Structure−Reactivity Relationships for β-Galactosidase (*Escherichia coli, lac Z*). 3. Evidence that Glu-461 Participates in Brønsted Acid−Base Catalysis of β-D-Galactopyranosyl Group Transfer†

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Received April 30, 1996; Revised Manuscript Received July 10, 1996⊗

ABSTRACT: Experiments are reported to determine the role of Glu-461 in the β-D-galactopyranosyl group transfer reaction catalyzed by β-galactosidase. E461G β-galactosidase catalyzes the hydrolysis of 4-nitrophenyl β-D-galactopyranoside through a galactosyl−enzyme intermediate that shows a high reactivity toward the anionic nucleophile azide ion, but no detectable reactivity toward the neutral nucleophile trifluoroethanol. By contrast, the galactosylated wild type enzyme is reactive toward trifluoroethanol but not anions. The change in specificity observed for the E461G mutant can be rationalized by a mechanism in which Glu-461 participates in general acid−base catalysis at the leaving group/nucleophile. The observed low activity of E461G β-galactosidase for hydrolysis of 2,2,2-trifluoroethyl β-D-galactopyranoside is due entirely to a wild type enzyme contaminant in our preparation of the mutant enzyme, and the mutant enzyme itself has essentially no catalytic activity for cleavage of this substrate. The substitution of glutamate at position 461 by glycine leads to a more than 500 000-fold reduction in the rate constant for enzymatic cleavage of the glycosidic bond to the strongly basic trifluoroethoxide leaving group ($pK_a = 12.4$), but to a smaller 1300-fold reduction in the rate constant for cleavage of the bond to the more weakly basic 4-nitrophenoxide leaving group ($pK_a = 7.1$). This corresponds to a more than 3.5 kcal/mol *greater stabilization* by Glu-461 of the transition state for the reaction of the substrate with the more basic trifluoroethoxide leaving group. These data are consistent with the conclusion that Glu-461 provides general acid catalysis of leaving group departure, which is most effective for cleavage of the relatively strong bonds to basic alkoxide leaving groups.

A number of amino acid residues and a magnesium ion cofactor have been shown to be essential for expression of the full catalytic activity of β-galactosidase (*Escherichia coli, lac Z*) for cleavage of β-D-galactopyranosyl derivatives (Sinnott, 1990) and/or have been located at the active site cleft by X-ray crystallography (Jacobson et al., 1994). However, the roles of most of these residues in catalysis are not well understood.

Degradation of the stable covalent complex formed between β-galactosidase and 2-deoxy-2-fluoro-β-D-galactopyranoside has shown that the carboxylate group of Glu-537 is the site of covalent attachment of the enzyme to this substrate analog (Gebler et al., 1992). This result is consistent with the conclusion that Glu-537 participates in covalent catalysis of the cleavage of the physiological substrate. Site-directed mutagenesis of Glu-537 has confirmed that a carboxylate group at this site is required for the observation of enzymatic activity (Yuan et al., 1994).

Tyr-503 has been shown by site-directed mutagenesis to be essential for full catalytic activity (Ring et al., 1985, 1988). Both this residue and Arg-388 have been located by X-ray crystallographic analysis of the free enzyme, where they are hydrogen-bonded to Glu-537 in the active site cleft (Jacobson et al., 1994). However, the role of this assembly of residues in the catalysis of glycoside cleavage is unclear. Met-502 is adjacent to Tyr-503 on the β-galactosidase polypeptide chain and undergoes chemical modification by β-D-galactopyranosylmethyl-4-nitrophenyltriazine (Sinnott & Smith, 1978). However, it is not clear what role, if any, the side chain of Met-502 plays in catalysis.

Glu-461 has been shown to undergo chemical modification by an epoxide substrate analog (Herrchen & Legler, 1984) and this residue has been located by X-ray crystallography at the active site cleft, where it lies in close proximity to Glu-416, His-391, and a bound Mg^{2+} ion (Jacobson et al., 1994). Site-directed mutagenesis of Glu-461 has shown that this residue is essential for full catalytic activity (Cupples et al., 1990; Huber & Chivers, 1993). However, the results of the initial mutagenesis study were interpreted within the framework of a role for Glu-461 in covalent catalysis (Cupples et al., 1990; Sinnott, 1990). The subsequent assignment of Glu-537 as the catalytic nucleophile and the suggestion that Glu-461 may function in acid−base catalysis at the leaving group (Gebler et al., 1992) have led to the studies reported in this and the following paper (Richard et al., 1996), which were directed toward the assignment of the role of Glu-461 in catalysis, and to the detailed examination of the mechanism for the reaction of nucleophilic anions with galactosylated E461G and E461Q (E = Glu, G = Gly, Q = Gln) β-galactosidases.

† This work was supported by Grant GM 47307 from the National Institute of General Medical Sciences.

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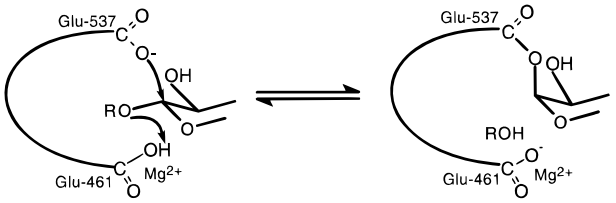
⊗ Abstract published in *Advance ACS Abstracts,* September 1, 1996.

S0006-2960(96)01028-8 CCC: $12.00

Scheme 1



Scheme 1 presents a hypothetical mechanism of action for β-galactosidase, which was arrived at by application of the paradigm established for the lysozyme-catalyzed cleavage of glycosides (Blake et al., 1967). This mechanism involves the participation of Glu-537 in nucleophilic catalysis of glycoside cleavage to give a covalent glycosyl−enzyme intermediate, the participation of Glu-461 in reversible acid−base catalysis at the alkoxy leaving group/nucleophile, and an enzyme-bound magnesium ion whose role in catalysis has not yet been defined. An alternative mechanism which is fully consistent with the available data for the mechanism of action of this enzyme utilizes the magnesium dication to provide electrophilic stabilization of a developing negative charge at the leaving group/alkyl alcohol nucleophile (Selwood & Sinnott, 1990; Sinnott, 1990).

We report here experiments on the E461G and E461Q mutants of β-galactosidase that were designed to evaluate the role of Glu-461 in the β-galactosidase-catalyzed hydrolysis of β-D-galactopyranosyl derivatives. These experiments test two predictions which must be fulfilled if this residue does participate in general acid−base catalysis at the leaving group/alkyl alcohol nucleophile:

(1) These mutations should have a very large effect on k_{cat} (s^{-1}) for cleavage of alkyl β-D-galactopyranosides, for which there is a large requirement for stabilization of developing negative charge at the basic alkoxide ion leaving group. However, the effect on k_{cat} for cleavage of aryl β-D-galactopyranosides should be smaller, because of the lesser requirement for stabilization of the negative charge at the less basic aryloxide ion leaving groups.

(2) The 6-fold greater nucleophilic reactivity of trifluoroethanol than of solvent water toward the galactosylated wild type enzyme (Richard et al., 1995b) is consistent with an interaction between the enzyme and the nucleophilic reagent which leads to more effective stabilization of the transition state for the reaction of trifluoroethanol than for the reaction of water. If this interaction corresponds to partial transfer of a proton from the alcohol to the carboxylate group of Glu-461 and to the more effective stabilization of negative charge by a trifluoroethoxy than by a hydroxy group, then a normal low nucleophilic reactivity should be observed for the reaction of trifluoroethanol with the galactosylated E461G enzyme, in which this carboxylate residue is absent.

Both of these predictions are confirmed by the data reported in the present work. The results provide strong evidence that Glu-461 functions directly in acid−base catalysis at the leaving group/nucleophile, and that Mg^{2+} plays a secondary role in ensuring that such catalysis is optimal.

MATERIALS AND METHODS

Unless stated otherwise, the sources of the chemicals and enzymes used here are as described in earlier work (Richard et al., 1995a,b). *N*-Tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES)[1] was purchased from Sigma, and ethylenediaminetetraacetic acid (EDTA) was purchased from Aldrich. β-D-Galactopyranosyl pyridinium bromide was prepared by a published procedure (Sinnott & Withers, 1974). Galactose dehydrogenase from *E. coli* containing the gene for the *Pseudomonas fluorescens* enzyme on a plasmid was purchased as an ammonium sulfate precipitate from Sigma. E461G and E461Q β-galactosidases were prepared and purified by a published procedure (Cupples et al., 1990).

Enzyme Assays. Unless stated otherwise, enzyme assays were carried out at 25 °C and pH 8.6 in 25 mM sodium pyrophosphate buffers that contained 1.0 mM $MgCl_2$ or 10 mM EDTA. Galactose dehydrogenase was freed of ammonium sulfate by dialysis against 25 mM sodium pyrophosphate buffer (pH 8.6) that contained 1 mM EDTA, and its activity was assayed as described in earlier work (Richard et al., 1995a). Magnesium-free E461G β-galactosidase was prepared by extensive dialysis against 10 mM EDTA, and the magnesium-free enzyme was assayed in the presence of 10 mM EDTA (Tenu et al., 1972).

The initial velocities of enzyme-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside at pH 8.6 (25 mM sodium pyrophosphate) and pH 7.0 (30 mM TES, 140 mM NaCl, 1.0 mM $MgCl_2$) were determined by monitoring the increase in absorbance at 405 nm using $\Delta\epsilon = 18\,300\ M^{-1}\ cm^{-1}$ (pH 8.6) and $\Delta\epsilon = 8900\ M^{-1}\ cm^{-1}$ (pH 7.0) determined for complete reaction of a known concentration of the substrate, as described in earlier work (Richard et al., 1995a).

The initial velocities of enzyme-catalyzed hydrolysis of alkyl β-D-galactopyranosides, β-D-galactopyranosyl azide, and the β-D-galactopyranosyl pyridinium ion to give D-galactose were determined in a galactose dehydrogenase coupled enzyme assay by following the formation of NADH at 340 nm ($\Delta\epsilon = 6200\ M^{-1}\ cm^{-1}$), as described in earlier work (Richard et al., 1995a). The initial velocities of cleavage of 2,2,2-trifluoroethyl β-D-galactopyranoside by E461G β-galactosidase in the presence of increasing concentrations of trifluoroethanol or sodium azide were determined using a substrate concentration of 0.6 mM.

The initial velocities of the substrate cleavage (v_{PNP}) and hydrolysis (v_{gal}) reactions of 4-nitrophenyl β-D-galactopyranoside (0.07 mM) catalyzed by wild type and E461G β-galactosidase were determined by monitoring the formation of 4-nitrophenoxide at 405 nm and the formation of D-galactose coupled to the formation of NADH at 340 nm in a single cuvette, using the galactose dehydrogenase coupled enzyme assay described in earlier work (Richard et al., 1995b), except that the changes in absorbance at 405 and 340 nm were monitored simultaneously. The ratio of the velocities of formation of D-galactose and 4-nitrophenoxide/4-nitrophenol (v_{gal}/v_{PNP}) were reproducible to ±3%.

Values of K_m and V_{max} for hydrolysis of β-D-galactopyranosyl derivatives catalyzed by E461G and E461Q β-galactosidases at pH 8.6 were determined from the nonlinear least squares fit of the observed initial velocities, v, to eq 1,

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

using the SigmaPlot curve fitting program from Jandel

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid; TFE, 2,2,2-trifluoroethanol.

Table 1: Kinetic Parameters for Cleavage of β-D-Galactopyranosyl Derivatives Catalyzed by E461G, E461Q, and Wild Type β-Galactosidases at 25 °C and pH 8.6 in the Presence of 1.0 mM Mg^{2+} [a]

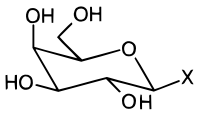
		E461G[b]			E461Q[b]	wild type[c]	
leaving group	pK_a [d]	$(k_{cat})_{obsd}$ [e] (s^{-1})	k_{cat} [f] (s^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat} (s^{-1})	K_m (mM)
Cl_2CHCH_2O	12.9	0.020	~0	0.30		230	0.31
CF_3CH_2O	12.4	0.035	<0.002[g]	0.54	<0.002[h]	410	0.61
						$k_3 = 970\ s^{-1}$ [i]	
4-NO_2-C_6H_4O	7.1	0.10	0.090	0.033	0.23	120	0.051
					$k_3 = 10\ s^{-1}$ [i,j]		
$C_6H_5N^+$	5.1	0.4	0.4	0.06		0.61[k]	1.3[k]
			$k_3 > 0.4\ s^{-1}$ [i]				
N_3	4.7	0.11	0.11	0.81		25[l]	1.6[l]

[a] In 25 mM sodium pyrophosphate buffer (pH 8.6), unless noted otherwise. Values of k_{cat} and K_m were reproducible to ±5%. [b] Data from this work. [c] Data from Richard et al. (1995a), unless noted otherwise. [d] pK_a of the conjugate acid of the leaving group taken from Jencks and Regenstein (1976). [e] Observed value of k_{cat} for catalysis by our preparation of E461G β-galactosidase. [f] Absolute value of k_{cat} for catalysis by E461G β-galactosidase, calculated from $(k_{cat})_{obsd}$ using eq 4 with $f_{wt} = 8.5 \times 10^{-5}$ for the fraction of wild type enzyme present in our preparation of the E461G mutant (see text). [g] Upper limit calculated with the assumption that less than 5% of the activity of our preparation of E461G β-galactosidase for cleavage of Gal-OCH_2CF_3 is due to catalysis by the mutant enzyme (see text). [h] Upper limit calculated from the experimental limit of $k < 0.0009\ s^{-1}$ at [S] = 0.6 mM, with the assumption that K_m for this substrate is similar to that for the wild type enzyme. [i] First-order rate constant for cleavage of the glycosidic bond of enzyme-bound substrate (Scheme 2). [j] Data from Richard et al. (1996). [k] Data for reaction at pH 7.0 (Sinnott & Withers, 1974). [l] Data from Richard et al. (1995b).

Scientific. Initial velocities, v, for hydrolysis of 4-nitrophenyl β-D-galactopyranoside at pH 7.0 and of this and other β-D-galactopyranosyl derivatives at pH 8.6 were determined for the reactions catalyzed by a *fixed* concentration of the E461G or E461Q enzyme at saturating or nearly saturating (≥5.5 K_m) concentrations of substrate. Relative values of V_{max} were calculated from the values of v and the Michaelis constant K_m for the respective substrates using eq 1. Values of k_{cat} (s^{-1}) were calculated from the relative values of V_{max} and the published values of $k_{cat} = 0.10$ and $0.34\ s^{-1}$ for cleavage of 4-nitrophenyl β-D-galactopyranoside by E461G and E461Q β-galactosidase at pH 7.0, respectively (Cupples et al., 1990). Values of k_{cat} and K_m were reproducible to ±10%.

RESULTS

Table 1 lists the *observed* kinetic parameters for cleavage of β-D-galactopyranosyl derivatives (Gal-X) catalyzed by our



Gal-X

preparations of E461G and E461Q β-galalctosidase at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}, and Table 2 gives data for the magnesium-free E461G enzyme in the presence of 10 mM EDTA. It has been shown in earlier work that the E461G enzyme is essentially 100% activated by 1.0 mM Mg^{2+} for reactions at pH 7.0 (Edwards et al., 1990). A control experiment under our reaction conditions (25 mM sodium pyrophosphate at pH 8.6) showed that E461G β-galactosidase is fully activated for cleavage of Gal-OC_6H_4-4-NO_2 by a total magnesium concentration of 1.0 mM.[2]

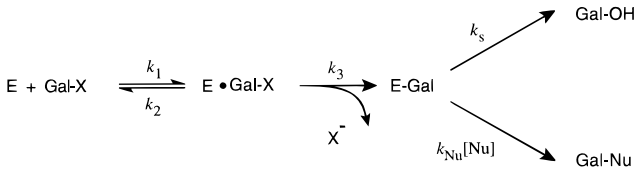
[2] The observed activity of E461G β-galactosidase for cleavage of 1 mM Gal-OC_6H_4-4-NO_2 increases to a maximum as the total concentration of Mg^{2+} is increased from 0 to 0.6 mM and then remains constant as the metal ion concentration is increased to 10 mM.

Table 2: Kinetic Parameters for Cleavage of β-D-Galactopyranosyl Derivatives Catalyzed by Magnesium-Free E461G and Wild Type β-Galactosidases at 25 °C and pH 8.6[a]

		E461G[b]		wild type
leaving group	pK_a [c]	$(k_{cat})_{obsd}$ [d] (s^{-1})	k_{cat} [e] (s^{-1})	k_{cat} (s^{-1})
CF_3CH_2O	12.4	7.0×10^{-4}	$<7.0 \times 10^{-4}$ [f]	8.1[g]
4-NO_2-C_6H_4O	7.1	0.045	0.043 (K_m = 0.51 mM)	26[g]
N_3	4.7	0.11	0.11	0.12[h]

[a] In 25 mM sodium pyrophosphate buffer containing 10 mM EDTA. Values of k_{cat} and K_m were reproducible to ±5%. [b] Data from this work. [c] pK_a of the conjugate acid of the leaving group taken from Jencks and Regenstein (1976). [d] Observed value of k_{cat} for catalysis by our preparation of E461G β-galactosidase. [e] Absolute value of k_{cat} for catalysis by E461G β-galactosidase, calculated from $(k_{cat})_{obsd}$ using eq 4 with $f_{wt} = 8.5 \times 10^{-5}$ for the fraction of wild type enzyme present in our preparation of the E461G mutant (see text). [f] The cleavage of Gal-OCH_2CF_3 in the presence of Mg^{2+} is due exclusively to the wild type enzyme contaminant in our preparation of the E461G mutant (see text), and the removal of Mg^{2+} results in the same 50-fold reduction in $(k_{cat})_{obsd}$ for both the E461G and the wild type enzyme. Therefore, most or all of the activity of our Mg^{2+}-free E461G mutant preparation must be due to this wild type contaminant. [g] Data from Richard et al. (1995a). [h] Data from Richard et al. (1995b).

Scheme 2



Tables 1 and 2 also give literature values of the kinetic parameters for cleavage of β-D-galactopyranosyl derivatives catalyzed by wild type β-galactosidase at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) as marked (Richard et al., 1995a,b).

For most of the reactions in Tables 1 and 2, k_{cat} is significantly smaller than k_s for hydrolysis of the respective galactosyl−enzyme intermediates, so that $k_{cat} \approx k_3$ for cleavage of the enzyme-bound substrate (Scheme 2). The values of k_s at pH 8.6 are $710\ s^{-1}$ for the wild type enzyme (Richard et al., 1995a), $0.35\ s^{-1}$ for the E461G enzyme

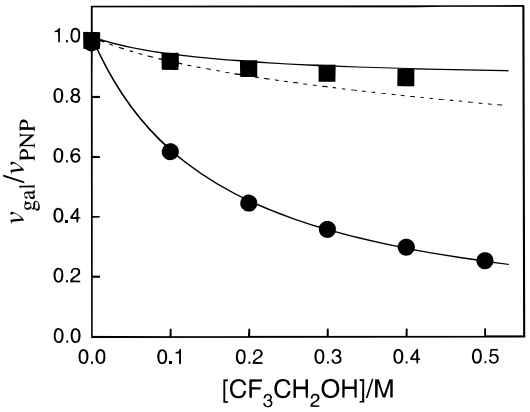


FIGURE 1: Dependence of the ratio of the initial velocities of formation of D-galactose (v_{gal}) and 4-nitrophenoxide/4-nitrophenol (v_{PNP}) from the reaction of 4-nitrophenyl β-D-galactopyranoside (0.07 mM) catalyzed by wild type (●) and E461G (■) β-galactosidase at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+} on the concentration of trifluoroethanol. The line through the data for the wild type enzyme was calculated as described in the text. The upper solid line shows the fit of the data for the E461G enzyme to eq 2, calculated using $(f_{act})_{wt} = 0.15$ for the fraction of the cleavage reaction of 4-nitrophenyl β-D-galactopyranoside catalyzed by the wild type enzyme present in the mutant preparation (Richard et al., 1996), $k_{TFE}/k_s = 6.0\ M^{-1}$ for partitioning of the galactosylated wild type enzyme between reaction with trifluoroethanol and solvent water (Richard et al., 1995b), and with the assumption that the galactosylated E461G enzyme is reactive toward water but not trifluoroethanol. The dashed line shows the expected fit using $k_{TFE}/k_s = 0.3\ M^{-1}$ for partitioning of the galactosylated E461G enzyme.

(Richard et al., 1996), and 0.23 s^{-1} for the E461Q enzyme (Richard et al., 1996). Table 1 gives the values of k_3 for two cases where k_s is partly or wholly rate-determining for k_{cat}: the wild type enzyme-catalyzed cleavage of 2,2,2-trifluoroethyl β-D-galactopyranoside (Gal-OCH_2CF_3) [k_3 = 970 s^{-1}, Richard et al. (1995a)], and the E461Q enzyme-catalyzed cleavage of 4-nitrophenyl β-D-galactopyranoside (Gal-OC_6H_4-4-NO_2) [k_3 = 10 s^{-1}, Richard et al. (1996)]. Hydrolysis of the galactosylated E461G enzyme is completely rate-determining for reaction of the β-D-galactopyranosyl pyridinium ion (Gal-Pyr^+), but the value of k_3 for this reaction was not determined.

A comparison of the kinetic parameters for cleavage of Gal-OC_6H_4-4-NO_2 catalyzed by E461G β-galactosidase determined at pH 8.6 (Table 1) with $k_{cat} = 0.10\ s^{-1}$ and K_m = 0.014 mM determined at pH 7.0 (Cupples et al., 1990) shows that the change from pH 7.0 to 8.6 has no effect on k_{cat} but leads to a small increase in K_m for this substrate.

Figure 1 shows the effect of increasing concentrations of trifluoroethanol on v_{gal}/v_{PNP} for the reaction of Gal-OC_6H_4-4-NO_2 (0.07 mM) catalyzed by wild type (●) and E461G (■) β-galactosidase in the presence of 1.0 mM Mg^{2+}, where v_{PNP} is the total velocity of cleavage of the substrate to give 4-nitrophenoxide/4-nitrophenol and v_{gal} is the velocity of hydrolysis of the substrate to give D-galactose. The difference between v_{PNP} and v_{gal} determined in this assay is equal to the velocity of formation of the nucleophile adduct Gal-OCH_2CF_3, while the ratio v_{gal}/v_{PNP} is equal to the fractional yield of D-galactose from enzyme-catalyzed cleavage of Gal-OC_6H_4-4-NO_2 (Richard et al., 1995b). The data show that the addition of trifluoroethanol leads to a much larger decrease in the fractional yield of D-galactose from reaction of the galactosyl−enzyme intermediate for wild type β-galactosidase than for the E461G enzyme. Therefore, the E461G mutation results in a decrease in the reactivity of trifluoroethanol, compared with water, toward the galactosyl−enzyme intermediate.

The data in Figure 1 for the wild type enzyme were fit to a simple equation (not given), derived for the mechanism shown in Scheme 2 (Nu = TFE), using $k_{TFE}/k_s = 6.0\ M^{-1}$ for partitioning of the galactosyl−enzyme intermediate between reaction with trifluoroethanol and solvent water (Richard et al., 1995b). The upper solid line in Figure 1 shows the fit of the data for the E461G enzyme to eq 2,

$$v_{gal}/v_{PNP} = 1 - \{(f_{act})_{wt}/[1 + k_s/(k_{TFE}[TFE])]\} \quad (2)$$

which was derived with the assumption that the galactosylated E461G enzyme is reactive toward water but *not* trifluoroethanol, using $(f_{act})_{wt} = 0.15$ as the fraction of the total cleavage reaction of Gal-OC_6H_4-4-NO_2 that is catalyzed by contaminating wild type enzyme in our preparation of the E461G mutant (Richard et al., 1996), and $k_{TFE}/k_s = 6.0\ M^{-1}$ for partitioning of the wild type galactosyl−enzyme intermediate. The agreement between the experimental data and the fit calculated using eq 2 (±3%) is within the estimated experimental error in v_{gal}/v_{PNP} (±3%), so that these results are *consistent* with the proposal that 15% of the observed activity of our E461G mutant preparation for cleavage of Gal-OC_6H_4-4-NO_2 is due to the presence of a very small amount of contaminating wild type enzyme (Richard et al., 1996).

Wild Type Enzyme in E461G Mutant Preparation. The observed activity of our preparation of E461G β-galactosidase for cleavage of Gal-OCH_2CF_3 (Tables 1 and 2) may be due to the E461G enzyme itself or to contaminating wild type enzyme. We can distinguish the wild type and E461G enzymes by their specificity for transfer of the β-D-galactopyranosyl group from the respective galactosylated enzymes to neutral and anionic nucleophiles. The wild type enzyme catalyzes the efficient transfer of the galactosyl group from the galactosyl−enzyme intermediate to neutral alcohols such as trifluoroethanol, but not to anions (Richard et al., 1995b), while for the E461G enzyme there is efficient transfer of the galactosyl group from the galactosyl−enzyme intermediate to anions (Huber & Chivers, 1993; Richard et al., 1996), but not to neutral alcohols (Figure 1). The efficiencies of galactosyl transfer from the galactosyl−enzyme intermediates of the wild type and E461G enzyme-catalyzed reactions of Gal-OCH_2CF_3 were evaluated by analysis of the inhibition of the enzyme-catalyzed *hydrolysis* of this substrate by added trifluoroethanol and azide ion (Richard et al., 1995b). Figure 2A shows that increasing concentrations of added trifluoroethanol lead to the *same* fractional decrease in the normalized velocities v_{obsd}/v_o for *hydrolysis* of Gal-OCH_2CF_3 (0.6 mM) to give D-galactose catalyzed by wild type (□) and E461G (●) β-galactosidase in the presence of 1.0 mM Mg^{2+}, where v_o is the velocity of hydrolysis in the absence of trifluoroethanol. The data for both reactions show a good fit to eq 3 which was derived

$$v_{obsd}/v_o = \frac{1}{1 + k_{TFE}[TFE]/k_s} \quad (3)$$

for Scheme 3, using $k_{TFE}/k_s = 6.0\ M^{-1}$ determined for

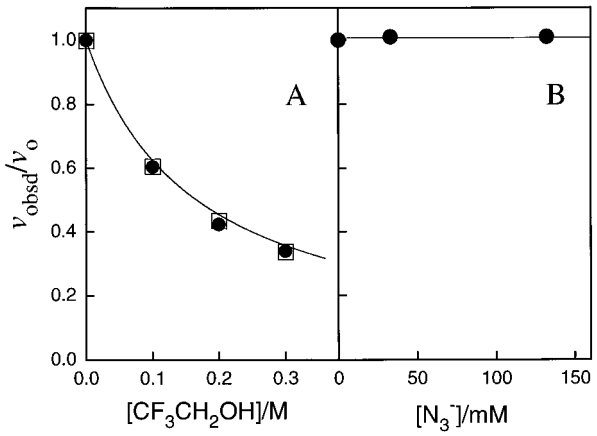


FIGURE 2: A. Dependence of v_{obsd}/v_o for hydrolysis of 2,2,2-trifluoroethyl β-D-galactopyranoside (0.6 mM) catalyzed by wild type (□) and E461G (●) β-galactosidase at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+} on the concentration of trifluoroethanol. The solid line shows the fit of the data to eq 3, calculated using $k_{TFE}/k_s = 6.0\ M^{-1}$ for partitioning of the galactosylated wild type enzyme between reaction with trifluoroethanol and solvent water (Richard et al., 1995b). B. Dependence of v_{obsd}/v_o for hydrolysis of 2,2,2-trifluoroethyl β-D-galactopyranoside (0.6 mM) catalyzed by E461G β-galactosidase at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+} on the concentration of azide ion.

Scheme 3

$$\text{E + Gal-OCH}_2\text{CF}_3 \underset{k_{TFE}[TFE]}{\overset{k_{cat}/K_m}{\rightleftharpoons}} \text{E-Gal} \xrightarrow{k_s} \text{E + Gal-OH}$$

partitioning of the galactosylated wild type enzyme between reaction with trifluoroethanol and solvent water (Richard et al., 1995b).

Figure 2B shows the effect of increasing concentrations of added sodium azide on the normalized velocity v_{obsd}/v_o for *hydrolysis* of Gal-OCH_2CF_3 (0.6 mM) to give D-galactose catalyzed by our preparation of E461G β-galactosidase in the presence of 1.0 mM Mg^{2+}, where v_o is the velocity of hydrolysis in the absence of azide ion. Any reaction of the galactosylated E461G enzyme with azide ion to form β-D-galactopyranosyl azide (Gal-N_3) will occur at the expense of formation of D-galactose and would cause the velocity of the hydrolysis reaction to decrease. For example, the velocity of the hydrolysis reaction of Gal-OC_6H_4-4-NO_2 catalyzed by our preparation of E461G β-galactosidase decreases by *ca.* 50% as $[N_3^-]$ is increased from zero to 0.1 mM (Richard et al., 1996). By contrast, Figure 2B shows that the addition of 130 mM N_3^- leads to no detectable change (<1%) in the velocity of formation of D-galactose from the reaction of Gal-OCH_2CF_3 catalyzed by our preparation of E461G β-galactosidase, so that there is *no detectable reaction* of azide ion with the galactosyl−enzyme intermediate of this reaction.

The data in Figure 2 show that the galactosyl−enzyme intermediate of the reaction of Gal-OCH_2CF_3 catalyzed by our preparation of E461G β-galactosidase cannot be the galactosylated E461G enzyme because the latter exhibits a large selectivity for reaction with azide ion (Huber & Chivers, 1993; Richard et al., 1996) and a very small selectivity for reaction with trifluoroethanol (Figure 1). In fact, the observed reaction intermediate has the same chemical selectivity for reaction with trifluoroethanol as that for the wild type enzyme and a negligible selectivity for reaction with azide ion (Figure 2), so that, by these criteria, it is indistinguishable from the galactosylated wild type enzyme (Richard et al., 1995b). These results provide strong evidence that the activity of our preparation of E461G β-galactosidase for cleavage of Gal-OCH_2CF_3 is due *entirely* to contaminating wild type enzyme.[3] The following considerations provide additional support for this conclusion:

(1) The values of K_m (at pH = 8.6) for hydrolysis of Gal-OC_6H_4-4-NO_2 and Gal-N_3 catalyzed by our preparation of E461G β-galactosidase are 1.5- and 2.0-fold smaller than the corresponding values of K_m for the wild type enzyme (Table 1), but, within experimental error, $K_m = 0.54$ mM for hydrolysis of Gal-OCH_2CF_3 catalyzed by the E461G mutant preparation is not different from $K_m = 0.61$ mM for the same reaction catalyzed by the wild type enzyme. These data are consistent with the conclusion that it is the E461G enzyme which is primarily responsible for catalysis of the cleavage of Gal-OC_6H_4-4-NO_2 and Gal-N_3 but that the cleavage of Gal-OCH_2CF_3 is catalyzed primarily by a small amount of contaminating wild type enzyme.

(2) The removal of Mg^{2+} from the enzyme leads to a 50-fold decrease in $(k_{cat})_{obsd}$ for hydrolysis of Gal-OCH_2CF_3 catalyzed by our preparation of E461G β-galactosidase, which is identical to the 50-fold effect of the removal of Mg^{2+} on the same reaction catalyzed by the wild type enzyme (Tables 1 and 2).

(3) The change from a trifluoroethoxy to a dichloroethoxy leaving group at Gal-X results in a 1.8-fold decrease in $(k_{cat})_{obsd}$ for glycoside cleavage catalyzed by our preparation of E461G β-galactosidase (Table 1), which is identical to the 1.8-fold effect of this change in leaving group on k_{cat} for the wild type enzyme (Richard et al., 1995a). By contrast, the change from a trifluoroethoxy to a 4-nitrophenoxy leaving group at Gal-X results in a 2.9-fold *increase* in $(k_{cat})_{obsd}$ for glycoside cleavage catalyzed by our preparation of E461G β-galactosidase, but a 3.4-fold *decrease* in k_{cat} for catalysis by the wild type enzyme (Table 1).

(4) Low concentrations of azide ion lead to sharp decreases in the yield of D-galactose and the formation of Gal-N_3 from the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461G β-galactosidase (Richard et al., 1996). However, the yield of D-galactose levels off at 15% and remains constant as the concentration of azide ion is increased to above 0.01 M (Richard et al., 1996). These results are consistent with the conclusion that 85% of the cleavage reaction of Gal-OC_6H_4-4-NO_2 is catalyzed by E461G β-galactosidase but that the remaining 15% is catalyzed by contaminating wild type enzyme in a reaction that is insensitive to azide ion (Richard et al., 1996). The fraction of contaminating wild type enzyme in our preparation of the E461G mutant that is calculated with the assumption that 100% of the activity of this preparation for cleavage of Gal-OCH_2CF_3 is due to the wild type enzyme is $f_{wt} = 0.035\ s^{-1}/410\ s^{-1} = 8.5 \times 10^{-5}$, where 0.035 and 410 s^{-1} are the observed activities of our mutant and wild type preparations, respectively, for cleavage of enzyme-bound Gal-OCH_2CF_3 (Table 1). This is somewhat smaller than $f_{wt} = 1.5 \times 10^{-4}$ that is calculated with the assumption that the limiting yield of 15% D-galactose

[3] The data in Figure 2A for inhibition of the E461G β-galactosidase-catalyzed hydrolysis of Gal-OCH_2CF_3 by added trifluoroethanol have been reproduced for two different preparations of the mutant enzyme, which effectively rules out the possibility that the wild type enzyme was introduced adventitiously during purification of the E461G mutant.

from the E461G enzyme-catalyzed cleavage of Gal-OC_6H_4-4-NO_2 at $[N_3^-] \geq 0.01$ M (Richard et al., 1996) is due to the presence of the wild type enzyme.[4] We prefer the value of $f_{wt} = 8.5 \times 10^{-5}$ (0.0085%) because it is obtained from a more direct analysis of the experimental data.

(5) E461Q β-galactosidase catalyzes the cleavage of Gal-OC_6H_4-4-NO_2 with $k_{cat} = 0.23$ s^{-1}, which is 2.3-fold larger than the observed value of k_{cat} for cleavage of this substrate by the E461G mutant (Table 1). By contrast, the lack of any detectable activity of the E461Q mutant for cleavage of Gal-OCH_2CF_3 was used to set the limit of $k_{cat} < 0.002$ s^{-1} for this reaction, so that k_{cat} for cleavage of Gal-OCH_2CF_3 by the E461Q mutant is more than 18-fold smaller than the *observed* value of k_{cat} for cleavage of this substrate by the E461G mutant (Table 1). This provides good evidence that Glu-461 is essential for cleavage of Gal-OCH_2CF_3, so that the observed cleavage of this substrate by our preparation of the E461G β-galactosidase must result from the presence of a wild type enzyme contaminant.

The observation of essentially constant velocities of hydrolysis of Gal-OCH_2CF_3 catalyzed by our preparation of E461G β-galactosidase in the presence of 0−0.13 M azide ion (Figure 2B) was used to set an upper limit on k_{cat} for the cleavage of this substrate by the E461G mutant in the following manner: at $[N_3^-] > 0.01$ M, the yield of D-galactose from reaction of the galactosylated E461G enzyme is less than 1% (Richard et al., 1996). Therefore, the failure of 0.13 M azide ion to cause any detectable decrease in the velocity of formation of D-galactose from the enzyme-catalyzed cleavage of Gal-OCH_2CF_3 shows that there is *no detectable* cleavage of this substrate by E461G β-galactosidase. The limit of $k_{cat} < 0.002$ s^{-1} for the E461G enzyme-catalyzed hydrolysis of Gal-OCH_2CF_3 was calculated from $(k_{cat})_{obsd} = 0.035$ s^{-1} (Table 1) with the assumption that a 5% decrease in the velocity of the *hydrolysis* reaction on increasing $[N_3^-]$ from zero to 0.13 M, corresponding to catalysis of 5% of the reaction by the E461G mutant, could have been detected in this experiment.

The absolute values of k_{cat} (s^{-1}) for cleavage of other Gal-X catalyzed by E461G β-galactosidase were calculated from the observed kinetic parameters in Tables 1 and 2 using eq 4, where $f_{wt} = 8.5 \times 10^{-5}$ is the fraction of wild type

$$(k_{cat})_{E461G} = (k_{cat})_{obsd} - f_{wt}(k_{cat})_{wt} \quad (4)$$

enzyme in our preparation of E461G mutant β-galactosidase (see above), and $(k_{cat})_{wt}$ is the kinetic parameter for cleavage of Gal-X by wild type β-galactosidase (Tables 1 and 2).

[4] Calculated from the following expression, which is the ratio of the activity of our preparation of E461G β-galactosidase attributed to the wild type enzyme contaminant to the total activity that would have been observed if 100% of the protein were wild type enzyme:

$$f_{wt} = 0.15\frac{(k_{cat})_{mut}\{[S] + (K_m)_{wt}\}}{(k_{cat})_{wt}\{[S] + (K_m)_{mut}\}} = 0.000\ 15$$

In this expression, 0.15 is the fraction of the total cleavage reaction of Gal-OC_6H_4-4-NO_2 (at [S] = 0.07 mM) attributed to catalysis by the wild type enzyme in our preparation of E461G β-galactosidase (Richard et al., 1996), $(k_{cat})_{mut} = 0.10$ s^{-1} and $(k_{cat})_{wt} = 120$ s^{-1} are the *observed* values of k_{cat} for the mutant and wild type enzymes, respectively (Table 1), and $(K_m)_{mut} = 0.033$ mM and $(K_m)_{wt} = 0.051$ mM are the Michaelis constants for the reactions catalyzed by the E461G and wild type enzymes, respectively (Table 1).

DISCUSSION

Wild Type or Mutant? It is generally difficult to determine whether the low levels of activity of site-directed enzyme mutants are due to the activity of the mutant enzyme itself, or to the wild type enzyme which forms by spontaneous reversion in one of the steps leading to protein synthesis. For example, preparations of Asp-461, Gly-461, and Gln-461 mutants of β-galactosidase show a low activity for hydrolysis of lactose, a substrate with a strongly basic 4-glucosyl leaving group (Cupples et al., 1990). However, it is not clear that these mutant enzymes catalyze the hydrolysis of lactose, since the observed activity may be due to the presence of a small amount of the wild type enzyme (Cupples et al., 1990).

We are able to distinguish E461G mutant from wild type β-galactosidase on the basis of the different specificities of the respective galactosylated enzymes for reaction with neutral and anionic nucleophiles: the galactosylated wild type enzyme is reactive toward neutral alcohols but unreactive toward azide ion (Richard et al., 1995b), while the galactosylated E461G enzyme is reactive toward azide ion (Huber & Chivers, 1993; Richard et al., 1996), but unreactive toward the neutral alcohol trifluoroethanol (Figure 1). Our preparation of E461G β-galactosidase catalyzes the cleavage of Gal-OC_6H_4-4-NO_2 through a galactosyl−enzyme intermediate which shows a high selectivity for reaction with azide ion (Richard et al., 1996) but only a very small selectivity for reaction with trifluoroethanol (Figure 1). By contrast, the same preparation of mutant enzyme catalyzes the cleavage of Gal-OCH_2CF_3 through an intermediate whose reactivity toward azide ion and trifluoroethanol is indistinguishable from that of the galactosyl−enzyme intermediate for the wild type enzyme (Figure 2). We therefore conclude that E461G β-galactosidase has essentially no activity for cleavage of Gal-OCH_2CF_3, and that the observed activity of this preparation for cleavage of this substrate is due entirely to contaminating wild type enzyme. The low level of wild type enzyme observed in a preparation of K12M triosephosphate isomerase has also been attributed to spontaneous reversion during protein synthesis (Lodi et al., 1994).

Our experimental results are consistent with an error that occurs at a rate of once every 12 000 insertions of Gly-461 into the growing polypeptide chain to give 0.0085% wild type enzyme. This lies within the range of the experimentally determined rates of translational error in *E. coli* (Schimmel, 1989). Our preparation of E461Q β-galactosidase shows no detectable activity toward Gal-OCH_2CF_3 and appears to be essentially free of the wild type enzyme (Richard et al., 1996). The rate of synthesis of wild type β-glucosidase from *Agrobacterium faecalis* by misincorporation of glutamate into a Gly-358 mutant is much faster than by misincorporation of glutamate into a Ala-358 mutant (Wang et al., 1994). These results suggest that misincorporation of glutamate for glycine may be more common than other translational errors for reasons which we do not understand.

Reactions of Trifluoroethanol. Figure 1 (■) shows the effect of increasing concentrations of trifluoroethanol on v_{gal}/v_{PNP} (the fractional yield of D-galactose) for the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by our preparation of E461G β-galactosidase. The presence of 0.0085% of the wild type enzyme in this preparation accounts for about 15% of the

Table 3: Effect of an E461 Mutation and/or the Removal of Mg^{2+} on k_3 for Cleavage of the Glycosidic Bond of Enzyme-Bound β-D-Galactopyranosyl Derivatives by β-Galactosidase (Scheme 2)[a]

form of β-galactosidase	$k_3/(k_3)_{wt}$ [b] (change in stability of transition state for cleavage of enzyme-bound substrate)[c] Gal-OCH_2CF_3 ($pK_a = 12.4$)[d]	Gal-OC_6H_4-4-NO_2 ($pK_a = 7.1$)[d]	Gal-N_3 ($pK_a = 4.7$)[d]
wild type enzyme (+ Mg^{2+})	1.00	1.00	1.00
E461G enzyme[e] (+ Mg^{2+})	$<2 \times 10^{-6}$ (>7.7 kcal/mol)	7.5×10^{-4} (4.2 kcal/mol)	0.0044 (3.2 kcal/mol)
E461Q enzyme[e] (+ Mg^{2+})	$<2 \times 10^{-6}$ (>7.7 kcal/mol)	0.083 (1.5 kcal/mol)	
wild type enzyme[f] (− Mg^{2+})	0.0084 (2.8 kcal/mol)	0.22 (0.9 kcal/mol)	0.0048 (3.2 kcal/mol)
E461G enzyme[g] (− Mg^{2+})	$<7 \times 10^{-7}$ (>8.4 kcal/mol)	3.6×10^{-4} (4.7 kcal/mol)	0.0044 (3.2 kcal/mol)

[a] At 25 °C and pH 8.6 (25 mM sodium pyrophosphate). [b] Calculated from the data in Tables 1 and 2. Except where noted otherwise in Tables 1 and 2, the rate constants k_3 for cleavage of the glycosidic bond of these enzyme-bound substrates are equal to k_{cat} for the overall reaction. [c] Calculated as $\Delta\Delta G^{\ddagger} = -RT \ln[k_3/(k_3)_{wt}]$. [d] pK_a of the conjugate acid of the leaving group at Gal-X taken from Jencks and Regenstein (1976). [e] Effect of E461 mutation on k_3 for cleavage of Gal-X by β-galactosidase in the presence of Mg^{2+}. [f] Effect of removal of Mg^{2+} on k_3 for cleavage of Gal-X by wild type β-galactosidase. [g] Effect of E461G mutation *and* removal of Mg^{2+}on k_3 for cleavage of Gal-X by β-galactosidase.

total enzyme activity for cleavage of Gal-OC_6H_4-4-NO_2, and the subsequent reaction of trifluoroethanol with the galactosylated wild type enzyme results in small decreases in v_{gal}/v_{PNP} with increasing concentrations of trifluoroethanol (Figure 1, ■). The dashed line in Figure 1 was calculated assuming $k_{TFE}/k_s = 0.3\ M^{-1}$ for the relative reactivities of trifluoroethanol and solvent water toward the galactosylated E461G enzyme; it lies up to 6% below the experimental data, which is comparable with the estimated experimental error in v_{gal}/v_{PNP} (±3%). We conclude that the E461G mutation results in a decrease in the selectivity of the galactosylated enzyme for reaction with trifluoroethanol from $k_{TFE}/k_s = 6.0\ M^{-1}$ for wild type β-galactosidase (Richard et al., 1995b) to $k_{TFE}/k_s < 0.3\ M^{-1}$ for the E461G enzyme.

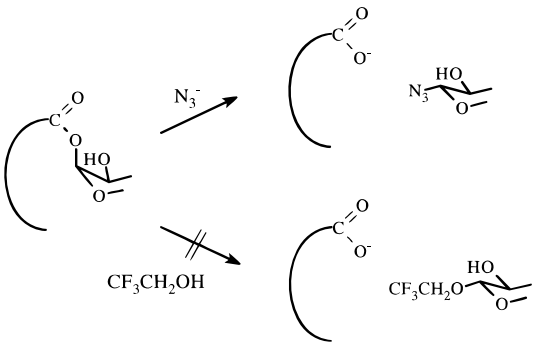
It is usual for trifluoroethanol to be less reactive than water in nucleophile addition reactions, because of the lower basicity of the alkoxy oxygen (Harris et al., 1978; Richard & Jencks, 1984; Ta-Shma & Jencks, 1986). Thus, the abnormally large nucleophilic reactivity of trifluoroethanol toward galactosylated wild type β-galactosidase ($k_{TFE}/k_s = 6.0\ M^{-1}$) is consistent with pronounced proton transfer from the nucleophile to the enzyme in the transition state and the development of negative charge at the alkoxy oxygen of the nucleophile, which is more effectively stabilized by the electron-withdrawing trifluoroethyl group than by a hydrogen or a simple alkyl group (Richard et al., 1995a,b). The more normal small selectivity for reaction of galactosylated E461G β-galactosidase with trifluoroethanol, $k_{TFE}/k_s < 0.3\ M^{-1}$, suggests that there is no general base catalysis of the reaction of nucleophilic alcohols, and it strongly implicates the propionate side chain of Glu-461 at the wild type enzyme in general base catalysis of the reaction of trifluoroethanol with the galactosyl−enzyme intermediate.

Role of Glu-461 in Catalysis. The mechanism of action of β-galactosidase shown in Scheme 1 follows the paradigm established for lysozyme (Blake et al., 1967) in that it utilizes carboxylate side chains to interact with the glycosidic carbon of the β-D-galactopyranoside (Glu-537) (Gebler et al., 1992) and to provide general acid−base catalysis at the leaving group/nucleophile (Glu-461) (Gebler et al., 1992). However, there is good evidence that Mg^{2+} also plays a role in electrophilic catalysis at the leaving group (Selwood & Sinnott, 1990; Sinnott, 1990; Sinnott et al., 1978). Structural analyses by X-ray crystallography and other methods is critical to the development of rational assignments of the roles of amino acid side chains and metal cofactors in catalysis. The crystal structure of β-galactosidase shows that Glu-461 and Mg^{2+} lie in close proximity in the free enzyme (Jacobson et al., 1994), so that either of these could function directly or indirectly in electrophilic catalysis at the leaving group. Distance measurements on the Mn^{2+} enzyme show that this metal ion is bound too distant from the methyl groups of methyl β-D-thiogalactopyranoside and β-D-galactopyranosyl trimethylammonium ion inhibitors to interact directly with the substituent at the galactopyranosyl ring (Loeffler et al., 1979). However, it has been suggested that "these inhibitors binding to the Mn^{2+}-enzyme are inaccurate models for substrates binding to the Mg^{2+}-enzyme" (Sinnott, 1990).

Table 3 summarizes the effects of an E461 mutation and/or the removal of Mg^{2+} on k_3, the first-order rate constant for β-galactosidase-catalyzed cleavage of the glycosidic bond of enzyme-bound β-D-galactopyranosyl derivatives (Scheme 2). These data expand considerably the evidence, summarized below, in support of a direct role for Glu-461 in general acid−base catalysis at the leaving group/nucleophile and an indirect role for Mg^{2+} that is yet to be clearly defined.

(1) The effects of E461 mutations on the stability of the transition state for cleavage of the glycosidic bond of Gal-X are much larger than the effects of removal of Mg^{2+}. For example: (a) The E461G and E461Q mutations cause a 1300- and a 12-fold reduction, respectively, in k_3 for cleavage of enzyme-bound Gal-OC_6H_4-4-NO_2 and more than 500 000-fold reductions in k_3 for cleavage of enzyme-bound Gal-OCH_2CF_3 (Table 3). By comparison, the removal of Mg^{2+} causes only a 5- and a 120-fold reduction, respectively, in k_3 for cleavage of enzyme-bound Gal-OC_6H_4-4-NO_2 and Gal-OCH_2CF_3 (Table 3). (b) The value of k_{cat} (= k_3) for cleavage of Gal-OCH_2CF_3 by the Mg^{2+}-free wild type enzyme is more than *4000-fold larger* than that for cleavage of this substrate by the E461G or E461Q enzymes (Table 3). These data show that, if Mg^{2+} *does* provide direct electrophilic catalysis of the cleavage of Gal-OCH_2CF_3, then either the contribution of this interaction to the catalytic rate acceleration is relatively small, or there is an alternative acidic amino acid side chain which functions to provide electrophilic stabilization of the

Scheme 4



leaving group anion in the Mg^{2+}-free enzyme.

(2) The E461G mutation causes a change in the specificity of the galactosylated enzyme from galactosyl transfer to neutral alcohols but not to anions for the wild type enzyme (Richard et al., 1995b), to galactosyl transfer to anions (Huber & Chivers, 1993; Richard et al., 1996) but not to alcohols (Figure 1) for the mutant enzyme (Scheme 4). These observations are readily rationalized by the participation of Glu-461 in general base catalysis of the reaction of alcohol nucleophiles with the galactosyl−enzyme for the following reasons: (a) General base catalysis of the reaction of neutral alcohols provides significant stabilization of the transition state for their reaction which is not available for an enzyme which lacks this basic amino acid side chain (Scheme 4). Therefore, the removal of this basic side chain should result in a large decrease in the reactivity of the weakly nucleophilic alcohol trifluoroethanol, for which the requirement for general base catalysis is greatest (see above). (b) The anionic carboxylate side chain of Glu-461 may create an electrostatic barrier to the binding and reaction of anionic nucleophiles such as azide ion. This barrier is presumably relieved upon excision of this anionic side chain.

(3) The value of $k_{TFE} = 4200\ M^{-1}\ s^{-1}$ for reaction of the galactosylated wild type enzyme with trifluoroethanol (Richard et al., 1995b) is similar to $k_{az} = 4900\ M^{-1}\ s^{-1}$ for reaction of the galactosylated E461G enzyme with azide ion (Richard et al., 1996). This startling observation localizes the effect of the E461G mutation on breakdown of the galactosyl−enzyme intermediate at the nucleophile, and shows that the net rate of galactosyl transfer from the mutant enzyme can even exceed that for transfer from the wild type enzyme, provided that the differing nucleophilic specificities of the two reactions are taken into account.

(4) The removal of Mg^{2+} leads to relatively small changes in $(\beta_{lg})_{k_3}$ and $(\beta_{lg})_{k_{cat}/K_m}$ for the wild type enzyme, from -0.49 to ~ -0.66 and -0.75 to ~ -0.93, respectively [Figures 2 and 3 in Richard et al. (1995a)]. This result is not easily rationalized by the direct chelation of Mg^{2+} to the developing alkoxide ion in the reaction transition state because removal of the chelated dication would be expected to result in a larger change in the "effective" negative charge (Hupe & Jencks, 1977) at the alkoxy oxygen than these observed *ca.* 0.2 unit increases (Richard et al., 1995a). For example, the addition of a proton to the transition state for the spontaneous cleavage of glycosides results in a *ca*. 1.0 unit decrease in effective negative charge at the oxygen of the leaving group [see Richard et al. (1995a)].

(5) The E461G mutation causes the equilibrium constant for transfer of the galactosyl group from the galactosylated enzyme to azide ion to increase by more than 8000-fold (Richard et al., 1996).[5] This is difficult to reconcile with a mechanism in which Glu-461 plays a relatively passive role as a ligand for Mg^{2+} (Edwards et al., 1990). The difference in these equilibrium constants may be a direct consequence of the coupling of galactosyl transfer to the thermodynamically unfavorable protonation of the carboxylate side chain of Glu-461 and the absence of such coupling for the E461G mutant. This could arise if there were an increase in the pK_a of the carboxylate group of Glu-461 on moving from the galactosylated enzyme, where the side chain would exist mostly as the anion required for general base catalysis of the reaction of water, to the free enzyme, where it would exist mostly in the protonated form required for general acid catalysis of glycoside cleavage (Richard et al., 1996).

The data for cleavage of Gal-N_3 and Gal-Pyr^+ by β-galactosidase (Tables 1 and 2) are also consistent with a primary role for Glu-461 in general acid−base catalysis:

(1) Electrophilic catalysis of expulsion of the weakly basic azide leaving group from Gal-N_3 is expected to be relatively unimportant. It is therefore surprising, at first sight, that both the E461G mutation and the removal of Mg^{2+} lead to similar increases of 3.2 kcal/mol in the activation barrier to cleavage of Gal-N_3 by β-galactosidase (Table 3). However, we have proposed in earlier work that the cleavage of Gal-N_3 to give the galactosylated enzyme and bound azide ion is readily reversible so that the rate-determining step is the protonation of the azide ion leaving group by Glu-461, which is assisted by Mg^{2+} because the metal ion leads to an increase in the acidity of the carboxylic acid of Glu-461 (Richard et al., 1995b). Thus, the observation that excision of this side chain results in a large decrease in the rate of hydrolysis of Gal-N_3 (Table 3), is consistent with its proposed role in protonation of the leaving group. Furthermore, there is no activation of the E461G enzyme for cleavage of Gal-N_3 by Mg^{2+} (Table 3). This is consistent with the proposal that the activation of β-galactosidase for cleavage of this substrate by Mg^{2+} is a consequence of an interaction between the metal cation and the carboxylate side chain of Glu-461, rather than simple electrophilic catalysis by the metal ion.

(2) The removal of Mg^{2+} from the wild type enzyme leads to a small increase in k_{cat} for cleavage of Gal-Pyr^+ at pH 7.0, from $k_{cat} = 0.61\ s^{-1}$ at $[Mg^{2+}] = 1.0$ mM (Sinnott & Withers, 1974) to $k_{cat} = 2.8\ s^{-1}$ for the magnesium-free enzyme (Sinnott et al., 1978). Similarly, the E461G mutation has only a small 1.5-fold effect on k_{cat} for hydrolysis of Gal-Pyr^+ at pH 8.6 (Table 1). However, this mutation is accompanied by a change in the rate-determining step for the reaction from k_3 for the wild type enzyme, to k_s for the mutant enzyme (Scheme 2). This result requires that the E461G mutation cause a large, but undetermined, *increase* in k_3 for cleavage of enzyme-bound Gal-Pyr^+. We conclude that Mg^{2+} provides little or no assistance to the cleavage of enzyme-bound Gal-Pyr^+ and that excision of the propionate side chain of Glu-461 results in a smaller barrier to cleavage of this substrate. Therefore, the removals of Mg^{2+} or Glu-461 have very different effects on enzymatic catalysis of the cleavage of substrates with oxygen leaving groups and of substrates such as Gal-Pyr^+ which lack an electron pair that

[5] These equilibrium constants were calculated from a Haldane relationship and kinetic data for the β-galactosidase-catalyzed synthesis and cleavage of β-D-galactopyranosyl azide (Richard et al., 1996).

can interact with an electrophile at the enzyme.

Interactions between Glu-461 and Mg^{2+}. A study of the dependence of the activation of β-galactosidase for cleavage of Gal-OC_6H_4-4-NO_2 on [Mg^{2+}] has shown that the E461G mutation causes the concentration of Mg^{2+} required for 50% activation of the enzyme to increase 31-fold, from 0.64 μM for the wild type enzyme to 20 μM for the E461G mutant (Edwards et al., 1990). This result is consistent with a direct interaction between Glu-461 and Mg^{2+} that assists in the binding of Mg^{2+} to the enzyme. However, the following observations are difficult to reconcile with a mechanism in which the primary role of Glu-461 is simply to anchor Mg^{2+} to β-galactosidase.

(1) If Glu-461 acts primarily to anchor the metal ion to the enzyme, then it is difficult to rationalize the more than 500 000-fold smaller value of k_3 for cleavage of enzyme-bound Gal-OCH_2CF_3 by the E461G than by wild type β-galactosidase (Table 3), *both of which are saturated with magnesium at [Mg^{2+}] = 1.0 mM*, because the simple removal of the carboxylate side chain of Glu-461 from the coordination sphere of Mg^{2+} should not lead to a larger decrease in k_3 than that observed for the complete removal of Mg^{2+} from the wild type enzyme (120-fold, Table 3).

(2) The E461G mutation may lead to a change in the location of the enzyme-bound Mg^{2+}, which might reduce the electrostatic stabilization of the transition state by this metal ion. However, the observation that the inclusion of Mg^{2+} results in similar increases in k_{cat} for cleavage of bound Gal-OC_6H_4-4-NO_2 by wild type and E461G β-galactosidase of 4.6-fold and 2.1-fold, respectively (Tables 1 and 2), is consistent with a similar stabilization of the transition state by bound Mg^{2+} for both enzymes.

(3) The value of $k_{cat} = 4\ s^{-1}$ for cleavage of Gal-OC_6H_4-4-NO_2 by E461H β-galactosidase at pH 7.0 (Martinez-Bilbao et al., 1995) is 40-fold larger than $k_{cat} = 0.10\ s^{-1}$ for cleavage of this substrate by the E461G mutant at the same pH (Cupples et al., 1990). These data suggest that the placement of an acidic side chain (the imidazolium ion of histidine) at position 461 greatly attenuates the effect of mutation of Glu-461 on catalytic activity, and are therefore consistent with a role for side chains at this position in general acid catalysis. In addition, the E461H mutant exhibits optimal activity in the *absence* of Mg^{2+}, and the inhibitory effect of Mg^{2+} increases with increasing pH (Martinez-Bilbao et al., 1995). This suggests that the E461H enzyme is fully active when the imidazole group of His-461 is protonated but that deprotonation of this residue leads to the uptake of Mg^{2+} and the loss of catalytic activity. This is consistent with the proposal that an acidic side chain in its protonated form at position 461 is required for optimal enzymatic activity, but that the complex in which the basic side chain of His-461 is chelated to Mg^{2+} is inactive.

We suggest that the effects of Glu-461 mutations on metal ion binding (Edwards et al., 1990) may be due to subtle changes in the shape of the binding pocket for the metal ion rather than the simple excision of a metal ligand.

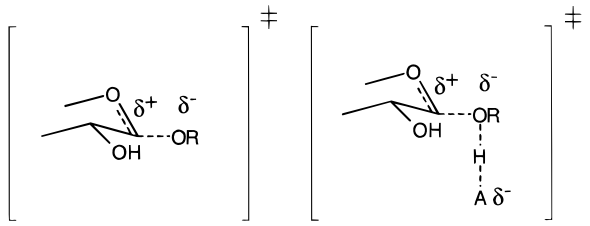
The conclusion from this work that Glu-461 functions directly in general acid−base catalysis at the leaving group/nucleophile does not detract from the significance of earlier work which implicates Mg^{2+} in electrophilic catalysis at the leaving group (Sinnott, 1990; Sinnott et al., 1978). The simplest proposal that reconciles these apparently conflicting conclusions is that the enzyme-bound Mg^{2+} functions to ensure optimal acid−base catalysis by Glu-461. We present evidence in the following paper that the carboxylic acid side chain of Glu-461 undergoes a substantial decrease in pK_a upon conversion of the wild type enzyme to its galactosylated form, and we offer the speculative possibility that this change in pK_a is caused by movement of bound Mg^{2+} toward Glu-461 on proceeding to the galactosylated enzyme. If the decrease in the pK_a of this group were partially expressed in the transition state for transfer of the galactosyl group from the substrate to the enzyme, then, as is observed, the presence of Mg^{2+} would result in an increase in the reactivity of Glu-461 as a general acid catalyst.

Contribution of General Acid Catalysis to the β-Galactosidase-Catalyzed Cleavage of Alkyl β-D-Galactopyanosides. Any quantitative analysis of our data is complicated by the observation that the rate constant k_3 for cleavage of the glycosidic bond of enzyme-bound Gal-OC_6H_4-4-NO_2 is 110-fold larger for E461Q than for E461G β-galactosidase (Table 1). Therefore, the stability of the transition state for this enzyme-catalyzed reaction is sensitive to small changes in the local environment at the active site, which are not well understood. However, a more detailed analysis of the effect of the E461G and E461Q mutations on k_3 for cleavage of enzyme-bound Gal-OCH_2CF_3 and Gal-OC_6H_4-4-NO_2 does allow for an interesting *estimate* of the relative contribution of general acid catalysis at the leaving group to stabilization of the transition state for enzymatic cleavage of these substrates and for a further refinement of the structure of this transition state (Richard et al., 1995a,b).

The rate constant for the spontaneous (uncatalyzed) hydrolysis of Gal-OC_6H_4-4-NO_2 at pH 8.6 is expected to be *ca.* $10^{6.6}$-fold larger than that for the spontaneous hydrolysis of Gal-OCH_2CF_3.[6] However, this very large difference in the *chemical* reactivity of these substrates is eliminated upon their binding to β-galactosidase, because $k_3 = 970\ s^{-1}$ for cleavage of the glycosidic bond of enzyme-bound Gal-OCH_2CF_3 by wild type β-galactosidase at pH 8.6 is 8-fold *larger* than that for cleavage of bound Gal-OC_6H_4-4-NO_2 (Table 1). This corresponds to a 10.2 kcal/mol larger stabilization of the transition state for cleavage of the former substrate by the enzyme. Part of this much larger apparent stabilization of the transition state for cleavage of Gal-OCH_2CF_3 than for cleavage of Gal-OC_6H_4-4-NO_2 may result from the requirement for the loss of nonproductive binding interactions between the enzyme and the hydrophobic 4-nitrophenyl group of Gal-OC_6H_4-4-NO_2 on proceeding from the enzyme-bound substrate to the transition state (Richard, 1995b). However, the remaining additional stabilization of the transition state for cleavage of Gal-OCH_2CF_3 provided by the enzyme must be due to the more effective electrophilic catalysis of cleavage of the glycosidic bond to the strongly basic trifluoroethoxide (pK_a = 12.4) than to the more weakly basic 4-nitrophenoxide (pK_a = 7.1) leaving group.

The data in Table 3 allow for a rough estimate of the difference in the stabilization of the enzymatic transition state for glycoside cleavage at E461G and E461Q β-galactosidases (transition state **1**) that is obtained from general acid catalysis

[6] Estimated from the difference in the pK_as of the two leaving groups [pK_a = 12.4 for trifluoroethanol and pK_a = 7.1 for 4-nitrophenol, taken from Jencks and Regenstein (1976)] and $\beta_{lg} = -1.25$, which is an average of the values of β_{lg} for the cleavage of β-D-galactopyranosyl derivatives summarized in Table 2 of Richard et al. (1995a).



1 **2**

by Glu-461 at the wild type enzyme (transition state **2**). The substitution of glutamate for either glycine or glutamine at position 461 leads to a much larger stabilization of the transition state for cleavage of Gal-OCH_2CF_3, $|(\Delta\Delta G^{\ddagger})_{TFE}|$ > 7.7 kcal/mol, than for cleavage of Gal-OC_6H_4-4-NO_2, $|(\Delta\Delta G^{\ddagger})_{ArO}|$ = 4.2 kcal/mol (glycine) or 1.5 kcal/mol (glutamine) (Table 3). Equation 5 relates the *difference* in

$$(\Delta\Delta G^{\ddagger})_{TFE} - (\Delta\Delta G^{\ddagger})_{ArO} = \{f_p\}\{-RT \ln[(K_a^{\ddagger})_{ArO}/(K_a^{\ddagger})_{TFE}]\} \quad (5)$$

these transition state stabilizations on moving from E461G or E461Q β-galactosidase to the wild type enzyme, $(\Delta\Delta G^{\ddagger})_{TFE} - (\Delta\Delta G^{\ddagger})_{ArO}$, to f_p, the fractional extent of proton transfer from the enzyme to the leaving group in the transition state **2**, where $(K_a^{\ddagger})_{TFE}$ and $(K_a^{\ddagger})_{ArO}$ are the effective acidity constants of the partially protonated leaving groups in the respective transition states (Jencks, 1972). The relationship defined by eq 5 is based on the assumption that the observed effect is due to a stronger interaction of the general acid catalyst with the highly basic trifluoroethoxy group than with the 4-nitrophenoxy leaving group. The maximum possible stabilization $|(\Delta\Delta G^{\ddagger})_{TFE} - (\Delta\Delta G^{\ddagger})_{ArO}| = 7.2$ kcal/mol will be observed for a "late" transition state in which there is both complete cleavage of the glycosidic C−O bond, so that $(K_a^{\ddagger})_{ArO}/(K_a^{\ddagger})_{TFE} = 10^{-7.1}/10^{-12.4} = 10^{5.3}$, and complete proton transfer to the leaving group, so that $f_p = 1.0$. The *observed* values of $|(\Delta\Delta G^{\ddagger})_{TFE} - (\Delta\Delta G^{\ddagger})_{ArO}|$ are more than 3.5 (≥7.7−4.2) kcal/mol for the E461G mutant and more than 6.2 (≥7.7−1.5) kcal/mol for the E461Q mutant. These correspond to the expression of more than 49% (E461G) or 86% (E461Q) of the maximum stabilization available from proton transfer from Glu-461 to the leaving group in the transition state for glycoside cleavage. These observations are entirely consistent with a transition state in which there is a very large fractional cleavage of the glycosidic C−O bond, and extensive proton transfer from the enzyme to the anionic leaving group.

ACKNOWLEDGMENT

We wish to dedicate this pair of manuscripts to William P. Jencks.

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BI961028J