E461H-β-Galactosidase (*Escherichia coli*): Altered Divalent Metal Specificity and Slow but Reversible Metal Inactivation

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ABSTRACT: β-galactosidase (*Escherichia coli*) with a His substituted for Glu-461 retained about 10% of its normal activity in the absence of divalent metals but was inactivated rather than activated by Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, and Co^{2+}. Since Zn^{2+}, Ni^{2+}, Cu^{2+}, and Co^{2+} do not interact with wild type β-galactosidase while Mg^{2+} and Mn^{2+} activate and Ca^{2+} binds but has no effect on wild type β-galactosidase activity, the substituted enzyme has very different divalent metal interactions. A much larger amount of Mg^{2+} than of the other divalent metal ions was needed to inactivate the substituted enzyme at pH 7 (half-maximal activity was at 12.5 mM Mg^{2+} while the half-maximal activities with the other metals were at micromolar levels) compared to the amount of Mg^{2+} needed to activate the wild type enzyme. The inactivation of E461H-β-galactosidase caused by Mg^{2+} took about 20 min. Reactivation by removal of the divalent metal took about 60 min. Interaction with Mg^{2+} was about 10^7-fold stronger at pH 9 than at pH 7, and inactivation occurred in less than 2 min at higher pH values. "Galactosylation" (k_2, cleavage of the glycosidic bond) seemed to be rate-limiting for E461H-β-galactosidase at pH values above 6 with both *o*-nitrophenyl β-D-galactopyranoside and *p*-nitrophenyl β-D-galactopyranoside in both the presence and absence of Mg^{2+}. Mg^{2+} caused decreases (about 50-fold) of the k_2 values of E461H-β-galactosidase (apparent pK_a was about 6.8). The substitution of Glu-461 by His, therefore, causes galactosylation to decrease, and the addition of divalent cation to the enzyme causes a further pH dependent decrease of the rate. Analysis of the results indicated that Glu-461 may function to orient and possibly stabilize an intermediate galactosyl cation and that Mg^{2+} might align and modulate the effect of Glu-461. The Mg^{2+} binding site also seems important for the overall geometry of the binding site since addition of Mg^{2+} to E461H-β-galactosidase caused increases of about 10-fold in the K_s values.

Glutamic acid 461 is important at the active site of β-galactosidase. Herrchen and Legler (1984) showed that conduritol-C (a reactive galactose analog) reacts covalently with Glu-461 and inactivates β-galactosidase. Substitutions for Glu-461 affect the k_{cat} values and the rates of "galactosylation" and "degalactosylation", as well as the binding of substrates and inhibitors by β-galactosidase (Cupples et al., 1990). Also, when Glu-461 is replaced by neutral residues, the formation of adducts between D-galactose and nucleophiles is catalyzed (Huber & Chivers, 1993). Edwards et al. (1990) showed that Glu-461 is probably a ligand of Mg^{2+}, and recent structural work has shown that, indeed, Glu-461 along with His-418 and Glu-416 are the ligands to Mg^{2+} (Jacobson et al., 1994; Roth & Huber, 1994). Gebler et al. (1992), who discovered that Glu-537 has a nucleophilic role at the active site, speculated that Glu-461 may act as an acid catalyst during the reaction.

The properties of a β-galactosidase with a His replacement for Glu-461 are unique (Cupples et al., 1990). It is more active than enzymes with other substitutions at position 461, and it loses activity in a pH dependent manner. In addition, the imidazole group of His has the potential to act as an acid/base catalyst, a nucleophile, an electrostatic catalyst, a metal chelator, and/or a substrate binding ligand, and this prompted us to study the effects of this substitution in detail.

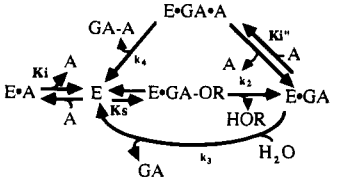
MATERIALS AND METHODS

Chemicals. ONPG,[1] PNPG, TES, EDTA, IPTG, PETG, and xylitol were from Sigma, Boehringer-Mannheim, or similar sources. Other reagents were obtained from Fisher or similar sources. The purest reagents available were used.

Enzyme Purification. The E461H-β-galactosidase was isolated as described in Cupples et al. (1990) except that the enzyme was passed through an FPLC Superose 6 column as a last step. Purity was determined by SDS−PAGE.

Kinetic Mechanism. The probable mechanism of β-galactosidase with acceptors present is:

Scheme 1. Postulated Reactions of β-Galactosidase in the Presence of an Acceptor (Inhibitor)[a]



[a] The acceptor (inhibitor) is thought to bind to both the free and the "galactosylated" enzyme. The dots indicate that some sort of complex exists with the enzyme: E, β-galactosidase; GA-OR, β-galactosyl substrate; GA, galactose; HOR, aglycone product; A, acceptor (inhibitor); GA-A, galactosyl-acceptor adduct.

In the absence of acceptors, $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_m = [k_3/(k_2 + k_3)]K_s$. The rate constant for the glycosidic

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cleavage step (galactosylation) is k_2, while k_3 is the rate constant for the hydrolysis part of the reaction (degalactosylation). Inspection of the above equations shows that, when k_2 is rate-limiting, $k_{cat} = \sim k_2$ and $K_m = \sim K_s$, but when k_3 is rate-limiting, $k_{cat} = \sim k_3$ and $K_m \ll K_s$. Alcohol and sugar acceptors (designated as A) can react (k_4) in place of water to form galactosyl adducts (GAL-A) with the acceptor. If $k_4 > k_3$ and if k_3 is partially or fully rate-limiting, the apparent k_{cat} of the reaction in the presence of the acceptor will increase as a function of the acceptor concentration. If $k_4 > k_3$ and if k_2 is rate-limiting, there will be no change in rate. If k_4 is smaller than k_3, the rate of the reaction will slow down regardless of which of k_2 or k_3 is slower. The rate change that results from the presence of an acceptor can be quantified by plotting apparent k_{cat} vs {(apparent k_{cat} − k_{cat})/[acceptor]}. The vertical intercept of such a plot (which is the theoretical k_{cat} at infinite acceptor concentration) is $k_2k_4/(k_2 + k_4)$ (Deschavanne et al., 1978). The acceptors are also usually competitive inhibitors as indicated on Scheme 1. The acceptor action must be considered when obtaining K_i values. The K_i values were, therefore, calculated using Eadie−Hofstee plots (least squares) and an equation described by Deschavanne et al. (1978) to account for the

$$\frac{\text{apparent } K_m}{\text{apparent } k_{cat}} = \frac{K_m}{k_{cat}}\left(1 + \frac{[A]}{K_i}\right)$$

acceptor reaction. Experiments at different concentrations of inhibitor (acceptor) were carried out, and the K_i values were averaged.

Enzyme Assays with ONPG and PNPG. The assay solution consisted of TES buffer (30 mM, pH 7.0) with 145 mM NaCl and substrate. The Mg^{2+} concentrations in the assays were varied, and when the Mg^{2+} was to be absent, Mg^{2+} was left out and 20 mM EDTA was added. When necessary, total Mg^{2+} concentrations were determined by flame ionization absorption and specific concentrations of EDTA were added to achieve desired concentrations of free Mg^{2+}. Other metals were also tested. The reactions were started by addition of enzyme. For most assays, the reactions were followed at 420 nm as a function of time in a Shimadzu UV-2101 PC spectrophotometer (25 °C). Extinction coefficients used were 2.65 mM^{-1} cm^{-1} for oNP and 6.50 mM^{-1} cm^{-1} for pNP. For the pH profiles, fixed time assays were used. The reactions were stopped with 2 volumes of 1 M Na_2CO_3, which increased the pH to 11 and stopped the reaction. Extinction coefficients for these assays were determined with oNP and pNP standards. The K_m and k_{cat} values were determined from Eadie−Hofstee plots. During purification, 2 mM ONPG was used routinely for the assays.

Enzyme was usually incubated at specific ion concentrations for at least 1 h before the assays to allow equilibration. To follow activity changes resulting from Mg^{2+} binding, however, the enzyme was incubated in buffer with 20 mM EDTA for 1 h and then a very small volume was transferred into assay solution with 20 mM Mg^{2+} (only a small volume was transferred to ensure that very small amounts of EDTA were carried over). Enzyme was also incubated in buffer with 20 mM Mg^{2+} for 1 h, and a small volume was transferred to assay solution with 20 mM EDTA (no added Mg^{2+}). Also, enzyme was incubated in buffer with 20 mM EDTA for 1 h and assayed with 20 mM EDTA, and the other enzyme was incubated with 20 mM Mg^{2+} for 1 h and assayed with 20 mM Mg^{2+}. These controls were required because the rate changes in response to changes of the Mg^{2+} concentration were slow and significant enough amounts of substrate were used up during the experiments to affect the rate.

pH Studies. The values of k_{cat}, K_m, and K_i of E461H-β-galactosidase were determined at various pH values. The effects of Mg^{2+} concentration at pH 7 and 9 were also followed. Other metals were studied only at pH 7.0.

Thin Layer Chromatography. To determine if the substrate itself was an acceptor, small amounts of reaction mixture from two assays, the first containing 20 mM ONPG and the second containing 1.6 mM ONPG, were spotted onto 5 × 5 cm Whatman Silica Gel 60A plates with galactose and ONPG standards and eluted with 1-butanol/acetic acid/water (2/1/1). For studies with methanol as an acceptor, methyl β-D-galactopyranoside was also used as a standard and the solvent system was acetic acid/1-butanol/ethyl acetate/2-propanol/water (1.35/1.35/3.85/2.3/1.15). Plates were developed with 2% orcinol in 10% sulfuric acid with heat. The intensities of the spots were estimated by eye.

RESULTS

Enzyme Purification. After purification of E461H-β-galactosidase, only one band was seen on SDS−PAGE, and it migrated to the same position as the protein band for normal β-galactosidase. The protein was at least 98% pure. Earlier studies have shown that the enzyme is present as a tetramer in the native form (Cupples et al., 1990), and the elution pattern of the protein from the Superose 6 column confirmed this. The properties of this substituted enzyme were so different from the properties of the wild type enzyme (especially in regard to the inactivation by Mg^{2+}) that there is little likelihood that there was any significant contamination by wild type enzyme.

Rate Changes Resulting from Changes in Mg^{2+} Concentrations. When E461H-β-galactosidase was incubated for 1 h in buffer with either 20 mM Mg^{2+} or 20 mM EDTA and then small volumes were transferred for assay under the same Mg^{2+} conditions, the rates were initially constant but then decreased slowly (Figure 1) at both pH 7.0 and 7.75. The most probable reason for the decreases in these controls is that the substrate concentrations were decreasing over the long time periods being followed (1 h). It is also possible that the product buildup (galactose and/or oNP) may have inhibited the activity. When enzyme that was incubated with 20 mM EDTA in assay buffer was added to assay solution with 20 mM Mg^{2+} in TES buffer, the rate decreased at pH 7.0 and 7.75 (the rate decrease was much faster at pH 7.75), and the final rates were like those found when the enzyme was incubated in buffer with 20 mM Mg^{2+} and then assayed with 20 mM Mg^{2+}. When enzyme was incubated in buffer with 20 mM Mg^{2+} and assayed in 20 mM EDTA, there was an initial rapid rate increase, and the rate then rose only slowly. The rate of reaction with enzyme initially incubated

[1] Abbreviations: ONPG, *o*-nitrophenyl β-D-galactopyranoside; PNPG, *p*-nitrophenyl β-D-galactopyranoside; IPTG, isopropyl β-D-thiogalactopyranoside; oNP, *o*-nitrophenol; pNP, *p*-nitrophenol; PETG, phenylethyl β-D-thiogalactopyranoside; DEAE, diethylaminoethyl; TES, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

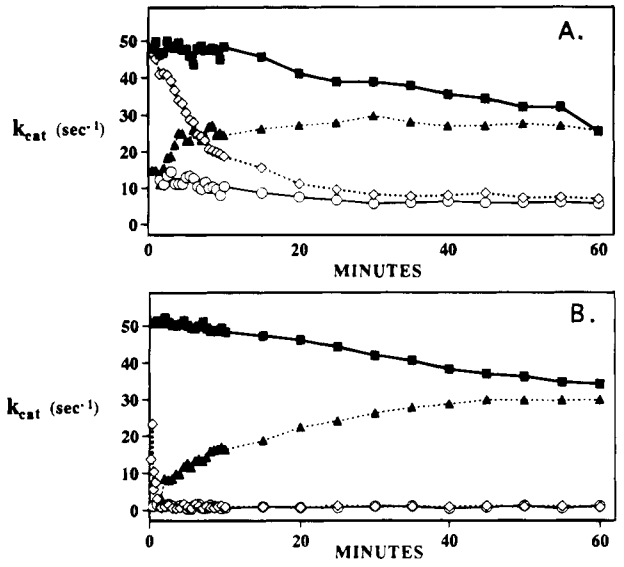


FIGURE 1: Changes in k_{cat} as a function of time. All of the experiments were done with 2 mM ONPG and in TES buffer (30 mM, 25 °C) with 145 mM NaCl: (A) pH 7.0 and (B) pH 7.75. (■) E461H-β-Galactosidase was first incubated for 1 h in 20 mM EDTA and then assayed in assay solution with 20 mM EDTA with no added Mg^{2+}. (◇) E461H-β-Galactosidase was incubated for 1 h in 20 mM EDTA and then assayed in assay solution with 20 mM Mg^{2+}. (○) E461H-β-Galactosidase was incubated for 1 h in 20 mM Mg^{2+} and then assayed in assay solution with 20 mM Mg^{2+}. (▲) E461H-β-Galactosidase was incubated for 1 h in 20 mM Mg^{2+} and then assayed in assay solution with 20 mM EDTA with no added Mg^{2+}. These experiments were repeated several times, and the same results were obtained in each case. Instead of doing replicates, we took many points over short periods of time, and the variation was small.

in buffer with 20 mM EDTA and then transferred into assay solution with 20 mM EDTA was, however, decreasing (as discussed above), and therefore, in the end, the rate was very similar to that when enzyme was incubated and assayed in 20 mM EDTA. The activation was not faster at pH 7.75 than at pH 7.0. Indeed, the activation curves were similar at the two pH values.

Kinetic Constants. The Eadie–Hofstee plots of the reactions of ONPG and PNPG with enzyme that was preincubated for at least 1 h in 20 mM Mg^{2+} or 20 mM EDTA yielded lines that curved downward at high substrate (not shown). The point at which the lines curved and the degree to which they bent depended on the Mg^{2+} concentration, the pH, and the substrate. We postulated that this "substrate inhibition" occurred because the substrate itself was an acceptor with a small k_4 value (see Scheme 1). Thin layer chromatography which confirmed this was carried out. At high substrate concentrations, a carbohydrate positive spot was present on a thin layer plate that did not correspond to either galactose or oNP and migrated to a position expected for a digalactoside with a nitrophenyl group attached. If the substrate inhibition effects (the downward bends) on the Eadie–Hofstee plots were ignored, the k_{cat} and K_m values shown in Table 1 were obtained. These values compare to a k_{cat} of 580 s^{-1} and a K_m of 0.12 mM for wild type β-galactosidase with 1 mM Mg^{2+} and a k_{cat} of 36 s^{-1} and a K_m of 0.57 mM in the presence of 20 mM EDTA. For PNPG with wild type enzyme, the k_{cat} was 98 s^{-1} and the K_m was 0.034 mM with 1 mM Mg^{2+} and 13 s^{-1} and 0.12 mM in the presence of EDTA.

Table 1: Experimental Values of K_m and k_{cat} for E461H-β-Galactosidase at Different Mg^{2+} Concentrations at pH 7, for ONPG and PNPG

	ONPG		PNPG	
	K_m (mM)	k_{cat} (s^{-1})	K_m (mM)	k_{cat} (s^{-1})
100 mM EDTA	0.15 ± 0.01	44 ± 0.9	0.13 ± 0.01	3.8 ± 0.1
20 mM EDTA	0.17 ± 0.01	50 ± 0.6	0.15 ± 0.02	4.0 ± 0.1
1 mM Mg^{2+}	0.17 ± 0.01	48 ± 0.8	0.18 ± 0.01	4.0 ± 0.05
5 mM Mg^{2+}	0.29 ± 0.03	37 ± 0.9	0.22 ± 0.02	2.2 ± 0.04
10 mM Mg^{2+}	0.26 ± 0.02	28 ± 0.9	0.24 ± 0.02	1.9 ± 0.03
20 mM Mg^{2+}	0.38 ± 0.02	20 ± 0.8	0.40 ± 0.04	1.6 ± 0.04
50 mM Mg^{2+}	0.93 ± 0.15	11 ± 0.6	0.85 ± 0.08	1.2 ± 0.03
100 mM Mg^{2+}	1.8 ± 0.34	5.7 ± 0.4	2.0 ± 0.4	1.5 ± 0.05

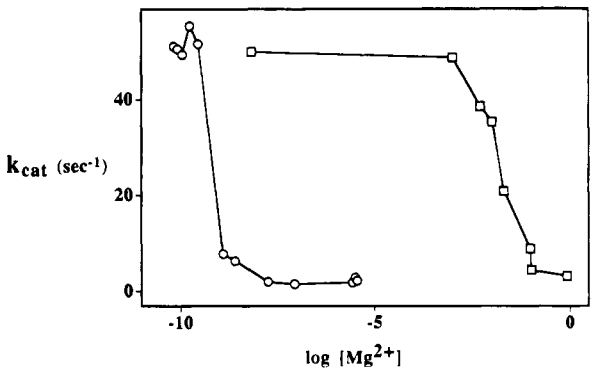


FIGURE 2: k_{cat} values of E461H-β-galactosidase at pH 7.0 and 9.0 in the presence of various free Mg^{2+} concentrations. The low free [Mg^{2+}] values were calculated from the total amount of Mg^{2+} found in the assay media by flame ionization absorption and the amount of EDTA added: (□) pH 7 and (○) pH 9. The errors were less than 4% in every case.

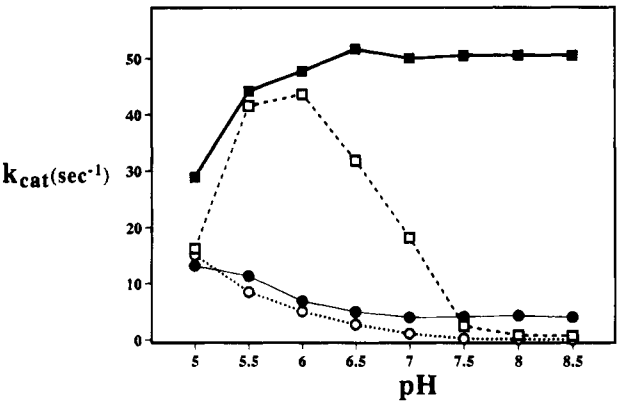


FIGURE 3: k_{cat} values of E461H-β-galactosidase with ONPG and PNPG at various pH values in the presence of 20 mM Mg^{2+} or 20 mM EDTA: (■) ONPG with 20 mM EDTA, (□) ONPG with 20 mM Mg^{2+}, (●) PNPG with 20 mM EDTA, and (○) PNPG with 20 mM Mg^{2+}. The errors were less than 4% in every case.

Figure 2 shows the effect of Mg^{2+} on the k_{cat} values of the His-substituted enzyme at two fixed pH values. At pH 7.0, the Mg^{2+} concentration needed to lower the k_{cat} to half-maximal was about 12.5 mM. At pH 9.0, the Mg^{2+} concentration needed was only 5×10^{-10} M. Figure 3 shows how the k_{cat} values of the enzyme for ONPG and PNPG varied with pH in the presence of 20 mM Mg^{2+} and of 20 mM EDTA. Between pH 5 and 6, the k_{cat} values for ONPG in 20 mM EDTA increased but then remained essentially constant as a function of pH. With 20 mM Mg^{2+} present, the rate also increased on the acid side between pH 5 and 6 in a way similar to the increase in the presence of 20 mM EDTA, but the rate then fell to very low values with a

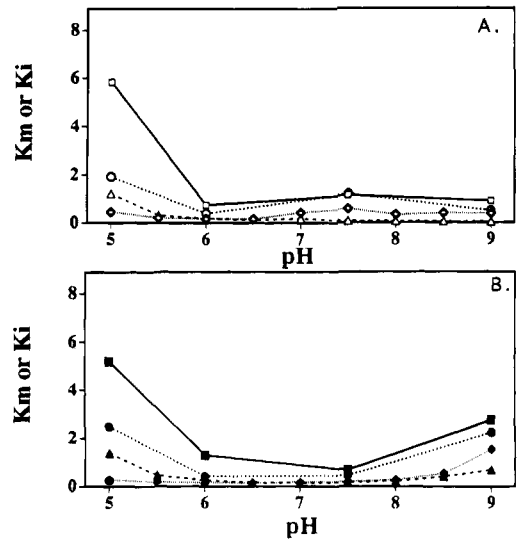


FIGURE 4: K_m values of E461H-β-galactosidase with ONPG and PNPG at various pH values in the presence of 20 mM Mg^{2+} and 20 mM EDTA. Also shown are K_i values for IPTG and PETG. The K_m values for both substrates and the K_i values for IPTG are in millimolar. The K_i values for PETG are in millimolar but were multiplied by 5. (A) Values of K_m or K_i in 20 mM Mg^{2+}: (◇) ONPG, (△) PNPG, (○) IPTG, and (□) PETG. (B) Values of K_m or K_i in 20 mM EDTA: (◆) ONPG, (▲) PNPG, (●) IPTG, and (■) PETG. The errors were less than 10% in every case.

midpoint at about pH 6.8. When PNPG was the substrate, the k_{cat} values changed differently. Except for the k_{cat} values at pH 5 and 5.5, the k_{cat} values for PNPG were much smaller than those for ONPG, and instead of increasing between pH 5 and 6, the k_{cat} values for PNPG decreased in both the presence and absence of Mg^{2+}. At about pH 6.5, the k_{cat} values for PNPG in the presence of EDTA leveled out. With Mg^{2+}, the k_{cat} values continued to decrease to low values after pH 6.5 with a midpoint (relative to the leveling out effect with 20 mM EDTA) at about pH 6.8.

Figure 4 shows how the K_m values for ONPG and PNPG and the K_i values for IPTG and PETG changed with pH in the presence and absence of Mg^{2+}. The K_m values for both substrates and the K_i values were small between pH 6 and 7.5. At the lower end of the pH scale, all the K_m and K_i values increased and the presence or absence of Mg^{2+} made little difference. At the high pH values without Mg^{2+}, the K_m and K_i values increased, while there was no change in the values with Mg^{2+}.

Rates of Reaction as a Function of the Type of Divalent Metal. Effects of other metals were studied at pH 7.0 (Figure 5). The Mn^{2+} needed for half-maximal activity of the His-substituted β-galactosidase was 5 μM. Zn^{2+}, Ni^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, and Cu^{2+} were also investigated, and all but Fe^{2+} and Ca^{2+} inactivated the enzyme. Concentrations needed for half-maximal activity (0.6, 14, 1.3, and 4.6 μM for Zn^{2+}, Ni^{2+}, Co^{2+}, and Cu^{2+}, respectively) were low compared to the amount of Mg^{2+} needed. In comparison to the other metals, the activity fell sharply as a function of the Cu^{2+} concentration (Figure 5).

Acceptors. Xylitol slowed the rates of reaction with both substrates in the absence of Mg^{2+} at pH 7. The extrapolation point of a plot of apparent k_{cat} vs {(apparent k_{cat} − k_{cat})/[xylitol]} to infinite concentrations of xylitol for ONPG was at about 15 s^{-1}. For PNPG, it was about 3.3 s^{-1}. These extrapolated values are equivalent to $k_2k_4/(k_2 + k_4)$ (Deschavanne et al., 1978). Since, in the absence of an acceptor $k_{cat} = k_2k_3/(k_2 + k_3)$, it follows that the k_4 for xylitol is smaller than the k_3 value. If it is assumed that k_2 is rate-limiting for PNPG (as discussed below) and, therefore, that the k_2 value for PNPG with 20 mM EDTA is 4 s^{-1} (Table 1), the values of the intercepts would be compatible with a value for k_4 of about 20 s^{-1} and a value for the k_2 of ONPG of about 50 s^{-1}. Methanol and 1,2-dihydroxypropane, which are very good acceptors for the normal enzyme, did not change the rate of the ONPG reaction. It is possible that they are not good acceptors for this enzyme, but since xylitol is an acceptor and even the substrate is an acceptor, that is not likely. It is more likely that the k_4 values of these acceptors are larger than k_2 and k_3 and that the rate of reaction with ONPG is limited by k_2. To check this, we looked at the production of methyl β-D-galactopyranoside when 1 M methanol was added, and thin layer chromatography showed that much more methyl-β-D-galactoside than galactose was formed.

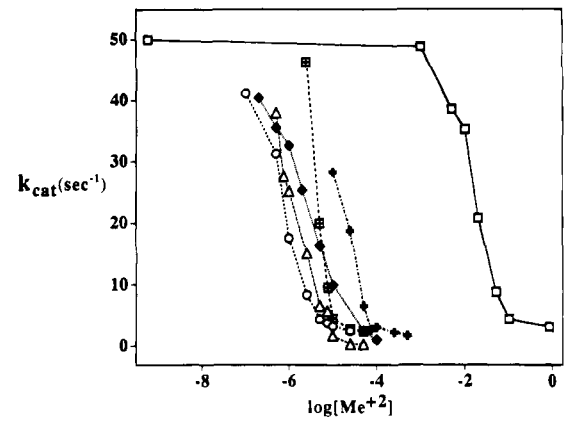


FIGURE 5: k_{cat} values of E461H-β-galactosidase at pH 7.0 in the presence of different divalent metals: (□) Mg^{2+}, (◆) Mn^{2+}, (○) Zn^{2+}, (△) Co^{2+}, (+) Ni^{2+}, and (cross-hatched □) Cu^{2+}. The errors were less than 4% in every case.

DISCUSSION

Histidine shares some properties with Glu, and therefore, substitution by a His is logical when one is investigating the properties of a Glu that is a ligand to Mg^{2+} (Edwards et al., 1990; Jacobson et al., 1994), that might act as a general acid/base catalyst (Gebler et al., 1992), that could act as a nucleophile, and that could be involved in the binding of substrate and in the stabilizing of an electron deficient transition state. Also, the His-substituted enzyme was unusual compared to β-galactosidases with other substitutions for Glu-461 in initial studies (Cupples & Miller, 1988; Cupples et al., 1990). For these reasons, it was singled out for detailed study.

E461H-β-galactosidase is inactivated by divalent metals. This is significant since wild type enzyme is activated by divalent metal ions. The inactivation was slow at pH 7.0 but much faster at pH 7.75 (Figure 1). The activation that occurred when the Mg^{2+} was removed was slower than the inactivation and was similar at each pH. These differences in apparent "on" and "off" rates probably account for the fact that the concentration of Mg^{2+} required for half-maximal activity (Figure 2) of the enzyme (after sufficient incubation time to equilibrate with Mg^{2+}) is highly pH dependent (12.5 mM at pH 7.0; only 5 × 10^{-10} M at pH 9.0). These results

indicate that Mg^{2+} binding must depend on the protonation state of the substituted His, and the pH dependence of the inactivation suggests that the pK_a of the His in the presence of 20 mM Mg^{2+} is near 6.8 (Figure 3). It follows that Glu-461 of the wild type enzyme must also be unprotonated to best coordinate the divalent metal (as is also predicted from chemical principles). Thus, it is highly unlikely that Glu-461 would act as a general acid catalyst by donating a proton to the glycosidic bond at pH 7.0 in the presence of Mg^{2+}.

Besides being inactivated by Mg^{2+} and Mn^{2+}, E461H-β-galactosidase was also inactivated by Zn^{2+}, Ni^{2+}, Cu^{2+}, and Co^{2+} (Figure 5) at pH 7. The loss of activity with Cu^{2+} was different from the activity losses with the other divalent ions. The Cu^{2+} caused a sharp activity decrease as a function of the Cu^{2+} concentration. Surface denaturation rather than specific interaction may have caused this inactivation in the presence of Cu^{2+} (Edwards & Huber, 1992). The concentration of Mg^{2+} needed for 50% inactivation (about 12.5 mM Mg^{2+}) was much higher (Figure 3) than the amount of the other ions needed for inactivation (micromolar range). When an oxygen ligand is replaced by a nitrogen ligand, improved binding of Mn^{2+}, Zn^{2+}, Ni^{2+}, Cu^{2+}, and Co^{2+} and decreased binding of Mg^{2+} and Ca^{2+} is expected (Hanzlik, 1976). Wild type enzyme binds only Mg^{2+}, Mn^{2+}, and Ca^{2+} (Huber et al., 1979). The substitution of a His for a Glu, therefore, dramatically changes the divalent metal binding site. A change in divalent metal specificity has also been observed with alkaline phosphatase (*Escherichia coli*). Replacement of an Asp by a His in that case resulted in the conversion of a Mg^{2+} site to a Zn^{2+} site (Murphy et al., 1993).

The loss of activity as a function of the Mg^{2+} concentration at pH 7 for both ONPG and PNPG is an effect of both a decrease in k_{cat} and an increase in K_m (Table 1). The K_m values were almost identical for the two substrates and increased proportionately as the Mg^{2+} concentration increased. The k_{cat} values for PNPG were about 10 times lower than those for ONPG, but the decreases of the k_{cat} values with PNPG were proportional to the decreases of the k_{cat} values with ONPG as the Mg^{2+} concentrations were increased.

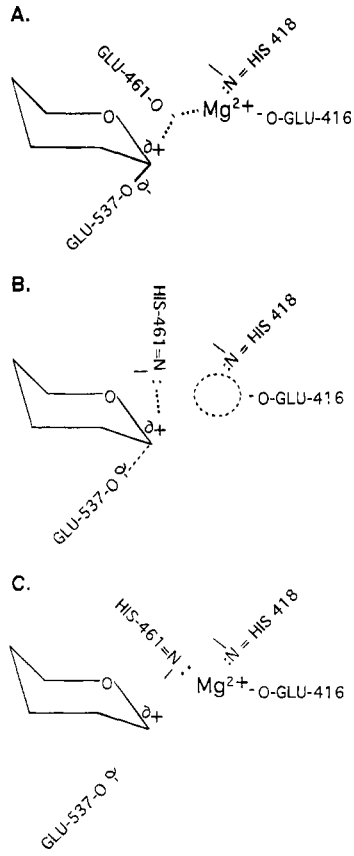
The K_i and K_m values obtained showed that binding is adversely affected at the low end of the pH scale in both the absence and presence of Mg^{2+} (Figure 4). Binding was also decreased at the high end of the pH scale in the absence of Mg^{2+}, while binding did not change with pH in the presence of Mg^{2+}. These effects could be due to conformational differences mediated by Mg^{2+}.

At pH values greater than 6, k_2 seems to be rate-limiting for both ONPG and PNPG in both the presence and absence of Mg^{2+} (Table 1, Figure 3). With ONPG as the substrate in the presence of 20 mM EDTA and at pH 7.0, k_{cat} was approximately 50 s^{-1}, and therefore, since $k_{cat} = k_2k_3/(k_2 + k_3)$, the k_2 (glycolytic bond cleavage, Scheme 1) and the k_3 (hydrolytic step, Scheme 1) both have values of at least 50 s^{-1} for ONPG. The k_{cat} with PNPG was about 4 s^{-1}. Thus, since k_3 is common to both substrates and has a value of at least 50 s^{-1} and since the k_{cat} with PNPG is only about 4 s^{-1}, the k_2 value for PNPG must be essentially rate-limiting and equal to about 4 s^{-1}. Similar arguments could be made to show that the k_2 of PNPG is rate-limiting at all pH values tested above 6 in both the presence and absence of Mg^{2+} since the k_{cat} for ONPG was always at least 1 order of magnitude larger than the k_{cat} for PNPG. The k_2 values with ONPG are also probably rate-determining. As stated above, the k_2 value with ONPG is greater than or equal to 50 s^{-1} at pH 7. Methanol and 1,2-dihydroxypropane, which are very good galactose acceptors with wild type β-galactosidases, did not change the rate of the ONPG reaction of the substituted enzyme in the absence of Mg^{2+} at pH 7 even though thin layer chromatography showed that methanol, at least, is also a good acceptor with this substituted enzyme. This shows that the rate of reaction with ONPG is probably limited by k_2 in the absence of Mg^{2+} at pH 7 (see discussion of Scheme 1). Also, the k_{cat} values with ONPG and PNPG decreased proportionately (Table 1) as the Mg^{2+} concentration was increased at pH 7, and this implies that the same step is rate-determining (i.e. it is unlikely that k_2 and k_3 have proportional changes in values as a function of Mg^{2+}, and thus only one or the other can be rate-determining). The evidence that k_2 is rate-limiting for ONPG at all pH values tested above 6 is not as strong. However, the k_{cat} values with ONPG and PNPG at all pH values above 6 are constant and proportional in the absence of Mg^{2+}, while they both drop with a similar apparent pK_a in the presence of Mg^{2+}. Further evidence was that neither methanol nor 2,3-propanediol increased (or decreased) the rates of reaction with ONPG in the presence or absence of Mg^{2+} when tested at pH values higher than 6 (data not shown). Again, thin layer chromatography of the products produced in the presence of methanol showed that a significant amount of transferolysis was occurring at each pH. Also, the K_m values were relatively large (in the millimolar range, Table 1 and Figure 4). If k_3 is rate-limiting, the K_m values should be small (i.e. $K_m = [k_3/(k_2 + k_3)]K_5$).

The k_{cat} values (Figures 3) for ONPG increased between pH 5 and 6, while the k_{cat} values for PNPG decreased regardless of whether Mg^{2+} was present (the curves with and without Mg^{2+} are not exactly identical, but they are quite similar). These data can best be explained if the rate-determining step for ONPG is different from that for PNPG between pH 5 and 6.

The loss of activity (lowering of the k_2 values) of the substituted enzyme in response to Mg^{2+} with an apparent pK_a of 6.8 implies that His has to be in the unprotonated form to interact with Mg^{2+}. The inactivation could simply result from a different conformation at the active site that occurs because of different interactions between His and Mg^{2+} as compared to those that normally occur between Glu and Mg^{2+}. The fact that substrate binding was dramatically decreased by Mg^{2+} (the K_m values in Table 1 are equal to K_s because k_2 is rate-limiting) indicates that the geometry of the substrate binding site is very sensitive to changes at the Mg^{2+} site. We feel, however, that effects in addition to conformation and geometry are important. There is quite good evidence that Glu-461 interacts with a galactosyl cation transition state. Wild type and E461D-β-galactosidase are inhibited strongly by positively charged amino derivatives of galactose (Cupples et al., 1990). This inhibition is absent when Glu-461 is replaced with residues that do not have negative charges. Those results indicate that, despite being a ligand of Mg^{2+}, Glu-461 must still have enough residual negative charge to interact with a positively charged galactosyl ion at the active site. Since His-418 and Glu-416 are also Mg^{2+} ligands (Jacobson et al., 1994), it is reasonable that there is some residual negative charge present. It is obvious, however, that stabilization of a galactosyl cation

Scheme 2. (A) Putative Action of Glu-461 in the Presence of Mg^{2+}, (B) Putative Action of His-461 in the Absence of Mg^{2+}, and (C) Putative Action of His-461 in the Presence of Mg^{2+} [a]



[a] For this scheme, the interaction between Glu-537 and the galactose is probably covalent (Gebler et al., 1992).

by the carboxyl of a Glu would be weak because the carboxyl group is also complexing to the Mg^{2+}. It is, therefore, more likely that Glu-461 mainly orients the cation properly for further reaction. Some weak stabilization of the cation would, however, probably also occur. A means by which the orientation or stabilization by Glu could occur is shown in part A of Scheme 2. When His is substituted for Glu-461 and Mg^{2+} is absent, His might be able to bring about some of the proper orientation (part b of Scheme 2). When Mg^{2+} is present, His could not orient the galactosyl cation because of coordination to Mg^{2+} (part C of Scheme 2). That could cause the inactivation. The structure of β-galactosidase (Jacobson et al., 1994) shows that Glu-461 could be in a position to orient the galactosyl cation, depending on how the cation is positioned at the active site.

Other explanations are possible. Mg^{2+} could act as a Lewis acid (Suh, 1992) and interact with the glycosidic oxygen of the substrate. It also is possible that a Lewis type of interaction between Mg^{2+} and water could cause the proton of the water molecule to be an acid catalyst by addition to the glycosidic oxygen. Neither of these explanations is totally satisfactory since they do not explain why the enzyme is inactivated by Mg^{2+} and also because Mg^{2+} is not really a good Lewis acid catalyst (Suh, 1992).

Earlier studies showed that k_3 (rather than k_2) is rate-limiting with substitutions of amino acids other than His for Glu-461 (Cupples et al., 1990). The k_3 value is obviously quite high in the His-substituted enzyme, and the His must in some way, therefore, allow degalactosylation to occur whereas other residues substituted for Glu-461 do not. It is possible that the His substituted for Glu-461 acts as a base catalyst in the degalactosylation reaction, or there could again simply be either a conformation or an orientation effect.

In summary, substitution of a His for Glu-461 resulted in the conversion of a site that is specific for Mg^{2+}, Mn^{2+}, or Ca^{2+} in the wild type to one that is broadly specific and binds Mg^{2+} poorly and does not bind Ca^{2+} at all at pH 7. The enzyme is inactivated rather than activated by divalent metals, and the changes in activity that result from divalent metal binding are slow and pH dependent. Both the k_{cat} and K_m values change. The results are best explained by a mechanism in which the residue at position 461 acts to orient a galactosyl cation.

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