# Evolution of Enzymatic Activities: Multiple Pathways for Generating and Partitioning a Common Enolic Intermediate by Glucarate Dehydratase from *Pseudomonas putida*

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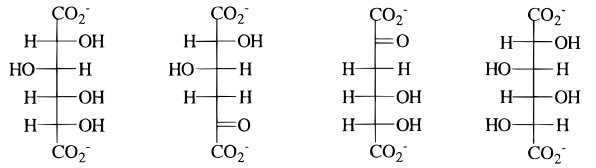
The evolution of a new enzyme presumably occurs by duplication of the gene for an existing enzyme followed by optimization of the chemical mechanism and substrate specificity to achieve the new catalytic activity. Whether the ancestral enzyme is selected on the basis of its chemistry or specificity is a matter of continuing interest and relevance to the design of new catalysts. In strong support of the proposal that chemistry rather than specificity is the more important factor,[1] we, together with others,[2] recently described the enolase superfamily of enzymes, each member of which shares the ability to catalyze the abstraction of the α-proton of a carboxylic acid to form a stabilized enolic intermediate. The intermediates partition to products *via* different mechanisms, depending upon the exact reaction that is catalyzed. Within the enolase superfamily, the structurally characterized mandelate racemase from *Pseudomonas putida* (MR) is the prototype of a subgroup that contains both a Lys and a His as general basic residues that initiate reactions by abstraction of the α-proton. MR catalyzes a 1,1-proton transfer reaction: Lys 166 is the (*S*)-specific acid/base catalyst, and His 297 is the (*R*)-specific acid/base catalyst.[3]

Glucarate dehydratase from *P. putida* (GlucD) is a member of the MR subgroup of the enolase superfamily.[2] In 1969,[4] GlucD was reported to catalyze the regio*selective* dehydration of D-glucarate (**1**) to yield a 92:8 mixture of 5-keto-4-deoxyglucarate (3-deoxy-L-*threo*-2-hexulosarate, **2**) and 2-keto-3-deoxyglucarate (3-deoxy-D-*erythro*-2-hexulosarate, **3**).[5] The

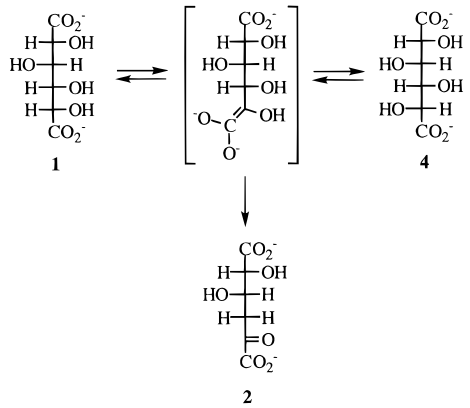


**1** **2** **3** **4**

product identification was based upon the fate of the 1- and 6-carboxylate carbons (labeled with $^{14}C$) following degradation of the 3-deoxy-2-hexulosarate product(s) into metabolites in the glucarate catabolic pathway[6] and not upon rigorous structural characterization.

Although the three-dimensional structure of GlucD is not yet available, alignment of its primary sequence with that of MR suggests that Lys 213 is a potential (*S*)-specific acid/base catalyst and His 345 is a potential (*R*)-specific acid/base catalyst.[2] Since the absolute configuration of carbon-5 of D-glucarate is *S* and that of carbon-2 is *R*, in analogy to the MR-catalyzed reaction, one explanation for the reported product distribution is that Lys 213 initiates the dehydration reaction that yields **2** and His 345 initiates the reaction that yields **3**. We report that this explanation is incorrect. Instead, GlucD catalyzes the dehydration of both D-glucarate and L-idarate (**4**; diastereomers at carbon-5) to yield **2** as well as their epimerization (Scheme 1).

**Scheme 1**



We expressed the gene for GlucD in *Escherichia coli* (BL21) and purified the protein to homogeneity. Using D-glucarate as substrate ($k_{cat}$ = 3 $s^{-1}$, $K_m$ = 65 $\mu M$[7]), a single product was isolated by ion chromatography on Dowex AG1-X8.[8] This product was identified as **2** by $^1H$ and $^{13}C$ NMR spectroscopies and comparison with the properties of an authentic sample of **3**.[9] No **3** was detected in the chromatogram of the enzymatic product, although **2** and **3** are well-resolved by the ion exchange chromatography. In contrast to the proposal in the literature,[4] GlucD catalyzes the regio*specific* dehydration of D-glucarate (Scheme 1). We hypothesize that this reaction is initiated by Lys 213, the (S)-specific acid/base identified by the sequence alignment with MR.[2]

The presence of a potential (*R*)-specific base, His 345, suggested that GlucD also might utilize L-idarate[10] as substrate. L-Idarate is, in fact, a good substrate ($k_{cat}$ = 4 $s^{-1}$, $K_m$ = 180 $\mu M$[7]), and the single expected product, **2**, was isolated by ion exchange chromatography (Scheme 1). We hypothesize that the dehydration of L-idarate is initiated by His 345.

The literature report that GlucD is a regio*selective* catalyst[4] and our conclusion that the enzyme is a regio*specific* catalyst for dehydration of D-glucarate can be reconciled if GlucD catalyzes the epimerization of D-glucarate and L-idarate (i.e., a 1,1-proton transfer reaction analogous to that catalyzed by MR (Scheme 1)). We have confirmed this hypothesis with two experiments. First, [1-$^{13}C$]-D-glucarate[11] was subjected to GlucD-catalyzed dehydration. The labeled **2** was isolated as its 3,6-lactone (**5**) in equilibrium with small amounts of α- and

(1) Petsko, G. A.; Kenyon, G. L.; Gerlt, J. A.; Ringe, D. A.; Kozarich, J. W. *Trends Biochem. Sci.* **1993**, *18*, 372.

(2) Babbitt, P. C.; Hasson, M.; Wedekind, J. E.; Palmer, D. R. J.; Barrett, W. C.; Rayment, I.; Ringe, D.; Kenyon, G. L.; Gerlt, J. A. *Biochemistry*, in press.

(3) Kenyon, G. L.; Gerlt, J. A.; Petsko, G. A.; Kozarich, J. W. *Acc. Chem. Res.* **1995**, *28*, 178.

(4) Jeffcoat, R.; Hassall, H.; Dagley, S. *Biochem. J.* **1969**, *115*, 969.

(5) A partially purified GlucD from *E. coli* was reported to yield an 85:15 mixture of **2** and **3** from D-glucarate and also utilize L-idarate as a substrate at a rate half of that observed for D-glucarate (Blumenthal, H. J. *Methods Enzymol.* **1966**, *IX*, 660).

(6) D-Glucarate is converted to **2** by GlucD; **2** is converted to α-ketoglutarate semialdehyde, $H_2O$, and $CO_2$ by a dehydratase/decarboxylase; and α-ketoglutarate semialdehyde is oxidized to α-ketoglutarate.

(7) Dehydration of both D-glucarate and L-idarate was performed in 50 mM Tris−HCl, pH 8.0, containing 10 mM $MgCl_2$, and assayed by end point detection of **2** as its semicarbazone (MacGee, J.; Doudoroff, M. *J. Biol. Chem.* **1954**, *210*, 617−624).

(8) Compounds **2** and **3** were chromatographed using a 3 × 16 cm column of AG1-X8 (formate) and eluted with a gradient of 0−6 M formic acid at a flow rate of 0.8 mL/min; 8 mL fractions were collected. The compounds were detected by semicarbazone assay.[7]

(9) Compound **3** was prepared by conversion of D-mannose to D-mannarodilactone (Haworth, W. N.; Heslop, D.; Salt, E.; Smith, F. *J. Chem. Soc.* **1944**, 217−224) followed by sodium methoxide-catalyzed elimination and hydrolysis. The product was purified by ion exchange chromatography.[8]

(10) L-Idarate (**4**) was prepared by reduction of L-sorbose to L-iditol (purified as its hexaacetyl derivative: Abdel-Akher, M.; Hamilton, J. K.; Smith, F. *J. Am. Chem. Soc.* **1951**, *73*, 4691−4692) followed by $HNO_3$ oxidation (Mehltretter, C. L. *Methods Carbohydr. Chem.* **1963**, *2*, 46−48).

(11) [1-$^{13}C$]-D-Glucarate was prepared by $HNO_3$ oxidation[10] of 99% [1-$^{13}C$]-D-glucose (Cambridge Isotope Laboratories).

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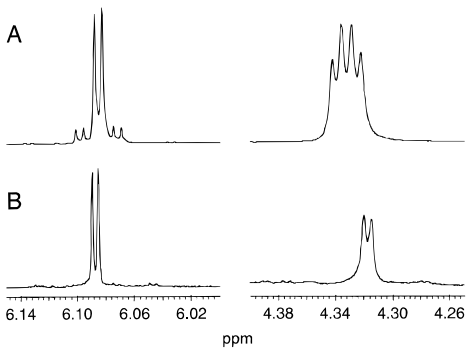
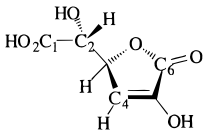



**Figure 1.** Partial $^1$H 400 MHz NMR spectra of **5** derived from labeled (panel A) and unlabeled (panel B) **1**. The resonance at 6.09 ppm is associated with the vinyl proton, and the resonance at 4.32 ppm is associated with the proton α to the carboxylic acid group.

$\beta$-hemiketals. $^1$H and $^{13}$C NMR spectra of **5**[12] revealed that



**5**

the GlucD-catalyzed reaction is accompanied by scrambling of the $^{13}$C label between the carboxylic acid and lactone carbons (observed ratio 85:15, respectively). For example, a comparison of the $^1$H NMR spectra of **5** obtained from labeled and unlabeled samples of D-glucarate (Figure 1) reveals the presence of $^1$H−$^{13}$C satellites in the labeled sample that allow quantitation of the $^{13}$C enrichment in both the carboxylic acid (unscrambled) and lactone (scrambled) carbons. This isotopic scrambling can be explained by the transient formation of the symmetrical L-idarate which is then converted to **2** (Scheme 1). Second, when the GlucD-catalyzed dehydration of D-glucarate was halted at ∼30% conversion to **2**, the hexaric acids recovered by ion exchange chromatography[13] included both L-idarate (19%) and D-glucarate (81%). GlucD catalyzes the epimerization of D-glucarate and L-idarate in competition with their dehydrations. We hypothesize that these reactions occur *via* a common, stabilized enolic intermediate (Scheme 1).

The demonstration that GlucD catalyzes two reactions that are cryptic in the catabolism of D-glucarate, the dehydration of L-idarate and the epimerization of D-glucarate, is surprising since L-idarate is not a naturally occurring compound; therefore evolutionary factors could not have required that GlucD be able to catalyze these reactions. That GlucD catalyzes the formation of a common enolic intermediate from both D-glucarate and L-idarate and is able to partition the intermediate between dehydration and epimerization (1) suggests that GlucD may have evolved from a racemase progenitor that acquired the ability to catalyze the $\beta$-elimination reaction and (2) provides additional, persuasive evidence for the proposal that chemistry, not substrate specificity, is the important factor in the evolution of new enzymatic activities.[1]

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(12) Lactone **5**: $^1$H NMR (500 MHz, $D_2O$) $\delta$ 6.09 (1H, d{$J$ = 2 Hz}, H-4), 5.21 (1H, dd, H-3), 4.32 (1H, d{$J$ = 2.5 Hz}, H-2); $^{13}$C NMR $\delta$ 173.5 (C-1), 171 (C-6), 142 (C-5), 117 (C-4), 79.5 (C-3), 69 (C-2). In $^{13}$C-enriched **5**, $J_{C1-H2}$ = 5.5 Hz and $J_{C6-H4}$ = 10.5 Hz.

(13) Compounds **1** and **4** were isolated by ion exchange chromatography[8] using a gradient of 0−2 M formic acid. The hexaric acids were detected as semicarbazones following dehydration with GlucD.