Bioorganic Chemistry **29,** 146–155 (2001)
doi:10.1006/bioo.2001.1206, available online at http://www.idealibrary.com on IDEAL®

Effect of an E461G Mutation of β-Galactosidase (*Escherichia coli, lac Z*) on pL Rate Profiles and Solvent Deuterium Isotope Effects[1]

John P. Richard,*,[2] Reuben E. Huber,† and Deborah A. McCall*

**Department of Chemistry, University at Buffalo, SUNY, Buffalo, New York 14260-3000 and †Division of Biochemistry, Faculty of Science, University of Calgary, Calgary, Alberta, TN2 IN4, Canada*

Received October 13, 2000

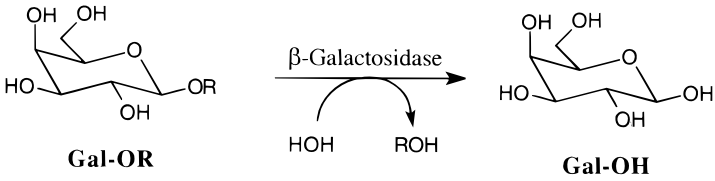
An E461G mutation of β-galactosidase results in the disappearance of the high pL (L = H, D) downward break in the rate profiles for k_{cat}/K_m for wild-type enzyme-catalyzed hydrolysis of 4-nitrophenyl β-D-galactopyranoside (Gal-OPNP) and a decrease from $(k_{cat})_{HOH}/(k_{cat})_{DOD}$ = 1.7 to $(k_{cat})_{HOH}/(k_{cat})_{DOD}$ = 1.2 in the solvent deuterium isotope effect. These observations provide evidence that the propionic acid side chain of Glu 461 is protonated at catalytically active free β-galactosidase and they are consistent with a role for this residue in Brønsted acid catalysis at the leaving group. The earlier observation that this same E461G mutation results in the loss of a downward break at high pH in the rate profile for k_s for transfer of the β-D-galactopyranosyl group from β-galactosidase to water cannot be simply explained by a mechanism in which the single side chain of Glu 461 functions to provide general acid catalysis in the rate limiting step for formation of the β-D-galactopyranosyl intermediate and general base catalysis of breakdown of this intermediate. Evidence is presented that there may be different catalytic mechanisms, with different roles for the side chain for Glu-461, for nucleophilic addition of water and of small alkyl alcohols to the β-D-galactopyranosyl reaction intermediate.

INTRODUCTION

The mechanism for enzymatic catalysis of glycoside cleavage, exemplified by β-galactosidase catalyzed cleavage of glycosides (Scheme 1), is defined by the imperatives for catalysis of nucleophilic substitution of a poor alkoxide ion leaving group at glycosides, and these imperatives will probably ensure the presence of the following essential catalytic residues or metal cofactors at all enzymes that catalyze glycosyl transfer with retention of configuration at the glycosidic carbon: (1) A nucleophilic residue that participates either by providing assistance to expulsion of the leaving group from the anomeric carbon, and/or electrostatic stabilization of an oxocarbenium

[1] This work was supported by a grant from the National Institutes of Health (GM 39754).
[2] To whom correspondence and reprint requests should be addressed. Fax: (716) 645-6963. E-mail: jrichard@chem.buffalo.edu.

0045-2068/01 $35.00

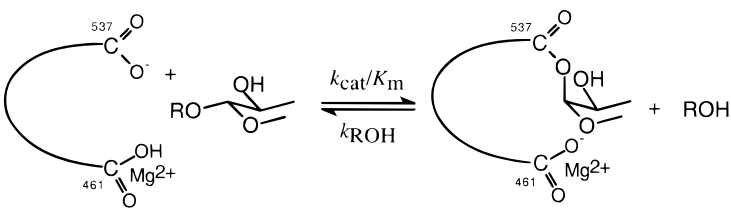


SCHEME 1.

ion reaction intermediate. (2) An acidic residue (*e.g.*, carboxylic acid or a metal ion) that provides stabilization of negative charge at the oxygen leaving group.

A consideration of such imperatives for enzyme catalysis is essential to assigning roles to the active amino-acid side chains. However, the problem of proving tentative assignments is particularly difficult when several groups with the capacity to serve the same function are identified at the active site. This is the case for β-galactosidase-catalyzed cleavage of glycosides (Scheme 1), where the following active site side chains and a metal cofactor have the potential to provide Brønsted acid/base catalysis of glycoside cleavage/synthesis: (a) the phenol group of Tyr 503 (*1*, *2*); (b) the propionic acid side chain of Glu 461 (*3*, *4*); and (c) an enzyme-bound Mg^{2+} cofactor (*5*, *6*). The results of studies on E461G and E461Q mutant forms of β-galactosidase provide evidence that the propionic acid side chain of Glu-461 acts as a Brønsted acid to catalyze cleavage of the bond to the oxygen leaving group (*7*) and as a Brønsted base to catalyze the formation of this bond (*8*). However, the observed differential activation of β-galactosidase by Mg^{2+} for cleavage of substrates with anionic oxygen leaving groups and neutral nitrogen leaving groups is consistent with a role for the metal cofactor in stabilizing negative charge at alkoxide ion leaving groups (*6*); and, the results of two-dimension NMR studies and molecular modeling to determine the conformation of enzyme-bound C-lactose, a stable substrate analog, have been interpreted within the context of a mechanism where the phenol side chain of Tyr 503 provides Brønsted acid catalysis of cleavage of the physiological substrate lactose (*9*).

We report here the results of further experiments on E461G β-galactosidase designed to test two predictions for the effect of excising a propionic acid side chain from the enzyme which functions to donate a proton to the nitrophenoxide ion leaving group during enzyme-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside (Gal-OPNP, Scheme 2).



SCHEME 2.

(1) The falloff in k_{cat}/K_m at high pH for wild-type β-galactosidase-catalyzed hydrolysis of Gal-OPNP observed in earlier work is consistent with a pK_a of 8.3 for an essential amino-acid residue at the free enzyme (*10*). If this downward break is due to deprotonation of the carboxylic acid side chain of Glu 461, then a flat pH rate profile at high pH should be observed for the reaction catalyzed by the E461G enzyme.

(2) A solvent deuterium isotope effect (SDIE) of $(k_{cat})_{HOH}/(k_{cat})_{DOD} = 1.7$ has been reported for wild-type β-galactosidase-catalyzed cleavage of Gal-OPNP (*10*). If this isotope effect results from loss of the zero-point energy for a solvent-derived proton at a transition state in which there is partial proton-transfer from the propionic acid side chain of Glu 461 to the oxygen leaving group, then removal of the proton from the transition state for the reaction catalyzed by E461G β-galactosidase should reduce the SDIE to around 1.0.

We report the observation of both of these results for E461G β-galactosidase. These data provide classical evidence that Glu-461 participates directly in Brønsted acid/base catalysis at the leaving group. However, their interpretation is complicated by the earlier observation that the E461G mutation also results in the disappearance of the downward break on the pH rate profile for transfer of the β-D-galactopyranosyl group from β-galactosidase to the solvent water. Evidence is presented that there may be different catalytic mechanisms with different roles for the side chain for Glu-461, for nucleophilic addition of water and small alkyl alcohols to the β-D-galactopyranosyl reaction intermediate.

MATERIALS AND METHODS

Reagent grade organic and inorganic chemicals were obtained from commercial sources and were used without further purification. Water was distilled and passed through a Milli-Q water purification system. 4-Nitrophenyl β-D-galactopyranoside (Gal-OPNP) and β-galactosidase (Grade VIII from *Escherichia coli*.) were purchased from Sigma. E461G β-galactosidase was prepared and purified by a published procedure (*3*). Galactose dehydrogenase from *E. coli* that contains the gene for the *Pseudomonas fluoroesens* enzyme on a plasmid was purchased from Sigma. Deuterium oxide (99.9%) was purchased from Cambridge Isotope Laboratories. Solution pH was determined using an Orion Model 601A pH meter equipped with a Radiometer GK2321C combination electrode that was standardized at pH 7.00 and 10.00. Values of pD were obtained by adding 0.40 to the observed pH meter reading (*11*). The methods for the routine assays of galactose dehydrogenase and of β-galactosidase-catalyzed cleavage of Gal-OPNP and trifluoroethyl β-D-galactopyranoside (Gal-OTFE) have been described in earlier work (*12, 13*).

pH and pD rate profiles. E461G β-galactosidase was assayed at 25°C in buffered solutions of H_2O or D_2O that contained $MgCl_2$ and sufficient NaCl to maintain ionic strength at 0.18 $\pm$ 0.02. The following buffer and magnesium concentrations were used: (A) 33 mM sodium 2-(*N*-morpholino)ethanesulfonate (MES) that contains 10 mM $MgCl_2$ (pD 6.9); (B) 33 mM sodium phosphate that contains 5 mM $MgCl_2$ (pD 7.4, 7.9 and 8.4); (C) 15 mM pyrophosphate that contains 5 mM $MgCl_2$ (pD 8.9 and 9.4); and, (D) 33 mM sodium carbonate that contains 5 mM $MgCl_2$ (pD 9.9).

Values for the difference in the extinction coefficients of Gal-OPNP and the products of β-galactosidase-catalyzed hydrolysis of Gal-OPNP, $(\Delta\varepsilon_{405})_{obs}$, were determined at

12 different pH between pH 5.6 and 9.5 and at 12 different pD between pD 5.5 and 9.5 from the change in absorbance at 405 nm for β-galactosidase hydrolysis of known concentrations of Gal-OPNP. The fit of the values of $(\Delta\varepsilon_{405})_{obs}$ to a titration curve for 4-nitrophenol in H_2O gave values of 7.05 for the pK_a for nitrophenol and of $(\Delta\varepsilon_{405})_{max} = 18{,}400\ M^{-1}\ cm^{-1}$ and $(\Delta\varepsilon_{405})_{min} = 0\ M^{-1}\ cm^{-1}$ for the maximum and minimum changes in extinction coefficient observed at high and low pH, respectively. A similar fit for data in D_2O gave values of p$K_a = 7.60$, $(\Delta\varepsilon_{405})_{max} = 17{,}900\ M^{-1}\ cm^{-1}$ and $(\Delta\varepsilon_{405})_{min} = 0\ M^{-1}\ cm^{-1}$.

β-Galactosidase-catalyzed hydrolysis of 4-nitrophenyl β-D-galactopyranoside was monitored by following the increase in absorbance at 405 nm from formation of 4-nitrophenoxide anion. The observed initial velocities for these reactions (v_{obsd}) were calculated from the change in the molar extinction coefficient ($\Delta\varepsilon_{405}$) for hydrolysis of the substrate Gal-OPNP at each pH or pD, where the values for $\Delta\varepsilon_{405}$ were calculated from the experimentally determined values of $(pK_a)_{app}$ for 4-nitrophenol and $(\Delta\varepsilon_{405})_{max}$ (see above).

It has been shown that the observed velocity for cleavage of Gal-OPNP catalyzed by preparations of E461G β-galactosidase is the sum of the velocities for catalysis of this reaction by E461G β-galactosidase (v_{mut}) and the wild-type enzyme (v_{wt}), which is present at a low level as a *contaminant* of our preparation of E461G β-galactosidase (*7*). This wild-type enzyme presumably forms by spontaneous reversion of the engineered glycine mutation to glutamic acid (*14*). The mutant enzyme shows no detectable activity toward cleavage of Gal-OTFE (*7*). The concentration of wild-type enzyme in this preparation of E461G β-galactosidase ($[E]_{wt} = 0.012\%$ of the concentration of the mutant enzyme) was determined from the observed velocity for catalysis of cleavage of a saturating concentration of Gal-OTFE at pH 8.6 using $k_{cat} = 410\ s^{-1}$ for the wild-type enzyme-catalyzed reaction (*7, 12*).

$$v_{obsd} - v_{wt} = v_{mut} = \frac{V_{max}[S]}{K_m + [S]} \qquad [1]$$

$$v_{wt} = \frac{k_{cat}[E]_{wt}}{K_m + [S]} \qquad [2]$$

The velocity of E461G β-galactosidase catalyzed cleavage of Gal-OPNP (v_{mut}, [Eq. 1]) was determined by correcting the observed velocity (v_{obsd}) for the presence of wild-type enzyme (v_{wt}, Eq. [1]). Values of v_{wt} were determined according to Eq. [2] using published values for the kinetic parameters k_{cat} and K_m for the wild-type enzyme-catalyzed reaction under the given reaction conditions in H_2O and D_2O (*10*). Values for K_m and V_{max} for E461G β-galactosidase catalyzed hydrolysis of Gal-OPNP were determined from the nonlinear least squares fit of 6–8 values of v_{mut} to Eq. [1] using SigmaPlot from Jandel Scientific. Values of k_{cat} for the E461G enzyme-catalyzed reaction were calculated from the relative values of V_{max} and the published values of $k_{cat} = 0.10\ s^{-1}$ for cleavage of Gal-OPNP by β-galactosidase at pH 8.6 (*7*).

RESULTS

The observed initial velocities for cleavage of Gal-OPNP catalyzed by E-461G mutant β-galactosidase at 25°C have been corrected for the reaction catalyzed by the small amount (0.012%) of wild-type enzyme present in this mutant preparation (*7*). This correction corresponds to 15–30% of v_{obsd} depending upon substrate concentration. The difference in the correction for enzyme-catalyzed reactions in H_2O and in D_2O does not affect the shape of the pH and pD rate profiles in these two solvents.

$$k_{cat}/K_m = \left(\frac{(k_{cat}/K_m)_{lim}}{1 + [H^+]/K_a}\right) \quad [3]$$

Figure 1A shows logarithmic pH and pD rate profiles for values of $(k_{cat})_{obsd}$ for E461G β-galactosidase catalyzed hydrolysis of GalOPNP at 25°C in H_2O and D_2O; Fig. 1B shows the pH and pD rate profiles for values of k_{cat}/K_m. The values for k_{cat} in H_2O and D_2O are compared directly in Table 1. The pL (L = H, D) rate profiles for k_{cat} are nearly flat (Table 1), and an average SDIE of $(k_{cat})_{HOH}/(k_{cat})_{DOD} = 1.2$ was determined as the average of the isotope effects observed at pH, pD = 7.0, 7.5, 8.0, 8.6 (Table 1). The small decrease in the SDIE to 0.95 at pL = 9.5 probably reflects the onset of a downward break in the profiles for k_{cat}, which is expected to

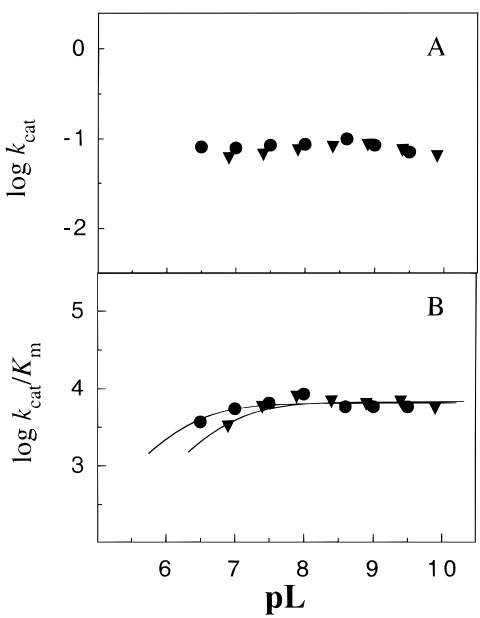
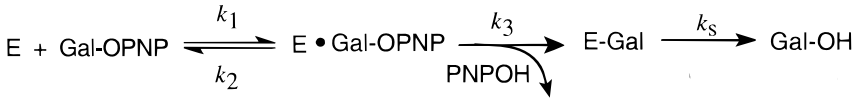


FIG. 1. pL rate profiles for the kinetic parameters k_{cat} and k_{cat}/K_m for E461G β-galactosidase-catalyzed cleavage of Gal-OPNP at 25°C. (A) ●, pH rate profile for k_{cat}; ▼, pD rate profile for k_{cat}. (B) ●, pH rate profile for k_{cat}/K_m; ▼, pD rate profile for (k_{cat}/K_m). The solid lines show the nonlinear least squares fit of the experimental data to Eq. [3] using the values of $(k_{cat}/K_m)_{lim}$ and K_a given in the text.



SCHEME 3.

occur at a lower pH than pD due to the higher pK_as of most acids in D_2O compared with H_2O (*15*).

The pL rate profiles for k_{cat}/K_m (Fig. 1B) are flat at high pL, but decrease about 0.3 log units from the maximum on moving to the lowest pL. The solid lines show the nonlinear least-squares fit of these experimental data to the logarithmic forms of Eq. [3] using values of $(k_{cat}/K_m)_{lim} = 425\ M^{-1}$ and $K_a = 10^{-6.3}$ M for the reaction in H_2O and $(k_{cat}/K_m)_{lim} = 420\ M^{-1}\ s^{-1}$ and $K_a = 10^{-6.9}$ M for the reaction in D_2O. These data give a value of 1.0 for the SDIE on $(k_{cat}/K_m)_{lim}$ for cleavage of Gal-OPNP by E461G β-galactosidase.

DISCUSSION

The rate-determining step for wild-type and E461G β-galactosidase catalyzed hydrolysis of Gal-OPNP is transfer of the β-D-galactopyranosyl group from substrate to Glu-537 to form a β-D-galactopyranosyl reaction intermediate (k_3, Scheme 3) (*16, 17*). The downward breaks at low pL observed for the rate profiles for k_{cat}/K_m for wild-type β-galactosidase-catalyzed cleavage of Gal-OPNP in H_2O and D_2O (*10*) are also seen in the corresponding pL rate profiles for E461G β-galactosidase (Fig. 1B). The downward breaks at pL 8.3 and 8.8 for reactions in H_2O and D_2O (*10*), respectively, observed for the rate profiles of k_{cat}/K_m for wild-type β-galactosidase-catalyzed hydrolysis of Gal-OPNP are consistent with the presence of an essential acidic residue

TABLE 1

Values of k_{cat} for E461G β-Galactosidase-Catalyzed Hydrolysis of Gal-OPNP in H_2O and D_2O at 25°C[a]

pH	k_{cat}/ s^{-1} [a]	pD	k_{cat}/ s^{-1} [a]	$(k_{cat})_{HOH}/(k_{cat})_{DOD}$[b]
6.5	0.081			
7.0	0.079	6.9	0.062	1.27
7.5	0.085	7.4	0.066	1.29
8.0	0.087	7.9	0.074	1.17
8.6	0.10	8.4	0.080	1.25
9.0	0.085	8.9	0.085	1.0
9.5	0.071	9.4	0.074	0.96
		9.9	0.063	

[a] Determined using the value of $k_{cat} = 0.10\ s^{-1}$ for the reaction at pH 8.6 in water *(7)* and the ratio of the values of V_m determined for hydrolysis of Gal-OPNP catalyzed by a constant concentration of β-galactosidase at pH 8.6 and at the given pH or pD.

[b] The ratio of the values of k_{cat} determined for reactions in HOH and DOD.

whose pK_a increases by 0.5 units with the change from H_2O to D_2O (*14*). The deletion of the propionic acid side chain of Glu-461 at E461G β-galactosidase results in pH and pD rate profiles for k_{cat}/K_m which are flat at high pL (Fig. 1B). The simplest explanation for this result is that the downward breaks observed at high pH and pD for wild-type enzyme are due to deprotonation of the essential carboxylic acid side chain of Glu 461.

Downward breaks at pL 9.2 and 9.7, respectively, were observed for the pL rate profiles for k_{cat} for β-galactosidase-catalyzed cleavage of Gal-OPNP in H_2O and D_2O (*10*). The correspoding pL rate profiles of k_{cat} for E461G β-galactosidase in H_2O and D_2O are nearly flat (Fig. 1A); but, the $\approx$30% decrease in k_{cat} observed at pH 9.5 may mark the onset of a downward break in this pH rate profile. The pH here is significantly higher than pH 9.2, which is the *center* of the downward break for k_{cat} for the wild-type enzyme-catalyzed reaction in H_2O. We do not know the explanation for the small falloff in k_{cat} at high pH observed for E461G β-galactosidase.

The SDIE of $(k_{cat})_{HOH}/(k_{cat})_{DOD} = 1.7$ for the wild-type enzyme catalyzed reaction shows that there is a significant decrease in the zero-point energy for a solvent-derived proton(s) that occurs on proceeding from the ES complex to the transition state for formation of the β-D-galactopyranosyl reaction intermediate (*10*). The decrease in the SDIE on k_{cat} to 1.2 for the E461G enzyme-catalyzed reaction is consistent with the conclusion that the most of the SDIE on the wild-type enzyme-catalyzed reaction represents loss of zero-point energies of the carboxylic acid proton at a transition state in is partly transferred to the 4-nitrophenoxide ion leaving group.

The difference in the SDIE on $(k_{cat})_{HOH}/(k_{cat})_{DOD} = 1.2$ and $(k_{cat}/K_m)_{HOH}/(k_{cat}/K_m)_{DOD} = 1.0$ for E461G β-galactosidase catalyzed hydrolysis of Gal-OPNP is smaller than the corresponding difference of $(k_{cat})_{HOH}/(k_{cat})_{DOD} = 1.7$ and $(k_{cat}/K_m)_{HOH}/(k_{cat}/K_M)_{DOD} = 1.0$ determined for wild-type β-galactosdase (*10*). The difference in the SDIE on k_{cat}/K_m and k_{cat} for the wild-type enzyme might reflect either the isotope effect on the dissociation constant K_d for substrate release (*18*) or a mechanism where substrate binding is partly rate-determining for k_{cat}/K_m, but not for k_{cat} (*19*). The change to a smaller difference between the SDIE on k_{cat}/K_m and k_{cat} for the E461G β-galactosidase might then represent an effect of this mutation on the SDIE for the dissociation constant K_d; however, we are not aware of any precedent for such an effect of a point mutation of the SDIE for substrate binding. If binding of Gal-OPNP were partially rate-limiting for k_{cat}/K_m for the reaction catalyzed by the wild-type enzyme, then the E461G mutation should result in a change to a rate-determining chemical step for k_{cat}/K_m, because the mutation will affect mainly the rate constants for the reaction of the bound substrate, rather than for substrate binding. This would have the effect of causing the SDIE on k_{cat} and k_{cat}/K_m to move toward the same value, because the same chemical step would limit the values of each kinetic parameter.

Other results. The pH rate profile for k_{cat} for E461G β-galactosidase-catalyzed cleavage of *o*-nitrophenyl β-D-galactopyranoside has been reported in earlier work (*3*) for reactions where the rate-determining step is hydrolysis of the β-D-galactopyranosyl intermediate (k_s, Scheme 3) so that $k_s \approx k_{cat} = 0.7\ s^{-1}$ (Scheme 3). The pH rate profiles for k_s for wild-type and E461G β-galactosidase are each flat at low pH (10).

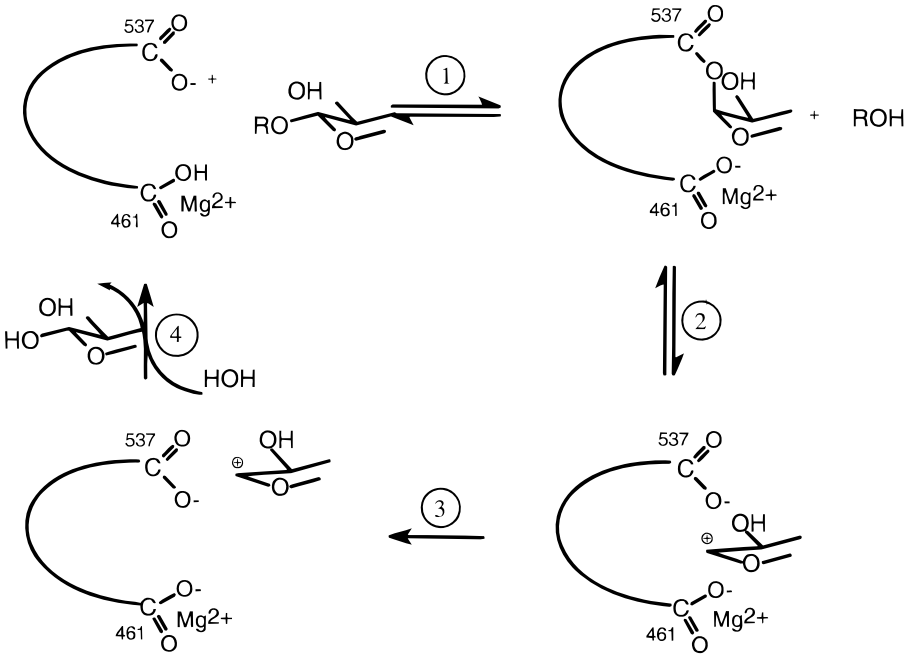
However, the pH rate profile for the wild-type enzyme shows a downward break at pH 8.8, while the profile for the mutant enzyme is flat at high pH (3).

One interpretation for the effect of the E461G mutation on the pH rate profile for k_s, which was favored for the effect of this mutation on the pL rate profiles for k_{cat}/K_m for hydrolysis of Gal-OPNP (see above), is that Glu 461 must be protonated at the transition state for k_s for the wild-type enzyme. In this case Glu-461 would be protonated in both the ground-state for formation (k_3) and hydrolysis (k_s) of the β-D-galactopyranosyl intermediate. This explanation would require a reinterpretation of experimental evidence cited in earlier work to support a role for Glu-461 in Brønsted acid/base catalysis at the leaving group/nucleophile for the reversible cleavage of Gal-OR (*7, 8*), because this mechanism requires that Glu-461 be protonated in the free enzyme, where it serves as a general acid to stabilize the transition state for cleavage of Gal-OR and deprotonated (basic form) at the β-D-galactopyranosyl intermediate, where it serves to provide Brønsted base catalysis of transfer of the intermediate to alkyl alcohols (Scheme 2).

There are at least two explanations for the effect of the E461G mutation on the pH rate profile for k_s: (1) The E461G mutation causes an upward perturbation in the pK_a of a second amino acid residue which must be protonated to observe full activity for hydrolysis of the β-D-galactopyranosyl intermediate. (2) The E461G mutation eliminates the effect of deprotonation of this second amino acid residue on k_{cat}. We cannot offer a mechanistic rationale for this effect of the E461G mutation on the pH rate profile for k_s. However, we suggest that it may be related to the following difference in the preferred pathways for formation of the β-D-galactopyranosyl intermediate by cleavage of β-D-galactopyranosides, and the breakdown of this intermediate by reaction with solvent water.

The mechanism for addition of water (HOH) to the β-D-galactopyranosyl intermediate to form Gal-OH may be different from the reverse of cleavage of sugar acetal substrates Gal-OR (that is, addition of alkyl alcohols ROH to E-Gal to form Gal-OR). For example, Glu-461 might provide Brønsted base catalysis of addition of alcohol nucleophiles (*13, 16*) at a relatively nonpolar active site which restricts the access of solvent water (Step 1, Scheme 4), while *movement* of a reactive galactosyl oxocarbenium ion away from this active site (Step 3, Scheme 4) may limit the rate of formation of galactose (Step 4, Scheme 4). Three observations show that there are significant differences in the changes in bonding between the enzyme catalyst and β-D-galactopyranosyl substrate on proceeding to the transition states for formation (k_3) and reaction (k_s) of the β-D-galactopyranosyl reaction intermediate. These provide evidence for the proposal that different steps are rate determining for formation of E-Gal by cleavage of Gal-OR (R = alkyl or aryl group) and for the reaction of E-Gal with the solvent water to form Gal-OH.

(a) There is a difference in the values of 1.04 for the α-deuterium isotope effect (α-DIE) on k_{cat} for β-galactosidase-catalyzed cleavage of Gal-OPNP, and of 1.25 for the α-DIE on k_s for hydrolysis of this reaction intermediate (*20*). The near-limiting α-DIE on k_s is consistent with essentially complete cleavage of the acylal in a preequilibrium step to form a galactosyl oxocarbenium ion (Step 2, Scheme 4) whose movement to reactive water (Step 3, Scheme 4) is rate-determining for k_s.



SCHEME 4.

(b) There are large solvent deuterium isotope effects of ≈1.7 on $k_{cat} \approx k_3$ (Scheme 3) for hydrolysis of Gal-OPNP (*10*) and Gal-OCH_2CF_3[3] for reactions where formation of E-Gal (Step 1, Scheme 4) is rate determining, but only a small solvent deuterium isotope effect of 1.1 on $k_{cat} \approx k_s$ for hydrolysis of 3,4-dinitrophenyl β-D-galactopyranoside with rate determining hydrolysis of E-Gal (*10*), which we suggest occurs by the stepwise mechanism shown in Scheme 4. This shows that there are substantial differences in the changes in zero-point energies at solvent derived hydrogen associated with movement to the transition states for formation and reaction of the β-D-galactopyranosyl intermediate.

(c) There is a >20-fold larger effect of the E461G mutation on k_{TFE} (M^{-1} s^{-1}) for addition of trifluorethanol to E-Gal to form Gal-OCH_2CF_3 compared with k_s for addition of water to form Gal-OH (*7*).[4] This result suggests that the $-CH_2CH_2CO_2^-$ side chain of Glu-461 serves an essential role of providing Brønsted base catalysis of addition of trifluoroethanol (Step 1, Scheme 4), but that it may play a different and less critical role in facilitating transfer of the β-D-galactopyranosyl group to water by the stepwise reaction proposed in Scheme 4.

[3] A solvent deuterium isotope effect of *ca* 2 has been determined for k_{cat} for β-galactosidase catalyzed cleavage of trifluoroethyl β-D-galactoctopyranoside (D. A. McCall, unpublished results).

[4] This limit was calculated from the difference in the rate constant ratios determined for partitioning of the intermediate of the wild-type (k_{TFE}/k_s = 6 M^{-1} (*13*)) and E461G β-galactosidase ($k_{TFE}/k_s < 0.3$ M^{-1} (*7*)) between addition of trifluoroethanol and water.

Summary. We have shown that the carboxylic acid/carboxylate side chain of Glu 461 at the active site of β-galactosidase is required to observe the downward breaks at high pL in the pL rate profiles of k_{cat}/K_m for cleavage of Gal-OPNP, and the solvent deuterium isotope effect of 1.7 on k_{cat} for enzyme-catalyzed cleavage of Gal-OPNP. These observations are predicted by the *simple* mechanism in which the $-CO_2H$ side chain of Glu-461 functions to provide classical Brønsted acid catalysis of cleavage of the bond between the sugar and the oxygen leaving group (Scheme 3).

The effect of the E461G mutation on the pH rate profile for hydrolysis of the β-D-galactopyranosyl intermediate (k_s, Scheme 3) (*3*) cannot be simply interpreted within the framework of a mechanism in which Glu 461 serves to provide both Brønsted general acid catalysis of cleavage of the bond to alkyl and aryl alcohol leaving groups, and Brønsted general base catalysis of transfer of this intermediate to water. We propose that the addition of water to the β-D-galactopyranosyl intermediate is not the simple microscopic reverse of formation of this intermediate by cleavage of Gal-OR, so the side chain of Glu-461 may play different roles in these two reactions (Scheme 4). There are several experimental observations which show that there are substantial differences in the transition states for formation and reaction of E-Gal, and which can be simply explained by the mechanism shown in Scheme 4. However, further work is needed to clarify this and other unresolved questions about the mechanism of action for β-galactosidase.

REFERENCES

1. Ring, M., Armitage, I. M., and Huber, R. E. (1985) *Biochem. Biophys. Res. Commun.* **131,** 675–680.
2. Ring, M., Bader, D. E., and Huber, R. E. (1988) *Biochem. Biophys. Res. Commun.* **152,** 1050–1053.
3. Cupples, C. G., Miller, J. H., and Huber, R. E. (1990) *J. Biol. Chem.* **265,** 5512–5518.
4. Huber, R. E., and Chivers, P. T. (1993) *Carb. Res.* **250,** 9–18.
5. Sinnott, M. L., Withers, S. G., and Viratelle, O. M. (1978) *Biochem. J.* **175,** 539–546.
6. Sinnott, M. L. (1990) *Chem. Rev.* **90,** 1171–1202.
7. Richard, J. P., Huber, R. E., Lin, S., Heo, C., and Amyes, T. L. (1996) *Biochemistry* **35,** 12377–12386.
8. Richard, J. P., Huber, R. E., Heo, C., Amyes, T. L., and Lin, S. (1996) *Biochemistry* **35,** 12387–12401.
9. Espinosa, J. F., Montero, E., Vian, A., García, J. L., Dietrich, H., Schmidt, R. R., Martín-Lomas, M., Imberty, A., Cañada, F. J., and Jiménez-Barbero, J. (1998) *J. Am. Chem. Soc.* **120,** 1309–1318.
10. Selwood, T., and Sinnott, M. L., (1990) *Biochem. J.* **268,** 317–323.
11. Glasoe, P. K., and Long, F. A. (1960) *J. Phys. Chem.* **64,** 188–190.
12. Richard, J. P., Westerfeld, J. G., and Lin, S. (1995) *Biochemistry* **34,** 11703–11712.
13. Richard, J. P., Westerfeld, J. G., Lin, S., and Beard, J. (1995) *Biochemistry* **34,** 11713–11724.
14. Schimmel, P. (1989) *Acc. Chem. Res.* **22,** 232–233.
15. Schowen, K. B., and Schowen, R. L. (1982) *Methods Enzymol.* **87,** 551–591.
16. Gebler, J. C., Aebersold, R., and Withers, S. (1992) *J. Biol. Chem.* **267,** 11126–11130.
17. Sinnott, M. L., and Viratelle, O. M. (1973) *Biochem. J.* **133,** 81–87.
18. Richard, J. P., and McCall, D. A. (2000) *Bioorg. Chem.* **8,** 49–56.
19. Rosenberg, S., and Kirsch, J. F. (1981) *Biochemistry* **20,** 3189–3196.
20. Sinnott, M. L., and Souchard, I. J. L. (1973) *Biochem. J.* **133,** 89–98.