Galactose Mutarotase: pH Dependence of Enzymatic Mutarotation†

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ABSTRACT: Here we report pH dependence of kinetic parameters for the mutarotation of α-D-glucose catalyzed by galactose mutarotase (GalM) from *Escherichia coli*. The values of k_{cat} and k_{cat}/K_m for the mutarotation of α-D-galactose were found to be 1.84×10^4 s^{-1} and 4.6×10^6 M^{-1} s^{-1}, respectively, at pH 7.0 and 27 °C. The corresponding values for α-D-glucose were 1.9×10^4 s^{-1} and 5.0×10^5 M^{-1} s^{-1}. Inasmuch as the value of k_{cat}/K_m for the reaction of α-D-galactose is 10 times that for α-D-glucose, and the diffusional rate constants should be essentially the same for the two sugars, the mutarotation of α-D-glucose should not be diffusion controlled. Therefore, pH−rate profiles should not be distorted by diffusion. The k_{cat} for the mutarotation of α-D-glucose is independent of pH. Therefore, either the enzyme−substrate complexes do not undergo ionization of catalytic groups, or the rate-limiting step is neither mutarotation nor diffusion. The profile of log k_{cat}/K_m versus pH is a distorted bell-shaped curve, with slopes of +1 on the acid side and −2 on the alkaline side. The values of pK_a are 6.0 and 7.5, and mutarotation depends on the ionization states of three functional groups in the free enzyme, one unprotonated and two protonated. On the acid side, ring opening of α-D-glucose limits the rate, and on the alkaline side, ring closure of the open-chain sugar limits the rate. A mutarotation mechanism is presented in which one of the catalytic groups shuttles a proton to and from the endocyclic oxygen and the other two shuttle protons to the anomeric oxygen atoms. In this mechanism, three catalytic groups overcome the problem of nonstereospecificity in mutarotation. The groups are postulated to be His 104, His 175, and Glu 309. Mutations of these residues grossly impair catalytic activity. Variants H104Q- and E309Q-GalM display sufficient activity to allow profiles of log k_{cat}/K_m versus pH to be constructed. Both profiles show breaks on the acid side corresponding to pK_a values of 5.8 for H104Q and 6.3 for E309Q. Apparently, ring opening of α-D-glucose limits the rate at low pHs, but ring closure does not become rate limiting at pHs up to 8.5 in reactions of these variants.

Galactose mutarotase (GalM)[1] from *Escherichia coli* (also known as aldose-1-epimerase) catalyzes the equilibration of α- and β-anomers of aldoses (*1*, *2*). The gene *galM* encodes this protein in *E. coli* and is a member of the *gal* operon (*3*). *E. coli* GalM participates in the metabolic conversion of β-D-galactose into α-D-glucose-6-P by way of the Leloir pathway (*4*). In lactose metabolism, β-galactosidase produces β-D-galactose, and the next step in galactose metabolism is phosphorylation to α-D-galactose-1-P catalyzed by galactokinase (GalK), another product of the *gal* operon. GalK requires α-D-galactose as its substrate, and GalM transforms β-D-galactose into α-D-galactose. GalM also catalyzes mutarotation of other sugars, including α-D-glucose.

A likely chemical mechanism for mutarotation by GalM would be related to that of the nonenzymatic reaction. Mutarotation of sugars in aqueous solution proceeds with general and specific acid or/and base catalysis of ring opening, followed by general and specific base or/and acid catalysis of ring closure to the anomer. In the nonenzymatic ring opening, base catalysis is required to remove a proton from the anomeric hydroxyl group, and acid catalysis is required to donate a proton to the endocyclic ether oxygen in the open-chain form of the sugar. Ring closure follows the microscopically reverse mechanism. In developing the concept of general acid and general base catalysis, Bronsted and Guggenheim used the mutarotation of glucose as their test system (*5*).

The acid and base catalysis of ring opening in solution is not concerted unless the acid and base groups are in the same molecule so that a two-body collision can allow both to occur simultaneously. In enzymatic catalysis, both acid and base groups are likely to be present in the correct orientations for concerted catalysis. Therefore, one expects, for the mutarotase mechanism, concerted general acid- and general base-catalyzed ring opening of the β-anomer, rotation about the C1−C2 bond of the open-chain sugar, and acid−base catalysis of ring closure to the α-anomer (*2*, *6*−*9*). Scheme 1 depicts this general mechanism.

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[1] Abbreviations: GalM, galactose mutarotase; ADA, *N*-(2-acetamido)-2-iminodiacetic acid; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-3-propanesulfonic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; GalK, galactokinase.

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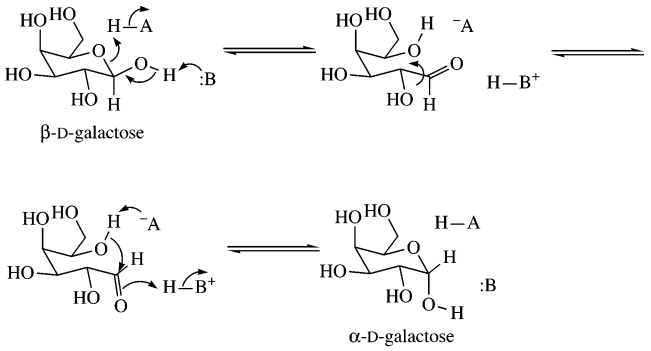
Scheme 1



Evidence from chemical modification experiments and pH dependence suggested the involvement of histidine residues in the mechanism (*2*, *10*). A sequence alignment and site-directed mutagenesis/kinetics experiments implicated His 104 and His 175 (*3*, *10*). The crystal structure of *E. coli* GalM shows that His 104 and His 175 are located in the active site, as is Glu 309.[2] The structure of the mutarotase from *Lactococcus lactis* shows a similar constellation of amino acid side chains at the active site (*11*).

In this paper, we present a pH−rate profile for the wild-type *E. coli* enzyme that differs somewhat from that presented previously (*2*). We also investigate the roles of His 104 and Glu 309 by obtaining the pH−rate profiles for H104Q- and E309Q-GalM and quantify the catalytic importance of Glu 309.

MATERIALS AND METHODS

Purification of Galactose Mutarotase. Wild-type *E. coli* GalM, H104Q-GalM, and E309Q-GalM were purified using previously described procedures (*10*). Unlike the wild-type enzyme and other mutated variants, E309Q-GalM emerged from the phenyl−Sepharose column at 3% ammonium sulfate and 10 mM HEPES (pH 7.5), rather than buffer alone.

Site-Directed Mutagenesis of Glutamate 309 to Glutamine. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene). Primers of 33 bases were used to change the Glu 309 codon (GAA) to the Gln codon (CAA). The DNA was transformed into GB87 cells (*3*, *12*) possessing a deletion in the gene for GalM. Cells incorporating these constructs were a gift from Dr. Sankar Adhya, National Cancer Institute. The nucleotide sequence of the full-length mutated gene was identical to that of *galM* (*3*) except for the change of CAA for GAA at codon 309.

Assays. The purification of the wild-type enzyme was monitored using the standard NAD and glucose dehydrogenase coupled assay (*3*, *10*, *13*). In this method, the initial rate of NADH formation by glucose dehydrogenase was measured at pH 7.5 (10 mM HEPES) in the presence of 0.05 mM α-D-glucose at 27 °C.

The pH−rate profile experiments for wild-type and H104Q-GalM were conducted using a polarimeter equipped with a water-jacketed cell maintained at 27 °C (*10*). Buffers (50 mM) were brought to the correct pH with NaOH and an ionic strength of 0.05 M with NaCl (*14*). The buffers included the following: acetate (pH 4.0, 5.0, 5.5), ADA (pH 6.0 and 6.5), HEPES (pH 7.0, 7.5, 7.75, and 8.0), and EPPS (pH 8.5). Control reactions were performed with identical buffers at 20 and 100 mM concentrations to ensure that the buffer was not inhibiting or accelerating the enzymatic or uncatalyzed reactions. The profile of pH−log k_{cat}/K_m for E309Q-GalM was measured using the coupled assay with NAD and glucose dehydrogenase. The initial rates of the background, uncatalyzed mutarotation were subtracted from the observed initial rates in all enzymatic reactions.

[2] J. G. Clifton, G. A. Petsko, et al., manuscript in preparation.

Table 1: Kinetic Parameters for Mutarotation of α-D-Glucose and α-D-Galactose[a]

substrate	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (mM)
α-D-glucose	$(1.9 \pm 0.2) \times 10^4$	$(5.0 \pm 0.6) \times 10^5$	38 ± 8
α-D-galactose	$(1.84 \pm 0.06) \times 10^4$	$(4.6 \pm 0.4) \times 10^6$	4.0 ± 0.5

[a] Parameters measured at pH 7.0 and 27 °C.

Data Analysis. Data were fitted with the KaleidaGraph (Synergy Software) curve-fitting program using eqs 1, 2 or 3, where values of y were the experimental pH-dependent values of k_{cat}/K_m and c were the pH-independent values resulting from the fitting procedure.

$$\log y = \log \frac{c}{1 + [H^+]/K_{a1} + K_{a2}/[H^+]^2} \quad (1)$$

$$\log y = \log \frac{c}{1 + [H^+]/K_a} \quad (2)$$

$$\log y = \log \frac{c}{1 + [H^+]/K_{a1} + K_{a2}/[H^+]} \quad (3)$$

RESULTS

Kinetic Parameters for the Mutarotation of α-D-*Galactose*. The interpretation of pH dependence in enzymatic reactions is simplified if diffusion is not rate limiting. To obtain information about possible rate limitation by diffusion in the action of mutarotase, the steady-state kinetic parameters for the enzymatic mutarotation of α-D-galactose and α-D-glucose were measured under the same conditions and compared. The parameters at pH 7 and 27 °C are listed in Table 1. If diffusion were rate limiting, values for the apparent second-order rate constants, k_{cat}/K_m, would be expected to be similar for α-D-galactose and α-D-glucose, which should diffuse at similar rates. However, the value of k_{cat}/K_m is about 9-fold larger for α-D-galactose. While no conclusion can be reached regarding rate limitation for the mutarotation of α-D-galactose, the results strongly suggest that the rate of mutarotation of α-D-glucose is not likely to be limited by the diffusion of the substrate. Moreover, the value of k_{cat}/K_m for the mutarotation of α-D-glucose is well below the range of 10^7-10^9 M^{-1} s^{-1} typical of the rate constants for molecules the size of sugars binding to enzymes. Therefore, the pH dependence for the mutarotation of α-D-glucose should not be complicated by diffusional effects.

pH Dependence for the GalM-Catalyzed Mutarotation of α-D-*Glucose*. For the pH−rate analysis, the α-anomer of glucose was chosen as the substrate because of the greater total change in optical rotation ($[\alpha]^t_D = 112.5°$ to $[\alpha]^t_D = 52.5°$) compared to the β-anomer ($[\alpha]^t_D = 18.7°$ to $[\alpha]^t_D =$

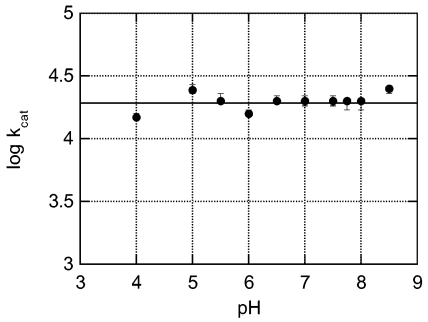


FIGURE 1: Plot of log k_{cat} against pH for wild-type GalM. Steady-state values of log k_{cat} for the mutarotation of α-D-glucose by wild-type GalM are plotted as a function of pH. Parameters were evaluated at 27 °C using buffers described in the Materials and Methods section. The average value of log k_{cat} is 4.2 ± 0.1 when fitted to a straight line and evaluated as the ordinal intercept.

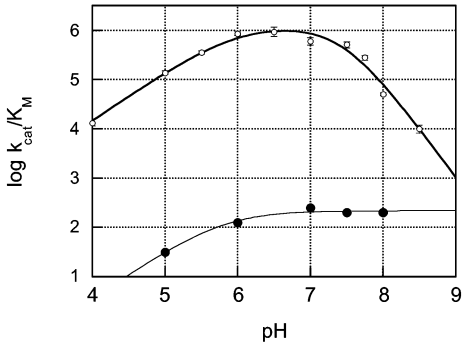


FIGURE 2: Plots of log k_{cat}/K_m against pH for wild-type GalM and H104Q-GalM. For wild-type GalM, we measured log k_{cat}/K_m as a function of pH, plotted the values against pH (○), and fitted the data to eq 1. The values of acid dissociation constants for the mechanism in Scheme 2 are $K_1 = (1.1 \pm 0.4) \times 10^{-6}$ M and $K_2 = K_3 = (3.5 \pm 0.7) \times 10^{-8}$ M. For H104Q-GalM, values of log k_{cat}/K_m measured as a function of pH are plotted against pH (●). The data are fitted to eq 2 and yield the dissociation constant $K_a = (1.7 \pm 0.3) \times 10^{-6}$.

52.5°), so that the sensitivity of the assay was maximized. Figure 1 is the plot of log k_{cat} against pH for the GalM-catalyzed mutarotation of α-D-glucose. The average measured value of k_{cat} was 19000 ± 3000 s^{-1}, and it did not display any pH dependence between pH 4 and pH 8.5. This result means that ionizations of the enzyme−substrate complexes do not exert detectable effects on the maximum rate in this pH range (*15*). Because acid−base catalysis is essential for the mutarotation of sugars, either the acid−base groups involved in catalysis are prevented from ionizing in the enzyme−substrate complexes, or the rate is limited by a conformational change. The catalytic groups could be prevented from ionizing in the Michaelis complexes if the values of their pK_as were shifted out of the range of the pH analysis.

Unlike k_{cat}, the value of k_{cat}/K_m is pH dependent. Figure 2 depicts the plot of log k_{cat}/K_m against pH, which is an unsymmetrical bell curve. The data fit eq 1, which specifies the ionization of three acid−base groups, one on the acid side and two on the alkaline side of the pH−log k_{cat}/K_m profile. Thus, k_{cat}/K_m is maximal when one group is unprotonated and two groups are protonated. The value of pK_a on the acid side is 6.0, and the data on the alkaline side correspond to a pK_a of 7.6 for both ionizing groups. The two groups cannot ionize with exactly the same acid dissociation constants, which must differ by a factor of at least 4 for statistical reasons (*16*). However, the available data do not permit an objective evaluation of two very similar values of pK_a.

Values of pK_a obtained from the pH dependence of k_{cat}/K_m correspond to ionizations of the free enzyme or free substrate and not to enzyme−substrate complexes. In this case, the substrate does not undergo ionization in the pH range under study, so the observed pH dependence must pertain to free GalM. We conclude that free GalM contains three ionizing groups that control the rate, one of which must be unprotonated and display a pK_a of 6.0 and two of which must be protonated and display the pK_a of 7.6.

pH Dependence for the H104Q-GalM-Catalyzed Mutarotation of α-D-*Glucose*. GalM contains two essential histidine residues, His 104 and His 175 (*10*). Mutation of His 175 to Asn decreases the activity to below the detectable limit (*10*), so pH−rate analysis is impossible for H175N-GalM. H104Q-GalM displays activity corresponding to 1/4000th that of wild-type GalM, and the rate cannot be saturated by increasing concentrations of α-D-glucose (*10*). Therefore, the observed rates should correspond to k_{cat}/K_m when divided by the enzyme concentration. Mutarotation of α-D-glucose by H104Q-GalM is pH dependent, and the values of log k_{cat}/K_m are plotted against pH as the lower curve in Figure 2. The plot displays a break on the acid side but not on the alkaline side. The data are fitted to eq 2 and correspond to a pK_a of 5.8, which is somewhat lower than the pK_a of the wild-type GalM on the acid side.

Importance of Glu 309. In the chemical modification of GalM, the enzyme activity proved to be sensitive to a water-soluble carbodiimide in the presence of aminomethanesulfonic acid, and the substrate provided protection against this reagent (*10*). The result was consistent with the importance of one or more acidic amino acids. To learn more about this, a Clustal W multiple sequence alignment (*17*, *18*) was performed using protein sequences of GalM (Figure 3). In a previous sequence alignment His 104 and His 175 were found to be conserved (*3*), and our results are consistent with those findings. We also found Glu 309 to be conserved, and it is located in the substrate binding pocket.[2] Consequently, we generated, expressed, and purified E309Q-GalM and examined it for activity in the standard coupled assay. The specific activity was 0.04 IU/mg of protein. This was 1/2000th the activity of wild-type GalM (79 IU/mg of protein) under the same conditions. The low activity documented the importance of Glu 309 in catalysis.

pH Dependence for the E309Q-GalM-Catalyzed Mutarotation of α-D-*Glucose*. In preliminary experiments to evaluate K_m in mutarotation catalyzed by E309Q-GalM, we found that the initial rate could not be saturated at increasing concentrations of α-D-glucose. Therefore, like H104Q-GalM, K_m for E309Q-GalM proved to be very high and was not evaluated. Instead, the initial rates of mutarotation of α-D-glucose were measured at a low concentration as a function of pH. From the data, values of k_{cat}/K_m were calculated and plotted as log k_{cat}/K_m against pH. The data were fitted to eqs 2 and 3 with the results shown in Figure 4. The fit to eq 3 is clearly superior to the fit to eq 2, indicating that two ionizing groups in free GalM control the rate. The values of pK_a arising from the fit of data to eq 3 are 6.2 ± 0.03 on the acid side and 8.7 ± 0.09 on the alkaline side. When fitted to eq 2, the

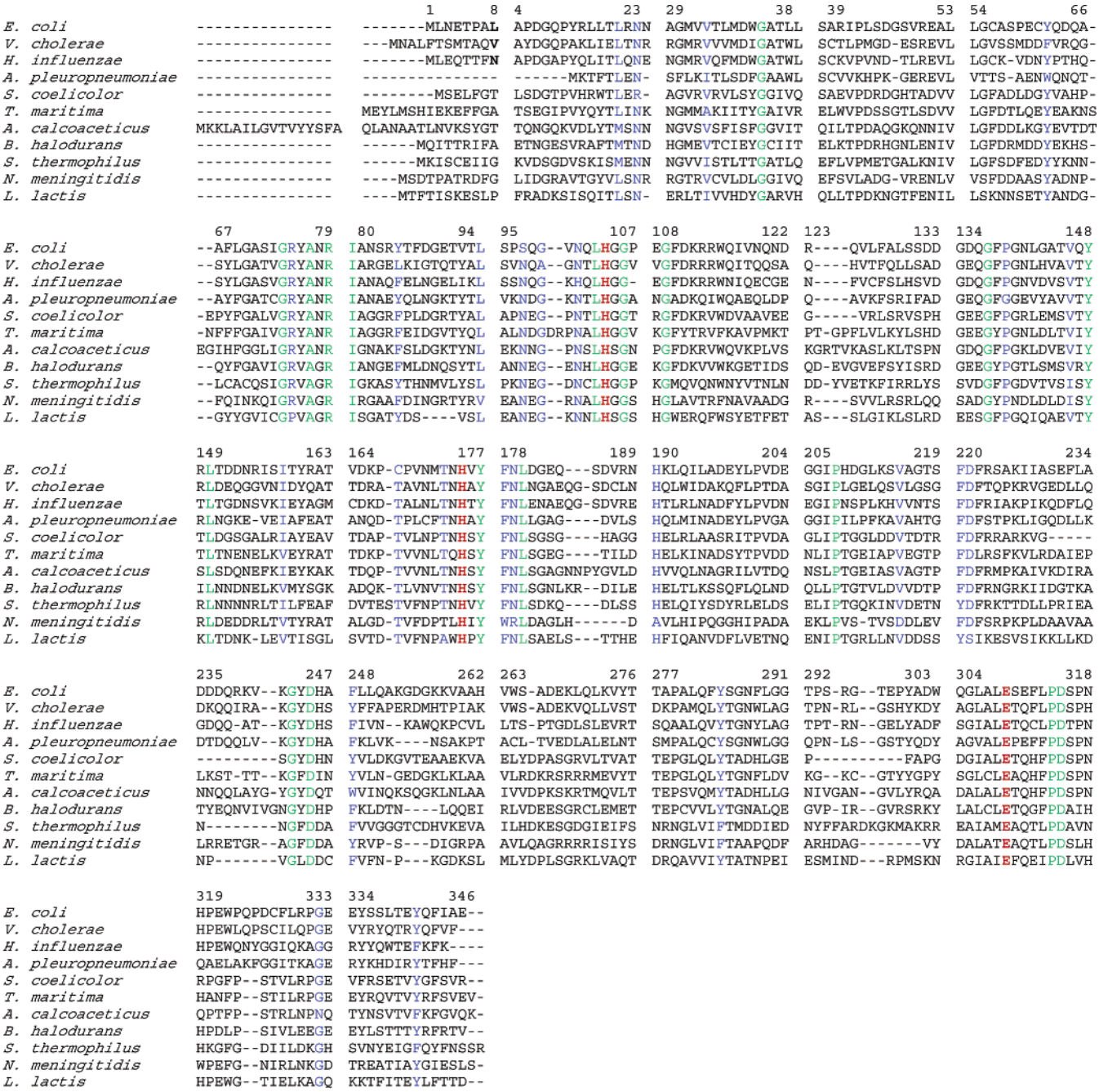


FIGURE 3: Multiple sequence alignment of 11 identified mutarotase proteins. The following mutarotase enzymes were used (accession numbers in parentheses): *Escherichia coli* (P40681), *Vibrio cholerae* (AAF94748), *Haemophilus influenzae* (C64096), *Actinobacillus pleuropneumoniae* (AAB37129), *Streptomyces coelicolor* (CAB62725), *Thermotoga maritima* (H72395), *Acinetobacter calcoaceticus* (A29277), *Bacillus halodurans* (BAB06474), *Streptococcus thermophilus* (B44509), *Neisseria meningitidis* (CAB85315), and *Lactococcus lactis* (AAD20257). Histidine 104, histidine 175, and glutamic acid 309 are colored red, while other fully conserved residues are in green. A MULTILIN multiple alignment using the pole Bio-Informatique Lyonnais was performed to identify residues >90% conseved as well as the IV, LM, and FY conserved positions, and they are colored in blue.

evaluated pK_a is 6.2 ± 0.08. Both fits give the same value of pK_a on the acid side.

DISCUSSION

pH Dependence of Wild-Type GalM. We conclude, on the basis of the present results, that three Bronsted acid−base groups are required to catalyze mutarotation at the active site of GalM. One group must initially be in its unprotonated, conjugate base state, and two must be protonated in the free enzyme. We further conclude that ionizations of the enzyme−substrate complexes in the range of pH 4−8.5 do not affect the activity of GalM. That is, the acid−base groups that participate in catalysis undergo ionization in the free enzyme over this pH range but not in the enzyme−substrate complexes. The results are compatible with the kinetic mechanism in Scheme 2.

The acid limb of the log(k_{cat}/K_m)−pH profile is governed by K_1, the activity is maximal at pHs above pK_1, and the value of pK_1 is 6.0. The alkaline side of the profile is governed by K_2 and K_3, and activity is maximal when these two groups are in their acidic forms. The data in Figure 2 are fitted well to eq 1, and the log form of eq 5 for k_{cat}/K_m

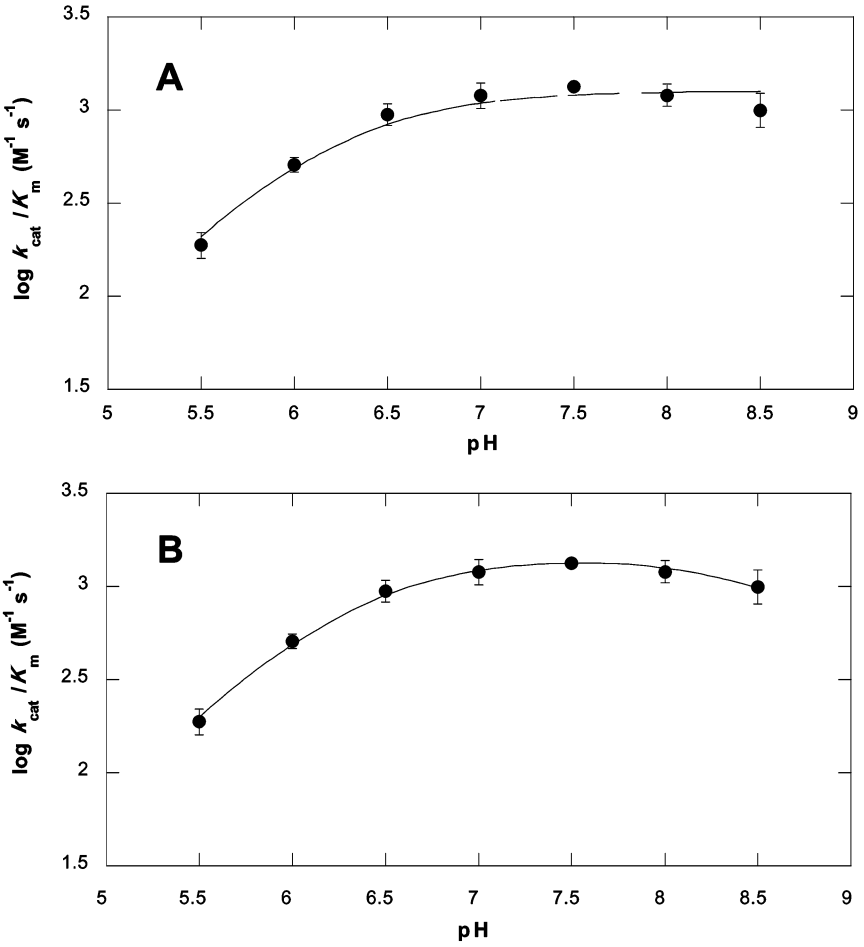
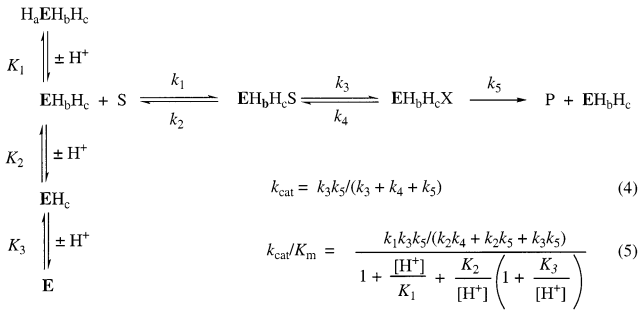


FIGURE 4: pH dependence for the activity of E309Q-GalM. The values of log k_{cat}/K_m for E309Q-GalM measured as a function of pH are plotted against pH in parts A and B. (A) The data are fitted to eq 2 and yield the value of pK_a = 6.2. (B) The data are fitted to eq 3 and yield the values of pK_{a1} = 6.2 and pK_{a2} = 8.7.

Scheme 2



$$k_{cat} = k_3k_5/(k_3 + k_4 + k_5) \quad (4)$$

$$k_{cat}/K_m = \frac{k_1k_3k_5/(k_2k_4 + k_2k_5 + k_3k_5)}{1 + \frac{[H^+]}{K_1} + \frac{K_2}{[H^+]}\left(1 + \frac{K_3}{[H^+]}\right)} \quad (5)$$

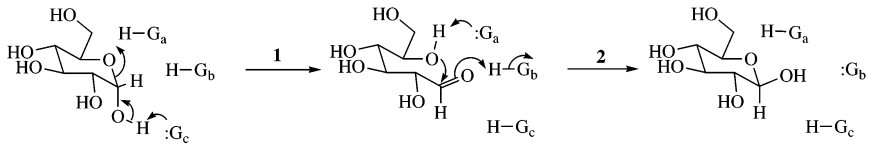
in Scheme 2 becomes equivalent to eq 1 when the values of pK_2 and pK_3 are the same. The values of K_2 and K_3 must differ by at least a factor of 4 for statistical reasons (*16*); however, the available data are insufficient to evaluate the difference from the plot in Figure 2. As shown by eq 4 in Scheme 2, the kinetic mechanism is also consistent with the pH independence of k_{cat} in Figure 1.

The results are compatible with stereochemical requirements for mutarotation at the active site of an enzyme. Mutarotation intrinsically includes a nonstereospecific process, and this presents a mechanistic barrier in an enzymatic site. Being chiral, enzymatic active sites catalyze reactions with a high degree of stereospecificity. Mutarotases, epimerases, and racemases operate by mechanisms that overcome the barriers to nonstereospecificity. In the case of GalM, it appears that this is accomplished by the actions of three acid−base residues, perhaps as outlined in the mechanism of Scheme 3 for the transformation of α- to β-anomers.

In Scheme 3 the acid−base catalysts are designated G_a, G_b, and G_c, and each has a specific function. G_a facilitates proton transfer to the endocyclic oxygen and proton abstraction from C5(OH) of the open-chain sugar intermediate. G_b facilitates proton transfer to and from the β-anomeric OH, and G_c catalyzes proton transfer to and from the α-anomeric OH. The reciprocating actions of G_b and G_c solve the stereochemical problem of nonstereospecific proton transfer at the anomeric center.

The mechanism in Scheme 3 is consistent with the profile of log k_{cat}/K_m versus pH in Figure 2 for wild-type GalM. The two legs of the profile are likely to represent a change

Scheme 3



in rate-limiting step on going from low to high pH or vice versa. At low pHs, glycosyl ring opening in step 1 probably limits the rate because the required base catalyst (G_c in Scheme 3) becomes protonated in the free enzyme at low pHs and would not be able to abstract another proton. At high pHs, ring closure of the aldehydic sugar in step 2 limits the rate because the two acid catalysts (G_b and G_c in Scheme 3) undergo ionization to their conjugate bases in the free enzyme.

An alternative to the mechanism in Scheme 3 is that two of the three catalytic groups function in acid−base catalysis, one in its protonated and another in its unprotonated form, and the third group must be in its protonated form for the substrate to bind. This latter mechanism is less satisfying from a stereochemical perspective because it would require a single catalytic group to interact as an acid−base catalyst with the α-anomeric OH group of α-D-glucose and the β-anomeric OH of β-D-glucose. However, such a mechanism cannot be excluded by the available data.

The three acid−base residues in GalM are postulated to be His 104, His 175, and Glu 309, all of which are in the active site and in contact with the substrate (*10*).[2] Because histidine and glutamate can ionize in the same pH range in enzymatic sites, the pH dependence for wild-type GalM does not allow the assignment of these groups in the mechanism of Scheme 3. However, the forthcoming crystal structures may shed light on the matter.

pH Dependencies of H104Q- and E309Q-GalM. In considering the behavior of a mutated form of GalM in which one of the catalytic groups is neutralized, it is necessary to recognize that an important step in the mechanism must take place without participation by the usual catalytic group. Most likely, another group at the active site takes over the function of the neutralized group. The group taking over may be one of the three required for the action of the wild-type GalM. In this scenario, one of the three catalytic groups may do double duty in the variants H104Q- and H309Q-GalM. For example, in step 1 of Scheme 3 two acid groups are normally present, $H-G_a$ and $H-G_b$. If $H-G_a$ is absent, step 1 may still occur if $H-G_b$ can take over the function of acid catalysis of ring opening. However, this would generate G_b at the wrong protonation level for step 2, which requires $H-G_b$ in Scheme 3. If $H-G_c$ can take over proton transfer to the β-anomeric oxygen in step 2, then G_b can abstract the proton from C5(OH) in place of G_a in step 2. In this case, G_b does double duty for G_a. Analogous scenarios can be constructed for the other variants.

A specific mutation of one of the three catalytic residues may lead to a change in the overall electrostatic charge at the active site relative to the wild-type GalM. Because two of the three catalytic residues must be protonated and the third unprotonated, and the residues are His 104, His 175, and Glu 309, the net charge of these residues in the most active form of free GalM must be +1. In the case of H104Q-GalM, replacement of His 104 by glutamine is likely to decrease the net charge of the most active form to zero (0). If a net charge of +1 were maintained in the variant, both catalytic groups would have to exist in their conjugated acid forms in neutral solution, and there would be no base to initiate the mechanism by abstracting a proton from an anomeric hydroxyl group. Moreover, there would be no group to undergo ionization at lower pHs, contrary to the requirements of the pH−rate profile in Figure 2. In the case of E309Q-GalM, the net charge may be retained at +1, which allows both an acid and a base at the active site, His 104 and His 175, as required for mutarotation and in agreement with the pH−rate profile in Figure 4.

The replacement of His 104 with glutamine greatly lowers the activity of GalM and also changes the pH−rate profile for k_{cat}/K_m. The acid leg of the wild-type profile is retained, but the alkaline leg is absent in the profile for H104Q-GalM. This likely results from a difference in rate limitation at high pHs in the mutated enzyme relative to the wild-type enzyme. The lower curve in Figure 2 suggests that ring closure never becomes rate limiting at higher pHs in the reaction of H104Q-GalM. Furthermore, the overall rate is seriously impaired by the mutation because the maximum value of k_{cat}/K_m for H104Q-GalM is less than 1/4000th that for wild-type GalM. In mutarotation, ring opening is intrinsically more difficult than ring closure, as shown by the predominance of the ring-closed α- and β-anomers of glucose at equilibrium (37% and 63%, respectively). It is possible that uncatalyzed ring closure could be occurring at high pHs in the reaction of H104Q-GalM. It seems clear that His 104 participates in an important way in the ring opening step 1 in Scheme 3, and its absence slows this step. Then, ring closure in step 2 does not become rate limiting within the accessible pH range.

The similarity between the values of pK_a for the acid legs of the two profiles in Figure 2 for wild-type (pK_a 6.0) and H104Q-GalM (pK_a 5.8) does not justify assignment to the same residue in the two proteins. This is because of the difference in net electrostatic charge at the active sites in wild-type (+1) and H104Q-GalM (0). The electrostatic difference is likely to lead to significant perturbations in the ionization constants of His 175 and Glu 309.

The pH dependence of E309Q-GalM suggests that the two histidine residues in the active site of this variant display pK_a values of 6.2 and 8.7. This difference is reasonable for two basic groups in proximity, where electrostatic repulsion between two closely spaced imidazolium groups would depress one of the dissociation constants. In these circumstances, one histidine residue could serve as the base catalyst and the other as the acid catalyst in the mechanism of Scheme 1, consistent with the pH−rate profile in Figure 4.

Correlation of Present and Past Results. In the earlier pH−rate study, a bell-shaped profile for log *V/K* was observed with less complete data than presented in this paper that did not distinguish the slope of −2 on the alkaline side (*2*). The present finding of two ionizing groups on the alkaline side is well correlated with the required catalytic groups and the stereochemical imperative for the mechanism. In the earlier work, a break on the acid side in the plot of log *V* against pH indicated an ionization of an enzyme−substrate complex. This was not observed in the present work, and we have no explanation for this discrepancy.

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