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

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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

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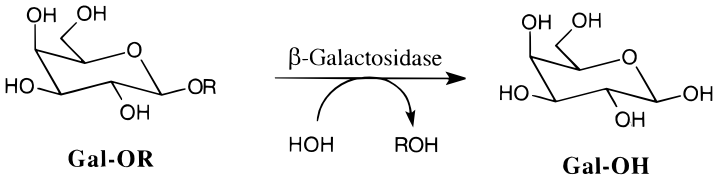
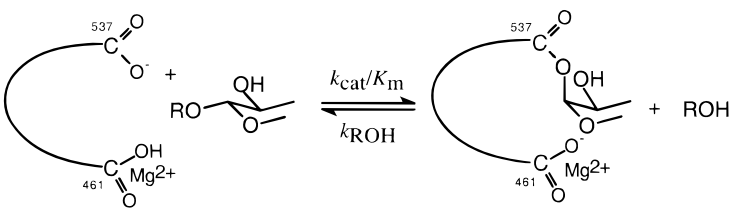


SCHEME 1.

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 $pK_a = 7.60$, $(\Delta\varepsilon_{405})_{max} = 17{,}900\ M^{-1}\ cm^{-1}$ 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

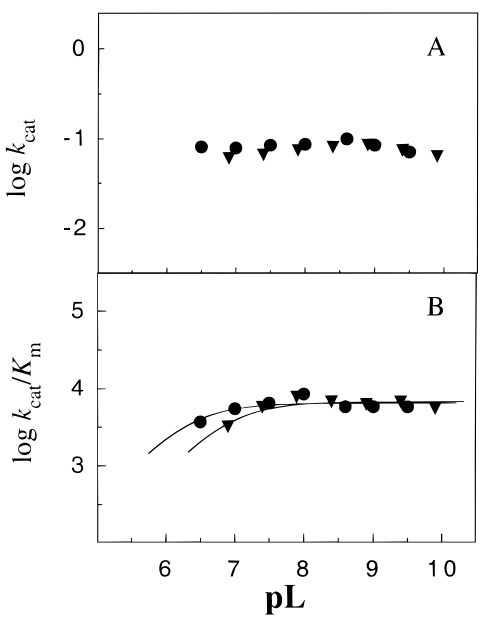


FIG. 1. pL rate profiles for the kinetic parameters k_{cat} and k_{cat}/K_m for E461G β-galactosidase-catalyzed cleavage of Gal-OPNP at 25°C. (A) ●, pH rate profile for k_{cat}; ▼, pD rate profile for k_{cat}. (B) ●, pH rate profile for k_{cat}/K_m; ▼, pD rate profile for (k_{cat}/K_m). The solid lines 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.