Stereochemistry and Kinetics of the Hydration of 2-Acetamido-D-glucal by β-*N*-Acetylhexosaminidases†

Ellen C. K. Lai and Stephen G. Withers*

Department of Chemistry, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z1

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ABSTRACT: Hydrolysis by three β-*N*-acetylhexosaminidases (human placenta, jack bean, and bovine kidney) is shown to occur with the retention of anomeric configuration, most likely via a double-displacement mechanism involving the formation and hydrolysis of a glycosyl−enzyme intermediate. 2-Acetamido-D-glucal is shown to be a slow, tight-binding substrate for the jack bean enzyme, with V_{max} and K_m values of 0.48 ± 0.01 unit/mg and 27 ± 2.8 μM, respectively. The same substrate is also bound very tightly by the human and bovine enzymes, with K_i values of 8 and 25 μM, respectively. All three enzymes hydrate 2-acetamido-D-glucal, yielding *N*-acetyl-D-glucosamine as the product in each case. This is the first time that proton transfer has been shown to occur from the top face during the hydration of a glycal by a retaining β-glycosidase. Kinetic studies of this hydration reaction with the jack bean enzyme demonstrate that the tight binding observed is due to the formation of a high-affinity, reversible complex, and not due to the accumulation of a reaction intermediate. This indicates that correctly substituted glycals might act as transition state mimics and suggests approaches to the design of high-affinity inhibitors of β-*N*-acetylhexosaminidases.

N-Acetylhexosaminidases (NAGases)[1] are enzymes of considerable importance in biological systems. This importance stems largely from the almost ubiquitous presence of *N*-acetylhexosaminides as components of glycoproteins and glycolipids in mammalian systems and from their frequent occurrence as components of bacterial cell walls. Enzymes capable of cleaving these sugars are therefore important, *inter alia*, in glycoprotein processing, glycolipid catabolism, and antibacterial action. Indeed, a deficiency in the lysosomal hexosaminidase A activity in humans results in Tay-Sachs disease.

Despite their importance, relatively little attention has been paid to mechanistic studies of NAGases, nor has much work been published on the design, synthesis, and testing of specific inhibitors. Since there is considerable evidence that some NAGases are produced at elevated levels and secreted into the extracellular medium by many different cell types *in vitro* (Bernacki et al., 1985), an interest in specific inhibitors is warranted. Two classes of inhibitors of these enzymes have, however, been described previously: aldonolactones and 5-amino-5-deoxy-D-hexopyranoses. 2-Acetamido-2-deoxy-D-gluconolactone is an NAGase inhibitor with K_i values between 0.1 and 5 μM (Conchie et al., 1967; Li & Li, 1970; Sandhoff & Wässle, 1971; Villar et al., 1984). However, like all glyconolactones, the 2-acetamido derivative is unstable in aqueous solution and therefore unsuitable for clinical applications. The 5-amino-5-deoxy-D-hexopyranose derivative of *N*-acetylglucosamine is an extremely effective NAGase inhibitor, with K_i values between 0.001 and 1 μM (Kappes & Legler, 1989). Unfortunately, these compounds are also unstable in aqueous solution, and their corresponding 1-deoxy analogues, although stable, are considerably less effective.

Glycals are a class of compounds whose potential to act as NAGase inhibitors has not really been addressed. These 1,2-unsaturated sugar derivatives are highly effective inhibitors of glycosidases in some cases, with K_i values in the micromolar range (Lee, 1969; Legler, 1990). The ability of glycals to act as inhibitors was originally attributed to their planar geometry at C-1, which mimics the geometry of the oxocarbonium ion-like transition state of glycosidase catalysis (Lee, 1969). However, further investigation of the interaction of D-galactal with *Escherichia coli* β-galactosidase invalidated this hypothesis, since D-galactal itself was found to bind rather poorly, with K_i values ≥10 mM (Wentworth & Wolfenden, 1974). D-Galactal is actually a substrate of the enzyme and is hydrated to yield 2-deoxy-D-galactose via a 2-deoxygalactosyl−enzyme intermediate. The strong inhibition observed is a consequence of the relatively rapid formation of the intermediate, coupled with its relatively slow rate of hydrolysis. This results in accumulation of the intermediate and inhibition of the enzyme. Several other glycosidases hydrate their corresponding glycals [reviewed in Legler (1990)], but relatively few of these reactions exhibit the high rate of formation of the intermediate and the low rate of its hydrolysis that together are required for good inhibition.

The stereochemistry of the enzyme-catalyzed protonation of C-2 of the glycal has been examined in a number of cases [reviewed in Legler (1990)]. Surprisingly, in all cases examined to date of β-glycosidases that hydrolyze glycosides with the net retention of anomeric configuration, the direction

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* Correspondence should be addressed to this author (telephone, 604-822-3402; Fax, 604-822-2847; e-mail, withers@unixg.ubc.ca).

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[1] Abbreviations: βGlcDNP, 2,4-dinitrophenyl β-D-glucoside; βGlcNAcPNP, 4-nitrophenyl *N*-acetyl-β-D-glucosaminide; D-glucal, 1,5-anhydro-2-deoxy-D-*arabino*-hex-1-enitol; DNP, dinitrophenol; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NAGase, β-*N*-acetylhexosaminidase; NAglucal, 2-acetamido-D-glucal (1,5-anhydro-2-deoxy-2-acetamido-D-*arabino*-hex-1-enitol).

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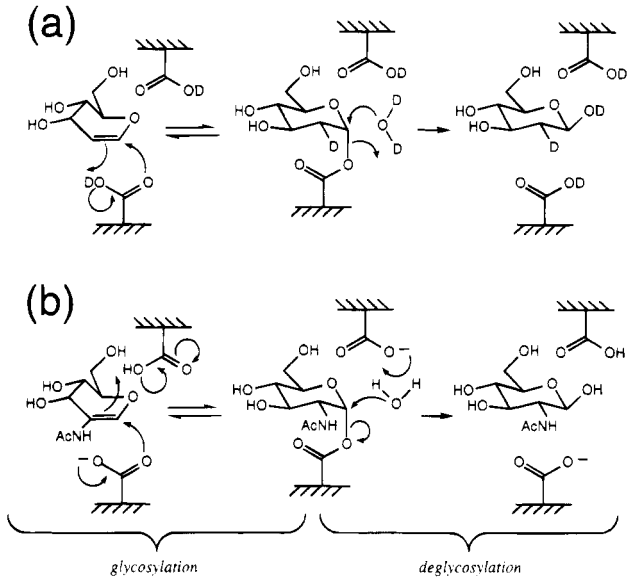
Scheme 1: Proposed Mechanisms for (a) the β-Glucosidase-Catalyzed Hydration of D-Glucal in D_2O and (b) the NAGase-Catalyzed Hydration of NAglucal



of protonation during the hydration of the corresponding glycal is opposite that expected on the basis of the normal mechanism. The same is also true of retaining α-glycosidases, with the one exception of coffee bean α-galactosidase (Weiser et al., 1992), which hydrates D-galactal via protonation from the bottom face. Thus, β- and α-glycosidases generally protonate glycals from the bottom and top faces of the sugar, respectively (Hehre et al., 1977; Legler, 1990). These results have been explained by the hypothesis that the first step of the hydration reaction involves the concerted *syn* addition of the carboxylic acid nucleophile across the double bond via a cyclic 6-membered transition state (Legler, 1990) (Scheme 1a), although an alternative mechanism involving an oxocarbonium ion has also been proposed (Matsui et al., 1993).

The poor reversible binding of glycals to their corresponding glycosidases, which is disappointing given the potential of these compounds to act as transition state analogues, is presumably a consequence of the absence of the C-2 hydroxyl group (Legler, 1990). This substituent is extremely important during catalysis, and noncovalent interactions between this hydroxyl and the enzyme stabilize the transition state by at least 8 kcal/mol (Roeser & Legler, 1981; Legler et al., 1980; Wolfenden & Kati, 1991; McCarter et al., 1992). The incorporation of a hydroxyl substituent at the C-2 of a glycal is not useful, since the resulting enol preferentially exists in the keto form. However, incorporation of an *N*-acetyl substituent at the C-2 of a glycal yields a stable derivative that is unaffected by tautomerism. This paper describes an investigation of 2-acetamido-D-glucal (NAglucal) as an inhibitor of three different NAGases and examines the effects of the *N*-acetyl substituent on the reversible binding, rate, and stereochemistry of the hydration of the glycal analogue of the normal substrate.

MATERIALS AND METHODS

Materials. β-*N*-Acetylhexosaminidases from human placenta, jack bean, and bovine kidney (catalogue numbers A-6152, A-2264, and A-2415, respectively), βGlcNAcPNP, GlcNAc, ManNAc, 3,4,6-tri-*O*-acetyl-D-glucal, and all buffer chemicals were obtained from the Sigma Chemical Company and were used without further purification. Deacetylation of 3,4,6-tri-*O*-acetyl-D-glucal using sodium methoxide in methanol (Sinnott & Souchard, 1973) yielded D-glucal (mp 57−58 °C; lit. mp 57−59 °C) (Roth & Pigman, 1963). βGlcDNP (mp 100−103 °C; lit. mp 99−101 °C) was synthesized from 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose and fluoro-2,4-dinitrobenzene (Ballardie et al., 1973; Koeners et al., 1980). Column chromatography was performed using Kieselgel 60 (230−400 mesh) silica gel. Inhibitors and substrates were obtained in crystalline form and characterized by melting point (uncorrected), ^{1}H NMR, ^{13}C NMR, and elemental analyses. The analytical data obtained for all of the compounds used in this work were consistent with the expected structures and literature data. Elemental analysis was performed by Mr. Peter Borda in the Microanalytical Laboratory, Department of Chemistry, at the University of British Columbia.

Deprotection of NAglucal. 1,5-Anhydro-2-deoxy-2-(*N*-acetylacetamido)-3,4,6-tri-*O*-acetyl-D-*arabino*-hex-1-enitol was synthesized from GlcNAc according to Pravdić et al. (1975) and purified by column chromatography (1:1 hexane/ethyl acetate). Deacetylation of 0.44 g of this starting material using sodium methoxide in methanol (Pravdić & Fletcher, 1967) removed one of the two *N*-acetyl groups and all three *O*-acetyl groups. Column chromatography (25:10:4 ethyl acetate/ethanol/water) yielded the crude product (0.4 g, 91%), which was recrystallized (2-propanol/CH_3CN/hexane) to yield NAglucal (0.25 g, 57%) (mp 120−122 °C; lit. mp 124−125 °C) (Pravdić & Fletcher, 1967). ^{1}H NMR (300 MHz, D_2O): δ 6.71 [d, 1 H, $J_{1,3}$ = 1 Hz, H(1)]; 4.29 [dd, 1 H, $J_{1,3}$ = 1 Hz, $J_{3,4}$ = 6.4 Hz, H(3)]; 4.02 [dt, 1 H, $J_{5,6}$ = $J_{5,6'}$ = 4.2 Hz, $J_{4,5}$ = 8.5 Hz, H(5)]; 3.89 (d, 2 H, $J_{5,6}$ = $J_{5,6'}$ = 4.2 Hz, H(6), H(6′)]; 3.79 [dd, 1 H, $J_{3,4}$ = 6.4 Hz, $J_{4,5}$ = 9.0 Hz, H(4)]; 2.10 [s, 3 H, H_3CCONH]. Anal. Calcd for $C_8H_{13}NO_5$ (203.20): C, 47.29; H, 6.45; N, 6.89. Found: C, 47.29; H, 6.39; N, 6.75.

Kinetic Studies. In all cases, 1 unit of NAGase activity represents the formation of 1 μmol of product per minute. Kinetic studies of hydrolysis reactions were performed by following changes in UV/vis absorbance on a Pye-Unicam PU-8800 spectrophotometer equipped with a circulating water bath that maintained the 1-cm cuvettes at 25 °C. Except as noted, reaction buffers contained BSA (0.1%), NaCl (100 mM), and citrate buffer (50 mM) and were adjusted to pH 5.0 (used with jack bean NAGase) or pH 4.25 (used with human placenta or bovine kidney NAGase). Molar extinction coefficients for 4-nitrophenol and βGlcNAcP-NP were determined at 25 °C by measuring the absorbance at 360 nm of carefully prepared stock solutions of each compound in the appropriate enzyme reaction buffer (pH 5 or 4.25). The molar extinction differences at 360 nm ($\Delta\epsilon_{360}$) determined for βGlcNAcPNP and 4-nitrophenol at pH 5.0 and 4.25 were 2280 and 2150 M^{-1} cm^{-1}, respectively.

The initial rates of βGlcNAcPNP hydrolysis were determined by incubating the reaction buffer at 25 °C in the thermostated cuvette holder of the spectrophotometer. An appropriate volume of stock substrate solution was added to the cuvette ~4 min before the reaction (to ensure negligible spontaneous hydrolysis of the substrate during the preincubation and reaction periods). Reactions were initiated by the addition of enzyme (in BSA-containing buffer) by

Table 1: Michaelis–Menten and Inhibition Parameters for NAGase-Catalyzed Reactions

enzymatic reaction	parameter	jack bean NAGase	bovine kidney NAGase	human placenta NAGase
βGlcNAcPNP hydrolysis	K_m (μM)	620 ± 36	1120 ± 136	580 ± 61
	V_{max} (units/mg)	50 ± 1.3	24 ± 1.5	8 ± 0.4
inhibition of βGlcNAcPNP hydrolysis by NAglucal	K_i (μM)	29 ± 1.5	~25	~8
NAglucal hydration	K_m (μM)	27 ± 2.8		
	V_{max} (units/mg)	0.48 ± 0.01		

syringe, and the reactions were monitored at 360 nm. Initial rates were determined using 6–7 different substrate concentrations, which ranged from about one-third to (in most cases) 3 times the value of the K_m ultimately determined. However, due to the high background absorbance of βGlcNAcPNP at 360 nm, its concentration was kept below 2 mM. The initial rates of βGlcDNP hydrolysis were determined using a comparable assay, except that the reaction was monitored at 400 nm. The $\Delta\epsilon_{400}$ determined for βGlcDNP and 2,4-dinitrophenol at pH 5.0 was 9750 M^{-1} cm^{-1}. Values of K_m and k_{cat} were determined from initial rates by a direct fit of the data to the Michaelis–Menten equation using the computer program GraFit (Leatherbarrow, 1990).

Inhibition constants (K_i values) were determined by measuring initial rates using a fixed βGlcNAcPNP concentration (1.4 mM) and widely varying inhibitor concentrations. A Dixon plot of this data yielded an approximate value for the K_i (from the intercept of this line with a horizontal line drawn at $1/V_{max}$). A full K_i determination (for jack bean NAGase) was carried out using a series of six different substrate concentrations bracketing the K_m value, together with each of five inhibitor concentrations bracketing the range-finding K_i value and in the absence of inhibitor. The nonlinear regression analysis computer program GraFit (Leatherbarrow, 1990) was used for the fitting of all such data.

HPLC-Based Stereochemical and Kinetic Studies. HPLC analyses were carried out using instrumentation from Waters, including the HPLC apparatus, Model 712 WISP autosampler (injector), Model 410 differential refractometer detector, Model 486 tunable absorbance detector, and an analytical DextroPak column (100 × 8 mm; operated using water as the eluant and used to separate anomers of GlcNAc). A Guard-Pak precolumn was used to remove protein before the sample entered the DextroPak column. A Bio-Rad Aminex HPX-87H column was also used (300 × 7.8 mm; operated using 13 mM H_2SO_4 as the eluant and used to separate GlcNAc and ManNAc). Data were collected using the Baseline 810 chromatography workstation, and analytes were identified by their retention times in comparison with authentic standards. Chromatographs from the workstation were exported as ASCII data files to the computer program GraFit (Leatherbarrow, 1990) for printing.

The stereochemical course of the enzymatic hydrolysis of βGlcNAcPNP was determined using 200-μL reactions that contained 2 mM βGlcNAcPNP, the appropriate buffer, and either 0.6, 1.2, or 9 μg of jack bean, bovine, or human NAGase, respectively. The anomeric stereochemistry of the hydration of NAglucal was determined using a 90-μL reaction that contained 60 μg of jack bean NAGase (freshly dialyzed to remove excess salt), 18 mM NAglucal, and the appropriate buffer (without BSA). The stereochemistry of the protonation of NAglucal was determined using 90-μL reactions that contained 5 mM NAglucal, the appropriate buffer, and ~20 μg of either of the NAGases under study.

The following procedures were used to determine kinetic parameters for NAglucal hydration catalyzed by jack bean NAGase. Seven 1.0-mL reaction buffer mixes containing citrate (5 mM, pH 5.0), BSA (0.01%), and one of seven different NAglucal concentrations were prepared. Reactions (at 25 °C) were started by injecting 2 μg of jack bean NAGase into each solution. Reactions were incubated at 25 °C for 8 min and then stopped by boiling for 30 s (the latter step irreversibly denatured the enzyme, but did not result in significant decomposition of other components of the reaction). Water was removed by lyophilization, and then the residue remaining in each reaction tube was resuspended with 0.100 mL of double-deionized water. Aliquots (0.080 mL) of each reaction were analyzed by HPLC using a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) with 13 mM H_2SO_4 as the eluant, which separated GlcNAc and ManNAc. A GlcNAc standard curve was prepared by following this procedure for the enzymatic reactions (but without the addition of enzyme) using eight different concentrations of GlcNAc standard. The concentration of the enzyme reaction product was determined by measuring the area of the GlcNAc peak, which was corrected by subtracting the area of the GlcNAc peak in appropriate controls (reactions with the same concentration of NAglucal, but without enzyme) and then comparing the corrected peak area with the standard curve.

RESULTS

βGlcNAcPNP Hydrolysis. Kinetic parameters for hydrolysis of the glycoside substrate, βGlcNAcPNP, were determined by direct spectrophotometric analysis, which involved monitoring the reaction at 360 nm. Even at the low pH values used for the assay there were sufficient differences in the molar extinction coefficients of substrate and product ($\Delta\epsilon_{360}$ = 2280 and 2150 M^{-1} cm^{-1} at pH 5.0 and 4.25, respectively) to permit direct monitoring of the reaction. The values of K_m and V_{max} determined for NAGases from human placenta, jack bean, and bovine kidney are presented in Table 1. The value of the K_m for jack bean NAGase determined using direct spectrophotometric monitoring agreed with that determined for jack bean NAGase (K_m = 0.64 mM) using a stopped assay (Li & Li, 1970).

The stereochemical course of the enzymatic hydrolysis of βGlcNAcPNP was determined by HPLC analysis using a DextroPak column. The ability of this method to separate the two anomers of GlcNAc (but not GlcNAc and ManNAc) is shown in Figure 1c, where the two anomers of GlcNAc in an equilibrated mixture are easily distinguishable. Assignment of the two peaks was achieved by loading a freshly prepared sample of αGlcNAc onto the column and measuring its retention time (3.55 min; data not shown). ^1H NMR analysis of a similar, freshly prepared sample of αGlcNAc

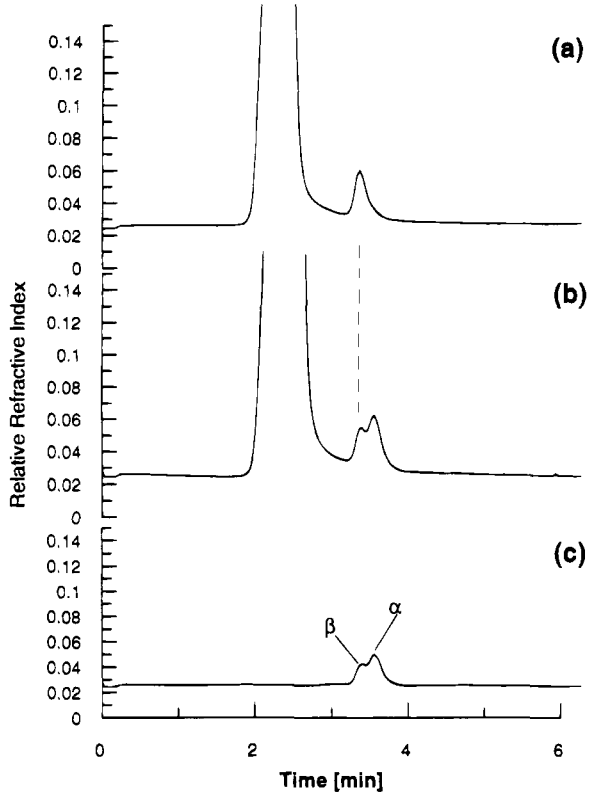


FIGURE 1: HPLC determination of the stereochemistry of βGlcNAcP-NP hydrolysis catalyzed by jack bean NAGase. Samples were from (c) an authentic GlcNAc standard (in water) or were aliquots from an enzymatic reaction (see Materials and Methods) analyzed after (a) 7 min or (b) 3 days.

confirmed the identity of the sample, while similar analysis of an equilibrated mixture confirmed that the α-anomer is indeed the major component. HPLC analysis of a sample of βGlcNAcPNP incubated for 7 min with jack bean NAGase revealed the initial production of *only* the β-anomer of GlcNAc (Figure 1a, retention time of 3.38 min). Repeating this analysis using a sample of the reaction mixture obtained after 3 days of incubation confirmed the reestablishment of equilibrium (Figure 1b). These results clearly indicate that jack bean NAGase catalyzes hydrolysis reactions with retention of the anomeric configuration of the sugar. Identical results were obtained with the other two NAGases studied (data not shown).

Inhibitor Studies Using NAglucal. Inhibition constants for NAglucal with the three enzymes were determined after first confirming that the compound did not cause irreversible inactivation. This was established by incubating the inhibitor with each enzyme and monitoring activity as a function of time. No time-dependent decreases in activity were observed. The inhibition constants (K_i values) determined for each enzyme are presented in Table 1. Data for jack bean NAGase are presented in the form of a double-reciprocal plot in Figure 2, demonstrating the competitive nature of the inhibition.

NAglucal Hydration. TLC analysis of samples of NAglucal incubated with jack bean NAGase indicated the formation of a product that comigrated with GlcNAc and ManNAc. Confirmation of this conversion and identification of the product were achieved by HPLC analysis of the reaction mixture using an Aminex HPX-87H column capable of separating the two epimers (Figure 3c). The sole product

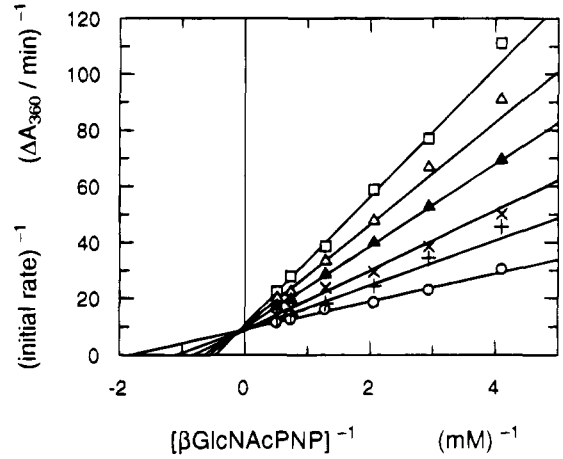


FIGURE 2: Double-reciprocal plot of the inhibition by NAglucal of βGlcNAcPNP hydrolysis catalyzed by jack bean NAGase. Reactions were performed as described in Materials and Methods. The concentrations of NAglucal in the reactions were (○) 0, (+) 8.2, (×) 16, (▲) 33, (△) 58, and (□) 82 μM.

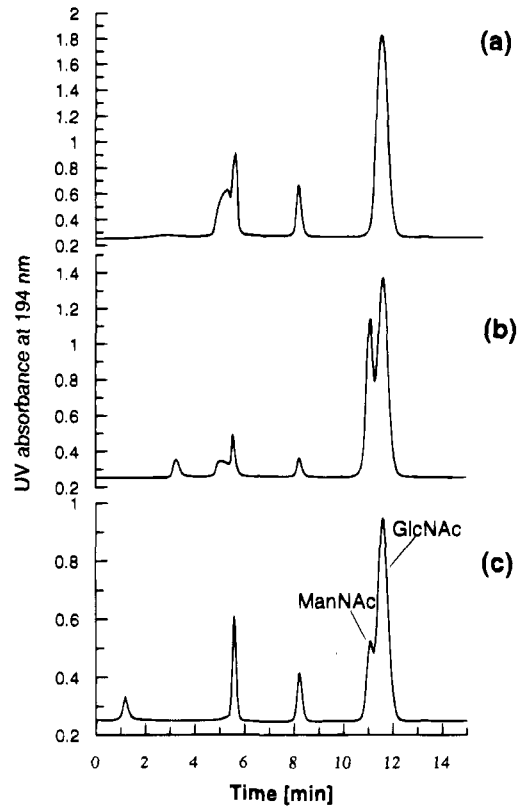


FIGURE 3: HPLC determination of the stereochemistry of NAglucal protonation catalyzed by jack bean NAGase. Samples were from (c) a mixture (in protein-free buffer) of authentic GlcNAc and ManNAc standards or (a and b) were aliquots from an enzymatic reaction (see Materials and Methods) analyzed after 2 days. Sample b was spiked with the ManNAc standard. Analysis of a control sample (without NAglucal; data not shown) showed that the enzyme and BSA eluted at ~5 min and that buffer components eluted at 5.7 and 8.3 min.

was clearly identified as GlcNAc by its elution at the same time (11.5 min) as an authentic sample (Figure 3a). Furthermore, the addition of ManNAc to this sample (Figure 3b) confirmed that ManNAc was *not* the product formed. Rates of hydration were determined using the same HPLC system. Since the sensitivity of detection was insufficient to permit direct analysis of reaction mixtures, given the low K_m value, it was necessary to concentrate reaction mixtures

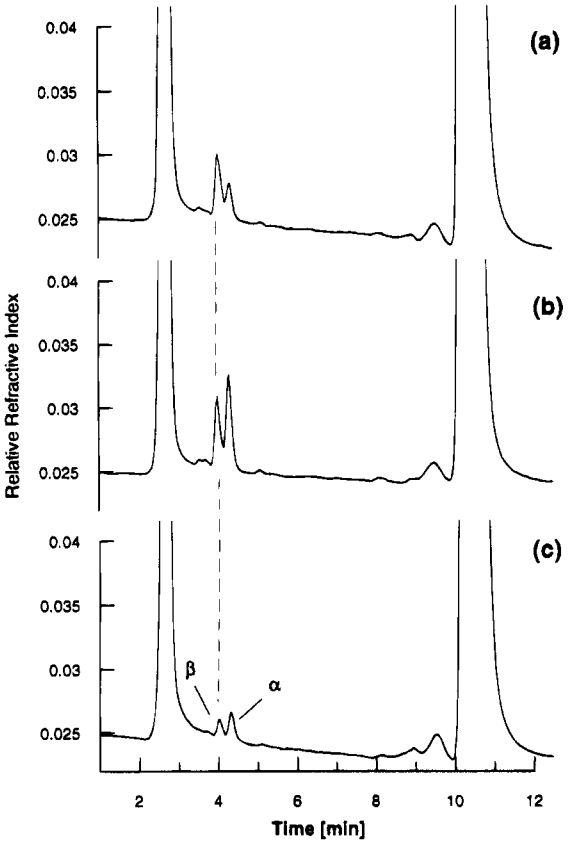


FIGURE 4: HPLC determination of the anomeric stereochemistry of NAglucal hydration catalyzed by jack bean NAGase. Samples were from (c) stock NAglucal (in protein-free buffer) or were aliquots from an enzymatic reaction (see Materials and Methods) that were (a) analyzed after 10 min or (b) removed after 40 min and then boiled for 30 s prior to analysis. The off-scale peak at ~10 min represents the elution of NAglucal.

10-fold prior to analysis. Integration of peaks allowed the measurement of released product as a function of time, and once controls had been performed to assess the background due to spontaneous hydrolysis under assay conditions, rates at each concentration were determined. The values of V_{max} and K_m were then determined by direct fit to the Michaelis–Menten equation. Values of V_{max} = 0.48 (±0.01) unit/mg and K_m = 27 (±2.8) μM were thereby determined for the hydration of NAglucal by jack bean NAGase. This K_m value agreed very well with the K_i value determined previously (Table 1).

The anomeric stereochemistry of the product of the hydration reaction (see Figure 4) was determined in a manner essentially identical to that used for monitoring βGlcNAcPNP hydrolysis. The NAglucal used in this experiment contained a small amount (0.4%) of contaminating GlcNAc, and separation of the equilibrium mixture of the two anomers is clearly seen in Figure 4c (the retention times for the anomers of GlcNAc differed slightly from those in Figure 1 because a new Dextropak column was used). Ten minutes after the addition of jack bean NAGase to a reaction mixture containing the appropriate buffer and NAglucal, an aliquot was analyzed by HPLC; the resulting chromatogram showed a large increase in the β-anomer peak, indicating the formation of βGlcNAc (Figure 4a). Confirmation of this result was obtained by removing another aliquot from the reaction mixture and then boiling this sample for 30 s prior to HPLC analysis; here the resulting chromatogram showed that anomeric equilibrium had been reestablished (Figure 4b).

The interaction of D-glucal with jack bean NAGase was also investigated to assess the importance of the *N*-acetyl substituent. A value of K_i = 38 mM was determined, and no conversion to 2-deoxy-D-glucose could be detected. A further indication of the importance of the C-2 substituent was our inability to detect NAGase-catalyzed hydrolysis of βGlcDNP, despite the superiority of the DNP leaving group compared with that of βGlcNAcPNP.

DISCUSSION

Tight Binding of NAglucal to NAGase. All three NAGases investigated have been shown to effect hydrolysis with net retention of the anomeric configuration. This therefore suggests that all three enzymes operate through a double-displacement mechanism involving a glycosyl–enzyme intermediate. This mechanism was originally proposed by Koshland (1953) and subsequently received considerable experimental support [reviewed in Sinnott (1990)]. The binding of NAglucal to each NAGase was shown to be high affinity, about 170-fold better than that of GlcNAc itself (K_i = 5 mM for jack bean NAGase; Li & Li, 1970), which is particularly noteworthy given the absence of the anomeric hydroxyl group on NAglucal.

There are two possible explanations for the tight binding observed. It could genuinely be a case of tight binding, i.e., due to some resemblance of NAglucal, with its planar geometry around C-1, to the structure of the oxocarbonium ion-like transition state. Alternatively, the tight binding could be a consequence of the accumulation of a 2-acetamido-2-deoxy-α-D-glucosyl–enzyme intermediate during the hydration reaction, comparable to that that occurs during the inhibition of *E. coli* β-galactosidase by D-galactal (Wentworth & Wolfenden, 1974), or of the inhibition of *Aspergillus wentii* β-glucosidase by D-glucal (Legler et al., 1979). The latter explanation is unlikely given the following observations. The deglycosylation step for the reaction of NAglucal must be identical to that for the hydrolysis of βGlcNAcPNP, since both involve the hydrolysis of a 2-acetamido-2-deoxy-α-D-glucosyl–enzyme intermediate. The V_{max} value for βGlcNAcPNP hydrolysis catalyzed by jack bean NAGase (50 μmol/min/mg) therefore represents a *minimum* estimate of the rate of the deglycosylation step. Since the V_{max} value for NAglucal hydration catalyzed by jack bean NAGase was only 0.48 μmol/min/mg, the second step cannot be rate-limiting in this case, and the value observed likely reflects the rate of formation of the intermediate.

The tight binding observed therefore is most likely a consequence of some critical structural features of the inhibitor resembling those of the transition state and the consequent exploitation of transition state-binding interactions. Such tight, reversible binding of a glycal to a glycosidase has never been observed previously, presumably because, in all cases examined, the key 2-hydroxyl substituent was necessarily missing. In fact, glycals typically bind 10–100-fold worse to glycosidases than do the corresponding glycosides (Legler, 1990). Comparable behavior was observed with D-glucal (which lacks the 2-acetamido substituent of NAglucal) and jack bean NAGase. The binding of D-glucal to jack bean NAGase was about 8-fold worse than that of GlcNAc (Li & Li, 1970) and 1300-fold worse than that of NAglucal.

The 1300-fold greater binding affinity of NAglucal compared with D-glucal establishes the contribution of the 2-acetamido functionality to the binding affinity of NAglucal as 4.2 kcal/mol and in turn establishes, at least to some extent, the contribution of this substituent to transition state stabilization in the normal reaction. NAglucal is, at best, an imperfect transition state analogue, given the absence of an anomeric oxygen atom and the imposed planarity around O-5, C-1, C-2, and C-3 rather than C-5, O-5, C-1, and C-2. The value of 4.2 kcal/mol therefore represents a *minimum* estimate of the contribution of the 2-acetamido group to transition state stabilization, as indeed is reflected in the complete absence of reaction of the enzyme with D-glucal or βGlcDNP, despite the superiority of the leaving group in the latter substrate.

Stereochemical Course of the Hydration of NAglucal. The stereochemistry of NAGase-catalyzed proton donation to the C-2 of NAglucal is without precedent in previous studies of retaining β-glycosidases. Previously, these enzymes have only been shown to effect proton donation to glycals from the bottom face, most likely through the concerted process shown in Scheme 1a (Legler, 1990). In the absence of other constraints, this more commonly observed stereochemistry presumably is a consequence of the known preference for the reaction of acetals via oxocarbonium ion-like transition states to occur in a preassociative process (Banait & Jencks, 1991). Such preassociation is most readily achieved in the concerted process shown. Further, with unsubstituted glycals, the intermediate formed involves a 2-deoxy sugar; thus, the correct stereochemical orientation of a bulky substituent at C-2 is not a consideration. However, if proton donation came from the bottom face during the catalytic hydration of NAglucal, it would result in the formation of an *N*-acetylmannosaminyl−enzyme intermediate in which the bulky C-2 substituent assumes the enzymatically unfavorable axial position. By contrast, the protonation of NAglucal at C-2 from the top face generates an *N*-acetylglucosaminyl−enzyme intermediate in which normal transition state-binding interactions with the equatorial 2-acetamido group can be exploited to assist the formation and subsequent hydrolysis of this intermediate (Scheme 1b). An alternative (but less likely) explanation is that the reaction proceeds through an oxazolidine intermediate[2] with the participation of the 2-acetamido group, as had at one time been proposed for lysozyme (Lowe et al., 1967). If this were the case, the preferred *trans*-diaxial addition to the double bond would require axial protonation at C-2. NAGase-catalyzed protonation of NAglucal from the top face, as observed, therefore is quite reasonable and reflects both the constraints and benefits of having the correct substituent at C-2. Finally, the formation of the β-anomer of the product is consistent with all previous observations and with the view that it is the product's configuration (rather than the substrate's) that is the conserved feature of catalysis by glycosylases (Weiser et al., 1992).

CONCLUSIONS

Our results provide the first determination of the stereochemical outcome of hydrolysis catalyzed by the NAGases under study. Indeed only one other NAGase (from boar epididymis) has previously been used for such an analysis (Ya Khorlin et al., 1972). More importantly, these results provide the first examples of the protonation of a glycal from the correct face by a retaining β-glycosidase. As such, they demonstrate firstly that the incorrect protonation observed in other cases is the consequence of an alternative pathway adopted in the absence of the 2-substituent, and secondly that protonation of glycals from the correct face is indeed possible. These results also suggest that binding interactions with the 2-acetamido substituent may assist in the recruitment of acid catalysis and the maintenance of the correct protonation state of the nucleophilic carboxylate.

The relatively tight, reversible binding of NAglucal suggests that glycals may indeed be regarded as transition state analogues for glycosidases, but that the relatively low affinities generally observed are a consequence of their lack of the critical 2-substituent. Indeed, these results suggest some interesting possibilities for the design of NAGase inhibitors of higher specificity and affinity, in which an oxygen atom is present at the anomeric center, possibly attached to another sugar moiety.

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