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Probing Synergy between Two Catalytic Strategies in the Glycoside Hydrolase *O*-GlcNAcase Using Multiple Linear Free Energy Relationships

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Abstract: Human *O*-GlcNAcase plays an important role in regulating the post-translational modification of serine and threonine residues with β-*O*-linked *N*-acetylglucosamine monosaccharide unit (*O*-GlcNAc). The mechanism of *O*-GlcNAcase involves nucleophilic participation of the 2-acetamido group of the substrate to displace a glycosidically linked leaving group. The tolerance of this enzyme for variation in substrate structure has enabled us to characterize *O*-GlcNAcase transition states using several series of substrates to generate multiple simultaneous free-energy relationships. Patterns revealing changes in mechanism, transition state, and rate-determining step upon concomitant variation of both nucleophilic strength and leaving group abilities are observed. The observed changes in mechanism reflect the roles played by the enzymic general acid and the catalytic nucleophile. Significantly, these results illustrate how the enzyme synergistically harnesses both modes of catalysis; a feature that eludes many small molecule models of catalysis. These studies also suggest the kinetic significance of an oxocarbenium ion intermediate in the *O*-GlcNAcase-catalyzed hydrolysis of glucosaminides, probing the limits of what may be learned using nonatomistic investigations of enzymic transition-state structure and offering general insights into how the superfamily of retaining glycoside hydrolases act as efficient catalysts.

Introduction

General acid/base and nucleophilic catalysis are among the most common mechanisms used by enzymes to increase rates of reaction above those of uncatalyzed reactions. Many enzymes catalyzing phosphoryl, acyl, or glycosyl group transfer reactions use these two catalytic strategies concomitantly.[1] Structure−reactivity studies of enzymes making use of altered substrates and mutant enzymes have firmly established the independent importance of both of these catalytic strategies. Experimental studies of enzymes that probe the interaction and synergy of these two mechanistic strategies, however, are notably lacking. Glycoside hydrolases are a vast superfamily of enzymes that can comprise up to 1% of the genome of species.[2] The pioneering determination of the X-ray structure of hen egg white lysozyme[3] has spurred sustained speculation and study into the catalytic and mechanistic roles of enzymic active site nucleophiles and general acids/bases in glycoside hydrolases.[4] Studies evaluating the contribution to catalysis by nucleophiles of glycosidases commonly use mutagenesis and the resulting decreases in k_{cat}/K_m values have been found to range from 10- to over 10^7-fold.[2,4] Of course, such analyses are hindered in their accuracy since deletion of the nucleophilic side chain results in a dramatic perturbation of the electrostatic character of an enzyme active site by changing, for example, the pK_a values of other active-site residues[5] and may even alter the stereochemical outcome of glycosyl transfer.[6] Owing to limitations in the number of naturally occurring amino acids, probing the role of the catalytic nucleophile in a more delicate manner, such as by attenuating nucleophilicity through a series of systematic changes, has not been realized. We were intrigued by this problem and, owing to our interest in the catalytic mechanism of a glycoside hydrolase known as *O*-GlcNAcase, recognized the feasibility of investigating this fundamental issue using this enzyme as a representative test case for the large superfamily of glycoside hydrolases.[2,4]

O-GlcNAcase (OGA) is a β-glucosaminidase from family GH84 responsible for removing 2-acetamido-2-deoxy-β-D-glucopyranose moieties from post-translationally modified serine and threonine residues of nucleocytoplasmic proteins.[7] This form of glycosylation has been implicated in various disease states,[8]

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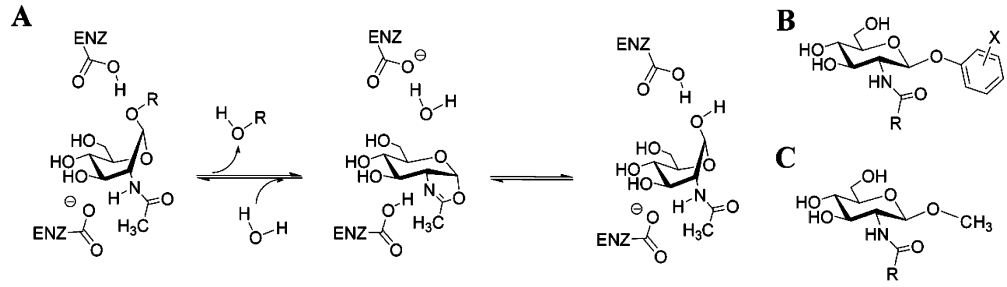

Figure 1. (A) *O*-GlcNAcase hydrolyses 2-acetamido-2-deoxy-β-D-glucopyranosides with retention of stereochemistry at the anomeric center using a substrate-assisted catalytic mechanism. (B) The series of aryl substrates synthesized and studied: R = CH_3, CH_2F, CHF_2, CF_3, and *n*-Bu and X = 3,4-DNP, 3-F-4-NO_2, 4-NO_2, 4-MU, 4-Cl-3-NO_2, 4-CN, 3-NO_2, 3,4-DF, 4-Cl, H, and 4-OMe. (C) The series of methyl glycosides synthesized and studied; R = CH_3, CH_2F, CHF_2, CF_3.

and an understanding of the catalytic mechanism of *O*-GlcNAcase[9] has led to the discovery of inhibitors that are proving useful in determining the role of the *O*-GlcNAc modification within a cellular context.[10−12] *O*-GlcNAcase uses a substrate-assisted catalytic mechanism in which the 2-acetamido group of the substrate acts as the catalytic nucleophile to form an oxazoline intermediate (Figure 1A), in contrast to the large majority of glycoside hydrolases that use an enzymic nucleophile to cleave their substrates with retention of configuration at the anomeric center. Notably, the structure of two bacterial homologues of human *O*-GlcNAcase have been solved and reveal a spacious active site which tolerates larger groups appended to the 2-acetamido group.[13,14] The biological substrates of *O*-GlcNAcase are hundreds of different *O*-GlcNAc-modified proteins,[8] and *O*-GlcNAcase must therefore tolerate many different leaving group structures. Consistent with this observation, *O*-GlcNAcase tolerates glucosaminides possessing various substituted phenolic leaving groups.[9,15,16]

We recognized that these tolerances of the active site to variation in these substituents of *O*-GlcNAcase substrates would enable us to concomitantly vary the electronic nature of both the nucleophile and leaving group in a quantifiable manner. In this way a series of linear free energy relationships could be generated that correlate different thermodynamic parameters of the substrates in solution, with the kinetic parameters governing their enzyme-catalyzed hydrolysis. Such linear free energy relationships are a widely used tool to study both enzyme catalysis and small molecule reactions in solution since they provide insight into how changes in the electronic properties of the substrate affect the sensitivity and, therefore, structure of the transition state of the reaction of interest. We speculated that, by using concomitant variation in two substituents of the substrate, the enzymic transition state could be investigated through multiple simultaneous free-energy relationships using an approach favored by Jencks to study small molecule systems.[17] Here we investigate the *O*-GlcNAcase-catalyzed hydrolysis of a large series of substrates (Figure 1B,C) using such multiple free energy relationships. These results allow us to evaluate the synergistic interaction between nucleophilic and acid catalysis and discuss the kinetic significance of an enzyme-sequestered oxocarbenium ion intermediate in glycosyl group transfer.

Materials and Methods

Substrate and Enzyme Preparation. Substrates were prepared according to previously reported synthetic schemes.[16] Spectroscopic characterization data for novel compounds may be found in the Supporting Information. Recombinant wild-type *O*-GlcNAcase was prepared according to previously reported procedures.[16]

Steady-State Kinetic Analysis. For the majority of substrates, enzyme specificity constants (k_{cat}/K_m) were determined by following substrate depletion using substrate concentrations at least 10 times lower than previously determined K_m values.[15] Stock solutions containing 100 μM substrate and 1% DMSO (v/v) in reaction buffer (pH 7.4 phosphate-buffered saline) were diluted 4-fold into the reaction buffer for kinetic assays. Reactions were followed spectrophotometrically at wavelengths reported in the Supporting Information (Table S1). First-order rate constants were determined from a nonlinear least-squares fit to the observed change in absorbance with time and the values reported represent the mean of at least three separate reactions. Where prior data were available, kinetic parameters determined using these DMSO-containing reaction volumes were identical (within error) to those previously determined in the absence of DMSO. For substrates having trifluorinated and difluorinated nucleophiles and for slow-reacting substrates possessing poor leaving groups with small absorbance changes occurring at wavelengths at which significant protein absorbance occurs, values of k_{cat}/K_m were evaluated by the initial rates method. The values of k_{cat}/K_m determined by substrate depletion and initial rates methods for the difluorinated compounds were identical within experimental error.

Investigation of the enzyme-catalyzed hydrolysis of methyl glycosides Me-GlcNAc-F_0 and Me-GlcNAc-F_1 were carried out as follows. Reactions were carried out in a total reaction volume of 200 μL using PBS (pH 7.4) as a buffer. Reactions contained a final concentration of 0, 10, 20, or 40 μM substrate and were initiated by the addition of enzyme (100 nM), after which the reaction mixture was incubated at 37 °C for 1 h. Reactions were terminated by the addition of 800 μL of 95% cold ethanol and stored at −20 °C for 30 min to allow protein to precipitate. Upon termination of

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the reaction, 2 μL of 1 mM fucose was added to each reaction as an internal standard. The suspension was centrifuged at 17 000 rpm (Eppendorf 5415C) for 20 min and the supernatant was collected and then dried by vacuum centrifugation. The residues were dissolved in 150 μL of ddH_2O, vortexed, and centrifuged at 17 000 rpm to remove residual insoluble debris. Carbohydrates were separated by high performance anion exchange chromatography (ASI 100 automated sample injector, Carbopack PA20 column, and ICS 3000; Dionex) and detected using an electrochemical detector (ED50, Dionex) using a gold working electrode and an Ag/AgCl reference electrode. An isocratic elution of 20 mM NaOH was used, which afforded optimal separation of fucose (3 min) and the hemiacetal products of the enzymatic reaction, GlcNAc (GlcNAc-F_0, 8 min) and GlcNAc-F_1 (12 min). Substrates (F_0 and F_1 methyl glycosides) eluted within the first 2 min. The amounts of the products produced in the reactions were determined by integration of the peak corresponding to GlcNAc-F_0 or GlcNAc-F_1 and corrected by comparison to the internal fucose standard. The absolute amount of each product formed was determined using standard curves constructed for GlcNAc-F_0 or GlcNAc-F_1 (the standard curve for GlcNAc-F_1 was generated by complete enzymatic hydrolysis of 3-fluoro-4-nitrophenyl-GlcNAc-F_1). All reactions were carried out in triplicate and the values of k_{cat}/K_m were determined by plotting initial rates obtained for each of the concentrations and taking the slope.

Solvent kinetic isotope effects were determined in phosphate-buffered saline at pD = 7.4. The stereochemistry of the first formed product of *O*-GlcNAcase-catalyzed *N*-acetylglucosaminide hydrolysis was determined in D_2O (pD = 7.4, phosphate buffered saline). Enzyme inhibition constants (K_i) were determined using previously reported procedures.[10]

Results

We prepared 52 substrates (Ar-GlcNAc-Rs, Figure 1B) in which both steric (R = *n*-butyl) and electronic [R = methyl (F_0), fluoromethyl (F_1), difluoromethyl (F_2), and trifluoromethyl (F_3)] perturbations are made to the 2-acetamido group of the substrate, while a variety of substituted phenols (Ar) were installed at the leaving group position (see Supporting Information for details of their synthesis and characterization). For all aryl glycosides prepared we avoided the use of ortho-substituted phenols, since these have been found to have effects on binding of substrates to glycoside hydrolases.[18] To investigate substrates having leaving groups with pK_a values closer to those of the natural substrates of *O*-GlcNAcase, we also prepared a series of methyl glycosides with corresponding changes to the acetamido group: Me-GlcNAc-F_0, Me-GlcNAc-F_1, Me-GlcNAc-F_2, Me-GlcNAc-F_3. We then investigated the *O*-GlcNAcase-catalyzed hydrolysis of these series of substrates (See Supporting Information for the complete data).

***O*-GlcNAcase-Catalyzed Hydrolysis of *N*-Acetylglucosaminides Occurs with Retention of Stereochemistry.** Previous studies have shown that deletion of the catalytic nucleophile in glycoside hydrolases can change the catalytic mechanism, resulting in an altered stereochemical outcome for the catalyzed reaction. To address this possibility, we carried out studies using ^1H NMR spectroscopy and monitored the time-dependent *O*-GlcNAcase-catalyzed hydrolysis of four representative Ar-GlcNAc-Rs in D_2O possessing different leaving groups (Ar = 3-fluoro-4-nitrophenyl or 3,4-difluorophenyl) and either good or poor nucleophiles (R = F_0 or F_3). The results show that for all substrates the first formed product is the β-anomer (data not shown), indicating no gross change in mechanism, such as change to an inverting mechanism, which would lead to formation of alternative products. Anomerization to a mixture containing predominantly the α-anomer was observed over time, and analysis of chemical shifts supports assignment of these products as GlcNAc-F_0 or GlcNAc-F_3 hemiacetals.

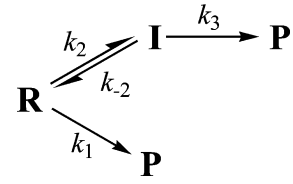
Free Energy Relationships. Variations in the $\log(k_{cat}/K_m)$ values obtained are illustrated as a function of leaving group ability, which are defined by the pK_a value of the conjugate acid of the leaving group aglycon, $(pK_a)_{lg}$. Plotting these two free energy terms against each other yields a series of Brønsted slopes that provide a measure of the relative negative charge build up on the leaving group oxygen.[4] Similarly, plotting the Taft σ^* parameter against $\log(k_{cat}/K_m)$ provides quantitative insight into *N*-acyl group nucleophilic involvement at the transition state.[4,9,19]

The quantitative description of the regimens of different gradients found in the Brønsted plots of Figure 2A may be found in Table 1, and the fits to these data are described by equations 1 and 2 outlined below. For substrates possessing the naturally occurring nucleophiles, reactivities are correlated by a single Brønsted slope as previously described.[15] Free energy relationships comprising a series of compounds that all react via the same mechanism and kinetically significant, rate-determining, transition state are linear. Equation 1 shows the correlation for such a typical Brønsted plot.

$$\log(k_{cat}/K_m) = \beta_1(pK_a)_{lg} + c_1 \quad (1)$$

The reactivities of fluorinated *N*-acylglucosaminides display multiphasic Brønsted plots with regimens (I and III) of negative gradient at high and low leaving group pK_a values, separated by a regimen (II) of positive gradient at intermediate leaving group pK_a values. The Brønsted plots for the reactivity of Ar-GlcNAc-F_2 and Ar-GlcNAc-F_3 series clearly display both upward (change in mechanism) and downward (change in rate-determining step) deflections (see Discussion). The minimal kinetic model used to correlate these data is given in Scheme 1.

Scheme 1. Minimal Kinetic Scheme for *O*-GlcNAcase Processing Ar-GlcNAc-F_2 and Ar-GlcNAc-F_3[a]



[a] A change in kinetically significant mechanism and kinetically significant rate-determining step are clearly demarked in the free energy relationships. R represents the *O*-GlcNAcase substrate, I represents a kinetically significant intermediate, and P represents the product of the reaction, in this case the oxazoline intermediate.

The observed rate of reaction (k_{cat}/K_m) may therefore be expressed in terms of individual rate constants according to equation 2.

$$k_{cat}/K_m = k_1 + \frac{k_2}{1 + (k_{-2}/k_3)} \quad (2)$$

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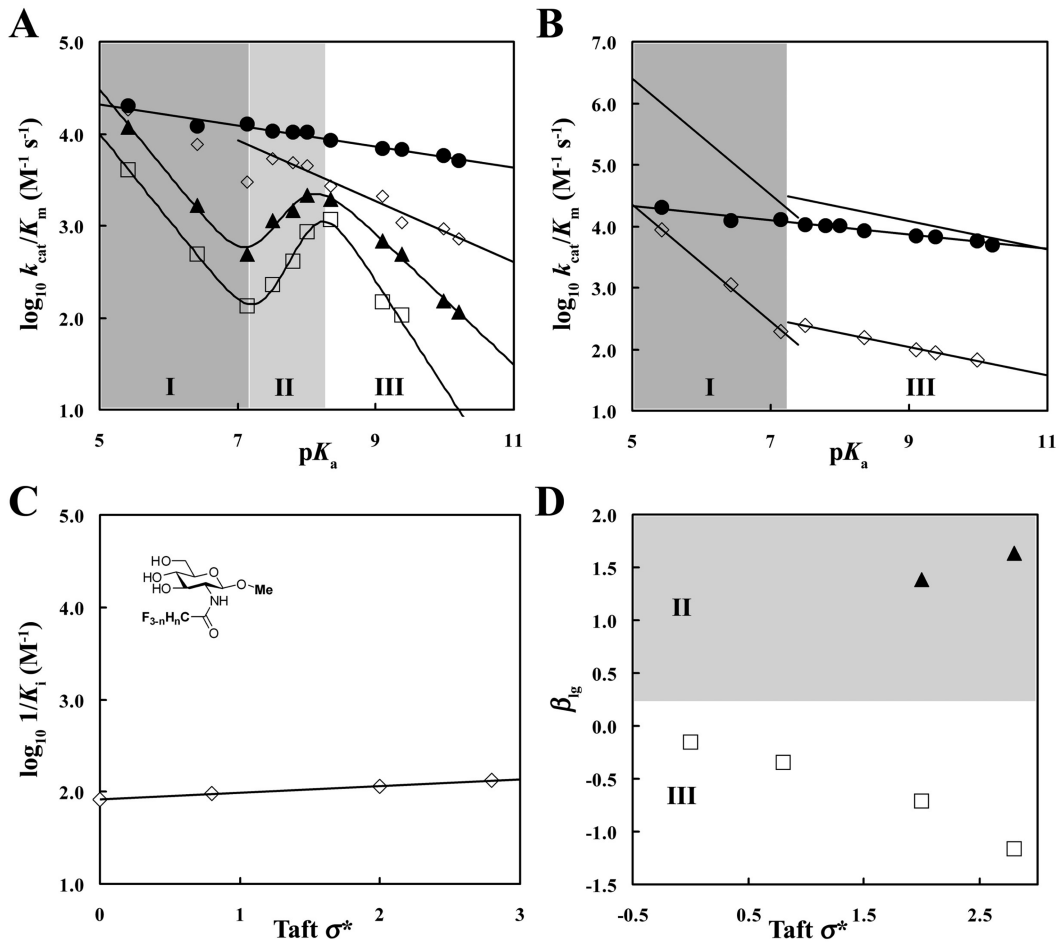

Figure 2. Effects of simultaneous variation of nucleophile and leaving group on the second-order rate constant of the *O*-GlcNAcase-catalyzed reaction, binding effects of fluorine substitution, and the dependence of β_{lg} values on σ^* values. (A) Brønsted plots showing the changes in k_{cat}/K_m with variation in $(pK_a)_{lg}$ as the nucleophile structure is varied. Regimen I (shaded dark gray) represents the rate-limiting departure of the leaving group as a phenolate anion (A_ND_N). The upward break passing from regimen I to II represents a change in mechanism to acid catalyzed leaving group departure. Positive slopes in regimen II (shaded light gray) reveal that the formation of an intermediate is rate-determining ($D_N^{\ddagger}{*}A_N$). The subsequent downward break between regimens II and III represents a change in rate-determining step such that in regimen III (unshaded) breakdown of an intermediate is rate-determining ($D_N{*}A_N^{\ddagger}$). (B) Observed rates for *n*-Bu (◇) and CH_3 hydrolysis (●) as well as the extrapolated rates for the chemical step of CH_3 hydrolysis (upper line lacking symbols). (C) Fluorine substitution has little effect on binding of methyl *N*-fluoroacetylglucosaminides to *O*-GlcNAcase. (D) β_{lg} values for hydrolysis of Ar-GlcNAc-F_n glycosides in regimens II and III vary depending on the strength of the *N*-acyl nucleophile (reflected by the σ^* value), revealing the synergistic interaction of general acid and nucleophilic catalysis. Fits shown are nonlinear fits determined as described in the text. The β_{lg} value for the CH_2F nucleophile in regimen II has been excluded due to limited data in this region.

Table 1. Brønsted Data for a Variety of Substrates Possessing Various Nucleophiles[a]

nucleophile	regimen	β_{lg}	c	r	n
CH_3[13]	–	−0.11 ± 0.01	+4.89 ± 0.06	0.984	11
CH_2F	III	−0.32 ± 0.03	+6.11 ± 0.29	0.980	8
CHF_2	I	−0.92 ± 0.09	+9.10 ± 0.54	0.997	11
	II	+1.38 ± 0.34	−7.46 ± 2.60		
	III	−0.71 ± 0.46	+9.30 ± 3.50		
CF_3	I	−0.93 ± 0.11	+8.66 ± 0.68	0.994	9
	II	+1.64 ± 0.48	−10.08 ± 3.69		
	III	−1.17 ± 0.62	+12.90 ± 4.82		
n-Bu	I	−0.95 ± 0.04	+9.10 ± 0.27	0.999	3
	III	−0.23 ± 0.01	+4.11 ± 0.07	0.998	5

[a] Where sufficient data exists and regimens I, II, and III are clearly defined (i.e., for Ar-GlcNAc-F_2 and Ar-GlcNAc-F_3), a least-squares fit to the appropriate kinetic expression (eq 3) was carried out. Where particular regimens are poorly delineated (e.g., regimens I and II for Ar-GlcNAc-F_1), linear fits to compounds falling within clearly defined regimens are carried out (e.g., regimen III for Ar-GlcNAc-F_1).

The (Brønsted) free energy relationship associated with $\log(k_{cat}/K_m)$ is therefore described by eq 3.

$$\log(k_{cat}/K_m) = \log\left[10^{\beta_1+c_1} + \frac{10^{\beta_2+c_2}}{1 + 10^{\Delta\beta+\Delta c}}\right] \qquad (3)$$

In which β_1 and c_1 are the gradients and offsets, respectively, associated with the linear free energy relationship of rate constant k_1 (similarly β_2 and c_2). $\Delta\beta$ (equal to $\beta_{-2} - \beta_3$) and Δc (equal to $c_{-2} - c_3$) report on differences in gradients and offsets of linear free energy relationships associated with the relevant rate constants.

To probe the chemical step for a nucleophile whose intrinsic strength is similar to that of the 2-acetamido group, the reactivity of a series of substrates possessing the bulkier *n*-butyl nucleophile was investigated. The Brønsted plot for these substrates was biphasic with two regimens (I and III) of negative gradient.

For this series of compounds, which displays a break in the free energy relationship, linear free energy relationships are used

to correlate data within the difference regimens. This approach is used because regions in which mechanism and rate-determining step change are not well-defined and a least-squares fit to a kinetic scheme representing solely a change in mechanism (upward break) is inappropriate. Since the data for the Ar-GlcNAc-F_1 are suggestive of, yet do not clearly define, a region having positive gradient, we treat the data for this series of compounds in the same manner.

***O*-GlcNAcase Cleavage of Methyl Glycosides.** We were able to determine the second order rate constants (k_{cat}/K_m) for the *O*-GlcNAcase-catalyzed hydrolysis of Me-GlcNAc-F_0 and Me-GlcNAc-F_1 (see Supporting Information for the complete data) using an HPLC assay in which the formation of the products are monitored; however, due to limitations in experimental design and assay sensitivity, we were unable to obtain kinetic data for Me-GlcNAc-F_2 and Me-GlcNAc-F_3. The rate constants obtained for the Me-GlcNAc-F_0 and Me-GlcNAc-F_1 substrates correlate well with the data obtained for the aryl glycosides in regimen III (Figure 4, see Conclusion).

Solvent Kinetic Isotope Effects (KIEs) Are Sensitive to Nucleophilic Strength. Small and normal solvent KIEs, $H(k_{cat}/K_m)/D(k_{cat}/K_m)$, were measured with several representative Ar-GlcNAc-Rs possessing different leaving groups (Ar = 3-fluoro-4-nitrophenyl or 3,4-difluorophenyl) and either good or poor nucleophiles (R = F_0 or F_3). Values of 1.48 ± 0.05 and 1.28 ± 0.06 were determined for 3-fluoro-4-nitrophenyl-GlcNAc-F_0 (pK_a = 6.4, regimen I) and 3,4-difluorophenyl-GlcNAc-F_0 (pK_a = 9.1, regimen III), respectively. Larger solvent KIEs were measured for the enzyme-catalyzed hydrolysis of 3-fluoro-4-nitrophenyl-GlcNAc-F_3 (2.2 ± 0.1, pK_a = 6.4, regimen I) and 3,4-difluorophenyl-GlcNAc-F_3 (2.8 ± 0.2, pK_a = 9.1, regimen III).

Substrate Binding Is Weakly Sensitive to the Nature of the Nucleophile. Because k_{cat}/K_m values reflect the free energy change on going from free enzyme and substrate to the transition state of the first irreversible step of the reaction, the reactivity parameters we attribute to on-enzyme chemical steps may be complicated by effects associated with substrate binding.[20] To address whether addition of fluorine to the 2-acetamido group caused significant perturbations to binding, a series of Me-GlcNAc-F_ns were tested as competitive inhibitors. As shown in Figure 2C, binding is well-correlated but very weakly sensitive to increasing fluorine substitution of the *N*-acyl group:

$$\log(1/K_i) = (0.07 \pm 0.01)(\sigma^*)_{nuc} + (1.91 \pm 0.01) \qquad (r = 0.999, n = 4)$$

On the basis of this finding, our subsequent analysis of the influence of different nucleophiles on reactivity can be interpreted without requiring corrections for steric or electronic effects on binding.

Discussion

The complex multiple free energy relationship measured here for *O*-GlcNAcase offers unique insight into the catalytic mechanism of glycoside hydrolases, enabling us to gain an understanding of the interplay between general acid catalysis and nucleophilic catalysis. The observed pattern of positive and negative inflections for substrates with poor nucleophiles and a single positive inflection for substrates with the natural good nucleophile are entirely unprecedented. Given their apparent complexity, we will discuss our interpretations for each regimen separately.

Substrates in Regimen I. Before embarking on an interpretation of the data in this regimen, several prior observations require consideration. Earlier mutagenesis studies of *O*-GlcNAcase have identified Asp175 as the general acid catalyst responsible for assisting departure of the aglycon leaving group,[15] and the X-ray structure of *Bacteriodes thetaiotaomicrometer* β-glucosaminidase (*Bt*GH84), a GH84 enzyme having conserved active site residues with human *O*-GlcNAcase, supports this assignment.[13] Key to the ensuing discussions, a kinetic pK_a value of approximately 7.8 has been assigned to Asp175.[15,16] Substrates possessing phenolic leaving groups having pK_a values below that of the general acid cannot benefit significantly from acid catalysis, as proton transfer is thermodynamically unfavorable.[21] Thus, the reactivity of compounds in regimen I (Figure 2A,B) must arise from a mechanism involving departure of phenolate anion without the benefit of acid catalysis, here termed "spontaneous departure".

The magnitude of the slope of plots of $\log(k_{cat}/K_m)$ against the $(pK_a)_{lg}$ indicates the extent of negative charge accumulation on the glycosidic oxygen in the transition state. The above analysis suggests that we should observe a large negative β_{lg} value for substrates having $(pK_a)_{lg}$ values below that of the general acid catalyst ($pK_a \approx 7.8$), since these must depart as the phenolate anion. The reactivities of all Ar-GlcNAc-F_0s (which span the pK_a of the enzymic general acid), however, are well-correlated by a single line, implying that no variation in mechanism or rate-determining step occurs with $(pK_a)_{lg}$. This observation, however, is inconsistent with the lack of general acid catalysis for substrates having low $(pK_a)_{lg}$. We speculated that this pattern of reactivity associated with Ar-GlcNAc-F_0s (Figure 2A) may be readily explained by a kinetically significant step (e.g., an enzyme conformational change) that precedes glycosidic bond cleavage, which is the first irreversible chemical step of the reaction. One piece of evidence supporting this interpretation is the observed solvent KIEs. For the *O*-GlcNAcase-catalyzed hydrolysis, significantly smaller KIEs were observed for substrates possessing the 2-acetamido nucleophile than for those KIEs found for substrates possessing the 2-trifluoroacetamido nucleophile, suggesting the KIE is partly masked by a significant contribution from a nonchemical step to the overall rate constant. Further support for this proposal is that the biphasic Brønsted plot of $\log(k_{cat}/K_m)$, previously determined for the Asp175Ala mutant-catalyzed hydrolysis of Ar-GlcNAc-F_0s, reveals a negligible gradient for substrates having $(pK_a)_{lg}$ values less than the pK_a of the general acid catalyst (regimen I), whereas for those substrates having $(pK_a)_{lg}$ values greater than 7.8, a significant negative slope is observed. These data suggest that, for the Asp175Ala mutant, a kinetically significant step precedes cleavage of the glycosidic linkage for good substrates, whereas for worse leaving groups cleavage of the glycosidic bond is the kinetically significant step.[15]

To test this hypothesis, we aimed to make the chemical step rate-determining while minimizing changes to the structure of the transition states associated with the chemical steps. Indeed, this strategy has been used with great success in other enzyme classes.[22] We therefore prepared a series of substrates having a

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bulky *n*-butyl alkyl chain, Ar-GlcNAc-*n*-Bu, which possess a nucleophile of similar intrinsic strength [σ^*(*n*-Bu) = −0.15] to the 2-acetamido group [σ^*(Me) = 0.00]. On the basis of previous studies with *O*-GlcNAcase,[23] the increased bulk of the *n*-butyl alkyl group raises by similar amounts the energy of both the rate-determining transition state and that of the Michaelis complex relative to free enzyme and substrate. The net result is that the chemical steps become rate-determining (Figure 2B). Consistent with our prediction, we find for this series of Ar-GlcNAc-*n*-Bu a β_{lg} value of −0.95 for those substrates having leaving groups that cannot benefit from general acid catalysis (regimen I) and a β_{lg} value of −0.23 for substrates with worse leaving groups that can benefit from general acid catalysis (regimen III). Notably, the steep negative slope (β_{lg} = −0.95) in regimen I for the Ar-GlcNAc-*n*-Bu series is comparable to that observed for the Asp175Ala mutant (β_{lg} = −1.0),[15] consistent with this process reflecting expulsion of the leaving group as a phenolate. This β_{lg} value also closely resembles those found for the Ar-GlcNAc-F_2 (−0.92) and Ar-GlcNAc-F_3 (−0.93) in regimen I. Thus, when values of β_{lg} report on the chemical step for spontaneous glycoside hydrolysis, rather than some preceding nonchemical rate-determining step, the extent of negative charge buildup on the leaving group is similar regardless of nucleophilic strength. These results indicate that the transition states for the spontaneous expulsion of phenoxides from glucosaminides occurring in aqueous and this enzymic environment are broadly similar: they both involve significant negative charge accumulation on the leaving group and significant nucleophilic participation.[24,25]

Using this data, and on the basis of prior studies investigating the influence of the *N*-acyl substituent on transition state and transition state analogue inhibitor binding, we estimate values of k_{cat}/K_m that would be observed for the hydrolysis of Ar-GlcNAc-F_0 if the chemical step were kinetically significant (Figure 2B and Supporting Information).[23] Throughout the paper we use these extrapolated k_{cat}/K_m values for the Ar-GlcNAc-F_0 series.

The significance of nucleophilic participation at the transition state for the chemical step is quantified by the Taft-reaction sensitivity (ρ^*). Obtaining ρ^* involves correlating the Taft parameter of each substrate (σ^*), which reflect the differing strengths of the *N*-acyl nucleophile of each substrate, against the log(k_{cat}/K_m) values obtained for each corresponding substrate in each series. For substrates in the Ar-GlcNAc-F_0, Ar-GlcNAc-F_2, and Ar-GlcNAc-F_3 series having leaving groups with $(pK_a)_{lg}$ values that place them in regimen I, the Taft-parameter is negative, suggesting significant nucleophilic participation that is fairly similar in all series [$(\rho^*)_{kcat/Km}$ = −0.85 ± 0.04; data not shown]. Collectively, the linear and parallel Brønsted (Figure 2A,B) and Taft plots obtained for substrates in regimen I, for which the chemical step is clearly rate-determining, are consistent with a concerted (S_N2-like, more clearly described as an A_ND_N mechanism[26]) transition state that remains relatively invariant with changing leaving group and nucleophile structure.

Substrates with Poor Nucleophiles in Regimens II and III. Inflections in linear free energy relationships, such as a Brønsted plot, can stem from two phenomena:[1] negative deviations reflect a change in rate-determining step, and positive deviations denote a change in mechanism. The positive deviations that we consistently observe as leaving group pK_a values increase beyond that of 4-nitrophenol (pK_a = 7.2) (regimen I passing to regimen II), therefore, reflect an enforced change in mechanism. More specifically, a change from spontaneous to acid-catalyzed glucosaminide cleavage seems most likely as proton transfer to the leaving group becomes thermodynamically favorable. Single positive deviations, as observed for the Ar-GlcNAc-*n*-Bu series, have previously been reported for a 1,3−1,4-β-D-glucan hydrolase.[27] However, the large positive β_{lg} values of regimen II, following the inflection, have not been observed in glycoside hydrolase-catalyzed reactions. Furthermore, the patterns of subsequent negative deviations on passing from regimen II to III are unprecedented in free energy relationships for glycosyl hydrolases, perhaps because attenuating the nucleophilicity of an enzymic group has been difficult or perhaps because such a downward break has not yet fallen into a region that is convenient to observe experimentally. This negative inflection, however, suggests a change in rate-determining step arising from the existence of a kinetically significant intermediate state (S_N1-like, more clearly described as an $D_N{*}A_N$ mechanism[26]). The microscopic nature of this intermediate state is of obvious interest and there is a compelling mechanistic rationale offering insight into its nature, as well as providing the basis for the trends in the Brønsted plots.

For glycoside hydrolases[4] and many ribosyl transferases[28] that possess preorganized active site nucleophiles, a mechanism in which motion of the anomeric carbon defines the reaction coordinate (Figure 1A) is invoked. This process may occur through either a concerted transition state (A_ND_N) or through a dissociative pathway possessing a kinetically significant, oxocarbenium ion-like intermediate ($D_N{*}A_N$). For glycosidases, the anomeric center is generally thought to move from bonded contact with the leaving group to bonded contact with the enzymic nucleophile, which both remain stationary.[4,29] Accordingly, if a kinetically significant oxocarbenium ion intermediate lies along the reaction coordinate, then the overall rate of reaction may be limited by either its rate of formation ($D_N^{\ddagger}{*}A_N$) or its collapse ($D_N{*}A_N^{\ddagger}$). The character of this intermediate would therefore be highly sensitive to the nature of the flanking nucleophile; decreasing nucleophile strength would increase the ionic character of the intermediate, contributing to its kinetic significance and giving shape to the Brønsted plots for poor nucleophiles. Electronically, this is consistent with decreasing

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(31) Alternative interpretations of the data that might account for the observed reactivity trends observed can be envisioned. For example, one alternative proposal is that regimen II might reflect rate-determining formation of a cationic intermediate and regimen III might reflect rate-determining diffusion of the phenol away from the cationic intermediate within the enzyme active site (rather than attack of the nucleophile). Although such a mechanism is not inconsistent with the data presented in this paper, we do not favor this alternative interpretation. These (S_Ni-type) mechanisms may operate for certain glycosyl transferases (and possibly even some mutant glycosyl hydrolases);[32] however, for *O*-GlcNAcase the presence of a preorganized nucleophilic species of reasonable strength on the opposing face of the incipient cationic intermediate to the leaving group suggests that an intermediate possessing significant covalent character will be formed.

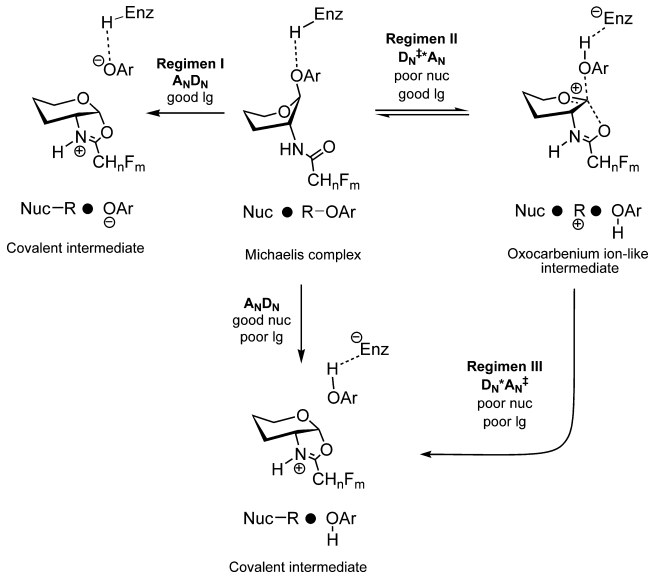

Figure 3. Proposal describing the formation and fate of intermediates in *O*-GlcNAcase-catalyzed glycosyl hydrolysis. The rate-determining step depends on the nature of the leaving group and nucleophile. For the best leaving groups, regimen I reflects a concerted (A_ND_N) pathway with departure of the leaving group as a phenolate being operative. For poor nucleophiles, regimen II reflects formation of an oxocarbenium ion being rate determining and regimen III reflects collapse of this intermediate ($D_N{}^{*}A_N$) pathways. For acid-assisted departure of the leaving group, either increasing nucleophile strength or decreasing leaving group ability will ultimately change the pathway followed from $D_N{}^{*}A_N$ to A_ND_N. For the different strength nucleophiles used in this study $n = 0-3$ and $m = 3 - n$, with increasing numbers of fluorine atoms decreasing nucleophilicity.

nucleophilic strength resulting in greater partial positive charge on the glycosyl moiety of the intermediate.

The reaction followed by compounds having poor nucleophiles in regimen II is represented in Figure 3 (poor nucleophile and good leaving group). The transition state that lies en route to the cationic intermediate is expected to be rate-determining. The rationale for the positive β_{lg} values is that protonation of the leaving group significantly leads glycosidic bond fission for all substrates in this regimen. Substrates with worse nucleophiles have more positive β_{lg} values (Figure 2D) and this can be explained by the progressively greater extent of partial positive charge on the glycosyl moiety of the intermediate. As the charge on the glycosyl moiety increases, the leaving group oxygen bears more relative positive charge, and this leads to consequent increases in β_{lg} values.

The reaction followed by compounds in regimen III is represented in Figure 3 (poor nucleophile and poor leaving group). This regimen defines a rate-determining step leading to collapse of the oxocarbenium ion intermediate. The *N*-acetyl group has been shown to be a nucleophilic participant in substitution reactions occurring even in solution, a condition where it must be disordered in the ground state.[30] This strongly suggests that ultimately a covalent oxazoline or oxazolinium ion intermediate will be formed in the enzyme active site. A $D_N{}^{*}A_N$ mechanism rationalizes observed reactivity trends: more basic phenols interact more strongly with the cationic intermediate in the active site. As a consequence, the back reaction becomes more favorable with more basic phenols and so formation of the cationic intermediate likely becomes reversible. Also, the rates of trapping of the cationic intermediate by the *N*-acyl nucleophile decrease with increasing pK_a (β_{lg} is negative). Therefore, the decreases in β_{lg} values observed for substrates having poorer nucleophiles (Figure 2D), which have a greater partial positive charge on the glycosyl moiety of the intermediate, is consistent with progressively tighter association between the glycosyl moiety and protonated leaving groups.[31]

Substrates with Better Nucleophiles and Poor Leaving Groups. The natural substrates of *O*-GlcNAcase possess a 2-acetamido group that is a significantly stronger nucleophile than fluorinated 2-*N*-acyl groups. *O*-GlcNAcase uses this nucleophile to displace serine or threonine leaving groups of polypeptide chains having $(pK_a)_{lg}$ values exceeding that of Asp175. Therefore, characterization of transition states falling within regimen III for substrates possessing the 2-acetamido group provide greatest insight into the biologically significant *O*-GlcNAcase mechanism.

Perhaps the most striking feature of the free energy relationships of regimens II and III for substrates bearing good nucleophiles is the absence of a clear negative deviation in the Brønsted relationship (Figure 2A,B). There are two possible explanations for the absence of such a break: the oxocarbenium ion-like intermediate state is no longer kinetically significant and a concerted A_ND_N mechanism is followed instead of a dissociative $D_N{}^{*}A_N$ pathway; in this scenario the break does not exist. Alternatively, the break does exist but now falls within regimen I, for which an entirely different mechanism operates. Although it is difficult to draw firm conclusions as to whether a change in mechanism from dissociative to concerted pathways has actually occurred or not in the specific case of the 2-acetamido nucleophile, trends of decreasingly positive values of β_{lg} in regimen II and decreasingly negative values of β_{lg} in regimen III are relatively clear (Figure 2D). The convergence of these values with increasing nucleophilic strength indicates that a change in mechanism from dissociative to concerted pathways very likely occurs for an appropriately strong nucleophile, perhaps, for example, for the acetamido group of the normal substrate of *O*-GlcNAcase.[33] Such a change in mechanism is consistent with the expectation that a positively charged glycosyl group will collapse much more rapidly with appropriately positioned strong nucleophiles. In any event, further investigations using comprehensive kinetic isotope effect studies are being undertaken to definitively clarify these and other detailed mechanistic proposals.[36]

Conclusion

The preorganized nature of the substrate within the active site of *O*-GlcNAcase involves strategic positioning of both nucleophile and general acid catalyst, a feature likely enabling concomitant acidic and nucleophilic catalysis. The potential synergy between these two modes of catalysis may be investigated by extrapolation of our data to a pK_a of 16, the approximate pK_a of the natural substrate of *O*-GlcNAcase

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(33) Symmetry consideration (i.e., the nucleophilic species in the forward direction is equivalent to the leaving group in the reverse direction and vice versa) suggests that for sufficiently poor leaving groups (strong nucleophiles in the reverse direction) a change in mechanism from dissociative ($D_N{}^{*}A_N$) to concerted (A_ND_N) pathways may also occur. This change in mechanism may be used to explain unusual upward breaks in the Brønsted plots observed for the acid-catalyzed hydrolysis of some acetals in solution.[34,35]
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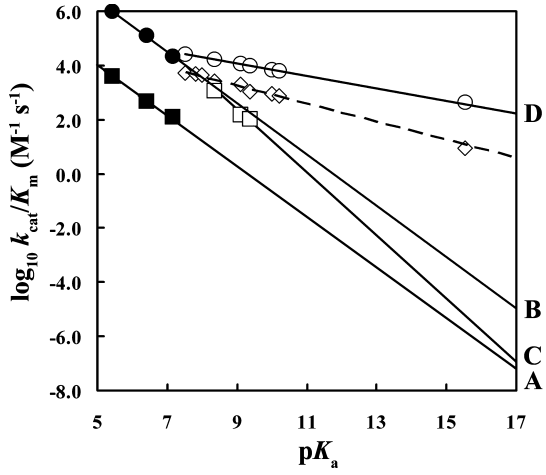

Figure 4. Evidence that *O*-GlcNAcase synergistically harnesses nucleophilic and general acid catalysis. The lines show rates of hydrolysis extrapolated to a leaving group pK_a of 16 for a mechanism involving (A) spontaneous departure of leaving group using a crippled nucleophile (Ar-GlcNAc-F_3s in regimen I, ■), (B) spontaneous departure of leaving group using the naturally occurring nucleophile (Ar-GlcNAc-F_0s in regimen I, ●), (C) acid-catalyzed leaving group departure using a crippled nucleophile (Ar-GlcNAc-F_3s in regimen III, □), (D) acid-catalyzed leaving group departure using the naturally occurring nucleophile (Ar-GlcNAc-F_0s, regimen III, ○). The extrapolated rates are likely reasonable since rates of hydrolysis of the methyl 2-*N*-acyl-*β*-glucopyranosides Me-GlcNAc-F_0 (bold line, D, ○) and Me-GlcNAc-F_1 (dashed line, ◇) correlate well with linear regressions for the processing of aryl substrates having either the acetamido or the monofluoracetamido nucleophile, respectively, that define regimen III.

(Figure 4). Notably, the observation that the data for the methyl glycosides (Me-GlcNAc-F_0 and Me-GlcNAc-F_1) correlate well with the data obtained for aryl glycosides of regimen III supports the validity of this extrapolation. As would be expected, when both forms of catalysis are impaired, the predicted rate of hydrolysis is slowest. It is notable, however, that the rate enhancement stemming from general acid or nucleophilic catalysis, on their own, do not come close to being additive to the rate enhancement observed when both modes of catalysis are operative. This difference highlights how the enzyme employs both general acid and nucleophilic catalysis in a synergistic manner and seems most likely to arise from the preorganized nature of the enzyme active site, a feature that makes this pathway favored by the enzyme whereas in solution its observation would likely prove impossible due to competing mechanistic pathways.

The linear free energy relationships we describe here for *O*-GlcNAcase are most readily interpreted as reflecting the kinetic significance of an oxocarbenium ion intermediate for substrates having poor nucleophiles. There is a long history of investigations into the existence of such carbocation-like intermediates in the hydrolysis of glycosides, with solution studies pointing to a lifetime dependent on subtle factors.[21] In enzymes, the existence of such a species has also long been contemplated,[38] and recent studies of a mutant glycosidase lacking the general acid catalyst support its viable existence.[39] Detailed microscopic interpretations of the Brønsted data are not essential for evaluating the extent of synergy. However, in the case of *O*-GlcNAcase acting on substrates with good nucleophiles and poor leaving groups, for which this enzyme has evolved, effective synergy of these catalytic strategies likely alter the reaction coordinate, such that the oxocarbenium ion intermediate ceases to be kinetically significant and the reaction possibly becomes concerted (Figure 3). Further investigation of this mechanistic proposal, using detailed kinetic isotope effect studies, are being undertaken to further assess these microscopic interpretations and will be reported in due course. More significantly, here we provide compelling evidence for synergy between nucleophilic and general acid catalysis, which, although widely speculated upon in many enzyme systems, has proven remarkably difficult to capture in small molecule models of enzymic catalysis,[40] including *N*-acetylglucosaminides,[30,41] or to experimentally demonstrate in enzyme systems. Such synchronization, however, is undoubtedly a key factor contributing to the proficient nature of enzyme catalysts, including glycoside hydrolases, as we reveal here experimentally for *O*-GlcNAcase.

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Supporting Information Available: Synthesis and characterization of compounds used in this study, measured rate data, procedure for NMR monitoring of reaction stereochemistry and the extrapolation of rates of chemical step for Ar-GlcNAc-F_0s. This material is available free of charge via the Internet at http://pubs.acs.org.

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(36) Kinetic isotope effects, and in particular the values of the anomeric carbon and endocyclic ring oxygen KIEs, are perhaps the most widely used means of differentiating between concerted (A_ND_N) and dissociative (D_N*A_N) mechanisms of group transfer.[37] Previous studies for dissociative mechanisms using models for the transition states that lack either the nucleophilic species ($D_N^{\ddagger}$**A_N) or the leaving group (D_N*$A_N^{\ddagger}$) have revealed good matching of experimental and computed KIEs, suggesting that these groups may be relatively insignificant in determining the vibrational environment of the transition state. Our results, however, show that values of k_{cat}/K_m determined in both regimens II ($D_N^{\ddagger}$**A_N) and III (D_N*$A_N^{\ddagger}$) are sensitive to both the nature of the leaving group and the nucleophile, indicating that these groups both contribute to the electrostatic environment of the transition state. Vibrational models of these transition states constructed using a comprehensive series of KIEs along with computational models should clarify details of the mechanistic proposal advanced here for human *O*-GlcNAcase.

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