Depolymerization and De-N-acetylation of Chitin Oligomers in Hydrochloric Acid

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The monosaccharide 2-amino-2-deoxy-D-glucose (glucosamine, GlcN) has recently drawn much attention in relation to its use to treat or prevent osteoarthritis in humans. Glucosamine is prepared from chitin, a process that is performed in concentrated acid, such as hydrochloric acid. This process involves two acid-catalyzed processes, that is, the hydrolysis of the glycosidic linkages (depolymerization) and of the *N*-acetyl linkages (de-N-acetylation). The depolymerization reaction has previously been found to be much faster compared to the deacetylation, with the consequence that the chitin chain will first be hydrolyzed to the monomer 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine, GlcNAc) which is subsequently deacetylated. We have found that the chitin disaccharide GlcNAc(1→4)GlcNAc could be completely hydrolyzed to the monosaccharide GlcNAc with negligible concomitant de-N-acetylation, and the chitin disaccharide and monosaccharide were further used to study the depolymerization reaction and the de-N-acetylation reaction, respectively. The reactions were performed in hydrochloric acid as a function of acid concentration (3−12 M) and temperature (20−35 °C), and ^{1}H− NMR spectroscopy was used to monitor the reaction rates. The ^{1}H NMR spectrum of GlcNAc in concentrated (12 M) and deuterated hydrochloric acid at 25 °C was assigned. The glucofuranosyl oxazolinium (**3**) ion was found to exist in equilibrium with the α- and β-anomers of the pyranose form of GlcNAc, where **3** was present in half the total molar concentrations of the two anomeric forms of GlcNAc. At lower acid concentration (3−6 M), only trace concentrations of **3** could be detected. The rate of de-N-acetylation of GlcNAc was determined as a function of hydrochloric acid concentration, showing a maximum at 6 M and decreasing by a factor of 2 upon decreasing or increasing the acid concentration to 3 or 12 M. The activation energy for hydrolysis of the *N*-acetyl linkage of GlcNAc was determined to be 102 ± 7, 116 ± 8, and 110 ± 8 kJ mol^{-1} at 3, 6, and 12 M hydrochloric acid concentration, respectively. The results are in accordance with the proposed S_N2 reaction mechanism of the acid-catalyzed hydrolysis of the *N*-acetyl linkage where the rate-limiting step is the addition of water to the carbonium ion. The ^{1}H NMR spectrum of the dimer GlcNAc−GlcNAc in concentrated (12 M) and deuterated hydrochloric acid at 25 °C was assigned. The rate of the acid-catalyzed cleavage of the glycosidic linkage of the dimer was determined as a function of hydrochloric acid concentration, showing a 6-fold increase from 3 to 6 M HCl concentration and a further 6-fold increase from 6 to 12 M HCl concentration, in contrast to the much smaller effect of acid concentration on the deacetylation reaction. Activation energy for hydrolysis of the glycosidic linkage of GlcNAc−GlcNAc was determined to be 110 ± 6, 111 ± 6, and 112 ± 4 kJ mol^{-1} at 3, 6 and 12 M hydrochloric acid concentration, respectively, that is, very similar to the activation energies determined for the deacetylation reaction. The results are in accordance with the proposed S_N1 reaction mechanism of the acid-catalyzed hydrolysis of the glycosidic linkage, where the rate-limiting step is the formation of the carbonium ion.

1. Introduction

Chitin is one of the most abundant organic materials on earth and is widely distributed in the exoskeleton of all animals with an outer skeleton, as well as in the cell walls of microorganisms such as fungi, yeast, and algae.[1] Chitin is mainly used as a raw material for production of chitosan and the de-N-acetylated monomer, glucosamine. A growing commercial market for glucosamine is the use of this monomer for treatment of osteoarthritis.[2,3] Glucosamine is produced by hydrolysis of chitin in concentrated acids such as hydrochloric acid. Falk et al.[4] found that the hydrolysis of chitin in concentrated acid occurred in three distinct steps: (1) degradation of the polysaccharide to smaller polymeric units, (2) production of *N*-acetylglucosamine from the latter, and (3) conversion of *N*-acetylglucosamine into glucosamine and acetic acid. These results were in agreement with those of Rupley.[5]

In our more recent publication,[6] the rate of hydrolysis of different chitosans with fractions of acetylated units (F_A) of 0.3−0.6 was investigated in both dilute and concentrated hydrochloric acid.[6] While the rate of de-N-acetylation was found to be less than $^1/_{10}$ the rate of depolymerization in concentrated acid, the two rates were found to be equal in dilute acid. It was suggested that this could be explained by different reaction mechanisms and rate-limiting steps in the reactions, that is, that the hydrolysis of the *N*-acetyl linkage is an S_N2 reaction (rate-limiting step: addition of water to the carbonium ion) while the hydrolysis of the glycosidic linkages is an S_N1 reaction where the rate-limiting step is the formation of the carbonium ion. Rupley[5] followed the hydrolysis of the chitin chain and the de-N-acetylation reaction by colorimetric procedures at different temperatures and acid concentrations. However, the interpretation of the data was complicated, as it was suggested that the rate of hydrolysis was governed by the structure of chitin, and no data on the activation energy of the de-N-acetylation reaction was reported.

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We here report studies of the depolymerization and de-N-acetylation reaction using the chitin dimer (GlcNAc−GlcNAc) and the monomer GlcNAc as model substances, respectively. The rates of the reactions were determined as a function of acid concentration and temperature. We find that while the depolymerization reaction increases strongly with increasing acid concentration, the deacetylation reaction is only moderately influenced by the acid concentration, while the activation energies of the two reactions were similar.

2. Experimental Section

2.1. NMR of Oligomers in Concentrated DCl. *N*-Acetylglucosamine and the chitin dimer (GlcNAc−GlcNAc) were purchased from Sigma and Seikagaku, respectively. The samples were dissolved at 0 °C in concentrated DCl (37 wt % from Sigma) at a concentration of 15 mg/mL. Proton solution-state NMR spectra were measured on a Bruker Avance DPX spectrometer at 300 or 400 MHz. Chemical shifts were measured relatively to sodium 3-(trimethylsilyl)propionate-d_4 (TSP) from Merck as an internal standard at 0.00 ppm. Due to the high ionic strength of the solvent, matching and tuning of the probe was a nontrivial task for samples in concentrated and deuterated hydrochloric acid. Because of the effect of radiation dampening, the rf pulses used had to be significantly longer than the pulses used with water as a solvent. To ensure that the relative areas of the observed resonances represent the relative amounts of the protons involved, that is, quantitative uptake parameters, the relaxation times (T_1) of the protons were determined by the inversion recovery method. All protons of the sugar rings showed relaxation times of about 1 s, while the protons from acetate had a relaxation time of about 3 s.

2.2. Determination of Rate Constant of De-N-acetylation of GlcNAc. The de-N-acetylation reaction of the monomer was monitored from the ratio of acetate protons to the total amount of protons from both acetyl and acetate. The fraction of acetylated units (F_A) as a function of time gave linear plots for all temperatures and acid concentrations since the change in F_A with time was relatively small and initial reaction velocities were determined. Reaction rate constants (k_{acetyl}) for the de-N-acetylation reaction were determined from the absolute values of the slopes of the linear trendlines of F_A as a function of time.

2.3. Determination of Rate Constant of Hydrolysis of the Glycosidic Bond in the Dimer (GlcNAc−GlcNAc). In order to be able to compare the rate constant of hydrolysis of the glycosidic bond in the dimer, k_{glyc}, with k_{acetyl}, reaction rates were determined on the basis of cleaved glycosidic linkages in the dimer sample and cleaved *N*-acetyl linkages in the monomer sample, respectively. The fraction of remaining glycosidic bonds in the dimer sample is in this context comparable to the F_A of the monomer sample. Since the hydrolysis of the glycosidic bond is much faster compared to the hydrolysis of the *N*-acetyl linkage in concentrated acid, resulting in reaction velocities deviating from the initial reaction velocities, the fraction of remaining glycosidic bonds as a function time did not give linear plots. Linear plots were obtained on a semilogarithmic scale, from which reaction rate constants of the hydrolysis (k_{glyc}) can be determined accurately as the slope of the trendline.

2.4. Determination of Activation Energies. Activation energies were calculated from the slopes of the trendlines in the Arrhenius plot, in which the reaction rate constants (k) (logarithmic scale) are plotted as a function of the inverse of the absolute temperature.

3. Results and Discussion

3.1. ^{1}H− NMR Spectra of Chitin Monomer and Dimer in Concentrated DCl. ^{1}H− NMR spectra of the dimer GlcNAc−GlcNAc and monomer GlcNAc in concentrated and deuterated hydrochloric acid at 25 °C are shown in Figure 1 A,B. In Figure 1C is shown the spectrum of the dimer that was incubated in concentrated DCl at 25 °C for 56 h, showing a spectrum essentially identical to the spectrum of the monomer in Figure 1B with negligible amounts of any de-N-acetylated units. This shows that all the glycosidic linkage of the dimer GlcNAc−GlcNAc can be hydrolyzed without almost any de-N-acetylation in concentrated DCl, in agreement with previous results on chitin hydrolysis.[4,5]

One advantage of using concentrated and deuterated hydrochloric acid as solvent is that the resonances of the solvent (HDO) do not interfere with any of the carbohydrate protons, as the solvent protons resonate at 9.2 ppm (probably a weighted average of the signals from water and acid protons due to the fast exchange between the two). The spectra show the characteristic resonances in the anomeric region of the α-anomer (5.43 ppm), the β-anomer (5.05 ppm), and the non-reducing-end anomer at 4.91 ppm. Resonances from acetyl protons are found at 2.62 ppm while the remaining ring protons appear between 3.6 and 4.4 ppm. The assignments of the resonances are given in Figure 1.

The relatively high intensity of protons from acetic acid (2.24 ppm) in the spectrum of the dimer originates from the presence of acetate in the dimer sample. The spectrum of the monomer GlcNAc contains additional resonances denoted X-1, X-2, X-3, Y-1, and Y-2. The different relative intensities of the resonances indicate they originate from two different compounds (X and Y).

Vincendon[7] observed a glucofuranosyl oxazolinium ion in ^{13}C− NMR spectra of GlcNAc in phosphoric acid. The resonances identified in the ^{13}C− NMR spectra of GlcNAc in concentrated DCl (spectra not shown) resembles the ^{13}C− NMR spectra of GlcNAc in phosphoric acid.[7] Bosso et al.[8] described the presence of a glucofuranosyl oxazolinium ion in samples of chitin in anhydrous hydrofluoric acid. These authors also detected a resonance in the ^{13}C− NMR spectra at 109.5 ppm that was assigned to a glucopyranosyl oxazolinium ion, which was not observed in our ^{13}C− NMR spectra of GlcNAc in concentrated and deuterated hydrochloric acid. We suggest that compound X is the glucofuranosyl oxazolinium ion existing in equilibrium with GlcNAc in concentrated HCl. The resonances X-1, X-2, and X-3 can be assigned to protons 1, 2, and 3 of the glucofuranosyl oxazolinium ion by correlation NMR [^{1}H−^{1}H correlation (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear single quantum coherence (HSQC) spectroscopy] and distortionless enhancement by polarization transfer (DEPT) 135 of samples of GlcNAc in concentrated DCl (data not shown). Proton 1 of the glucofuranosyl oxazolinium ion has a relatively high chemical shift at 7.03 ppm, which can be explained by deshielding of the proton by the presence of two electronegative oxygens on each side of carbon 1. Compound Y is present in lower concentrations than compound X, and we speculate that these resonances may be from the protons of the open ring form of the acetylated reducing-end residue of the dimer or of the monomer.

The resonances from compounds X and Y are not present in spectra of de-N-acetylated monomer (GlcN) in the same solvent (spectra not shown). The assumption that compound X is the glucofuranosyl oxazolinium ion is also consistent with the absence of compound X in the spectra of the dimer GlcNAc−GlcNAc, since the conversion of pyranose to furanose requires that carbon 4 of the reducing-end residue is not glycosidically bound.

Scheme 1 shows the 2-acetamido-2-deoxy-D-glucopyranose (**1**) that via the open ring form will exist in equilibrium with

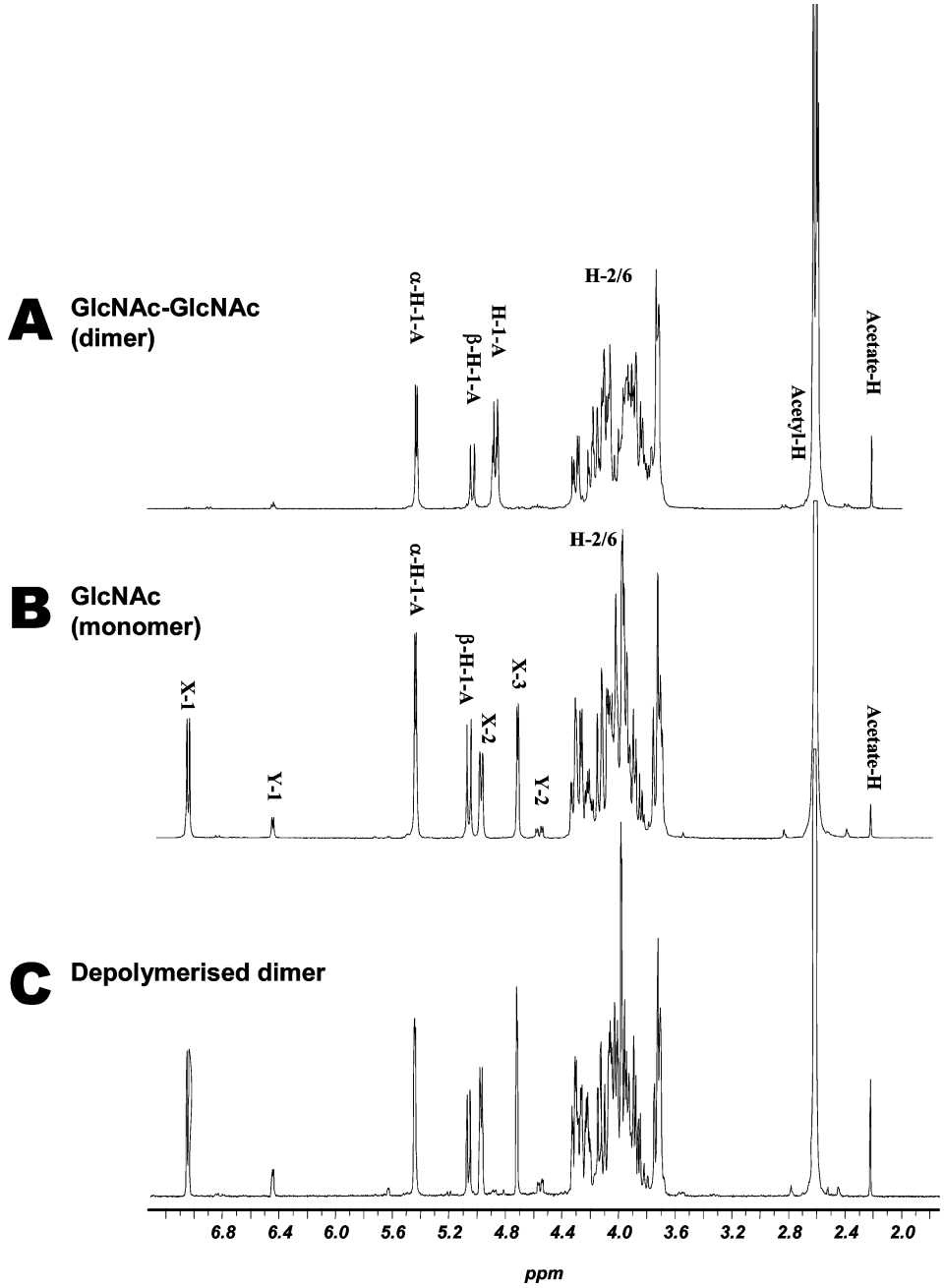
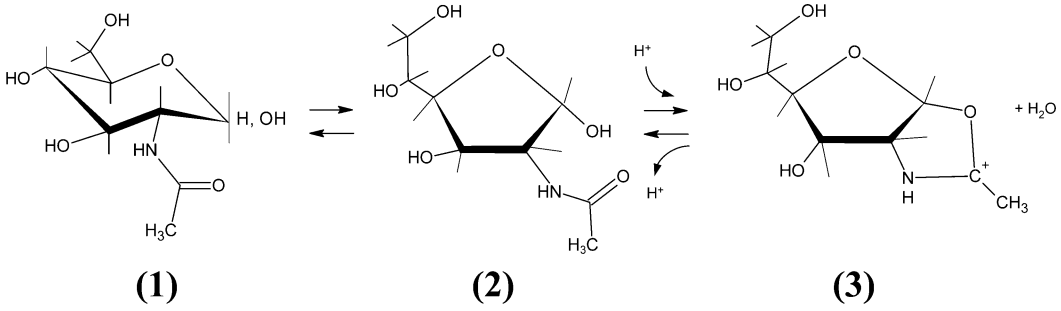


Figure 1. ^{1}H− NMR spectra (400 MHz) of solutions of GlcNAc−GlcNAc (A) and GlcNAc (B) in concentrated DCl at 25 °C. The spectrum of the dimer that was incubated in concentrated DCl at 25 °C for 56 h is shown in panel C. Chemical shifts are given relative to TSP at 0.00 ppm.

Scheme 1. Proposed Reaction for Formation of the Glucofuranosyl Oxazolinium Ion (**3**) in Concentrated HCl



(1) **(2)** **(3)**

the 2-acetamido-2-deoxy-D-glucofuranose (**2**), which can react to form the glucofuranosyl oxazolinium (**3**) ion in concentrated DCl.

To investigate the formation of the glucofuranosyl oxazolinium ion in samples of GlcNAc in concentrated HCl, ^{1}H NMR spectra of GlcNAc were obtained as a function of time after

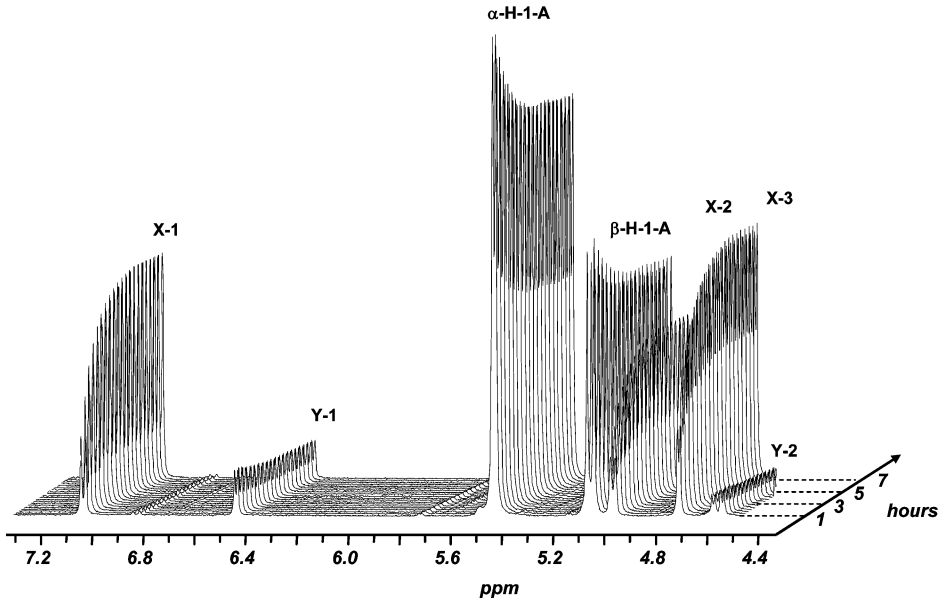


Figure 2. Anomer region of ^{1}H− NMR spectra of GlcNAc in concentrated DCl at 25 °C as a function of time (TSP as reference at 0.00 ppm).

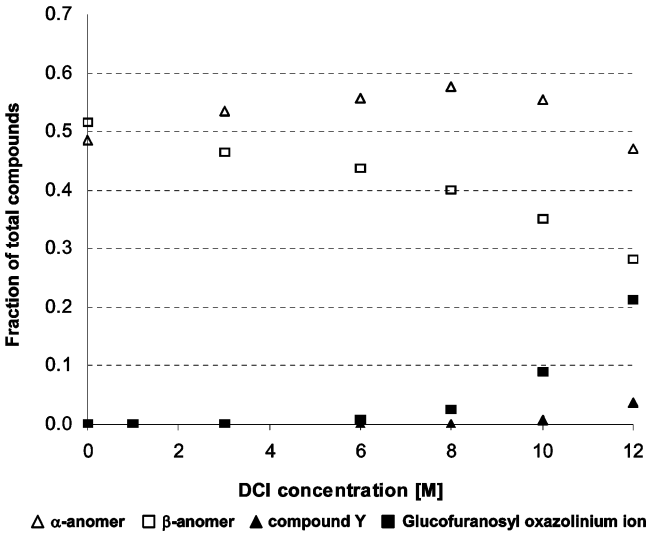


Figure 3. Relative concentrations of α- and *β*-anomers of GlcNAc, glucofuranosyl oxazolinium ion (compound X), and compound Y as a function of acid concentration.

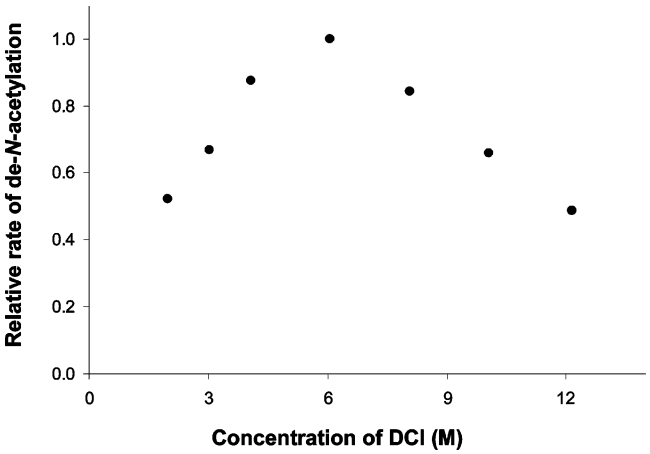


Figure 4. Relative rate of de-N-acetylation of monomer GlcNAc as a function of DCl concentration at 25 °C.

the sample was dissolved in acid, and Figure 2 shows the anomer region of ^{1}H NMR spectra of GlcNAc in concentrated DCl as a function of time.

The spectra shown in Figure 2 clearly show how the resonances of α- and *β*-anomers of GlcNAc decrease with time, while the concentration of the glucofuranosyl oxazolinium ion (compound X) increases. The results indicate it takes approximately 4 h to establish equilibrium between the glucofuranosyl oxazolinium ion and GlcNAc at 25 °C. When this equilibrium is established, the concentration of the oxazolinium ion slowly decreases toward zero due to deacetylation of the sample. It was also noted that the ^{1}H NMR spectra of GlcNAc in 12 M DCl showed an increase in the amount of the oxazolinium ion with increasing temperature of the sample: at 25 °C, 20% of the monomers are present as oxazolinium ion as compared to 30% at 35 °C (at equilibrium).

^{1}H NMR spectra of samples of GlcNAc at varying concentration of DCl were obtained, and a plot of the relative concentrations of the GlcNAc anomers and the glucofuranosyl oxazolinium ion as a function of acid concentration is given in Figure 3.

The relative concentrations of compound Y and the glucofuranosyl oxazolinium ion (compound X) decrease with decreasing concentration of DCl and are below the detection limit when the DCl concentration is less than 6 M.

When a sample of GlcNAc in 12 M DCl was diluted to 5 M DCl (with D_2O) 4 h after dissolving the sample, the ^{1}H NMR spectrum of the sample in 5 M DCl showed that resonances from compound Y immediately diminished below the detection limit, while resonances from the glucofuranosyl oxazolinium ion gradually decreased and were below the detection limit after 3 h. These results clearly show that the equilibrium shown in Scheme 1 can be reversed.

3.2. Rate of De-N-acetylation of GlcNAc as a Function of Acid Concentration. The effect of acid concentration on the rate of the de-N-acetylation of the monomer GlcNAc was first determined in relation to the previously reported formation of compound X and Y (see 3.1). The results are shown in Figure 4.

The rate of de-N-acetylation was found to increase only moderately with increasing acid concentration from 2 to ∼6 M. A further increase in the acid concentration led to a decrease in the rate of de-N-acetylation. The formation of glucofuranosyl oxazolinium ion at DCl concentrations above 6 M (Figure 3) will contribute to a decrease in the concentration of GlcNAc,

Table 1. Rate Constants[a] as a Function of Temperature and Acid Concentration

	25 °C		30 °C		35 °C	
HCl concn (M)	k_{acetyl}	k_{glyc}	k_{acetyl}	k_{glyc}	k_{acetyl}	k_{glyc}
3	26	57	53	127	109	250
6	39	392	85	819	177	1670
12	19	2220	36	4980	71	9430

[a] k_{acetyl}, rate constant for de-N-acetylation of GlcNAc; k_{glyc}, rate constant for hydrolysis of the glycosidic linkage in the dimer GlcNAc−GlcNAc. All rate constants are given $\times 10^8$ s.

and the decrease in the rate of de-N-acetylation at higher acid concentrations suggests that the glucofuranosyl oxazolinium ion is not important with respect to the deacetylation reaction. Thus, in a process of preparing glucosamine from chitin, where the deacetylation is the rate-limiting step, the use of HCl concentrations above 6 M will result in a less efficient formation of glucosamine.

3.3. Rate of De-N-acetylation and Depolymerization as a Function of Temperature and Acid Concentration. The rate of de-N-acetylation of GlcNAc was determined as a function of temperature at three different acid concentrations (3, 6, and 12 M) and the results are given in Table 1, where k_{acetyl} is the rate constant for the de-N-acetylation reaction. The rate of hydrolysis of the glycosidic linkage of the dimer GlcNAc−GlcNAc was determined as a function of temperature at three different acid concentrations (3, 6, and 12 M) and the results are given in Table 1, where k_{glyc} is the rate constant for cleavage of the glycosidic linkage.

The reaction rate constant of de-N-acetylation of GlcNAc (k_{acetyl}) is about 4 times higher at 35 °C than at 25 °C at all three acid concentrations, and k_{acetyl} is found to vary similarly as shown in Figure 4 at all three temperatures. Reaction rate constants of hydrolysis of the glycosidic linkage show a 6-fold increase from 3 to 6 M acid concentration and a further 6-fold increase from 6 to 12 M HCl concentration, in contrast to the much smaller effect of the acid concentration on k_{acetyl}. This different dependence on the reaction rates of the two reactions with respect to the acid concentration is in accordance with the previously proposed reaction mechanisms,[6] where the hydrolysis of the *N*-acetyl linkage was proposed to be a S_N2 reaction while the hydrolysis of the glycosidic linkage was a S_N1 reaction. However, the previous interpretation regarding the rate-limiting step in relation to the water concentration[6] seems misinterpreted, as our present results indicate that the important parameter is the proton concentration.

It has been found that most acid-catalyzed reactions proceed more readily in D_2O than in H_2O.[9] Since deuterium oxide has a smaller autoprotolysis constant than water, it is believed to be less basic than water,[9] and since the concentration of the conjugate acid of the substrate will then be higher in D_2O, the rate of the reaction should also be higher in D_2O than in H_2O. Most of the increase in reaction rate due to the use of D_2O as compared to H_2O as solvent is 1 to 3 times.[9] Absolute values of rate constants obtained in D_2O cannot be directly compared to values obtained in H_2O.

3.4. Activation Energies. The reaction rate constants (Table 1) were plotted as a function of the inverse of the absolute temperature (Arrhenius plot) in Figure 5.

The Arrhenius plots are linear, indicating that the reaction mechanism is the same within the temperature interval tested. Note the relatively small differences between k_{acetyl} as compared to k_{glyc} at the three acid concentrations, as discussed in section 3.3. Table 2 shows the activation energies for de-N-acetylation

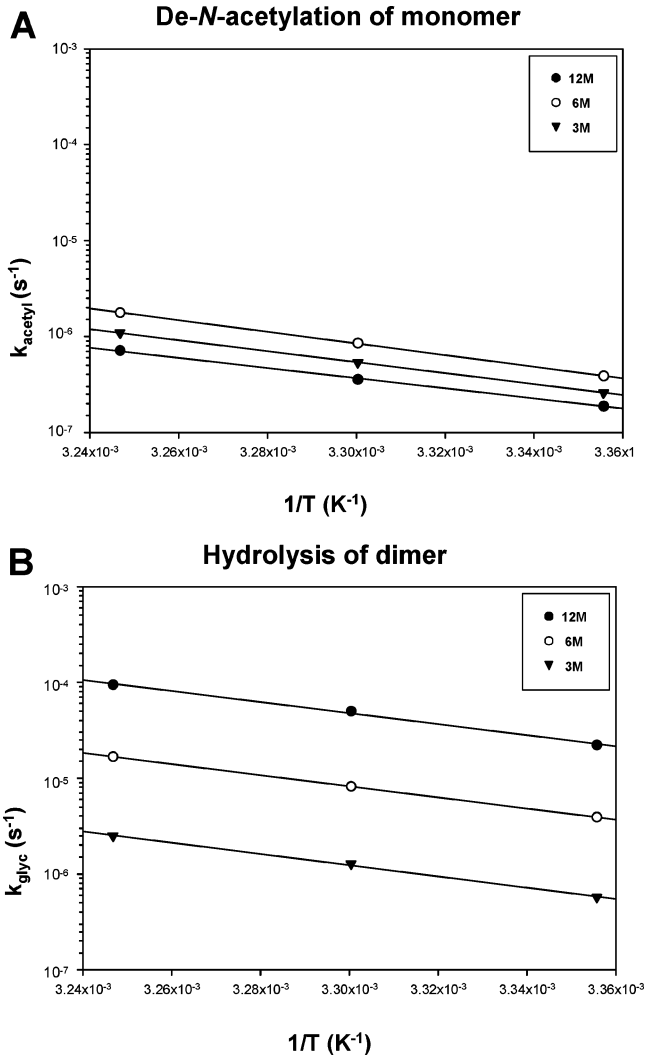


Figure 5. Rate constants of de-N-acetylation of GlcNAc (A) and rate constants of hydrolysis of the glycosidic linkage in the dimer (GlcNAc−GlcNAc) (B) (logarithmic scale) as a function of the inverse of absolute temperature at three different concentrations of hydrochloric acid (Arrhenius plot).

Table 2. Activation Energies[a] at Different Acid Concentrations

activation energy (kJ/mol)	3 M HCl	6 M HCl	12 M HCl
E_{acetyl}	102 ± 7	116 ± 8	110 ± 8
E_{glyc}	110 ± 6	111 ± 6	112 ± 4

[a] E_{acetyl}, activation energy for de-N-acetylation; E_{glyc}, activation energy for hydrolysis of the glycosidic linkage.

of GlcNAc (E_{acetyl}) and for hydrolysis of the glycosidic linkage in GlcNAc−GlcNAc (E_{glyc}) as determined from the slopes of the trendlines in Figure 5.

The values of the activation energies are the same within experimental error for both reactions and at all three acid concentrations. These activation energies are somewhat higher compared to the activation energies determined for the de-N-acetylation of chitin in alkali of 92 kJ/mol.[10] These authors did not report any activation energies of the deacetylation of the monomer GlcNAc in alkali, due to the formation of byproducts.

The activation energies are similar to that previously reported for acid hydrolysis of the model compound methyl-2-acetamido-2-deoxy-β-D-glucopyranose (in 2.5 M HCl) of 118.4 kJ/mol.[11] However, the reported activation energy of 94.1 kJ/mol for the hydrolysis of chitin in 11 M HCl[5] is somewhat lower as compared to the activation energies in Table 2. Also, the

activation energies for the hydrolysis of the A−A and A−D glycosidic linkage in chitosans (in dilute acid) of around 130 kJ/mol[6] are higher as compared to the values determined herein, which may be explained by the difference in acid concentrations.

Conclusion

By use of the model compounds GlcNAc and GlcNAc−GlcNAc, the rate of de-N-acetylation (k_{acetyl}) and depolymerization (k_{glyc}) have been determined as a function of acid concentration (3−12 M) and temperature (25−35 °C). It was found that k_{acetyl} and k_{glyc} were similar at the lowest acid concentration (3 M), while k_{glyc} was much higher than k_{acetyl} at the highest acid concentration (12 M). The activation energies of the de-N-acetylation and depolymerization reactions were, however, similar at all acid concentrations. Thus, the acid concentration but not the temperature can be used to control k_{glyc} relative to k_{acetyl} when the monosaccharides GlcNAc and GlcN are chemically prepared from chitin in hydrochloric acid.

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