Evolution of Enzymatic Activities in the Enolase Superfamily: Identification of a "New" General Acid Catalyst in the Active Site of D-Galactonate Dehydratase from *Escherichia coli*

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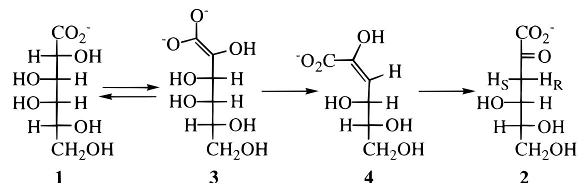
The members of the enolase superfamily catalyze different overall reactions related by conserved abstraction of the α-proton of a carboxylate anion substrate to generate an enolic intermediate stabilized by interactions with electrophilic groups, including a divalent metal ion.[1] The intermediates are directed to products via divergent chemistries determined by the specific active site. The structurally characterized members of the superfamily include yeast enolase, mandelate racemase (MR), and muconate lactonizing enzyme I (MLE). Additional members can be divided into three subgroups, designated enolase, MR, and MLE, based upon alignments of their sequences that allow prediction of (1) the ligands for a conserved divalent metal ion and (2) the basic catalysts that initiate the reactions by abstraction of the α-protons.

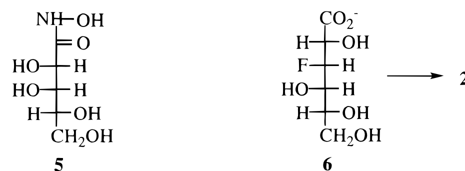
The MR subgroup includes D-glucarate dehydratases (GlucDs) from several eubacteria[2−4] and both L-rhamnonate[5] (RhamD) and D-galactonate[6] (GalD) dehydratases from *Escherichia coli*. Within this subgroup, the metal ion ligands (Figure 1) are predicted to include one Asp, one Glu in an **E**-[E/D]-P sequence motif, and usually one Glu in a G-**E** motif (the GlucDs are an exception in that the third ligand is an Asn).[7] In addition, each member is predicted to contain an His-Asp dyad, where the His is a basic catalyst; some, but not all, members contain a [K/R]-X-K motif, where the first cationic residue neutralizes the negative charge on the carboxylate group and the second, if present, also functions as a basic catalyst.

In addition to α-proton abstraction, the GlucDs, RhamD, and GalD catalyze β-elimination of OH^- (dehydration). To understand the evolution of this incremental function within the superfamily, we must identify the additional general acid(s), if any, required to accomplish the dehydration reaction. We have obtained kinetic and mechanistic evidence in support of His 185 as a "new" general acid in GalD that catalyzes the vinylogous β-elimination of the 3-OH group.

GalD catalyzes the dehydration of D-galactonate (**1**) to afford 2-keto-3-deoxy-D-galactonate (**2**), presumably via enolic (**3**) and enol (**4**) intermediates



Alignment of the sequences of the members of the MR subgroup allowed prediction of the ligands for the divalent metal ion (Asp 183, Glu 209, and Glu 235) as well as two essential catalysts in the active site of GalD:[1] His 285 hydrogen-bonded to Asp 258 was predicted to initiate the reaction by abstraction of the α-proton from **1**, and Glu 310 was predicted to assist in stabilization of **3** by hydrogen bonding. The kinetic phenotypes of the H285N and E310Q mutants (Table 1) support these predictions.[8] A structure of GalD complexed with D-lyxonohydroxamate (**5**), a competitive inhibitor and an analogue of **3**, has been solved at moderate resolution and supports the proposed roles of His 285 and Glu 310 in catalysis.[9] More importantly, this structure discloses the proximity of His 185 to the 2-OH of **5**, the homologue of the 3-OH group in **1**. This location allows the hypothesis that His 185 is a new general acid in the conversion of **3** to **4** and, perhaps, in the stereospecific tautomerization of **4** to **2**.



Consistent with the participation of His 185, the values of both k_{cat} and k_{cat}/K_m for the H185N and H185Q mutants are significantly decreased (Table 1). Chemical support for the importance of His 185 was obtained by determining the effect of substitution of F for OH at carbon-3 of **1**. The lithium salt of 3-fluoro-D-galactonate (3-FGal, **6**) was prepared via the diethylaminosulfur trifluoride (DAST)-mediated fluorination of 1,2:5,6-di-*O*-isopropylidene-α-D-gulofuranose, removal of the protecting groups, oxidation with bromine, and anion-exchange chromatography using LiCl as eluent.[10] **6** is a substrate and, like **1**, is converted to **2**. The values of both k_{cat} and k_{cat}/K_m measured for wild-type GalD, His 285N, and E310Q with **6** are comparable to those obtained with **1** (Table 1), providing further support for the importance of His 285 and Glu 310. However, in striking contrast, the values

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(8) The conversion of D-galactonate (**1**) and 3-FGal (**6**) to 2-keto-3-deoxy-D-galactonate was quantitated using a semicarbazide assay (MacGee, J.; Doudoroff, M. *J. Biol. Chem.* **1954**, *210*, 617−624).

(9) Clifton, J. G.; Wieczorek, S. J.; Gerlt, J. A.; Petsko, G. A., experiments in progress. The current structure with eight polypeptides in the asymmetric unit is refined at 3.2 Å resolution. The effective resolution of the structure was increased by 8-fold averaging of the electron density map. A full description of the structure will be published and coordinates deposited in the Protein Data Bank when refinement at higher resolution is completed. Until that time, coordinates are available without restriction by request to G.A.P. (E-mail: petsko@binah.cc.brandeis.edu).

(10) 3-FGal (**6**): ^{1}H NMR (400 MHz, D_2O) δ 4.74 (1H, ddd {$J_{F,3}$ = 45 Hz}, H-3), 4.12 (1H, dd {$J_{F,2}$ = 36 Hz}, H-2), 3.75 (1H, d {$J_{F,4}$ = 3.7 Hz}, H-4), 3.73 (1H, m, H-5), 3.53, (2H, m, H-6). ^{13}C NMR(100 MHz, D_2O) δ 177 (C-1), 91.4 (C-3, *J* = 178.7 Hz), 70.3 (C-4, *J* = 20.5 Hz), 69.4 (C-5), 67.1 (C-2, *J* = 27.0 Hz), 62.3 (C-6). ^{19}F NMR (376 MHz, D_2O) δ −207 (m).

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Figure 1. Partial alignment (GCG pileup) of the sequences of several members of the MR subgroup of the enolase superfamily showing the contexts of the three metal ion ligands.[13] The metal ion ligands are highlighted in blue; the position of His 185 in GalD is highlighted in red.

Table 1. Kinetic Phenotypes of GalD and Mutants

enzyme	substrate	k_{cat} (sec^{-1})	k_{cat}/K_m (M^{-1} sec^{-1})
wild type	D-galactonate, **1**	3.5 ± 0.033	2300
	3-FGal, **6**	3.7 ± 0.16	260
H285N	D-galactonate, **1**	0.000048 ± 0.0000014	0.0025
	3-FGal, **6**	0.000014 ± 0.0000014	0.0032
E310Q	D-galactonate, **1**	0.00047 ± 0.0000045	0.42
	3-FGal, **6**	0.00026 ± 0.000018	0.026
H185N	D-galactonate, **1**	0.00014 ± 0.000002	0.16
	3-FGal, **6**	0.31 ± 0.047	16
H185Q	D-galactonate, **1**	0.00024 ± 0.000011	0.18
	3-FGal, **6**	0.42 ± 0.041	28

of both k_{cat} and k_{cat}/K_m measured for both H185N and H185Q with **6** significantly exceed those obtained with **1** and are only modestly reduced from those observed with wild-type GalD. These differences are consistent with the requirement for a general acid for elimination of the 3-OH group but not of the 3-F group.

After departure of the 3-leaving group, an enol intermediate (**4**) is formed. As assessed by ^{1}H NMR analyses of **2** obtained in D_2O (in the α-pyranosyl hemiketal conformation), wild-type GalD catalyzes the replacement of both the 3-OH group of **1** (Figure 2, panels A and B) and the 3-F group of **6** (Figure 2, panel C) with a solvent-derived hydrogen with retention of configuration (H_S in **2**).[11] This outcome could be explained if His 185 also served as a general acid in the conversion of **4** to **2**. However, in both the H185N- (Figure 2, panel D) and H185Q-catalyzed (data not shown) reactions using **6** as substrate, a deuteron is incorporated with retention of configuration. Perhaps these substitutions allow a water molecule to enter the active site and function as this acidic catalyst; otherwise, an as-of-yet unidentified active site acid, including a positionally restricted water molecule, mediates the final partial reaction.

These observations identify His 185 as a new general acid/base catalyst in the enolase superfamily. The position of this residue, at the end of the third β-strand in the $(\beta/\alpha)_8$-barrel domain (Figure 3), is consistent with our analysis of the active site design of the superfamily, i.e., the functional groups are located at the ends of the β-strands where their identities can be conserved or diverged to generate new overall reactions.[1,12] On the basis of the locations of His 185 and His 285 (the ends of the third and seventh β-strands on opposite sides of the $(\beta/\alpha)_8$-barrel domain), we conclude that the GalD-catalyzed β-elimination reaction proceeds with an anti stereochemical course.

GlucD does not contain a homologue of His 185,[1,7] requiring that another group assume the role of the required general acid (Figure 1). However, the sequences of three members of the MR subgroup that have unknown functions (RspA, SpaA, and Yin2) contain homologues of His 185, allowing the prediction that these catalyze dehydration reactions on as-of-yet unidentified α,β-dihydroxycarboxylate anion substrates.[13,14]

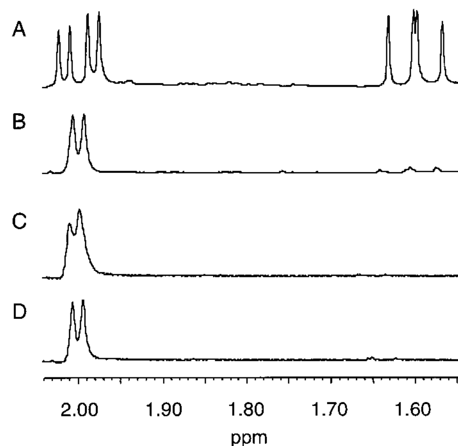

Figure 2. Panels A and B, partial 400 MHz ^{1}H NMR spectra at pD 8 of the hydrogens of carbon-3 in the hemiketals of **2** obtained from D-galactonate (**1**) with wild-type GalD in H_2O (panel A) and D_2O (panel B); the 3-pro*S* hydrogen of **2** in panel B is replaced with solvent deuterium.[8] Panel C, the spectrum of the hydrogens of carbon-3 of the hemiketals of **2** obtained from 3-FGal (**6**) in D_2O using wild-type GalD. Panel D, the spectrum of the hydrogens of carbon-3 of the hemiketals of **2** obtained from **6** in D_2O using H185N.

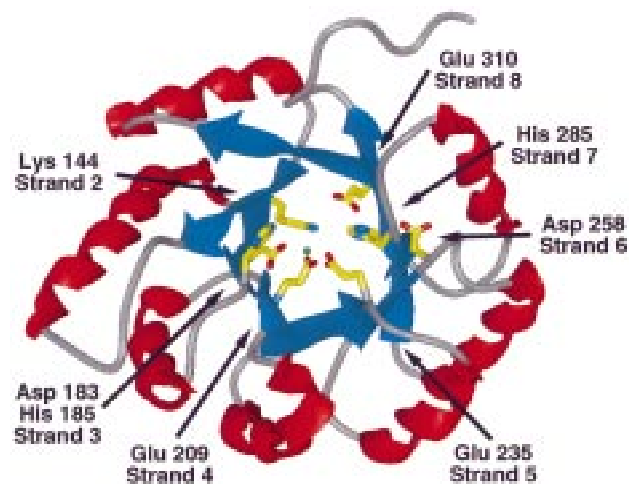

Figure 3. The $(\beta/\alpha)_8$-barrel domain of GalD.[9] Asp 183, Glu 209, and Glu 235 are metal ion ligands, the His 285−Asp 258 dyad is the general basic catalyst, Lys 144 and Glu 310 are electrophilic catalysts, and His 185 is the "new" general acidic catalyst that catalyzes the vinylogous β-elimination of the 3-OH group.

(13) RspA from *E. coli*, SwissProt P38104; Yin2 from *Streptomyces ambofaciens*, SwissProt P32436; and SpaA from *Streptomyces coelicolor*, PIR JC5178. RspA, Yin2, and SpaA each share ~32% sequence identity with GalD; the sequence identities relating RspA, Yin2, and SpaA are ≥60%, suggesting that these proteins may catalyze the same reaction.

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