b]	
	L,L-DAP	L,L-3-F (**1**)	D,L-3-F (**2**)
wild-type	100	+++	++
C73A	<0.05	−	++++
C217A	<0.05	+	−
C73S	3	++++	++++
C217S	2	++	+
C73S/C217S	<0.05	++	++

[a] Activity was measured with the coupled enzyme activity assay in the L,L → D,L direction.[8] [b] Normalized to the same protein concentration. ++++ complete HF elimination in <1 h; +++ <2 h; ++ <6 h; + <24 h.

For glutamate racemase, substrate analogues, *threo*-3-chloroglutamate and *N*-hydroxyglutamate, were used to assign Cys73 as the general base for proton abstraction in the D → L direction and Cys184 as the general base for the L → D directions.[5] Glutamate racemase mutants, C73A and C184A, which were inactive as racemases, catalyzed hydrogen chloride elimination of the L- and D-glutamate analogues, 2*S*,3*S*- and 2*R*,3*R*-3-chloroglutamate, respectively. The cysteine to serine mutants, C73S and C184S, were able to eliminate water more readily from L-*N*-hydroxyglutamate and D-*N*-hydroxyglutamate, respectively.[11] For *H. influenzae* diaminopimelate epimerase, on the basis of the observations of the ability or inability of the mutants to catalyze elimination of HF from either **1** or **2**, we assign Cys73 as the general base responsible for the abstraction of the C-2 hydrogen of the L,L-3-fluoro-DAP, and C217 is responsible for proton abstraction of the D,L-3-fluoro-DAP (Figure 2). Assuming that the 3-fluoro-DAP analogues bind in a fashion similar to that of the corresponding diaminopimelate substrates, the results of these experiments support the assignments of Cys73 and Cys217 as the general base for proton abstraction in the L,L → D,L and D,L → L,L directions, respectively. Currently under investigation are the steady-state kinetic and isotope effects studies of the C73S and C217S diaminopimelate epimerase mutants.

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