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A Covalent Succinylcysteine-like Intermediate in the Enzyme-Catalyzed Transformation of Maleate to Fumarate by Maleate Isomerase

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Abstract: Maleate isomerase (MI), a member of the Asp/Glu racemase superfamily, catalyzes cis−trans isomerization of the C2−C3 double bond in maleate to yield fumarate. Mutational studies, in conjunction with the structure of the C194A mutant of *Nocardia farcinica* MI cocrystallized with maleate, have revealed an unprecedented mode of catalysis for the superfamily in which the isomerization reaction is initiated by nucleophilic attack of cysteine at the double bond, yielding a covalent succinylcysteine-like intermediate.

The Asp/Glu racemase superfamily provides a model for the divergent evolution of enzymes wherein a progenitor protein fold is recruited for the development of structurally related biocatalysts capable of catalyzing a range of chemical reactions.[1,2] Bacterial aspartate and glutamate racemases (AspRs and GluRs, respectively) themselves furnish D-amino acids for peptidoglycan synthesis and are thus interesting targets for antimicrobial chemotherapy.[3,4] Hydantoin racemases (HydRs) are used in the industrial production of amino acids,[5] and arylmalonate decarboxylases (AMDs) have generated interest as biocatalysts for the production of enantiopure carboxylic acids.[6−8] The structures of the superfamily members consist of a pseudosymmetrical dyad, with each domain providing the active site with one catalytic cysteine, each of which, in the case of racemases, acts as a general acid/base in the racemization reaction.[9,10] In AMDs, only one cysteine is retained, and it effects stereospecific protonation of a decarboxylated enediolate intermediate.[11] The cis−trans isomerization of the carbon−carbon double bond in maleate to give fumarate catalyzed by bacterial maleate isomerase (MI) (Figure 1)[12−14] represents an extension of the catalytic portfolio of the superfamily [for sequence alignments and a phylogenetic tree, see Figures S1 and S2 in the Supporting Information (SI)]. As part of wider studies into the evolution of catalytic diversity in the Asp/Glu racemase superfamily, we were eager to examine the mechanistic basis of double-bond isomerization in MI as achieved through recruitment of the Asp/Glu racemase active site. In this report, the crystal structures of the MI from *Nocardia farcinica* IFM 10152 (NfMI) and its C194A mutant, in conjunction with kinetic analyses, reveal a mechanism initiated by nucleophilic attack of Cys76 to yield a succinylcysteine-like covalent intermediate. This represents an extension of the known catalytic repertoire of the superfamily and is an unprecedented enzyme mechanism demonstrated by the first crystallographic evidence for the succination of cysteine.

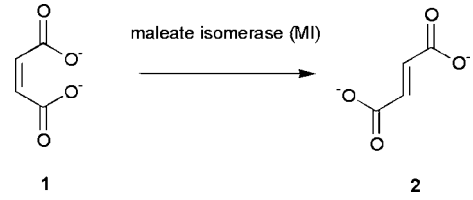

Figure 1. Cis−trans isomerization of maleate (**1**) to fumarate (**2**) catalyzed by NfMI.

Table 1. Summary of the Kinetic Parameters Obtained for NfMI and Active-Site Mutants[a]

	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} M^{-1})
WT	2.8 ± 0.7	4.6 ± 0.5	6.1×10^5
C76S	$(1.9 \pm 0.1) \times 10^{-3}$	3.7 ± 0.1	513
C194A	−[b]	N.D.[c]	−
C194S	$(3.5 \pm 0.5) \times 10^{-4}$	7.0 ± 2.9	50
Y133F	0.32 ± 0.05	0.53 ± 0.11	6.0×10^5

[a] Steady-state kinetic constants were determined by an HPLC assay performed at the NfMI optimum pH of 7.5. The data correspond to mean ± maximal error of two independent determinations of the constants. [b] The activity of C194A was beneath the measurable limits of detection. [c] Not determined.

The gene encoding NfMI was cloned and expressed in *Escherichia coli* and the enzyme purified. Dynamic light scattering experiments suggested that the enzyme was a dimer in solution (see the SI). NfMI displayed an optimum pH of 7.5 (see Figure S3) and a strict specificity for maleate, with no activity detected toward structurally related substrates including citraconate, mesaconate, dimethylmaleate, and maleamide, although bromomaleate was a weak inhibitor ($K_i = 1$ μM). NfMI was crystallized and the structure of the enzyme solved using molecular replacement (Figure S4). The structure revealed four molecules in the asymmetric unit, representing two dimers (Figure S5).

In common with other superfamily members,[6,10,15] each monomer consists of a pseudosymmetrical dyad of Rossmann fold domains, each contributing one of two cysteine residues, Cys76 and Cys194, to an active-site cleft formed at their interface, although Cys76 is oriented away from the putative ligand binding site (Figure S4). Additional electron density in the active site was successfully refined as a molecule of 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) from the crystallization buffer. This initial structure informed the design of active-site mutants (Table 1) that were used in conjunction with the C194A structure described below in formulating possible mechanisms for NfMI. Cys76 and Cys194 were crucial for activity, as mutants C76S and C194S displayed more than 1000-fold reduced turnover and C194A activity was not detectable.

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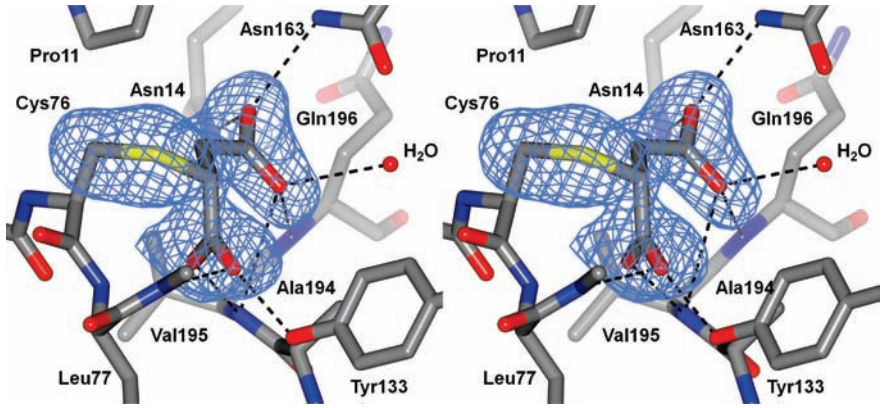

Figure 2. Stereoview of the C194A mutant active site containing the covalently bound intermediate. Ligand electron density corresponds to the omit map ($F_o - F_c$ map) contoured at a level of 3σ obtained after the final rounds of refinement in the absence of the ligand and the Cys76 side chain, which have been added to the figure for clarity.

C76S displayed 5-fold higher turnover than C194S, although each activity was extremely low. The K_M (maleate) for C76S and C194S was largely unchanged from the wild type (WT). While it was possible to invoke acid−base mechanisms for cis−trans isomerization that incorporate both cysteine residues, the mechanism of maleylacetoacetate isomerase,[16] in which cis−trans isomerization is initiated by the action of the thiol of glutathione as a Michael-type nucleophile at a C═C bond, prompted us to use electrospray ionization mass spectrometry (ESI-MS) to analyze mutant−ligand complexes for covalent modifications. A sample of C194A that had been mixed with 50 mM maleate displayed an increase of *m*/*z* 116 over the expected value of *m*/*z* 28664 (Figure S6), suggesting a possible covalent adduct of NfMI with maleate. Cocrystallization of C194A with 50 mM maleate resulted in a structure (refined to 1.95 Å resolution) in which the omit map revealed significant electron density attached to the γ-sulfur atom of Cys76. This density was successfully refined as either a saturated succinyl residue or an enediolate, with electrons delocalized from the double bond of maleate to the carboxylate (Figure 2).

The MS data and the trapped intermediate both provide evidence for a Michael addition-type mechanism in which Cys76 executes a nucleophilic attack on C2 of maleate. The kinetic data on the C76S mutant are consistent with those of previous studies on cytosine methyltransferase from *E. coli*, in which a serine mutant of a cysteine Michael nucleophile also displayed a K_M similar to that of the WT with a massively reduced k_{cat}.[17] Nucleophilic attack of Cys76 would require a deprotonated thiolate, which, when encountered in most other Asp/Glu racemase superfamily members as a base, is stabilized by electrostatic interactions with positively charged amino acids. In NfMI there are no positively charged residues near Cys76, but the N-terminus of helix D is oriented toward it, and this may instead provide the mechanism for thiolate stabilization in the mode observed for diaminopimelate epimerase.[18] Cys76 in the structure of the Tris complex is situated in a hydrophobic pocket formed by Met40, Pro11, Tyr74, and Met53, but in the C194A structure, its conformation is flipped to attack the double bond (Figure 3), with its previous position now being occupied by a water molecule. This is suggestive of a hydrophobic driving force for attack of the nucleophile once deprotonation has occurred, although the agent of the deprotonation has not yet been identified.

At the resolution obtained for the structure of the complex, the covalent intermediate is best-described as "succinylcysteine-like". Figure 4 outlines two mechanistic possibilities that entail different consequences for subsequent chemical steps, most notably for the intermediate structure and for the role of Cys194. In Figure 4a,

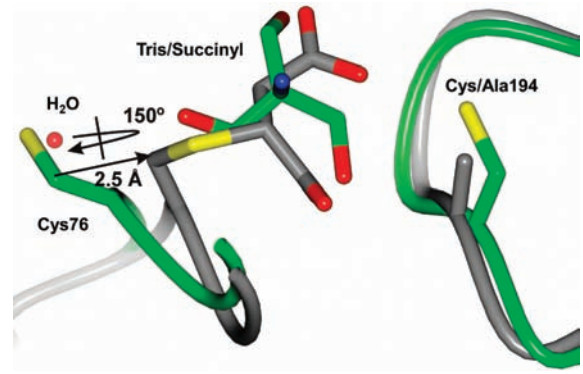

Figure 3. Superimposition of the active sites of NfMI with Tris bound to the WT (green) and the succinylcysteine-like intermediate to the C194A mutant (gray), illustrating the movement of the Cys76 side chain upon complex formation.

nucleophilic attack of Cys76 is accompanied by concerted protonation of the C2−C3 bond by an enzymatic acid, such as Cys194, to give a saturated succinylcysteine. Rotation around the C2−C3 bond is followed by deprotonation of the succinyl intermediate at a conformational juncture appropriate for the production of *trans*- fumarate, with concomitant release of Cys76. Evidence for "succination" of cysteine has been provided by MS studies on complexes of glyceraldehyde-3-phosphate dehydrogenase upon reaction with fumarate.[19] However, there is no acid residue within proton-donating distance of the C2−C3 bond in the complex structure, even accounting for the C194A mutation, perhaps ruling out Cys194 as a proton donor in this mode. In Figure 4b, attack of Cys76 at C2 results in an enediolate intermediate analogous to those described for both GluR and AMD.[11,20,21] The enediolate would be stabilized in a dioxyanion hole in which one of the partners is Cys194 acting as a catalytic acid donor to the C4 carboxylate. Rotation around the saturated C2−C3 bond is followed by an electron relay initiated by deprotonation of the C4 acid by Cys194, leading to the release of both fumarate and Cys76. Although it is a less likely candidate for the trapped intermediate on grounds of stability, evidence for a trapped 1,1-enediolate in the active site of fumarate reductase has been presented previously.[22] While this mechanism would require rotation around the C2−C3 bond with the enediolate being restrained by Cys194, the active-site flexibility required for such a rotation is evident through comparison of the Tris and maleate complexes and may be sufficient to permit it.

In other structures of Asp/Glu racemase superfamily members, the putative enediolate intermediate (formed by either substrate deprotonation in racemases or decarboxylation in AMDs) is thought

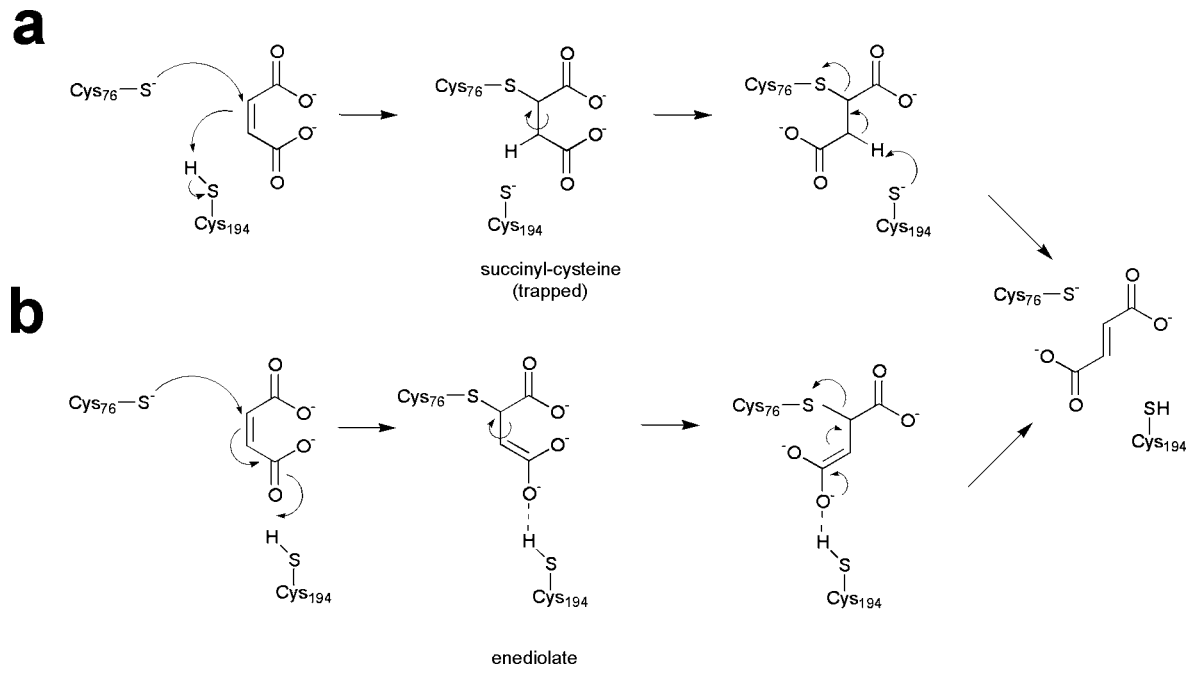

Figure 4. Two possible mechanisms for NfMI-catalyzed isomerization of maleate. In each, Cys76 performs a Michael-type nucleophilic attack on C2 of maleate, saturating the C2═C3 double bond to allow free rotation. (a) Concomitant with nucleophilic attack of Cys76, Cys194 protonates the C2═C3 double bond, forming a stable succinylcysteine intermediate. After C2−C3 bond rotation, deprotonation of C3 by Cys194 initiates release of fumarate and Cys76. (b) Nucleophilic attack by Cys76 initiates delocalization of electrons to the C3−C4 bond, giving an enediolate intermediate stabilized by the dioxyanion hole including Cys194. C2−C3 bond rotation is followed by the reverse electron relay from the enediolate to Cys76, initiated by deprotonation of the C4 carboxylate by Cys194.

to be stabilized by a conserved dioxyanion hole, which provides it with six hydrogen-bonding partners.[11] In NfMI, this dioxyanion hole is only partially conserved and binds the C1 carboxylate of the intermediate rather than the C4, as might be expected from the proposed mechanisms. Only four of the six H-bonding partners persist in NfMI, as Leu77 and Val78 are found in place of Asn and Thr (racemases) or Thr and Ser (AMDs) (Figure 2 and Figure S7) Of these four, mutation of Tyr133 to Phe (Table 1) resulted in a mutant that retained more than 10% of the WT activity, perhaps indicative of a less critical role in catalysis for this region of the active site in NfMI. The putative dienolate, or succinylcarboxylate of C4 in the C194A complex, is in fact bound by a dioxyanion hole on the other side of the active site and is formed by five H-bond partners: the side chains of Asn14 and Asn163, the backbone NHs of Val195 and Glu196, and a water molecule (Figure 2); this reveals a structural motif for intermediate stabilization unique to NfMI among the superfamily members described to date.

The discrimination of the two possible intermediates suggested by the C194A structure remains the focus of future work. The description of the nucleophilic cysteine and absence of a fully conserved dioxyanion hole in NfMI highlight a catalytic plasticity within the Asp-Glu racemase superfamily that is more extensive than envisaged previously. Overall, the results reveal an unprecedented biochemical mechanism for C═C bond isomerization and shed further light on the mechanisms of enzyme adaptation as achieved through divergent evolution.

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Supporting Information Available: Phylogenetic tree of Asp/Glu racemase superfamily; protocols for cloning, expression, purification, and crystallization of NfMI and mutants; data collection and refinement, with statistics on WT NfMI and the C194A mutant; and kinetics assays and pH/activity plot. This material is available free of charge via the Internet at http://pubs.acs.org.

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