Importance of Mechanistic Imperatives in Enzyme-Catalyzed β-Elimination Reactions: Stereochemical Consequences of the Dehydration Reactions Catalyzed by D-Galactonate Dehydratase from *Escherichia coli* and D-Glucarate Dehydratase from *Pseudomonas putida*

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The relevance of chemical imperatives in the evolution of the mechanisms of enzyme-catalyzed reactions is an important issue in mechanistic enzymology. The rates of some enzyme-catalyzed reactions (e.g., triose phosphate isomerase[1]) are limited by the diffusive processes by which substrates enter and/or products leave the active site. This observation suggests that enzyme-catalyzed reactions are optimized to take advantage of mechanistic pathways that would specify the orientation of catalytic groups in active sites. The stereochemical courses of enzyme-catalyzed β-elimination reactions reveal a remarkable conservation of stereochemistry:[2,3] reactions initiated by abstraction of the α-proton from a carboxylate anion substrate proceed, with one exception,[4] *via anti* stereochemical pathways; reactions initiated by abstraction of the α-proton from an aldehyde, ketone, or thio ester proceed *via syn* stereochemical pathways. These results have been rationalized on the basis of the pK_as of the α-protons:[2,5] those adjacent to carboxylate anions are >29 and those adjacent to aldehydes, ketones, and thio esters are <25. The *syn* stereochemical course is attributed to a "one-base mechanism" in which the conjugate acid of the base that abstracts the α-proton catalyzes the departure of the β-leaving group. The *anti* stereochemical course is attributed to a "two-base mechanism" since the conjugate acid of the base may not be sufficiently acidic to protonate the leaving group, and a second, more acidic, group shielded from the base by the substrate is required. However, Mohrig et al. have suggested that historical contingency rather than this chemical imperative determines the stereochemical courses:[6] the nonenzymatic hydrations of both fumarate (to form malate) and *S*-crotonyl-*N*-acetylcysteamine [to form *S*-(3-hydroxybutyryl)-*N*-acetylcysteamine] show modest biases toward *anti* addition, even though the analogous reactions catalyzed by fumarase and crotonase proceed with the "expected" *anti* and *syn* stereochemical courses, respectively.

The members of the recently described enolase superfamily catalyze different overall reactions that are each initiated by abstraction of the α-proton from a carboxylate anion substrate.[7] The mandelate racemase (MR) subgroup of the superfamily includes galactonate dehydratase from *Escherichia coli* (GalD) and glucarate dehydratase from *Pseudomonas putida* (GlucD). We have studied the stereochemical courses of the β-elimination reactions catalyzed by GalD and GlucD to provide additional insight into whether the pK_as of the α-protons of the carboxylate anion substrates are of primary importance in the evolution of the mechanisms of enzyme-catalyzed β-elimination reactions.

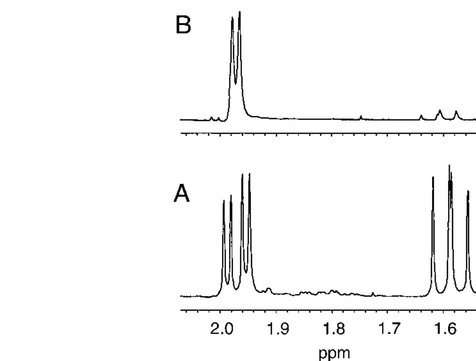
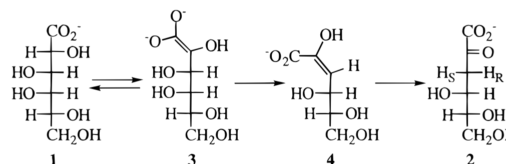


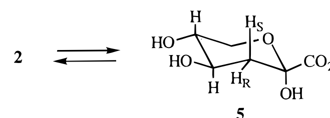
Figure 1. Partial 400 MHz ^{1}H NMR spectra at pD 8 of the hydrogens of carbon-3 of **5** obtained from D-galactonate (**1**) in H_2O (panel A) and D_2O (panel B). In panel B, the 3-*pro-S* hydrogen of **5** is replaced with solvent deuterium. The signals due to unlabeled hemiketal in panel B result from H_2O contaminating the sample of GalD.

GalD catalyzes the dehydration of D-galactonate (**1**) to afford 2-keto-3-deoxygalactonate (**2**), presumably *via* two enolic/enol intermediates (**3** and **4**). Alignment of the primary sequence



of GalD with that of MR allows the prediction that the active site contains a single, *R*-specific general basic catalyst (His 285) that mediates abstraction of the α-proton from the substrate.[7]

The product (**2**) exists as a single pyranosyl hemiketal (**5**) as assessed by both ^{1}H and ^{13}C NMR spectroscopies. When the



reaction is performed in D_2O,[8] one of the prochiral hydrogens of carbon-3 of **5** is stereospecifically deuterated (Figure 1), demonstrating that the conversion of **4** to **2** is enzyme-catalyzed.[9] Analysis of the vicinal ^{1}H−^{1}H coupling constants of **5** reveals that the 3-*pro-S* hydrogen of **2** is deuterated, so the departing 3-OH group is replaced with solvent deuterium with retention of configuration. Direct assignment of the absolute stereochemical course of the conversion of **1** → **4** is prevented because the vinylogous β-elimination of the 3-OH (**3** → **4**) generates an unstable, enzyme-bound enol that tautomerizes to the observed

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(8) The GalD-catalyzed reactions (1.0 mL) contained 10 mM D-galactonate, 50 mM Tris (protiated, pH 8 in H_2O; or d_7, pD 8 in D_2O), 10 mM $MgCl_2$, and 6.5 mM GalD. Upon completion of the reactions (as assayed by semicarbazone formation), the samples were dried by speed-vacuum centrifugation. One milliliter of D_2O was added, and ^{1}H NMR spectra were recorded immediately using a Varian U400 NMR spectrometer. ^{1}H NMR δ (D_2O): 1.58 (1H, dd{J = 5.1 Hz, 13.1 Hz}, H-3_S), 1.96 (1H, dd{J = 11.6 Hz, 13.1 Hz}, H-3_R), 3.40 (2H, m, H-5, H-6), 3.60 (1H, m, H-6), 3.66 (1H, m, H-4). ^{13}C NMR (D_2O): δ 38.7 (C-3), 62.6 (C-6), 68.6 (C-4), 70.4 (C-5), 96.3 (C-2), 176 (C-1).
(9) In the absence of GalD, the 3-*pro-S* and -*pro-R* hydrogens of **2** undergo chemical exchange at equivalent rates.

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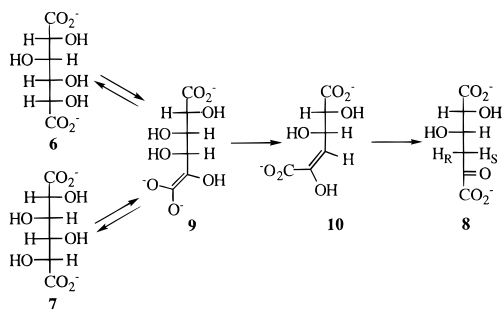
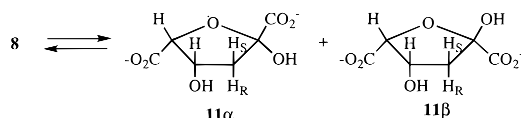
product **2**. However, the structure of the active site of the homologous MR suggests that **1** binds in the active site of GalD in an extended conformation; if correct, GalD catalyzes the expected[2,3] *anti* β-elimination reaction.

GlucD catalyzes the dehydration of both D-glucarate (**6**) and L-idarate (**7**) to yield the same product, 3-deoxy-L-*threo*-2-hexulosarate (**8**), presumably via common enolic/enol intermediates (**9** and **10**).[10] This unusual lack of stereospecificity as well



as the ability of GlucD to catalyze the epimerization of **6** and **7**[10] can be rationalized by alignment of the primary sequence of GlucD with that of MR. The active site of GlucD is predicted to contain both *R*-specific (His 345) and *S*-specific (Lys 213) general basic catalysts that mediate abstraction of the α-proton from opposite faces of bound substrate.[7] The epimerization of **6** and **7** is accompanied by quantitative incorporation of solvent-derived hydrogen into product,[11] consistent with the involvement of two acid/base catalysts in mediating proton-transfer reactions to and from the α-carbon of the substrate.

The dehydration product (**8**) exists as a mixture of α- and β-furanosyl hemiketals (**11**) as assessed by both ^{1}H and ^{13}C NMR spectroscopies. When the dehydration reaction of either



6 or **7** is performed in D_2O,[12] one prochiral hydrogen of carbon-4 of each of the anomers of **11** is stereospecifically deuterated (Figure 2), indicating that the conversion of **10** to **8** is enzyme-catalyzed.[13] *In both reactions, the same hydrogen is deuterated.* Analysis of the vicinal ^{1}H−^{1}H coupling constants of the anomers of **11** reveals that the proton giving rise to the larger coupling constant, which from the Karplus relationship is almost certainly

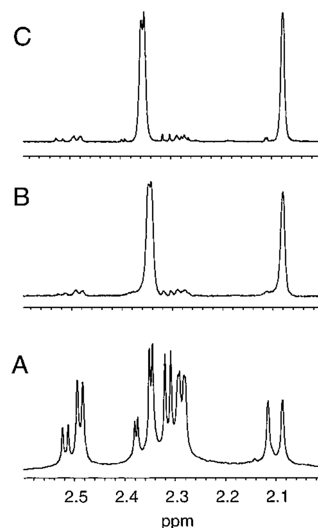


Figure 2. Partial 400 MHz ^{1}H NMR spectra at pD 2 of hydrogens of carbon-4 of the anomers of **11** obtained from D-glucarate (**6**) in H_2O (panel A), D-glucarate (**6**) in D_2O (panel B), and L-idarate (**7**) in D_2O (panel C). In panels B and C, the 4-*pro-S* hydrogen of the anomers of **11** is replaced with solvent deuterium. The signals due to the unlabeled hemiketals in panels B and C result from H_2O contaminating the sample of GlucD.

the 4-*pro-S* hydrogen, is deuterated, so the departing 4-OH group is replaced with solvent deuterium with retention of configuration.

Although the absolute stereochemical courses of the dehydration reactions catalyzed by GlucD cannot now be specified [the vinylogous β-elimination of the 4-OH (**9** → **10**) generates an unstable, enzyme-bound enol that tautomerizes to the observed product **8**], that GlucD does not utilize D-mannarate as substrate suggests that **6** and **7** bind in identical geometries in the active site, with the exception of the disposition of the 5-protons to Lys 213 and His 345, the putative *S*- and *R*-specific general basic catalysts, respectively. If both **6** and **7** bind in the active site in extended conformations, GlucD catalyzes a *syn* β-elimination reaction with D-glucarate (**6**) as substrate and an *anti* β-elimination reaction with L-idarate (**7**) as substrate. In any event, since the values for k_{cat} and k_{cat}/K_m for **6** and **7** are nearly identical,[10] GlucD must catalyze both *syn* and *anti* β-elimination reactions with equal facility.

High-resolution three-dimensional structures for both GalD[14] and GlucD[15] together with mechanistic studies of active site mutants should resolve the absolute stereochemical courses for the dehydration reactions as well as whether the *syn* reaction (GlucD with **6**) occurs *via* a one-base mechanism and the *anti* reactions (GalD with **1** and GlucD with **7**) occur *via* two-base mechanisms. However, that GlucD can catalyze the β-elimination of hydroxide anion (a poor leaving group whose departure presumably requires acid catalysis) in both *syn* and *anti* β-elimination reactions reveals that a chemical imperative based upon the pK_as of the α-proton of the carboxylate ion substrate has not dominated the evolution of mechanisms of β-elimination reactions in the enolase superfamily. We note that this conclusion is possible because our parallel, comparative investigations of GalD and GlucD allow the importance of mechanistic differences in the evolution of function to be defined.[7]

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(12) The GlucD-catalyzed reactions (800 mL) contained 0.1 M D-glucarate, 0.2 M potassium phosphate (pH 8.0 in H_2O or pD 8.0 in D_2O), 5 mM $MgCl_2$, and 5 mM GlucD. For the reaction in D_2O, upon completion of the reaction (as assayed by semicarbazone formation), the pD was lowered to 2 with concentrated 35% DCl in D_2O. For the reaction in H_2O, the completed reaction was frozen, lyophilized, dissolved in D_2O, and then adjusted to pD 2. [The resonances associated with H-4 of the anomers of **11** are not completely resolved at pD 8, but at pD 2, distinct ABX patterns can be observed for the 4-*pro-S* and 4-*pro-R* protons, as shown in Figure 2A.] ^{1}H NMR spectra were recorded immediately using a Varian U400 NMR spectrometer. ^{1}H NMR δ (D_2O, pD = 2): 4.75 (1H, d{J = 4.6 Hz}, H-2), 4.68 (1H, d{J = 4.4 Hz}, H-2), 4.63 (2H, m, H-3), 2.51 (1H, dd{J = 5.4 Hz, 14.5 Hz}, H-4$_S$), 2.37 (1H, dd{J = 3.1 Hz, 14.6 Hz}, H-4$_R$), 2.30 (1H, dd{J = 5.8 Hz, 14.6 Hz}, H-4$_S$), 2.10 (1H, dd{J = 0 Hz, 14.5 Hz}, H-4$_R$). ^{13}C NMR δ (D_2O, pD = 2): 173.6 (C-6), 173.1 (C-6), 172.4 (C-1), 172.2 (C-1), 103.4 (C-5), 103.0 (C-5), 83.9 (C-2), 82.2 (C-2), 71.7 (C-3), 71.4 (C-3), 44.2 (C-4), 43.7 (C-4). The signals associated with the α- and β-anomers cannot be unequivocally assigned.

(13) In the absence of GlucD, the rate of chemical exchange of the 4-*pro-R* hydrogen of **8** exceeds that of the 4-*pro-S* hydrogen by at least a factor of 4, stereoselectivity *opposite* to that observed in the GlucD-catalyzed reaction.

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