Structure−Reactivity Relationships for β-Galactosidase (*Escherichia coli, lac Z*). 4. Mechanism for Reaction of Nucleophiles with the Galactosyl-Enzyme Intermediates of E461G and E461Q β-Galactosidases†

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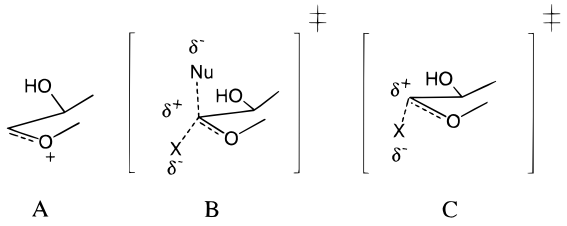
ABSTRACT: Second-order rate constants for transfer of the β-D-galactopyranosyl group from the galactosyl−enzyme intermediates of the galactosyl transfer reactions catalyzed by E461G and E461Q β-galactosidases to anionic nucleophiles have been determined. The second-order rate constant for reaction of the galactosylated E461G enzyme with azide ion is 4900 M^{-1} s^{-1}. By contrast, there is no detectable reaction of the galactosylated wild type enzyme with azide ion (Richard et al., 1995b), and the E461G mutation leads to a large decrease in the second-order rate constant k_{cat}/K_m for catalysis of cleavage of β-D-galactopyranosyl azide, which is the microscopic reverse of the reaction of azide ion with the galactosyl−enzyme intermediate. These data show that the E461G mutation causes a more than 8000-fold increase in the equilibrium constant for transfer of the β-D-galactopyranosyl group from β-galactosidase to azide ion. We propose that this change represents the requirement for the coupling of galactosyl transfer from the native enzyme to the thermodynamically unfavorable protonation of the carboxylate group of Glu-461, but the expression of the full chemical affinity of azide ion for galactosyl transfer from the mutant enzyme which lacks this ionizable side chain at position 461. The reactions of acetate, butyrate and methoxyacetate ions with the galactosylated E461G enzyme and of acetate with the galactosylated E461Q enzyme give both the corresponding β-galactopyranosyl derivatives and D-galactose, and the formation of the latter represents formal catalysis of the reaction of water with the galactosylated enzyme. However, the reaction of formate ion with the galactosylated E461G enzyme gives only D-galactose. These results suggest that carboxylate anions can take the place of the excised propionate side chain of Glu-461 to provide general base catalysis of the reaction of water with the galactosyl−enzyme intermediates. The relative reactivity of anionic nucleophiles toward the covalent galactosyl−enzyme intermediate of the reactions catalyzed by the E461G enzyme is similar to that observed for partitioning of stable carbocations in water. This suggests that replacement of the anionic side chain of Glu-461 by a hydrogen exposes an enzyme-stabilized oxocarbenium ion intermediate to reaction with external nucleophilic reagents.

Many of the details of enzymatic catalysis of glycosyl group transfer to neutral and anionic nucleophiles are not well understood. These reactions may proceed by either of the concerted A_ND_N (S_N2) and stepwise $D_N + A_N$ (S_N1) (Guthrie & Jencks, 1989; IUPAC Commission on Physical Organic Chemistry, 1989) reaction mechanisms that are observed for aliphatic nucleophilic substitution reactions in solution (Sinnott, 1987, 1990). The stepwise reaction mechanism is favored because the putative oxocarbenium ion intermediate is stabilized by electron donation from the α-oxygen (Chart 1A). However, substitution reactions at α-alkoxy derivatives may also follow a concerted reaction mechanism, through a transition state which avoids formation of the oxocarbenium ion reaction intermediate (Chart 1B) (Craze & Kirby, 1978; Knier & Jencks, 1980; Amyes & Jencks, 1989a; Banait & Jencks, 1991).

The large secondary α-deuterium isotope effects observed for enzyme-catalyzed glycosyl transfer (Dahlquist et al., 1969; Kirsch, 1977; Sinnott & Souchard, 1973) show that there is a substantial change from sp^3 to sp^2 hybridization at the glycosidic carbon on proceeding to the reaction transition state, and are consistent with extensive bond cleavage and development of positive charge at the glycosyl group in these transition states. These results serve to define the *appearance* of the transition state as carbocation-like, but they are not sufficient to prove that glycosyl oxocarbenium ions are formed as intermediates of stepwise substitution reactions. For example, large secondary α-deuterium isotope effects have been observed for both stepwise (Young et al., 1980) and concerted (Knier & Jencks, 1980) nucleophilic substitution reactions at acetals. Therefore, the transition states for

Chart 1



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these stepwise (Chart 1C) and concerted (Chart 1B) nucleophilic substitution reactions both appear to be highly oxocarbenium-ion-like, presumably because the *total* bonding of the leaving group and the nucleophile to the central carbon is similar for the two transition states. This reflects the open or "exploded" nature of the transition state for concerted nucleophilic substitution at acetals (Craze & Kirby, 1978; Knier & Jencks, 1980; Amyes & Jencks, 1989a; Banait & Jencks, 1991).

It is sometimes possible to detect reactive carbanion (Guthrie et al., 1984; Tapuhi & Jencks, 1982) and carbocation intermediates (Jencks, 1981; Richard et al., 1984) of organic reactions in solution by the trapping of these species with electrophilic or nucleophilic reagents, respectively. It is more difficult to trap reactive carbanion and carbocation intermediates of enzyme-catalyzed reactions, because the protein catalyst usually protects these intermediates from their reaction with external reagents to form nonphysiological products. However, there are several observations which show that the protection of carbanions from oxidative side reactions is not perfect. Class I and class II aldolases catalyze the oxidation of dihydroxyacetone phosphate by $Fe(CN)_6^{3-}$ (Grazi, 1975; Kuo & Rose, 1985; Pathy, 1978) and tetranitromethane (Christen & Riordan, 1968; Healy & Christen, 1972; Riordan & Christen, 1969), presumably by the oxidative trapping of carbanion or "carbanionoid" reaction intermediates, while the oxygenase reactions catalyzed by ribulose bisphosphate carboxylase, acetolactate synthase, pyruvate decarboxylase, class II aldolases, and glutamate decarboxylase (Abell & Schloss, 1991; Tse & Schloss, 1993) are consistent with the trapping of carbanion reaction intermediates by their reaction with singlet molecular oxygen.

By contrast, we are not aware of any reports of trapping of glycosyl oxocarbenium ion intermediates of wild type enzyme-catalyzed glycosyl transfer reactions by strongly nucleophilic anions. In fact, the potent nucleophile azide ion is more than 60-fold less reactive than the weakly nucleophilic alcohol trifluoroethanol toward the galactosyl−enzyme intermediate of the β-D-galactopyranosyl group transfer reaction catalyzed by β-galactosidase (Richard et al., 1995b). The failure to observe transfer of the galactosyl group from β-galactosidase to azide ion may reflect the destabilization of the transition state of this reaction by unfavorable electrostatic interactions between the nucleophilic anion and an anionic amino acid side chain which participates in general base catalysis of the reaction of water with the covalent galactosyl−enzyme intermediate. This interpretation is supported by the observations of (a) a more than 100-fold greater reactivity of azide ion toward galactosylated E461G β-galactosidase than toward the galactosylated wild type enzyme (Cupples et al., 1990; Richard et al., 1995b); (b) the effective trapping by azide ion of the α-D-glycopyranosyl reaction intermediate for E127A exoglucanase/xylanase from *Cellulomonas fimi* (McLeod et al., 1994); and (c) the effective trapping by azide ion of an intermediate of the reaction catalyzed by E358A *Agrobacterium* β-glucosidase (Wang et al., 1994). However, the mechanistic implications of the observed activation of these reaction intermediates for reaction with azide ion by the mutation of active site glutamate residues have not yet been examined in detail.

We report here the results of a study of the reactions of galactosylated E461G and E461Q β-galactosidases with nucleophilic anions. These experiments were designed to address several questions.

(1) What are the relative reactivities of nucleophilic anions toward galactosylated E461 mutant β-galactosidases? The different scales of nucleophile reactivity (Pearson et al., 1968; Ritchie, 1972, 1986) and the different relative nucleophile reactivities across a common reactivity scale (Ritchie, 1972, 1986; Knier & Jencks, 1980) that have been observed for nucleophilic addition to carbenium ions and concerted aliphatic nucleophilic substitution reactions in solution may serve as a basis for the distinction between these pathways for the reaction of nucleophiles with galactosylated E461 mutant β-galactosidases. We report here that both the order and the relative magnitude of the rate constant ratios for partitioning of galactosylated E461G β-galactosidase between reaction with nucleophilic anions and solvent water are similar to those observed for the addition of nucleophiles to carbenium ions in water, so that they are consistent with the reaction of these nucleophiles with an enzyme-bound glycosyl oxocarbenium ion intermediate.

(2) Can exogenous carboxylate anions bind to E461 mutant β-galactosidases and undergo reaction in place of the excised propionate side chain of Glu-461? The identification and characterization of such reactions might provide insight into the role of the propionate side chain of Glu-461 in catalysis. We report that formate anion is essentially unreactive as a nucleophile toward galactosylated E461G and E461Q β-galactosidases, but that this carboxylate anion provides effective general base catalysis of the reaction of water with the galactosyl−enzyme intermediate. These results suggest that the propionate side chain of Glu-461 plays a similar role in the activation of water for reaction with the galactosylated wild type enzyme.

(3) The dramatic effect of the E461G mutation on the reactivity of the galactosyl−enzyme intermediate toward azide ion shows that this mutation causes a large change in the equilibrium constant for transfer of the β-D-galactopyranosyl group from β-galactosidase to azide ion, $K_{az} = k_{az}/(k_{cat}/K_m)$ (Scheme 2), which we want to estimate and rationalize. We report that the E461G mutation results in a more than 8000-fold increase in the equilibrium constant for transfer of the β-D-galactopyranosyl group from β-galactosidase to azide ion, and we suggest that the smaller equilibrium constant for the wild type enzyme represents the coupling of galactosyl transfer to the thermodynamically unfavorable protonation of the carboxylate group of Glu-461.

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; Richard et al., 1996). Sodium azide, methoxyacetic acid, and the sodium salts of formic, acetic, butyric, and chloroacetic acids were purchased from Aldrich. Sodium cyanide was purchased from Fisher. 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).

Preparation of Solutions. Stock solutions of sodium azide (0.001−1.0 M), sodium formate (2.0 or 5.0 M), sodium acetate (1.0 or 4.0 M), and sodium chloroacetate (2.0 M)

were prepared by dissolving the sodium salts in water followed by the addition of small aliquots of 1 M HCl or 1 M NaOH to give pH ≈ 8.6. A stock solution of sodium methoxyacetate (3.0 M) was prepared by careful neutralization of methoxyacetic acid with sufficient volumes of ~10 and 1 M NaOH to give pH ≈ 8.6. A stock solution of sodium butyrate (0.15 M) was prepared by dissolving the sodium salt in 27.8 mM sodium pyrophosphate buffer (pH 8.6) containing 1.11 mM $MgCl_2$; the final pH of the stock solution was 8.3. A stock solution of sodium cyanide (0.50 M) at pH 9.2 was prepared IN A FUME HOOD by dissolving sodium cyanide in water followed by the cautious addition of 1 M HCl. A value of $pK_a = 9.2$ for hydrogen cyanide under our reaction conditions ($I \approx 0.22$, maintained with NaCl) was determined as the observed pH of 0.05−0.20 M 50% free base cyanide buffers at $I = 0.25$ (NaCl).

Enzyme Assays. Galactose dehydrogenase was freed of ammonium sulfate by dialysis against 25 mM sodium pyrophosphate buffer (pH 8.6) that contained 1 mM ethylenediaminetetraacetic acid (EDTA),[1] 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).

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. Assay mixtures were prepared by mixing 0.9 mL of 27.8 mM sodium pyrophosphate buffer (pH 8.6) that contained 1.11 mM $MgCl_2$ or 11.1 mM EDTA with the appropriate volumes of stock solutions of the substrate and other reactants or water to give a total volume of 1.0 mL. Assays in the presence of cyanide ion contained 1.0 mM $MgCl_2$; the concentrations of free cyanide anion were calculated from the observed pH of the assay mixtures and the total concentration of cyanide using $pK_a = 9.2$ for hydrogen cyanide determined under our reaction conditions (see above).

The initial velocities of hydrolysis of 2-nitrophenyl β-D-galactopyranoside (0.6 mM) at pH 8.6 and 9.2 (25 mM sodium pyrophosphate) and pH 7.0 (30 mM TES, 140 mM NaCl, 1.0 mM $MgCl_2$) catalyzed by *fixed* concentrations of E461G or E461Q β-galactosidase were determined by monitoring the appearance of 2-nitrophenoxide at 410 nm using $\Delta\epsilon = 4500$ M^{-1} cm^{-1} (pH 8.6), $\Delta\epsilon = 4600$ M^{-1} cm^{-1} (pH 9.2), or $\Delta\epsilon = 2100$ M^{-1} cm^{-1} (pH 7.0) determined for complete reaction of a known concentration of the substrate. Observed values of k_{cat} (s^{-1}) at pH 8.6 and 9.2 were calculated directly from the relative initial velocities ([S] ≫ K_m) and the published values of $k_{cat} = 0.71$ and 0.36 s^{-1} for cleavage of 2-nitrophenyl β-D-galactopyranoside at pH 7.0 in the presence of 1.0 mM Mg^{2+} catalyzed by E461G and E461Q β-galactosidase, respectively (Cupples et al., 1990).

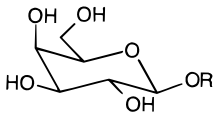
The initial velocities of reaction of 2-nitrophenyl β-D-galactopyranoside catalyzed by E461G and E461Q β-galactosidase in the presence of increasing concentrations of nucleophilic anions at pH 8.6 and pH 9.2 (reactions in the presence of cyanide) were determined using a substrate concentration of 0.6 mM for reactions in the presence of 1.0 mM $MgCl_2$, or 3.4 mM for the magnesium-free enzymes in the presence of 10 mM EDTA. The initial velocities of reaction of 4-nitrophenyl β-D-galactopyranoside catalyzed by E461Q β-galactosidase in the presence of increasing concentrations of acetate and formate ([S] = 0.07 mM) or azide ([S] = 2 mM) ions were determined by following the appearance of 4-nitrophenoxide at 405 nm using $\Delta\epsilon = 18\,300$ M^{-1} cm^{-1} determined at pH 8.6 (Richard et al., 1995a).

The initial velocities of formation of D-galactose (v_{gal}) and 4-nitrophenoxide (v_{PNP}) from the reaction of 4-nitrophenyl β-D-galactopyranoside (0.07 mM for reactions in the presence of 1.0 mM $MgCl_2$ or 0.15 mM for reactions in the presence of 10 mM EDTA) catalyzed by E461G, E461Q, and wild type β-galactosidase in the presence of increasing concentrations of nucleophilic anions at pH 8.6 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, 1996). Periodic control experiments were carried out to show that the velocity of formation of NADH at the highest concentration of nucleophile used was independent of the concentration of the galactose dehydrogenase coupling enzyme.

Linear and nonlinear least squares fits of kinetic data to the appropriate equations were determined using the SigmaPlot curve fitting program from Jandel Scientific. Values of k_{cat} and K_m were reproducible to ±5%.

RESULTS

The *observed* kinetic parameters for cleavage of enzyme-bound 2-nitrophenyl β-D-galactopyranoside (Gal-OC_6H_4-2-NO_2) by our preparation of E461G β-galactosidase at 25 °C



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and pH 8.6 and 9.2 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+} are $(k_{cat})_{mut} = 0.40$ and 0.33 s^{-1}, respectively. These rate constants can be converted to absolute values of $k_{cat} = 0.35$ and 0.32 s^{-1} for cleavage of this substrate by the E461G enzyme itself at pH 8.6 and 9.2, respectively, using eq 1 (Richard et al., 1996), where $f_{wt} =$

$$k_{cat} = (k_{cat})_{mut} - f_{wt}(k_{cat})_{wt} \quad (1)$$

8.5×10^{-5} is the fraction of wild type enzyme in our preparation of E461G β-galactosidase (Richard et al., 1996), and $(k_{cat})_{wt} = 590$ s^{-1} (Richard et al., 1995b) and *ca.* 100 s^{-1} (Tenu et al., 1971) are the kinetic parameters for cleavage of Gal-OC_6H_4-2-NO_2 by wild type β-galactosidase in the presence of 1.0 mM Mg^{2+} at pH 8.6 and 9.2, respectively. Kinetic parameters of $k_{cat} = 1.0$ s^{-1} and $K_m = 0.79$ mM were determined for cleavage of Gal-OC_6H_4-2-NO_2 by magnesium-free E461G β-galactosidase at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 10 mM EDTA.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; NAD, β-nicotinamide adenine dinucleotide; TES, *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid.

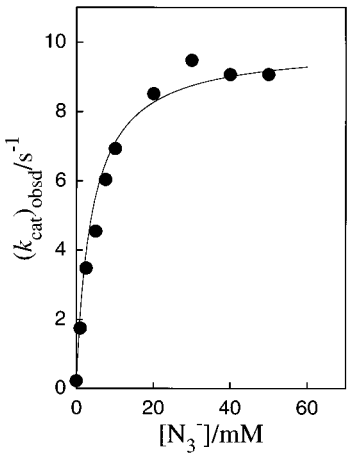
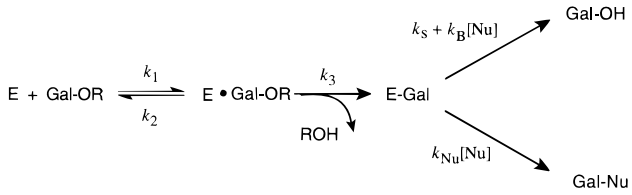


FIGURE 1: Dependence of $(k_{cat})_{obsd}$ for cleavage of enzyme-bound 4-nitrophenyl β-D-galactopyranoside (2 mM, [S] $\gg$ K_m) by E461Q β-galactosidase on the concentration of azide ion at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}.

Scheme 1



A value of $k_{cat} = 0.23\ s^{-1}$ was determined for cleavage of both Gal-OC_6H_4-2-NO_2 and 4-nitrophenyl β-D-galactopyranoside (Gal-OC_6H_4-4-NO_2) by E461Q β-galactosidase at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}. Figure 1 shows that the addition of azide ion leads to a large increase in $(k_{cat})_{obsd}$ for cleavage of Gal-OC_6H_4-4-NO_2 (2 mM, [S] $\gg$ K_m) by E461Q β-galacatosidase at pH 8.6 in the presence of 1.0 mM Mg^{2+}, from $(k_{cat})_o = 0.23\ s^{-1}$ at $[N_3^-] = 0$, to a limiting value of $(k_{cat})_{obsd} = 10\ s^{-1}$ at high concentrations of azide ion.

The initial velocities of the enzyme-catalyzed reactions of Gal-OC_6H_4-4-NO_2 to give D-galactose (v_{gal}) and 4-nitrophenoxide/4-nitrophenol (v_{PNP}) at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) were determined in a single cuvette using the galactose dehydrogenase coupled enzyme assay that was described in detail in earlier work (Richard et al., 1995b, 1996). In this assay, the velocity of formation of 4-nitrophenoxide is determined by monitoring the change in absorbance at 405 nm, and the velocity of formation of D-galactose is determined by coupling its formation to the reduction of NAD^+ using galactose dehydrogenase and monitoring the appearance of NADH at 340 nm. The kinetic data obtained from these experiments provide a quantitative measure of the velocity of formation of β-D-galactopyranosyl derivatives (k_{Nu}[Nu], Scheme 1), as the difference between the total velocity of cleavage of Gal-OC_6H_4-4-NO_2 (v_{PNP}) and the velocity of its hydrolysis reaction (v_{gal}) to give D-galactose ($k_s + k_B$[Nu], Scheme 1). The ratio v_{gal}/v_{PNP} is equal to f_{gal}, the fraction of the total reaction of Gal-OC_6H_4-4-NO_2 that gives D-galactose (Richard et al., 1995b, 1996).

There is no detectable change in the ratio $v_{gal}/v_{PNP} = 1.0$ for cleavage of Gal-OC_6H_4-4-NO_2 (0.07 mM) catalyzed by wild type β-galactosidase at pH 8.6 in the presence of 1.0 mM Mg^{2+} as the concentration of acetate or formate ion is increased from zero to 0.15 M, and it was shown in earlier work that this ratio does not decrease when the concentration of azide ion is increased from zero to 0.50 M (Richard et al., 1995b). These experiments show that there is no detectable reaction of good anionic nucleophiles with the galactosyl−enzyme intermediate of the cleavage reaction of β-D-galactopyranosyl derivatives catalyzed by wild type β-galactosidase.

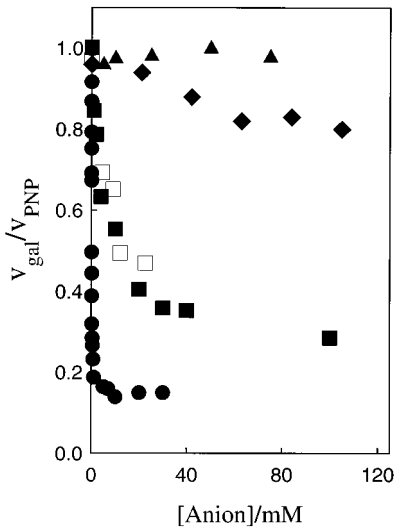


FIGURE 2: Dependence of the ratio of the initial velocities of formation of D-galactose (v_{gal}) and 4-nitrophenoxide/4-nitrophenol (v_{PNP}) from the reaction of 4-nitrophenyl β-D-galactopyranoside (0.07 mM) catalyzed by our preparation of E461G β-galactosidase on the concentration of azide (●), acetate (■), butyrate (□), methoxyacetate (◆), and formate (▲) ions at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}.

Figure 2 shows the dependence of v_{gal}/v_{PNP} for the reaction of Gal-OC_6H_4-4-NO_2 (0.07 mM) in the presence of increasing concentrations of azide (●), acetate (■), butyrate (□), methoxyacetate (◆), and formate (▲) ions catalyzed by E461G β-galactosidase at 25 °C and pH 8.6 in the presence of 1.0 mM Mg^{2+}. There was no change in the initial velocity of formation of 4-nitrophenoxide (v_{PNP}) in these experiments on increasing the concentrations of azide, acetate, and formate ions from zero to 100 mM, or the concentration of butyrate ion from zero to 15 mM. The addition of 105 mM methoxyacetate ion at [S] = 0.07 mM ($\approx 2K_m$) leads to a 20% decrease in v_{PNP}, but 90 mM of this ion at [S] = 0.2 mM ($\approx 6K_m$) leads to an even larger *ca.* 30% decrease in v_{PNP}. Therefore, the slight inhibition of E461G β-galactosidase by methoxyacetate ion is likely due to a salt effect on enzymatic activity, rather than to competitive binding of this anion to the free enzyme.

The data in Figure 2 show that $v_{gal}/v_{PNP} = f_{gal}$ for the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461G β-galactosidase decreases sharply with increasing $[N_3^-]$, but that there is a levelling off to a limiting constant value of $f_{gal} = 0.15 \pm 0.02$ at high concentrations of azide ion. We have presented evidence in the previous paper (Richard et al., 1996) that this limiting yield of 15% D-galactose is due mostly or entirely to the presence of a very small (*ca.* 0.0085% of total β-galactosidase) amount of the wild type enzyme in our preparation of E461G β-galactosidase, because the galactosylated wild type enzyme is unreactive toward azide ion and reacts with water to give D-galactose as the sole product (Richard et al., 1995b).

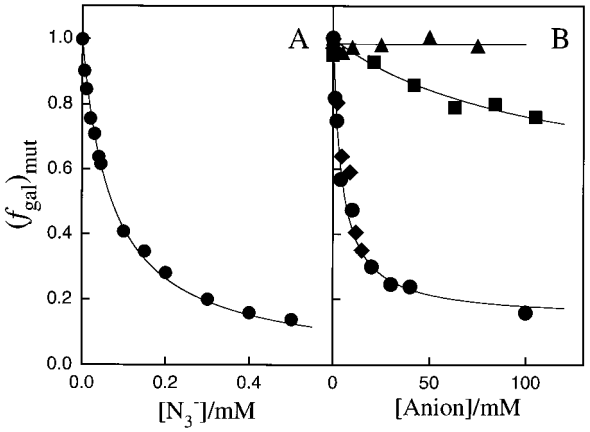


FIGURE 3: Dependence of the fractional yield of D-galactose, $(f_{gal})_{mut}$, from the reaction of 4-nitrophenyl β-D-galactopyranoside (0.07 mM) catalyzed by E461G β-galactosidase on the concentration of nucleophilic anions at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}. The values of $(f_{gal})_{mut}$ were calculated from the data in Figure 2 using eq 2 with $(f_{act})_{wt} = 0.15$, see text. A. Data for azide ion. B. Data for acetate (●), butyrate (◆), methoxyacetate (■), and formate (▲) ions.

The fractional yields of D-galactose from the E461G β-galactosidase-catalyzed reaction of Gal-OC_6H_4-4-NO_2 in the presence of increasing concentrations of nucleophilic anions, $(f_{gal})_{mut}$, were calculated from the data in Figure 2 using eq 2, where $(f_{act})_{wt} = 0.15$ is the fraction of D-galactose

$$(f_{gal})_{mut} = \frac{v_{gal}/v_{PNP} - (f_{act})_{wt}}{1 - (f_{act})_{wt}} \qquad (2)$$

obtained from catalysis by the contaminating wild type enzyme in a reaction that is insensitive to anionic nucleophiles, and $1 - (f_{act})_{wt}$ is the fraction of the cleavage reaction of Gal-OC_6H_4-4-NO_2 that is catalyzed by the E461G enzyme. Figure 3A shows the dependence of $(f_{gal})_{mut}$ for the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461G β-galactosidase at 25 °C and pH 8.6 in the presence of 1.0 mM Mg^{2+} on the concentration of azide ion, and Figure 3B shows data for the same reaction in the presence of increasing concentrations of acetate (●), butyrate (◆), methoxyacetate (■), and formate (▲) ions.

Figure 4A shows the dependence of $f_{gal} = v_{gal}/v_{PNP}$ for the reaction of Gal-OC_6H_4-4-NO_2 (0.07 mM) catalyzed by E461Q β-galactosidase at 25 °C and pH 8.6 in the presence of 1.0 mM Mg^{2+} on the concentration of azide ion. At $[N_3^-] \geq 5$ mM there was no detectable formation of D-galactose, which shows that essentially 100% of the cleavage of this substrate proceeds through a galactosyl−enzyme intermediate that can be trapped by azide ion, so that there is no detectable wild type enzyme in our preparation of E461Q β-galactosidase. Figure 4B shows data for the same reaction in the presence of increasing concentrations of acetate (●) and formate (■) ions.

Figure 5 shows the dependence of the ratio $(k_{cat})_{obsd}/(k_{cat})_o$ for cleavage of Gal-OC_6H_4-2-NO_2 (0.6 mM) catalyzed by E461G β-galactosidase at 25 °C in the presence of 1.0 mM Mg^{2+} on the concentration of butyrate (◆), formate (●), methoxyacetate (■) and chloroacetate (▼) ions at pH 8.6, and cyanide ion (▲) at pH 9.2, where $(k_{cat})_{obsd}$ and $(k_{cat})_o$ are the kinetic parameters for cleavage of this substrate by the E461G enzyme in the presence and absence of these added

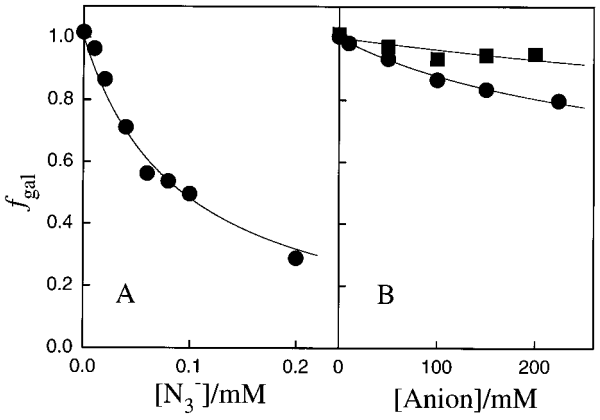


FIGURE 4: Dependence of the fractional yield of D-galactose, f_{gal}, from the reaction of 4-nitrophenyl β-D-galactopyranoside (0.07 mM) catalyzed by E461Q β-galactosidase on the concentration of nucleophilic anions at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}. A. Data for azide ion. B. Data for acetate (●) and formate (■) ions.

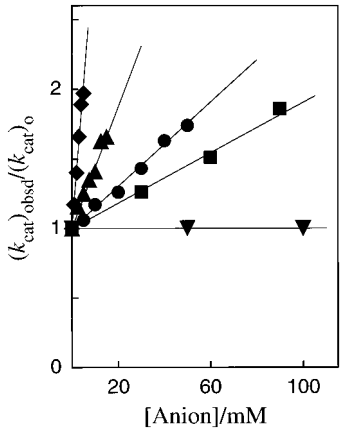


FIGURE 5: Dependence of the ratio $(k_{cat})_{obsd}/(k_{cat})_o$ for cleavage of 2-nitrophenyl β-D-galactopyranoside (0.6 mM, $[S] \gg K_m$) by E461G β-galactosidase on the concentration of nucleophilic anions at 25 °C and pH 8.6 or 9.2 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}. $(k_{cat})_{obsd}$ and $(k_{cat})_o$ are the observed kinetic parameters in the presence and absence of added nucleophile, respectively. Data at pH 8.6: butyrate (◆), formate (●), methoxyacetate (■), and chloroacetate (▼) ions. Data at pH 9.2: cyanide ion (▲).

anions, respectively. The plot of $(k_{cat})_{obsd}/(k_{cat})_o$ for cleavage of Gal-OC_6H_4-2-NO_2 (0.6 mM) catalyzed by E461Q β-galactosidase at 25 °C in the presence of 1.0 mM Mg^{2+} against the concentration of cyanide ion at pH 9.2 (not shown) has a slope of 23 M^{-1}.

Figure 6A shows the dependence of the ratio $(k_{cat})_{obsd}/(k_{cat})_o$ for cleavage of Gal-OC_6H_4-2-NO_2 (3.4 mM) catalyzed by magnesium-free E461G β-galactosidase at 25 °C and pH 8.6 in the presence of 10 mM EDTA on the concentration of azide (●) and formate (◆) ions. Figure 6B shows the dependence of the fractional yield of D-galactose, $(f_{gal})_{mut}$, obtained from the reaction of Gal-OC_6H_4-4-NO_2 (0.15 mM) catalyzed by magnesium-free E461G β-galactosidase at 25 °C and pH 8.6 in the presence of 10 mM EDTA on the concentration of azide (●), acetate (■), and formate (◆) ions. The values of $(f_{gal})_{mut}$ were calculated from the values of v_{gal}/v_{PNP} using eq 2 with $(f_{act})_{wt} = 0.035$ for the fraction of D-galactose obtained from catalysis by the contaminating wild type enzyme in the absence of Mg^{2+}.[2]

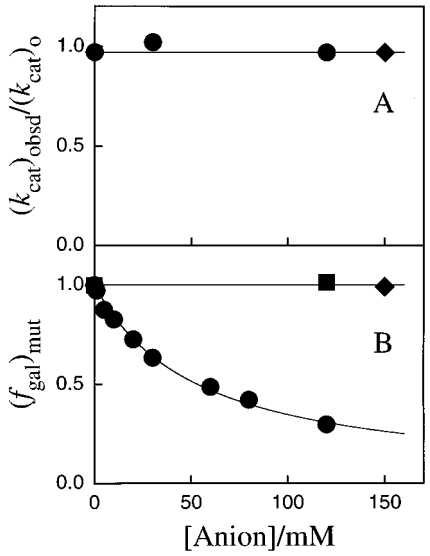


FIGURE 6: A. Dependence of the ratio $(k_{cat})_{obsd}/(k_{cat})_o$ for cleavage of 2-nitrophenyl β-D-galactopyranoside (3.4 mM) catalyzed by magnesium-free E461G β-galactosidase on the concentration of azide (●) and formate (◆) ions at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 10 mM EDTA. $(k_{cat})_{obsd}$ and $(k_{cat})_o$ are the observed kinetic parameters in the presence and absence of added nucleophile, respectively. B. The dependence of the fractional yield of D-galactose, $(f_{gal})_{mut}$, from the reaction of 4-nitrophenyl β-D-galactopyranoside (0.15 mM) catalyzed by magnesium-free E461G β-galactosidase on the concentration of azide (●), acetate (■), and formate (◆) ions at 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 10 mM EDTA. The values of $(f_{gal})_{mut}$ were calculated from eq 2 using $(f_{act})_{wt} = 0.035$, see text.

DISCUSSION

This work is a continuation of studies on the reaction of nucleophiles with galactosylated E461 β-galactosidases (Cupples et al., 1990; Huber & Chivers, 1993). The most important results from the earlier work on these reactions include (1) identification of β-D-galactopyranosyl nucleophile adducts by GLC chromatography and ^{1}H NMR and (2) characterization of the effect of nucleophilic anions on the rate of cleavage of enzyme-bound Gal-OC_6H_4-2-NO_2 by E461G β-galactosidase. The primary goal of the present work was to quantify the effect of nucleophilic anions on both the *rate* and the *distribution of products* of transfer of the β-D-galactopyranosyl group from Glu-461 mutants of β-galactosidase to these nucleophiles, in order to determine the dependence of the second-order rate constant k_{Nu} (M^{-1} s^{-1}) for galactosyl transfer on the nucleophilicity of the anion.

Nature of the Rate-Determining Step. Our analyses of the effect of increasing concentrations of nucleophilic reagents on the *rates* of degalactosylation of the covalent galactosyl−enzyme intermediates require that this step be rate-determining for the hydrolysis of enzyme-bound Gal-OC_6H_4-2-NO_2 and Gal-OC_6H_4-4-NO_2 ($k_3 \gg k_s$, Scheme 1). Therefore, we will consider briefly how the identity of the rate-determining step for cleavage of these enzyme-bound substrates depends on the presence or absence of the magnesium ion cofactor, the side chain of the amino acid at position 461, and the aryloxide leaving group at the substrate.

The addition of up to 100 mM azide ion has no effect on $k_{cat} = 0.09$ s^{-1} (Richard et al., 1996) for cleavage of Gal-OC_6H_4-4-NO_2 by E461G β-galactosidase (see Results), but it leads to an increase from $(k_{cat})_o = 0.71$ s^{-1} for cleavage of Gal-OC_6H_4-2-NO_2 at pH 7.0 (Cupples et al., 1990) to a limiting value of $(k_{cat})_{obsd} = 14$ s^{-1} (Huber & Chivers, 1993). These results show that the rate-determining steps for hydrolysis of enzyme-bound Gal-OC_6H_4-4-NO_2 and Gal-OC_6H_4-2-NO_2 by E461G β-galactosidase are $k_{cat} = k_3$ and $k_{cat} = k_s$, respectively.

The removal of Mg^{2+} from E461G β-galactosidase leads to a 2-fold decrease in $k_{cat} = k_3$ for hydrolysis of Gal-OC_6H_4-4-NO_2 (Richard et al., 1996). By contrast, removal of this metal ion results in a small increase from $k_{cat} = 0.35$ to 1.0 s^{-1} for cleavage of Gal-OC_6H_4-2-NO_2 (this work), and it causes k_{cat} to become independent of the concentration of azide ion (Figure 6A). Therefore, the removal of the metal ion cofactor leads to a change in rate-determining step for hydrolysis of enzyme-bound Gal-OC_6H_4-2-NO_2 by E461G β-galactosidase from $k_{cat} = k_s$ to $k_{cat} = k_3$ (Scheme 1), and to a large (≫3-fold) *increase* in k_s ($k_s \gg 1.0$ s^{-1}) for hydrolysis of the galactosylated E461G enzyme.

Figure 1 shows that the addition of azide ion leads to a large increase in $(k_{cat})_{obsd}$ for cleavage of enzyme-bound Gal-OC_6H_4-4-NO_2 by E461Q β-galactosidase, which shows that the rate-limiting step for this reaction is $k_{cat} = k_s = 0.23$ s^{-1} (Scheme 1). The data were fit to eq 3 (Figure 1, solid line),

$$(k_{cat})_{obsd} = \frac{1 + \{(k_B + k_{Nu})/k_s\}[Nu]}{1/(k_{cat})_o + \{(k_B + k_{Nu})/(k_3 k_s)\}[Nu]} \quad (3)$$

derived for the mechanism shown in Scheme 1, using $(k_{cat})_o = 0.23$ s^{-1} and $(k_B + k_{Nu})/k_s = 10{,}700$ M^{-1} (Table 1) that was determined from product analysis (see below), to give $k_3 = 10$ s^{-1} as the limiting value of $(k_{cat})_{obsd}$ for cleavage of this substrate at high concentrations of azide ion. Therefore, the substitution of glutamine for glycine at position 461 leads to a *ca.* 100-fold increase in k_3 for cleavage of enzyme-bound Gal-OC_6H_4-4-NO_2, and to a change in the rate-determining step for the overall hydrolysis reaction from k_3 to k_s (Scheme 1). By contrast, the same mutation leads to a small decrease in k_s from 0.35 to 0.23 s^{-1}.

While we are unable to provide a thorough rationalization for these changes in rate-determining step, we suggest the following interpretations which are in accord with our model for the mechanism of action of β-galactosidase.

(1) The removal of Mg^{2+} from the E461G enzyme may expose the anionic side chain of Glu-416 or another basic ligand of the metal ion (Jacobson et al., 1994). Such a group could then serve as a Brønsted general base catalyst to

[2] The value of $(f_{act})_{wt} = 0.035$ ([S] = 0.15 mM) was calculated from the following expression (Richard et al., 1996), where $f_{wt} = 0.00015$ is the fraction of the wild type enzyme present in our preparation of E461G β-galactosidase that was calculated (Richard et al., 1996) from the limiting yield of 15% D-galactose from cleavage of Gal-OC_6H_4-4-NO_2 ([S] = 0.07 mM) by our preparation of E461G β-galactosidase at pH 8.6 in the presence of 1.0 mM Mg^{2+} and $[N_3^-] \geq 0.01$ M (Figure 2).

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

In this expression, $(k_{cat})_{mut} = 0.045$ s^{-1} (Richard et al., 1996) and $(k_{cat})_{wt} = 26$ s^{-1} (Richard et al., 1995b) are the *observed* values of k_{cat} for the E461G and wild type enzymes, respectively, at pH 8.6 in the absence of Mg^{2+}, and $(K_m)_{mut} = 0.51$ mM (Richard et al., 1996) and $(K_m)_{wt} \approx 1.5$ mM are the Michaelis constants for the reactions catalyzed by the E461G and wild type enzymes, respectively, at pH 8.6 in the absence of Mg^{2+}. The value of $(K_m)_{wt} \approx 1.5$ mM was calculated from $k_{cat} = 26$ s^{-1} at pH 8.6 (Richard et al., 1995a) and $k_{cat}/K_m = 17\,900$ s^{-1} at pH 8.6 that was estimated using the equation for the dependence of this parameter on pH (Selwood & Sinnott, 1990).

Table 1: Rate Constant Ratios for Partitioning of Galactosylated E461G and E461Q β-Galactosidase between Reaction with Nucleophilic Anions and Solvent Water (Scheme 1)[a]

		E461G				E461Q			
nucleophile	pK_a [b]	$(k_B + k_{Nu})/k_s$ [c] (M^{-1})	k_B/k_s [d] (M^{-1})	k_{Nu}/k_s [e] (M^{-1})	k_{Nu} [f] (M^{-1} s^{-1})	$(k_B + k_{Nu})/k_s$ [c] (M^{-1})	k_B/k_s [d] (M^{-1})	k_{Nu}/k_s [e] (M^{-1})	k_{Nu} [f] (M^{-1} s^{-1})
H_2O	−1.7				$k_s = 0.35$ s^{-1} [g] ($k_s > 8$ s^{-1})[h]				$k_s = 0.23$ s^{-1} [g]
HCO_2^-	3.8	15	15	~0	~0	<0.5[i,j]	<0.5[k]	<0.5[k]	<0.1
$CH_3CO_2^-$	4.8		30 ± 10[l]	180 ± 20[l]	63	3.3[j]	1.7 ± 0.04	1.6 ± 0.04	0.37
$CH_3CH_2CH_2CO_2^-$	4.8	210	50 ± 10	160 ± 10	56				
$MeOCH_2CO_2^-$	3.5	9.0	4.5 ± 0.3	4.5 ± 0.3	1.6				
$ClCH_2CO_2^-$	2.9	<1[i]	<1[k]	<1[k]	<0.4				
CN^- [m]	9.2[n]	44	≤44[o]	≤44[o]	≤14	23	≤23[o]	≤23[o]	≤5.3
N_3^-	4.7		~0	14 000 ± 400[p] (19 ± 1)[p,q]	4900 (>150)[q]		~0	10 700 ± 700[p]	2500

[a] At 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}, unless noted otherwise. The quoted errors are the standard deviations that were obtained from the nonlinear least-squares fit of the data to the appropriate equation. [b] pK_a of the conjugate acid of the nucleophile taken from Jencks and Regenstein (1976). [c] Sum of the partitioning ratios for reaction of the galactosylated enzyme with the anion as a nucleophile (k_{Nu}, M^{-1} s^{-1}) and a general base catalyst (k_B, M^{-1} s^{-1}) and solvent water (k_s, s^{-1}), determined from the slope of a plot of $(k_{cat})_{obsd}/(k_{cat})_o$ for cleavage of 2-nitrophenyl β-D-galactopyranoside against [Nu] according to eq 6, unless noted otherwise. The error in the sum of these partitioning ratios is estimated to be ±5%. [d] Partitioning ratio for reaction of the galactosylated enzyme with the anion as a general base catalyst and with solvent water, determined from the nonlinear least-squares fit of the fractional yields of D-galactose from the reaction of 4-nitrophenyl β-D-galactopyranoside to eq 5 using $(k_B + k_{Nu})/k_s$ (M^{-1}) as a known parameter, unless noted otherwise. [e] Determined as the difference between $(k_B + k_{Nu})/k_s$ (M^{-1}) and k_B/k_s (M^{-1}), unless noted otherwise. [f] Second-order rate constant for transfer of the β-D-galactopyranosyl group from the enzyme to the anionic nucleophile, calculated from the partitioning ratio k_{Nu}/k_s (M^{-1}) and the pseudo-first-order rate constant for galactosyl transfer to solvent water, k_s (s^{-1}). [g] Pseudo-first-order rate constant for transfer of the β-D-galactopyranosyl group from the enzyme to solvent water, see text. [h] Estimated lower limit for the magnesium-free enzyme, see text. [i] Upper limit calculated with the assumption that a 10% increase in $(k_{cat})_{obsd}/(k_{cat})_o$ at the highest concentration of nucleophile used could have been detected in these experiments. [j] Determined using 4-nitrophenyl β-D-galactopyranoside as the substrate. [k] Based on the upper limit on $(k_B + k_{Nu})/k_s$. [l] Determined from the nonlinear least squares fit of the fractional yields of D-galactose from the reaction of 4-nitrophenyl β-D-galactopyranoside to eq 5. [m] Data at pH 9.2 where $k_s = 0.32$ s^{-1}. [n] Determined at 22 ± 2 °C and $I = 0.25$ (NaCl) (this work). [o] The upper limit is based on $(k_B + k_{Nu})/k_s$ because it was not possible to determine the fractional yields of D-galactose for reactions in the presence of cyanide ion, see text. [p] Determined from the nonlinear least-squares fit of the fractional yields of D-galactose from the reaction of 4-nitrophenyl β-D-galactopyranoside to eq 4, which was derived with the assumption that $k_B = 0$, see text. [q] Data for magnesium-free enzyme in the presence of 10 mM EDTA.

accelerate transfer of the galactosyl group from the mutant enzyme to solvent water, resulting in the observed increase in k_s. However, the side chain would be in the incorrect ionization state to provide general acid catalysis of cleavage of the glycosidic bond at Gal-OR and should not lead to an increase in k_3.

(2) There may be specific stabilization of the transition state for cleavage of Gal-OC_6H_4-4-NO_2 at the E461Q compared with the E461G enzyme which leads to the observed *ca*. 100-fold increase in k_3, by hydrogen-bonding or charge−dipole interactions between the leaving group anion and the propionamide side chain of Gln-461. However, such interactions would not accelerate degalactosylation (k_s) because the development of negative charge at the weakly acidic nucleophile water is highly unfavorable, so that its reaction would require formal proton transfer to an anionic general base such as the side chain of Glu-461.

Kinetic and Product Analyses. We report here experiments to determine the effect of nucleophilic reagents on both the rates of transfer of the galactosyl group from E461 β-galactosidases, determined as $(k_{cat})_{obsd}$ for reactions of Gal-OR where breakdown of the galactosyl−enzyme intermediate is rate-determining, and on the distribution of the products of these galactosyl transfer reactions.

Figure 5 shows the effect of increasing concentrations of anionic nucleophiles on $(k_{cat})_{obsd}/(k_{cat})_o$ for reaction of Gal-OC_6H_4-2-NO_2 catalyzed by E461G β-galactosidase in the presence of 1.0 mM Mg^{2+}, where $(k_{cat})_{obsd}$ and $(k_{cat})_o$ are the kinetic parameters for cleavage of this substrate in the presence and absence of these added anions, respectively. As discussed above, the rate of this enzymatic reaction is cleanly limited by the transfer of the galactosyl group from the enzyme to nucleophilic reagents (Cupples et al., 1990; Huber & Chivers, 1993), so that $(k_{cat})_{obsd}$ is effectively equal to the sum of the rate constants for all of the reactions of the galactosyl−enzyme intermediate, $k_s + k_B[Nu] + k_{Nu}[Nu]$ (Scheme 1). We use data for relatively low concentrations of these anions ([Nu] ≤ 100 mM), because this minimizes any nonspecific effects of high salt concentrations on enzyme activity, and it ensures that nucleophilic displacement at the galactosyl−enzyme intermediate remains fully rate-determining over the entire range of [Nu].

The distribution of the products of the enzyme-catalyzed reactions of Gal-OC_6H_4-4-NO_2 were determined from the relative velocities of formation of 4-nitrophenoxide/4-nitrophenol (v_{PNP}) and D-galactose (v_{gal}). The velocity of formation of any β-D-galactopyranosyl nucleophile adduct, Gal-Nu (Scheme 1), is then equal to the difference between v_{PNP} and v_{gal}. We have verified in earlier work (Richard et al., 1995b) that the yields of methyl β-D-galactopyranoside from reaction of galactosylated wild type β-galactosidase with methanol determined using this assay are in good agreement with the yields determined by direct analysis of the reaction products (Sinnott & Viratelle, 1973; Viratelle & Yon, 1973).

The structures of the products of the enzyme-catalyzed reactions of anionic nucleophiles with Gal-OC_6H_4-4-NO_2 are inferred to be the corresponding β-D-galactopyranosyl nucleophile adducts, Gal-Nu. The product of the reaction of Gal-OC_6H_4-2-NO_2 in the presence of azide ion catalyzed by E461G β-galactosidase has been isolated and characterized as β-D-galactopyranosyl azide (Gal-N_3) by ^{1}H NMR (Huber & Chivers, 1993), and the product of the reaction of this substrate in the presence of acetate ion has been isolated and detected by gas chromatography (Huber & Chivers,

1993). Our attempts to characterize the cyanide nucleophile adduct are discussed in the section which deals with the reactions of this anion.

Reactions of Azide Ion. Figure 2 (●) shows the effect of azide ion on $\nu_{gal}/\nu_{PNP} = f_{gal}$ (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 values of f_{gal} decrease sharply with increasing concentrations of azide ion, but level off at a limiting value of 0.15. Most, or all, of this limiting yield of 15% D-galactose arises from catalysis of the hydrolysis of Gal-OC_6H_4-4-NO_2 by a very small amount of the wild type enzyme that is present as a contaminant in our preparation of E461G β-galactosidase (see Results), but some of this limiting yield of D-galactose may also arise from general base catalysis by azide ion of the reaction of solvent water with the galactosyl−enzyme intermediate (k_B, Scheme 1).

The effect of azide ion on the fractional yield of D-galactose from the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461G β-galactosidase itself, $(f_{gal})_{mut}$, is shown in Figure 3A. The data were fit to eq 4 (solid line), derived for the mechanism shown in Scheme 1 with the assumption that $k_B = 0$, to give $k_{az}/k_s = 14\,000$ M^{-1} (Table 1) for partitioning of the galactosyl−enzyme intermediate between reaction with azide ion and solvent water. The good fit of these data to eq 4 shows that there is no significant formation of D-galactose by a pathway that is catalyzed by azide ion ($k_B \approx 0$, Table 1). The ratio $k_{az}/k_s = 14\,000$ M^{-1} can be combined with $k_s = 0.35$ s^{-1} (see Results) to give $k_{az} = 4900$ M^{-1} s^{-1} for transfer of the galactosyl group from E461G β-galactosidase to azide ion to give Gal-N_3. This is in fair agreement with $k_{az} = 7500$ M^{-1} s^{-1} calculated (Richard et al., 1995b) from the data of Huber and Chivers at pH 7.0 (Huber & Chivers, 1993), with the difference representing either experimental error or the effect of the change in pH.

Figure 4A shows the effect of azide ion on the fractional yield of D-galactose, f_{gal}, from the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461Q β-galactosidase. The observation that the yield of D-galactose decreases to zero at high concentrations of azide ion (see results) shows that there is essentially no wild type enzyme present in our preparation of E461Q β-galactosidase and that $k_B \approx 0$ (Table 1). The data were fit to eq 4 (solid line) to give $k_{az}/k_s = 10\,700$ M^{-1}

$$(f_{gal})_{mut} = \frac{1}{1 + (k_{Nu}/k_s)[Nu]} \qquad (4)$$

(Table 1) for partitioning of the galactosyl−enzyme intermediate between reaction with azide ion and solvent water.

Figure 6B (●) shows the effect of azide ion on the fractional yield of D-galactose, $(f_{gal})_{mut}$, from the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by magnesium-free E461G β-galactosidase in the presence of 10 mM EDTA. The data were fit to eq 4 (solid line) to give $k_{az}/k_s = 19$ M^{-1} (Table 1) for partitioning of the galactosyl−enzyme intermediate between reaction with azide ion and solvent water. Figure 6A (●) shows that there is no change in $(k_{cat})_{obsd} = 1.0$ s^{-1} for reaction of Gal-OC_6H_4-2-NO_2 catalyzed by magnesium-free E461G β-galactosidase when the concentration of azide ion is increased from zero to 120 mM, which shows that k_3 is effectively completely rate-determining for reaction of this substrate in the absence of Mg^{2+} (Scheme 1). A 10% increase in $(k_{cat})_{obsd}$ could have been detected in these experiments, so that $(k_{cat})_{obsd} < 1.1$ s^{-1} at $[N_3^-] = 0.12$ M. This limit was substituted into eq 3 with $k_B = 0$ and $k_{az}/k_s = 19$ M^{-1} to give the lower limits of $k_s > 8$ s^{-1} for galactosyl transfer from magnesium-free E461G β-galactosidase to solvent water and $k_{az} > 150$ M^{-1} s^{-1} for transfer of the galactosyl group to azide ion (Table 1). We conclude that, while Mg^{2+} is not required for activation of galactosylated E461G β-galactosidase for reaction with azide ion, the binding of this metal ion to the enzyme *may* cause up to a 30-fold increase in the second-order rate constant for transfer of the galactosyl group from the enzyme to azide ion (Table 1). Such an effect of Mg^{2+} might be attributed either to the direct stabilization of the Michaelis complex with the anion by its chelation to the metal ion, or to destabilization of the complex with the magnesium-free enzyme by unfavorable electrostatic interactions between the anion and basic side chains that are exposed as a result of removal of the metal ion (see above).

Table 2: Rate and Equilibrium Constants for Reversible Transfer of the β-D-Galactopyranosyl Group from Wild Type and E461G β-Galactosidases to Azide Ion (Scheme 2)[a]

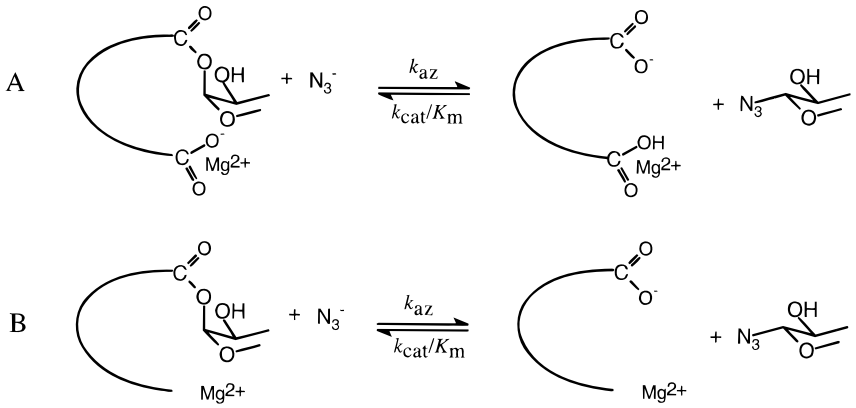
form of β-galactosidase	k_{az} [b] (M^{-1} s^{-1})	k_{cat}/K_m [c] (M^{-1} s^{-1})	$K_{az} = \frac{k_{az}}{(k_{cat}/K_m)}$ [d]
wild type	<70[e]	1.6×10^4 [f]	<0.0044
E461G	4900[g]	140[h]	35
	$(K_{az})_{E461G}/(K_{az})_{wt} > 8000$		

[a] At 25 °C and pH 8.6 (25 mM sodium pyrophosphate) in the presence of 1.0 mM Mg^{2+}. [b] Second-order rate constant for transfer of the β-D-galactopyranosyl group from the enzyme to azide ion. [c] Second-order rate constant for enzyme-catalyzed cleavage of β-D-galactopyranosyl azide. [d] Equilibrium constant for transfer of the β-D-galactopyranosyl group from the enzyme to azide ion to give β-D-galactopyranosyl azide and the free enzyme at pH 8.6. [e] Data from Richard et al. (1995b). [f] Calculated from $k_{cat} = 25$ s^{-1} and $K_m = 1.6$ mM (Richard et al., 1995b). [g] This work. [h] Calculated from $k_{cat} = 0.11$ s^{-1} and $K_m = 0.81$ mM (Richard et al., 1996).

Haldane Relationships. The substitution of glycine for glutamate at position 461 of β-galactosidase results in a more than 70-fold *increase* in the second-order rate constant k_{az} for transfer of the β-D-galactopyranosyl group from the enzyme to azide ion (Table 2). This result is unusual, because site-directed mutations seldom cause a significant stabilization of the transition state for an enzyme-catalyzed reaction. In fact, this same substitution causes k_{cat}/K_m for cleavage of Gal-N_3 to decrease by 120-fold (Table 2). That is, the E461G mutation *stabilizes* the transition state for the synthesis of Gal-N_3 from azide ion and the galactosylated enzyme, but it *destabilizes* the transition state for the reverse galactosylation of the free enzyme by Gal-N_3 (Scheme 2). The net result (Table 2) is that the E461G mutation causes transfer of the β-D-galactopyranosyl group from the enzyme to azide ion to change from a strongly thermodynamically unfavorable process, with $(K_{az})_{wt} = k_{az}/(k_{cat}/K_m) < 0.0044$ (Scheme 2A), to a thermodynamically favorable one, with $(K_{az})_{E461G} = k_{az}/(k_{cat}/K_m) = 35$ (Scheme 2B) (Table 2).[3]

[3] Only the steps on the reaction pathway up to and including the irreversible loss of the azide ion leaving group from the galactosyl−enzyme intermediate will affect k_{cat}/K_m (M^{-1} s^{-1}) for the β-galactosidase-catalyzed cleavage of Gal-N_3 (Ray, 1983). These steps correspond to the microscopic reverse of the reaction of azide ion with the galactosylated enzyme (k_{az}, Scheme 2). Consequently, K_{az} can be determined as the ratio of rate constants $k_{az}/(k_{cat}/K_m)$ (Scheme 2).

Scheme 2

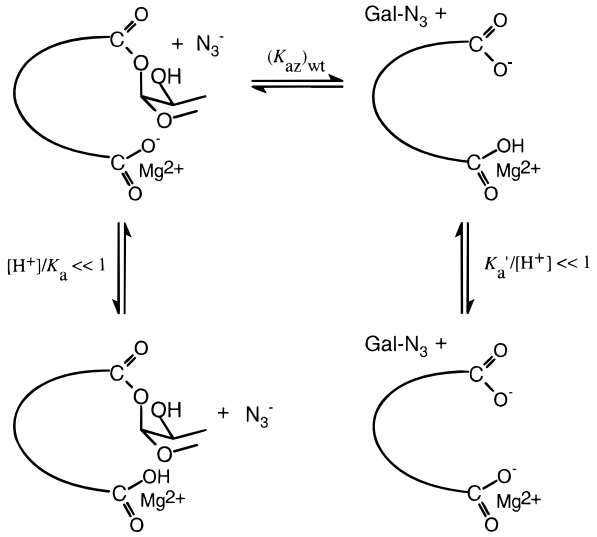


We conclude that the E461G point mutation leads to a more than 5.3 kcal/mol *destabilization* of the galactosylated enzyme relative to Gal-N_3 (Scheme 2). This result requires that the *chemical potential* for transfer of a β-D-galactopyranosyl group from β-galactosidase to azide ion be strongly controlled by the presence of the propionate side chain of glutamate at position 461.

This large effect of the E461G mutation on $K_{az} = k_{az}/(k_{cat}/K_m)$ for transfer of the β-D-galactopyranosyl group from β-galactosidase to azide ion might represent the *direct* chemical stabilization of the galactosylated enzyme by interactions between the galactosyl group and the propionate side chain of Glu-461. However, it is difficult to formulate a chemical interaction between these groups which would cause both the observed more than 70-fold *decrease* in the second-order rate constant for reaction of the galactosyl−enzyme intermediate with azide ion *and* the more than 40 000-fold *increase* in the second-order rate constant for reaction of this intermediate with the neutral nucleophile trifluoroethanol.[4] These observations require that the propionate side chain act to destabilize the transition state for galactosyl transfer to anions, but to stabilize the transition state for galactosyl transfer to neutral alcohols and water (Richard et al., 1996).

Azide ion is much more reactive than carboxylate ions toward carbocations (Richard et al., 1992; Richard & Jencks, 1984b; Ritchie, 1972). This greater nucleophilicity of azide ion than of carboxylate ions is consistent with a larger carbon basicity and it provides an explanation for the thermodynamically favorable transfer of the β-D-galactopyranosyl group from the carboxylate side chain of Glu-537 of E461G β-galactosidase to azide ion ($(K_{az})_{E461G} = 35$, Table 2). By contrast, $(K_{az})_{wt} < 0.0044$ for the wild type enzyme is an unusually small equilibrium constant for transfer of a galactosyl group between these two nucleophiles. This suggests that the full chemical affinity of azide ion for the β-D-galactopyranosyl group is expressed in the equilibrium constant for galactosyl transfer from the E461G enzyme, but that the galactosylated wild type enzyme is stabilized toward galactosyl transfer to azide ion by the requirement that this reaction be coupled to the thermodynamically unfavorable

Scheme 3

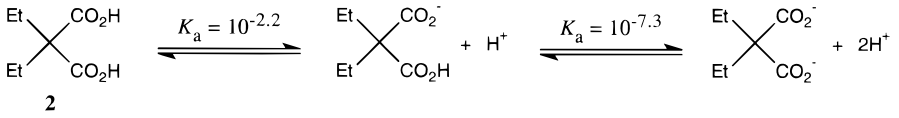


protonation of the carboxylate group of Glu-461 at the galactosylated enzyme (Scheme 3). This proposal is consistent with the evidence presented in the previous manuscript (Richard et al., 1996) that Glu-461 plays a direct role in Brønsted acid−base catalysis at the leaving group/nucleophile. It also provides a simple rationalization of the very different effects of the E461G mutation on the rate constants for galactosyl transfer from the galactosyl−enzyme intermediate to trifluoroethanol and azide ion, if it is accepted that the anionic general base Glu-461 is essential for the reaction of neutral nucleophiles (Richard et al., 1996), but presents an electrostatic barrier to the reaction of anionic nucleophiles such as azide ion (Richard et al. 1995b).

Our proposed mechanism of action of β-galactosidase is shown in Scheme 3. In this mechanism, Glu-461 is largely ionized at the galactosylated enzyme ($[H^+]/K_a \ll 1$), where it is required to provide general base catalysis of the reaction of neutral nucleophiles, but it is largely protonated at the free enzyme ($K_a'/[H^+] \ll 1$), where it is required to provide general acid catalysis of the cleavage of Gal-OR. We therefore propose that there is a large increase in the pK_a of the carboxylic acid of Glu-461 on proceeding from the galactosylated enzyme to the free enzyme, and that transfer of the galactosyl group from the enzyme to anions is tightly coupled to the protonation of this group. The mechanism shown in Scheme 3 also makes the testable prediction that a significant reaction of azide ion with galactosylated wild type β-galactosidase *will be observed* at low pH where the

[4] Calculated from $k_{TFE} = 4200\ M^{-1}\ s^{-1}$ for the galactosylated wild type enzyme (Richard et al., 1995b) and $k_{TFE} < 0.1\ M^{-1}\ s^{-1}$ for the galactosylated E461G enzyme. The latter was calculated from $k_{TFE}/k_s < 0.3\ M^{-1}$ for partitioning of galactosylated E461G β-galactosidase between reaction with trifluoroethanol and solvent water (Richard et al., 1996) and $k_s = 0.35\ s^{-1}$ (Table 1).

Scheme 4



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carboxylate group of Glu-461 at the galactosylated enzyme exists mostly in the protonated form.

The proposal that the propionate side chain of Glu-461 is mostly ionized at the galactosylated enzyme but mostly protonated at the free enzyme is consistent with the pH−rate profile for the cleavage of enzyme-bound Gal-OC_6H_4-4-NO_2 by wild type β-galactosidase, which shows a downward break at pH 9.2 at the high end, and the pH−rate profile for hydrolysis of the galactosylated wild type enzyme which remains flat through pH 5.5 (Selwood & Sinnott, 1990). If a single residue serves as both the essential catalytic acid at the free enzyme *and* the catalytic base at the galactosylated enzyme (Scheme 3), then it must undergo an increase in pK_a of greater than 3.7 units upon transfer of the β-D-galactopyranosyl group from the enzyme to nucleophilic reagents, in order to ensure that the enzyme shows optimal activity for catalysis of both glycoside cleavage and hydrolysis ofthe galactosyl−enzyme intermediate at pH values between pH 5.5 and 9.2 (Scheme 3).

We suggest that the pK_a of Glu-461 at the galactosylated enzyme is $\leq$5.5, which is normal for a carboxylic acid, but that transfer of the galactosyl group from Glu-537 to nucleophilic reagents exposes the carboxylate of Glu-537, and that the resulting destabilizing electrostatic interactions between the anionic side chains of Glu-537 and Glu-461 cause a more than 3.7 unit upward perturbation in the pK_a of the latter residue by a field effect (Hine, 1975). There is good precedent for highly perturbed pK_as of carboxylic acid side chains at protein catalysts (Fersht, 1985). For example, a value of pK_a = 8.0−8.5 has been estimated for Glu-35 at lysozyme complexed to the substrate glycol chitin (Parsons & Raftery, 1972). It has recently been shown by direct titration of the carboxylic acid residues of the 20 kDa xylanase from *Bacillus circulans* that the pK_a of Glu-172, the putative general acid−base catalyst, depends upon the state of protonation and covalent modification of Glu-78. The observed 2.5 unit decrease in the pK_a for this residue, from 6.7 in the free enzyme with Glu-78 ionized, to 4.2 in the covalent complex between Glu-78 and 2-deoxy-2-fluoro-β- xylobioside is a direct consequence of the reduction in electrostatic destabilization of charge at Glu-172 which occurs upon neutralization of charge at Glu-78 (McIntosh et al, 1996).

The upward perturbation in the pK_a of Glu-461 that results from the change in charge at the side chain of Glu-537 resembles the effect of the first ionization of a dicarboxylic acid on the pK_a for ionization of the second carboxyl group. This effect is large when the separation between the carboxyl groups is small and the charge−charge interactions are through a medium of low effective dielectric constant. For example, the difference between the first (pK_a = 2.2) and the second (pK_a = 7.3) pK_as of diethylmalonic acid (**2**) in water is 5.1 units (Jencks & Regenstein, 1976) (Scheme 4).

The pK_a of Glu-461 will also be affected by interactions with the enzyme-bound Mg^{2+} (Jacobson et al., 1994), but these interactions and their effects have not been well characterized. One possibility is that the changes in the relative position of the side chain of Glu-461 with respect to Mg^{2+} on proceeding from the galactosylated to the free enzyme may contribute to an upward perturbation in the pK_a of this residue.

Reactions of Carboxylate Ions as Nucleophiles and General Base Catalysts. Figure 3B (●) shows the effect of acetate ion on the fractional yield of D-galactose, $(f_{gal})_{mut}$, from the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461G β-galactosidase. These yields do not approach zero at high concentrations of acetate ion, as would be required for the simple partitioning of a reaction intermediate between nucleophilic substitution by acetate ion and solvent water. Rather, at high concentrations of acetate ion there is a limiting yield of *ca*. 15% D-galactose. This result requires that the reaction between acetate ion and the covalent galactosyl−enzyme give both Gal-O_2CCH_3 from reaction of acetate ion as a nucleophile (k_{RCOO}, Scheme 1) and D-galactose from reaction of acetate as general base catalyst of the reaction of water (k_B, Scheme 1). The data were fit to eq 5, which was derived for the mechanism shown in

$$(f_{gal})_{mut} = \frac{1 + (k_B/k_s)[Nu]}{1 + \{(k_B + k_{Nu})/k_s\}[Nu]} \qquad (5)$$

Scheme 1, to give k_B/k_s = 30 M^{-1} and k_{RCOO}/k_s = 180 M^{-1} (Table 1) for partitioning of galactosylated E461G β-galactosidase between its reactions with acetate ion as a general base (k_B, M^{-1} s^{-1}) and a nucleophile (k_{RCOO}, M^{-1} s^{-1}) and with solvent water (k_s, s^{-1}) (Scheme 1).

Figure 3B (▲) shows that the reaction of Gal-OC_6H_4-4-NO_2 in the presence of formate ion catalyzed by E461G β-galactosidase results in a quantitative yield of D-galactose and no detectable formation of the formate ion adduct Gal-O_2CH. This failure to observe formation of the formate ion adduct is not due to its rapid hydrolysis to give D-galactose for the following reasons: (a) The methoxyacetate ion adduct Gal-O_2CCH_2OMe can be detected using this assay (Figure 3B, ■). Gal-O_2CH should be at least as stable toward hydrolysis as Gal-O_2CCH_2OMe, because the pK_a of the methoxyacetate leaving group is slightly lower than that of the formate ion leaving group (Table 1). (b) The acetate ion adduct, Gal-O_2CCH_3, is stable to an overnight incubation in the presence of E461G β-galactosidase (Huber & Chivers, 1993). Therefore it is very unlikely that the formate ion adduct Gal-O_2CH undergoes quantitative hydrolysis in the duration of our enzymatic product assay (10 min), because the 1.0 pK unit lower basicity of the formate ion than of the acetate ion leaving group at the respective galactosyl derivatives should result in at most a 10-fold greater reactivity of Gal-O_2CH. We therefore conclude that there is no significant transfer of the β-D-galactopyranosyl group from E461G β-galactosidase to formate ion ($k_{RCOO} \approx 0$, Scheme 1).

Figure 5 (●) shows that $(k_{cat})_{obsd}/(k_{cat})_o$ for reaction of Gal-OC_6H_4-2-NO_2 catalyzed by E461G β-galactosidase, for which breakdown of the galactosyl−enzyme intermediate is rate-determining [$(k_{cat})_o = k_s$, Scheme 1], increases with

increasing concentrations of formate ion at pH 8.6. Similar results have been reported for this reaction at pH 7.0 (Huber & Chivers, 1993). These increases in $(k_{cat})_{obsd}$ are due entirely to general base catalysis by formate ion of transfer of the β-D-galactopyranosyl group from the enzyme to water (k_B, Scheme 1) because there is no detectable reaction of this ion as a nucleophile ($k_{RCOO} \approx 0$, Scheme 1). The linear dependence of $(k_{cat})_{obsd}/(k_{cat})_o$ on [Nu] is described by eq 6,

$$\frac{(k_{cat})_{obsd}}{(k_{cat})_o} = 1 + \left(\frac{k_B + k_{Nu}}{k_s}\right)[Nu] \qquad (6)$$

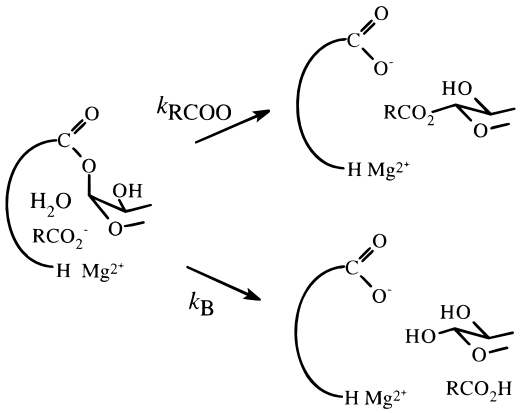
which was derived for the mechanism shown in Scheme 1 with the assumptions that $k_3 \gg k_s$ (the rate-limiting step for cleavage of this substrate is k_s, see above) and $(k_B + k_{Nu})[Nu] \approx k_s$; these assumptions lead the full rate equation, eq 3, to reduce to eq 6. The slope of the line through the data for formate ion in Figure 5 is $(k_B + k_{RCOO})/k_s = k_B/k_s = 15\ M^{-1}$ (Table 1).

Figures 3B and 5 also show the effects of butyrate and methoxyacetate ions on the fractional yield of D-galactose, $(f_{gal})_{mut}$, from the reaction of Gal-OC_6H_4-4-NO_2 and of butyrate, methoxyacetate, and chloroacetate ions on $(k_{cat})_{obsd}/(k_{cat})_o$ for the reaction of Gal-OC_6H_4-2-NO_2 catalyzed by E461G β-galactosidase, respectively. As described for formate ion, the values of $(k_B + k_{RCOO})/k_s$ (M^{-1}, Table 1) for the total reaction of these ions with the galactosyl−enzyme intermediate were determined from the slopes of the linear correlations in Figure 5, according to eq 6. The partitioning ratios k_B/k_s (M^{-1}, Table 1) for the reactions of butyrate and methoxyacetate ions were then determined from the fit of the data in Figure 3B to eq 5, using the values of $(k_B + k_{RCOO})/k_s$ (M^{-1}) determined from the data in Figure 5 as known parameters. This procedure results in more meaningful nonlinear least-squares fits of the data to eq 5, because it reduces the latter to an equation with a single unknown parameter. The partitioning ratios k_{RCOO}/k_s (M^{-1}, Table 1) for reaction of these anions as nucleophiles were then obtained as the difference between the ratios $(k_B + k_{RCOO})/k_s$ (M^{-1}) and k_B/k_s (M^{-1}).

Figure 4B shows the effect of acetate (●) and formate (■) ions on the fractional yield of D-galactose, f_{gal}, from the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461Q β-galactosidase. The slope of the plot of $(k_{cat})_{obsd}/(k_{cat})_o$ for this reaction (not shown), for which breakdown of the galactosyl−enzyme intermediate is rate-determining [$(k_{cat})_o = k_s$, Scheme 1], against the concentration of acetate ion gave the ratio $(k_B + k_{RCOO})/k_s$ (M^{-1}, Table 1), and the individual values of k_B/k_s (M^{-1}, Table 1) and k_{RCOO}/k_s (M^{-1}, Table 1) were obtained from the fit of the product data to eq 5 as described above. The upper limit on $(k_B + k_{RCOO})/k_s$ (M^{-1}, Table 1) for reaction of formate ion with the galactosyl−enzyme intermediate was calculated with the assumption that a 10% increase in $(k_{cat})_{obsd}/(k_{cat})_o$ at the highest concentration of formate ion used could have been detected in these experiments.

We propose that the catalysis by carboxylate ions of the reaction of water with galactosylated E461G β-galactosidase follows the same mechanism as for the wild type enzyme, with the enzyme-bound carboxylate ions acting as general bases to replace the excised propionate side chain of Glu-461 (Scheme 5). The functional replacement of excised amino acid side chains by small molecule analogs has been

Scheme 5



reported for galactose-1-phosphate uridylyltransferase (Kim et al., 1990) and for aspartate aminotransferase (Toney & Kirsch, 1989).

The observation that formate ion reacts exclusively as a general base to catalyze transfer of the β-D-galactopyranosyl group from E461G β-galactosidase to water suggests that its preferred orientation in the enzyme active site is similar to that of the excised propionate side chain of Glu-461. By contrast, the greater reactivity of acetate and butyrate ions, and the similar reactivity of methoxyacetate ion, as nucleophilic acceptors of the β-D-galactopyranosyl group than as general bases may reflect steric hindrance to the binding of these anions in a position that is favorable for their participation in Brønsted base catalysis (*i.e.*, at the site of the excised propionate side chain of Glu-461), so that they are forced to bind in ways that are more favorable for their direct reaction with the galactosyl−enzyme intermediate as nucleophiles.

Reactions of Cyanide Ion. The total reactivity of cyanide ion toward galactosylated E461 β-galactosidases was evaluated from the effects of increasing concentrations of this anion on $(k_{cat})_{obsd}/(k_{cat})_o$ for the reactions of Gal-OC_6H_4-2-NO_2 catalyzed by E461G (Figure 5, ▲) and E461Q (data not shown) β-galactosidase at pH 9.2, for which breakdown of the galactosyl−enzyme intermediate is rate-determining [$(k_{cat})_o = k_s$, Scheme 1]. There is no detectable reaction of this nucleophile at pH 7.0 (Huber & Chivers, 1993), where the equilibrium strongly favors conversion of cyanide anion to hydrogen cyanide ($pK_a = 9.2$). This shows that the observed increases in $(k_{cat})_{obsd}/(k_{cat})_o$ at pH 9.2 are due to the reaction of cyanide anion and that hydrogen cyanide is unreactive toward the galactosyl−enzyme intermediates. Values of $(k_B + k_{CN})/k_s$ (M^{-1}, Table 1) were obtained from the slopes of the plots of the data, according to eq 6.

A variety of problems were encountered in our attempts to determine the product distribution of the reaction of cyanide ion with the galactosylated E461G enzyme. It was not possible to determine the yield of cyanide ion adduct from the difference in the velocities of formation of 4-nitrophenoxide/4-nitrophenol and D-galactose from the reaction of Gal-OC_6H_4-4-NO_2 using our coupled enzyme assay, because cyanide ion undergoes rapid addition to the NAD^+ cofactor. A series of experiments were carried out in which the yield of D-galactose from the reaction of Gal-OC_6H_4-4-NO_2 catalyzed by E461G β-galactosidase at pH 9.2 was determined after complete reaction of the substrate, by acidification of the reaction mixture to pH 7 and removal of

hydrogen cyanide by aspiration, followed by an assay of the concentration of D-galactose using galactose dehydrogenase. However, these experiments did not give reproducible results, with the apparent yield of D-galactose decreasing with increasing delays between the end of the reaction and the removal of hydrogen cyanide, which suggests that there is significant conversion of D-galactose to its cyanohydrin. We have also isolated the products of a large-scale reaction of the E461G enzyme-catalyzed reaction of Gal-OC_6H_4-2-NO_2 (3 mM) in the presence of 50 mM cyanide anion and 1.0 mM Mg^{2+} at pH 9.2, which were shown by ^{1}H NMR spectroscopy to be neither D-galactose nor its cyanohydrin. However, the structure of the major product of this reaction was not rigorously characterized as β-D-galactopyanosyl cyanide. In conclusion, we have been unable to obtain reliable yields of the products of reaction of the galactosylated enzymes in the presence of cyanide ion, so that the values of $(k_B + k_{CN})/k_s$ (M^{-1}, Table 1) are upper limits on k_{CN}/k_s (M^{-1}) for transfer of the galactosyl group from the enzyme to cyanide ion and solvent water.

Comparison of E461G and E461Q β-Galactosidases. The galactosylated E461G and E461Q β-galactosidases show similar reactivities toward transfer of the β-D-galactopyranosyl group to water and to the small anionic nucleophiles azide and cyanide ion (Table 1). However, the addition of the propionamide side chain at position 461 of the E461G mutant to give the E461Q mutant leads to a 170-fold decrease in k_{RCOO} (M^{-1} s^{-1}) for transfer of the β-D-galactopyranosyl group from the enzyme to acetate ion, and a more than 40-fold decrease in k_B (M^{-1} s^{-1}) for catalysis by formate ion of transfer of the β-D-galactopyranosyl group to water. These data show that the interactions of the small linear nucleophiles azide and cyanide ion with the propionamide side chain at the E461Q enzyme do not significantly affect their reactivity toward the enzyme-bound β-D-galactopyranosyl group, but that, for reasons that we do not understand, the interactions of carboxylate ions with this side chain result in a significant decrease in their reactivity toward the galactosyl−enzyme intermediate.

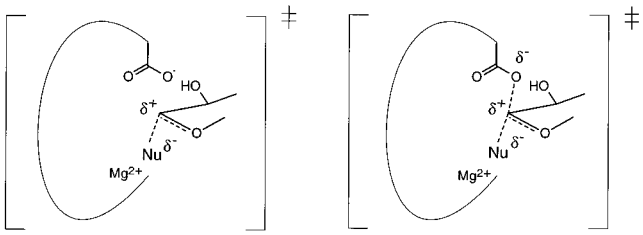
Mechanism of Transfer of the β-D-Galactopyranosyl Group to Nucleophilic Anions. The rate constants for the nucleophilic substitution reactions of anions at the glycosidic carbon of galactosylated E461G and E461Q β-galactosidase are expected to be at least partly controlled by the chemical reactivity of these anions, in the same fashion that the rate constants for transfer of the β-D-galactopyranosyl group to neutral alkyl alcohols are controlled by the chemical reactivity of these neutral nucleophiles (Richard et al., 1995b). The variation in the rate constants k_{Nu} (M^{-1} s^{-1}, Table 1) is not due simply to differences in the binding affinity of these anions for the galactosylated enzymes for the following reasons:

(1) The low specificity for the leaving group and the similar Michaelis constants for cleavage of a wide range of β-D-galactopyranosyl derivatives by wild type β-galactosidase show that the binding pocket accepts a wide variety of leaving groups/nucleophiles.

(2) Azide, acetate, and cyanide ions are all expected to show relatively weak noncovalent binding interactions with β-galactosidase, because these nucleophiles are small and contain a similar number of atoms.

(3) The observed differences in the reactivities of small linear nucleophiles such as azide ion and cyanide ion toward the enzyme-bound β-D-galactopyranosyl group (Table 1) might be explained by the differences in the symmetry of these ions, which would allow for formation of a chelate between one terminal nitrogen of azide ion and the magnesium ion and for a nucleophilic reaction at the other terminal nitrogen. However, the large values of $k_{az}/k_s \geq 10\,700$ M^{-1} determined here are consistent with the expression of the very large intrinsic nucleophilicity of azide ion; they are not easily rationalized by reaction of azide ion that is chelated to an enzyme-bound magnesium ion, because this chelate would be chemically less nucleophilic than the free azide anion. The rapid falloff in the reactivity of substituted carboxylate ions toward the enzyme-bound β-D-galactopyranosyl group with decreasing pK_a of the nucleophile (Table 1) provides additional support for the conclusion that the variations in the rate constants for the reactions of nucleophilic anions (Table 1) are due primarily to changes in the nucleophilicity of these anions rather than to differences in their interactions with the bound magnesium ion.

The relative barriers to reaction of nucleophilic anions with the *covalent* intermediate (Sinnott & Souchard, 1973) of the reactions catalyzed by E461G and E461Q β-galactosidase (Table 1) are similar to the relative barriers observed for the reactions of these anions with *carbocations* in water. That is, kinetically, these anions behave as though they are undergoing reaction with an enzyme-bound oxocarbenium ion intermediate of a stepwise $D_N + A_N$ reaction (Guthrie & Jencks, 1989; IUPAC Commission on Physical Organic Chemistry, 1989) (see **3**). The following considerations lend



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support to this conclusion:

(1) Carbocations with lifetimes in the seconds to microseconds range exhibit selectivities for reaction with azide ion and largely aqueous solvents of up to $k_{az}/k_s \approx 10^7$ M^{-1} (Richard, 1995; Richard & Jencks, 1984b; Ritchie, 1972, 1986). Therefore the value of $k_{az}/k_s = 14\,000$ M^{-1} for partitioning of the intermediate of the reaction catalyzed by E461G β-galactosidase is consistent with the partitioning of an electrophilic glycosyl oxocarbenium ion between reaction with azide ion and solvent water.

(2) The relative reactivities of azide and cyanide ion toward the enzyme-bound β-D-galactopyranosyl group, $k_{az}/k_{CN} \geq 350$ (Table 1), are similar to those observed for partitioning of carbocations in water (Ritchie, 1972, 1986). This large ratio effectively excludes concerted displacement reactions of the type observed for nucleophilic substitution at primary and secondary aliphatic carbon, because the transition states for such reactions are more effectively stabilized by interaction with cyanide than with azide ion ($k_{CN} > k_{az}$) (Pearson et al., 1968).

(3) The rate constants k_{Nu} (Table 1) exhibit a large dependence on nucleophilicity. For example, there is a 14 000-fold difference in the reactivities of azide ion and

solvent water, an 80-fold difference in the reactivities of azide and acetate ions, and a more than 350-fold difference between the reactivity of azide and cyanide ions toward the intermediate of the reaction catalyzed by the E461G enzyme. These results are consistent with a transition state in which there is extensive bond development between the nucleophile and the glycosidic carbon. They are inconsistent with concerted nucleophilic substitution through an "exploded" transition state (see **4**) such as has been observed for the reaction of nucleophiles with *N*-(methoxymethyl)-*N*,*N*-dimethylanilinium ions (Knier & Jencks, 1980) (Scheme 6) and related compounds (Craze & Kirby, 1978; Amyes & Jencks, 1989a). The second-order rate constants for reaction of nucleophiles with these substrates show only a very small dependence on nucleophilicity (*e.g.*, $k_{az}/k_s = 1.2$ M^{-1}, $k_{az}/k_{CN} = 5.3$, and $k_{az}/k_{AcO} = 11.6$) for the reaction of **5** (Knier & Jencks, 1980) because only weak bonding interactions are developed between the nucleophile and the central carbon in the transition state.

(4) Acetate, butyrate, and methoxyacetate ions react with the covalent intermediate of E461G β-galactosidase both as nucleophiles to form adducts Gal-Nu (k_{RCOO}) and as Brønsted bases (k_B) to catalyze the formation of D-galactose (Scheme 1). Similar competing pathways for nucleophilic addition of carboxylate anions and for general base catalysis of the addition of solvent have been observed for the reactions of ring-substituted 1-phenylethyl carbocations (Richard & Jencks, 1984a,b; Ta-Shma & Jencks, 1986).

Comparison of Glycosyl Transfer in Solution and at β-Galactosidase. The *solvolyses* of glycosyl derivatives in water are thought to proceed by a stepwise mechanism (Amyes & Jencks, 1989b; Banait & Jencks, 1991) through glycosyl oxocarbenium ion intermediates which lie in a shallow potential energy well (Figure 7A) with lifetimes of *ca*. 10^{-12} s (Amyes & Jencks, 1989b). The selectivities for reaction of glycosyl oxocarbenium ions with nucleophilic reagents must be very small, because the rate constants for their reaction with solvent are already within a factor of 10 of the limiting vibrational rate constant for nucleophilic addition to a carbocation of *ca*. 10^{13} s^{-1}. In fact, the small nucleophilic selectivities of $k_{Nu}/k_s = 3.7$ and 0.55 M^{-1}, respectively, determined for the reaction of azide and acetate ions and solvent water with α-D-glucopyranosyl fluoride (Banait & Jencks, 1991) pertain to the bimolecular nucleophilic substitution reactions of these nucleophiles with the neutral substrate. These concerted reactions avoid the formation of the glycosyl oxocarbenium ion intermediate, and they are probably enforced because the barrier to collapse of the highly unstable oxocarbenium ion−nucleophile complex to products is smaller than the barrier to a bond vibration (Jencks, 1980; Jencks, 1981).

It is likely (but not proven) that at least part of the rate acceleration for enzyme-catalyzed glycosyl transfer is due to the specific stabilization of the oxocarbenium-ion-like transition state, which would also be expressed at an enzyme-bound glycosyl oxocarbenium ion (Sinnott, 1987, 1990). Such stabilization of the intermediate of a stepwise reaction should cause a further weakening of the already loose interactions of the nucleophile and leaving group with the glycosidic carbon in the transition state for the bimolecular nucleophilic substitution reaction, as the result of an anti-Hammond effect (Thornton, 1967; Jencks, 1985). This would result in either a *decrease* in the already small

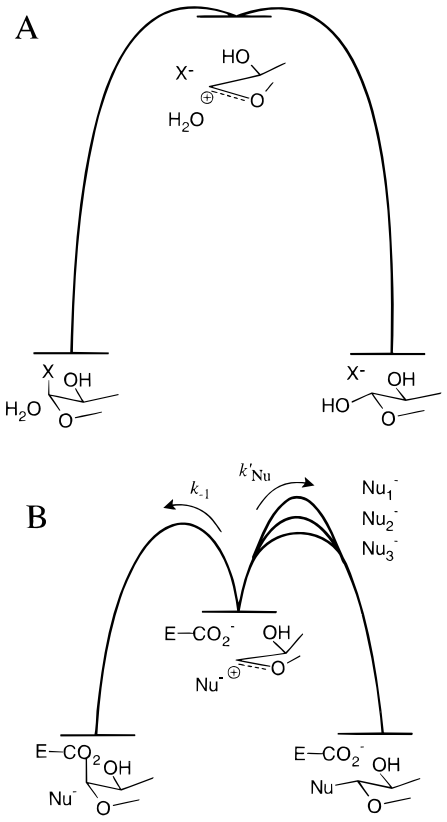
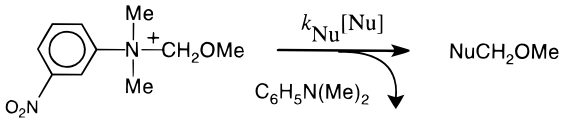


FIGURE 7: Hypothetical free energy profiles for (A) the solvolysis reaction of a simple glycoside in water through a glycosyl oxocarbenium ion intermediate which lies in a shallow potential energy well and for (B) the nucleophilic substitution reactions of galactosylated E461G β-galactosidase through an enzyme-bound glycosyl oxocarbenium ion intermediate which shows a large discrimination between reaction with strongly and weakly nucleophilic anions. The rate constants k_{-1} and k'_{Nu} are defined in Scheme 7.

Scheme 6



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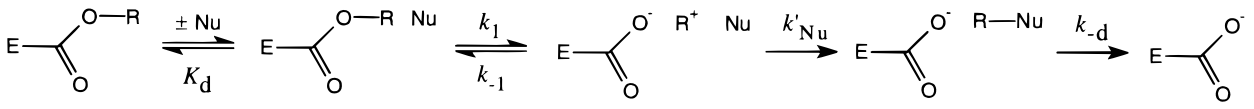
nucleophile selectivities, or a change to a stepwise mechanism for the reaction of nucleophilic anions. In fact, the nucleophile selectivities for reaction of the β-D-galactopyranosyl group attached to E461G and E461Q β-galactosidase (Table 1) are *much larger* than those for bimolecular nucleophilic substitution at glycosides (Banait & Jencks, 1991) and other acetals (Craze & Kirby, 1978; Knier & Jencks, 1980; Amyes & Jencks, 1989a) in aqueous solution. This increase in selectivity toward nucleophiles is the opposite of the decrease that would be expected for an anti-Hammond shift in the position of the transition state for concerted bimolecular nucleophilic substitution (Thornton, 1967; Jencks, 1985).

The large nucleophile selectivities for reaction of the enzyme-bound β-D-galactopyranosyl group (Table 1) are consistent with a stepwise reaction through a galactosyl oxocarbenium ion intermediate in which there are differing degrees of stabilization of the transition state for its capture from interactions with nucleophilic reagents of differing nucleophilicity. This is illustrated by the changes in the

Scheme 7



barriers (k'_{Nu}) to the reaction of a series of nucleophiles of increasing nucleophilicity, Nu_1^-, Nu_2^-, and Nu_3^-, shown in Figure 7B. The results suggest that the enzyme−ligand interactions which stabilize the enzyme-bound glycosyl oxocarbenium ion relative to the covalent adduct also cause a significant increase in the kinetic barrier for reaction of the oxocarbenium ion with solvent and other nucleophilic reagents (Figure 7B), which contrasts the almost barrierless reactions of these nucleophiles with glycosyl oxocarbenium ions in water (Figure 7A).

Other Structure−Reactivity Relationships. Scheme 7 shows a stepwise mechanism for transfer of the β-D-galactopyranosyl group (R) from Glu-537 of β-galactosidase to nucleophilic reagents, and eq 7 gives the relationship

$$k_{Nu} = \left(\frac{1}{K_d}\right)\left(\frac{k_1 k'_{Nu}}{k_{-1} + k'_{Nu}}\right) \qquad (7)$$

between the observed second-order rate constant for nucleophilic substitution, k_{Nu}, and the microscopic rate and equilibrium constants shown in Scheme 7. The *second-order* rate constant for the nucleophilic reaction of acetate ion with galactosylated E461G β-galactosidase is 80-fold smaller than that for reaction of azide ion (Table 1). This lower reactivity of acetate ion is consistent with rate-determining reaction of the galactosyl oxocarbenium ion intermediate with acetate ion (k'_{Nu}, Scheme 7), which requires $k_{-1} \gg k'_{Nu}$ for reaction of the enzyme-bound oxocarbenium ion. Now, if the trapping of this oxocarbenium ion by the carboxylate side chain of Glu-537 (k_{-1}, Scheme 7) is faster than its reaction with bound acetate ion (k'_{Nu}), then there must be a significant perturbation of the relative chemical reactivity of these two carboxylate ions at the enzyme, because they are expected to have similar reactivities in water. The nucleophilicity of azide ion in water is much greater than that of carboxylate ions, so that ionization of the galactosyl−enzyme intermediate to give the galactosyl oxocarbenium ion in the presence of azide ion may be irreversible ($k'_{Nu} \gg k_{-1}$) and hence rate-determining for reaction of this nucleophile ($k_{az} = k_1/K_d$, Scheme 7).

The sharp >180-fold decrease in the reactivity of carboxylate ions as nucleophiles as the pK_a is decreased from 4.8 for acetate to 2.8 for chloroacetate (Table 1) is unlikely to be due to an unfavorable steric effect on the binding of chloroacetate, because the same trend in reactivity is observed for the reaction of methoxyacetate and butyrate ions, which have very similar steric bulk. The results are consistent with $\beta_{nuc} > 1.0$ and the development of significant bonding interactions between these carboxylate ions and the glycosidic carbon in the transition state for the glycosyl transfer reaction (Figure 7B). This might reflect either an exceptionally late transition state, if chemical bond formation (k'_{Nu}, Scheme 7) is rate-determining for reaction of these anions, or the rate-determining release of product, where the bond between the carboxylate ion and the galactosyl group is fully formed (k_{-d}). However, it is important to emphasize that the observation of $\beta_{nuc} > 1.0$ does not absolutely require that there be a fully-formed bond between the glycosidic carbon and the carboxylate ion in the transition state. For example, a value of $\beta_{eq} = 1.56$ has been determined for the equilibrium transfer of the galactosyl group from wild type β-galactosidase to alkoxide ions (Richard et al., 1995b).

The rate constant for hydrolysis of the acylal (galactosyl−enzyme) intermediate for wild type β-galactosidase at pH 7.0 is $k_s = 1300\ s^{-1}$ (Sinnott & Souchard, 1973), which is 10^8-fold larger than the rate constants for the spontaneous hydrolysis of simple glycosyl acylals in water (Brown & Bruice, 1973). This shows that β-galactosidase acts to stabilize the transition state for transfer of a β-D-galactopyranosyl group both to and from Glu-537, presumably by stabilizing the bound glycosyl oxocarbenium ion intermediate that is common to both reactions. It is interesting that the observed second-order rate constant for transfer of the β-D-galactopyranosyl group from E461G β-galactosidase to azide ion, $k_{az} = 4900\ M^{-1}\ s^{-1}$ (Table 1), is comparable to the second-order rate constants for reaction of the galactosylated wild type enzyme with neutral alcohols (Richard et al., 1995b). This near-equality of the second-order rate constants for the transfer of the β-D-galactopyranosyl group from the wild type enzyme to neutral nucleophiles and for its transfer from the E461G mutant to nucleophilic anions suggests the following:

(1) The primary effect of the E461G mutation on transfer of the β-D-galactopyranosyl group from β-galactosidase to nucleophilic acceptors is to change the specificity from reaction with neutral alcohols for the wild type enzyme, to reaction with nucleophilic anions for the E461G mutant.

(2) Wild type and E461G β-galactosidase provide similar stabilization of the transition state for degalactosylation of the galactosyl−enzyme intermediate (k_1, Scheme 7), and of the galactosyl oxocarbenium ion intermediate, when the specific acceptor of the β-D-galactopyranosyl group is bound to the enzyme. In other words, our studies of trapping of the intermediate for E461G β-galactosidase are directly relevant to the mechanism for the wild type enzyme, because the two enzyme-catalyzed reactions proceed through similarly stabilized galactosyl oxocarbenium ions.

(3) The catalytic residues that are responsible for providing general acid−base catalysis at the leaving group/nucleophile function independently of those responsible for stabilization of the glycosyl oxocarbenium ion reaction intermediate.

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

REFERENCES

Abell, L. M., & Schloss, J. V. (1991) *Biochemistry 30*, 7883−7887.

Amyes, T. L., & Jencks, W. P. (1989a) *J. Am. Chem. Soc. 111*, 7900−7909.

Amyes, T. L., & Jencks, W. P. (1989b) *J. Am. Chem. Soc. 111*, 7888−7900.

Banait, N. S., & Jencks, W. P. (1991) *J. Am. Chem. Soc. 113*, 7951−7958.

Brown, A., & Bruice, T. C. (1973) *J. Am. Chem. Soc. 95*, 1593−1601.
Christen, P., & Riordan, J. F. (1968) *Biochemistry 7*, 1531−1538.
Craze, G.-A., & Kirby, A. J. (1978) *J. Chem. Soc., Perkin Trans. 2*, 354−356.
Cupples, C. G., Miller, J. H., & Huber, R. E. (1990) *J. Biol. Chem. 265*, 5512−5518.
Dahlquist, F. W., Rand-Meir, T., & Raftery, M. A. (1969) *Biochemistry 8*, 4214−4221.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman and Company, New York.
Gebler, J. C., Aebersold, R., & Withers, S. (1992) *J. Biol. Chem. 267*, 11126−11130.
Grazi, E. (1975) *Biochem. J. 151*, 167−172.
Guthrie, J. P., Cossar, J., & Klym, A. (1984) *J. Am. Chem. Soc. 106*, 1351−1360.
Guthrie, R. D., & Jencks, W. P. (1989) *Acc. Chem. Res. 22*, 343−349.
Healy, M. J., & Christen, P. (1972) *J. Am. Chem. Soc. 94*, 7911−7916.
Hine, J. (1975) *Structural Effects on Equilibria in Organic Chemistry*, Wiley-Interscience, New York, NY.
Huber, R. E., & Chivers, P. T. (1993) *Carbohydr. Res. 250*, 9−18.
IUPAC Commission on Physical Organic Chemistry (1989) *Pure Appl. Chem. 61*, 23−56.
Jacobson, R. H., Zhang, X.-J., DuBose, R. F., & Matthews, B. W. (1994) *Nature 369*, 761−766.
Jencks, W. P. (1980) *Acc. Chem. Res. 13*, 161−169.
Jencks, W. P. (1981) *Chem. Soc. Rev. 10*, 345−375.
Jencks, W. P. (1985) *Chem. Rev. 85*, 511−527.
Jencks, W. P., & Regenstein, J. (1976) in *Handbook of Biochemistry and Molecular Biology, Physical and Chemical Data*, 3rd ed. (Fasman, G. D., Ed.) Vol. 1, pp 305−351, CRC Press, Cleveland, OH.
Kim, J., Ruzicka, F., & Frey, P. A. (1990) *Biochemistry 29*, 10590−10593.
Kirsch, J. F. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrup, D. B., Eds.) pp 100−121, University Park Press, Baltimore, MD.
Knier, B. L., & Jencks, W. P. (1980) *J. Am. Chem. Soc. 102*, 6789−6798.
Kuo, D. J., & Rose, I. A. (1985) *Biochemistry 24*, 3947−3952.
McIntosh, L. P., Hand, G., Johnson, P. E., Joshi, M. D., Körner, M., Plesniak, L. A., Ziser, L., Wakarchuk, W. W., & Withers, S. G. (1996) *Biochemistry 35*, 9958−9966.
McLeod, A. M., Lindhorst, T., Withers, S. G., & Warren, R. A. J. (1994) *Biochemistry 33*, 6371−6376.
Pathy, L. (1978) *Eur. J. Biochem. 88*, 191.
Parsons, L. M., & Raftery, M. A. (1972) *Biochemistry 11*, 1633−1638.
Pearson, R. G., Sobel, H., & Songstad, J. (1968) *J. Am. Chem. Soc. 90*, 319−326.
Ray, W. J. (1983) *Biochemistry 22*, 4625−4637.
Richard, J. P. (1995) *Tetrahedron 51*, 1535−1573.
Richard, J. P., & Jencks, W. P. (1984a) *J. Am. Chem. Soc. 106*, 1396−1401.
Richard, J. P., & Jencks, W. P. (1984b) *J. Am. Chem. Soc. 106*, 1373−1383.
Richard, J. P., Rothenberg, M. E., & Jencks, W. P. (1984) *J. Am. Chem. Soc. 106*, 1361−1372.
Richard, J. P., Amyes, T. L., & Vontor, T. (1992) *J. Am. Chem. Soc. 114*, 5626−5634.
Richard, J. P., Westerfeld, J. G., & Lin, S. (1995a) *Biochemistry 34*, 11703−11712.
Richard, J. P., Westerfeld, J. G., Lin, S., & Beard, J. (1995b) *Biochemistry 34*, 11713−11724.
Richard, J. P., Huber, R. E., Lin, S., Heo, C., & Amyes, T. L. (1996) *Biochemistry 35*, 12377−12386.
Riordan, J. F., & Christen, P. (1969) *Biochemistry 8*, 2381−2386.
Ritchie, C. D. (1972) *Acc. Chem. Res. 5*, 348−354.
Ritchie, C. D. (1986) *Can. J. Chem. 64*, 2239−2250.
Selwood, T., & Sinnott, M. L. (1990) *Biochem. J. 268*, 317−323.
Sinnott, M. L. (1987) in *Enzyme Mechanisms* (Page, M. I., & Williams, A., Eds.) pp 258−297, The Royal Society of Chemistry, London.
Sinnott, M. L. (1990) *Chem. Rev. 90*, 1171−1202.
Sinnott, M. L., & Souchard, I. J. L. (1973) *Biochem. J. 133*, 89−98.
Sinnott, M. L., & Viratelle, O. M. (1973) *Biochem. J. 133*, 81−87.
Ta-Shma, R., & Jencks, W. P. (1986) *J. Am. Chem. Soc. 108*, 8040−8050.
Tapuhi, E., & Jencks, W. P. (1982) *J. Am. Chem. Soc. 104*, 5758−5765.
Tenu, J.-P., Viratelle, O. M., Garnier, J., & Yon, J. (1971) *Eur. J. Biochem. 20*, 363−370.
Tenu, J.-P., Viratelle, O. M., & Yon, J. (1972) *Eur. J. Biochem. 26*, 112−118.
Thornton, E. R. (1967) *J. Am. Chem. Soc. 89*, 2915−2927.
Toney, M. D., & Kirsch, J. F. (1989) *Science 243*, 1485−1488.
Tse, J. M.-T., & Schloss, J. V. (1993) *Biochemistry 32*, 10398−10403.
Viratelle, O. M., & Yon, J. M. (1973) *Eur. J. Biochem. 33*, 110−116.
Wang, Q., Graham, R. W., Trimbur, D., Warren, R. A. J., & Withers, S. G. (1994) *J. Am. Chem. Soc. 116*, 11594−11595.
Young, P. R., Bogseth, R. C., & Rietz, E. G. (1980) *J. Am. Chem. Soc. 102*, 6268−6271.

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