Characterization of Ligand Binding to *N*-Acetylglucosamine Kinase Studied by STD NMR[†]

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ABSTRACT: Saturation transfer difference (STD) NMR experiments on human *N*-acetylglucosamine kinase (GlcNAc kinase) have been used to determine binding epitopes for the GlcNAc and ATP substrates and their analogues. The study reveals that during the enzyme reaction the binding mode of both substrates is conserved, although the binding affinity of the sugar is reduced. This suggests that the protein does not undergo any significant structural changes during catalysis. Our experiments also demonstrate that GlcNAc kinase has residual activity in the absence of Mg^{2+}. Furthermore, our experiments clearly show that the GlcNAc kinase predominately, if not exclusively, produces the β anomer of phosphorylated sugars. To identify the minimum requirements for substrate binding, a detailed analysis of different natural occurring as well as synthetic sugars was employed. Modifications at the 1, 2, 3, 4 and 6 position as well as the *N*-acetyl group greatly reduce the binding affinity. In addition, the binding mode of these substrate analogues is often also changed. The high β anomeric preference of GlcNAc kinase along with the drastically reduced binding affinity for sugars other than GlcNAc, suggests that GlcNAc kinase phosphorylates β-GlcNAc in cells.

N-Acetylglucosamine (GlcNAc)[1] is a major component of complex carbohydrates. It is found in *N*- and *O*-glycans (*1, 2*) as well as in glycolipids (*3*). Glycosaminoglycans (*4*) and the glycosyl phosphatidylinositol anchor of membrane bound glycoproteins (*5*) also contain GlcNAc moieties. GlcNAc is derived from the degradation of glycoconjugates by glycosidases and from nutritional sources. GlcNAc is then converted into GlcNAc-6-phosphate by the *N*-acetylglucosamine kinase (GlcNAc kinase; EC 2.7.1.59). GlcNAc-6-phosphate can enter a catabolic pathway that ultimately leads to the formation of fructose-6-phosphate (*6*), or it can enter an anabolic pathway leading to the formation of UDP-GlcNAc (*7*). UDP-GlcNAc is a substrate for different GlcNAc transferases (*2*) or is further metabolized to *N*-acetylneuraminic acid (*7*). UDP-GlcNAc is also substrate for *O*-GlcNAc transferase, which modifies cytosolic and nuclear proteins at serine or threonine residues by addition of a single GlcNAc. These *O*-GlcNAc modifications are associated with regulatory functions ranging from transcription, translation, cell signaling and stress response to carbohydrate metabolism (*8*).

GlcNAc kinase was first characterized in the 1970s (*9, 10*) and has been isolated from several mammalian species (*9*−*11*). Purification of rat liver GlcNAc kinase to homogeneity revealed that it is a homodimeric enzyme of 39 kDa subunits (*12*). Identification of the amino acid sequence of GlcNAc kinase from different organisms demonstrated that GlcNAc kinase is a member of the sugar kinase/heat shock protein 70/actin superfamily, which is characterized by a common ATPase domain (*13*). This was confirmed by the recently published crystal structure of human GlcNAc kinase (*14*). But so far no experimental data concerning the structure or mechanistic aspects of the enzymatic reaction of GlcNAc kinase are available in aqueous solution at atomic resolution. Furthermore, GlcNAc kinase's function as a "rescue enzyme" for other kinases is discussed in literature (*15*).

NMR spectroscopy offers a variety of techniques to identify and characterize the binding of different ligands to enzymes in solution (*16*), including saturation transfer difference (STD) NMR experiments for the analysis of binding epitopes at atomic resolution (*17*). In this study we characterize the interactions of a number of different ligands with GlcNAc kinase in aqueous solution by employing STD NMR. In addition to the natural substrates GlcNAc and ATP, several natural and synthetic substrate analogues were investigated for their binding epitopes. Competitive STD NMR titration experiments rank the ligands according to their relative binding affinity yielding detailed structure-binding relations. To complement the data, the conversion rates of the ligands by GlcNAc kinase were also studied. This study not only provides a key for the design of inhibitors or compounds for metabolic engineering but also furthers our

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[1] Abbreviations: GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; GlcNBut, *N*-butylglucosamine; ManNAc, *N*-acetylmannosamine; STD, saturation transfer difference.

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understanding of mechanistic aspects of the GlcNAc kinase reaction and the biological role of this enzyme.

MATERIALS AND METHODS

Materials. All ligands were obtained from Sigma (Germany). Methyl-α-D-GlcNAc, methyl-β-D-GlcNAc and *N*-butylglucosamine were synthesized following published procedures (*18, 19*). 3-OMe-GlcNAc and 3,4-OMe-GlcNAc were generous gifts from Prof. A. Giannis (University of Leipzig, Germany) and were synthesized as described (*20, 21*). The c-myc Thr58-*O*-GlcNAc peptide (KKFELLPT(-*O*-GlcNAc)PPLSPSRR) was kindly provided by Prof. G. W. Hart (Johns Hopkins University, Baltimore, MD).

Expression and Purification of GlcNAc Kinase. Human GlcNAc kinase was recombinantly expressed as a GST fusion protein in *Escherichia coli* cells and purified as described (*14*). Enzyme activity was monitored with a colorimetric GlcNAc kinase assay and purity was controlled by SDS−PAGE analysis. Typically, 10 mg of homogeneous protein was obtained from 1 L of *E. coli* culture.

As the planned STD experiments require deuterated buffers, the purified GlcNAc kinase was dialyzed for 3 h against D_2O at room temperature using a 10 kDa MWCO membrane. The dialyzed protein was then applied to a PD-10 column (Amersham) and eluted using 5 mL of 50 mM sodium phosphate, pH* 7.5 (uncorrected pH reading for the presence of $^2H^+$) containing 100 mM sodium chloride in D_2O. Fractions of 5 drops were collected and protein concentration was determined using the Bradford assay. When nucleotides were measured in the absence and presence of Mg^{2+}, a buffer containing 50 mM d_{11}-Tris-HCl instead of 50 mM sodium phosphate was used for purification on the PD-10 column to prevent the formation of insoluble $MgHPO_4$.

NMR Experiments. STD NMR spectra were obtained at 5 °C using a Bruker DRX 500 spectrometer equipped with a triple resonance probehead, incorporating gradients in the *z*-axis. Samples contained 15 μM protein and a ligand concentration of 200 μM nucleotide and 1 mM or 5 mM sugar, respectively. For the acquisition of STD NMR spectra a 1D pulse sequence incorporating a $T_{1\rho}$ filter was used. On-resonance irradiation was performed at −1 ppm and off resonance at 20 ppm. Irradiation was performed using 50 Gaussian pulses with a 1% truncation, 49 ms duration, and separated by a delay of 1 ms to give a total saturation time of 2 s. The duration of the $T_{1\rho}$ filter was 15 ms. STD NMR spectra were acquired with a total of 3072 transients in addition to 32 scans to allow the sample to come to equilibrium. Spectra were performed with a spectral width of 5 kHz and 32768 data points. Reference spectra were acquired using the same conditions but with only 1536 transients. All ligands were assigned (see Supporting Information) under the same conditions as the STD NMR spectra through the use of $^1H-^1H$ TOCSY, $^1H-^{13}C$ HSQC and $^1H-^{13}C$ HMBC spectra.

In order to determine the size of the STD effect the observed signal intensities were integrated with respect to the corresponding signal in the reference spectrum. For group epitope mapping these effects are then normalized against the largest STD effect observed, thus, 100% corresponds to the signal with the largest STD effect. In the interpretation and comparison of the resulting epitopes, it is the general trend that is important, not the absolute values.

Titrations to determine relative binding affinities were performed under the conditions described above, by repetition of the STD spectra in the presence of a second ligand at molar ratios of 1 to 5 with respect to the first ligand. The observed STD signals were then plotted against the molar ratios of the ligands in order to determine which of the ligands had the higher affinity.

The conversion rates of the synthetic GlcNAc analogues were determined by incubating 15 μM GlcNAc kinase with 5 mM GlcNAc analogue and 5 mM ATP. For enzyme kinetics samples with 15 μM enzyme and 1 mM GlcNAc and 1 mM ATP were measured in the presence and absence of different $MgCl_2$ concentrations. Each time point consists of a 1D "pulse and acquire" NMR experiment with 32 or 16 scans for acquisition and 8 dummy scans. The sweep width and recycle delay were as for the STD experiments. The resulting data were analyzed for decreasing signal intensities of isolated ATP signals and product formation.

RESULTS

STD NMR is a powerful method that provides information about interactions between a protein and different ligands. STD NMR experiments can be used to detect ligand binding, as well as to determine ligand binding epitopes at an atomic resolution. With this aim we characterized the interactions of several ligands with GlcNAc kinase.

NMR Data Reflect Binding of GlcNAc and Enzymatic Activity. In the presence of ATP GlcNAc kinase phosphorylates GlcNAc by forming GlcNAc-6-phosphate. Significant STD effects were obtained for GlcNAc in the presence of GlcNAc kinase (Figure 1), indicating that GlcNAc is bound by the enzyme under these conditions. The STD experiments were performed in the absence of ATP and therefore show that GlcNAc binds to the enzyme in the absence of a nucleotide triphosphate. The STD spectrum also shows that both anomers, the α and β, bind to the enzyme. To validate the activity of GlcNAc kinase under STD NMR conditions 1D NMR spectra of GlcNAc in the presence of GlcNAc kinase and ATP were acquired. Formation of GlcNAc-6-phosphate and ADP as products clearly indicated that the GlcNAc kinase is active under the NMR conditions employed. This ^1H NMR activity assay also revealed that the enzymatic conversion of GlcNAc to GlcNAc-6-phosphate predominately yields β-GlcNAc-6-phosphate. The equilibrium ratio of α- to β-GlcNAc-6-phosphate is reached after several hours due to mutarotation.

Binding Epitopes and Binding Affinities of GlcNAc, GlcNAc-6-phosphate and Natural GlcNAc Derivatives. From the STD amplification factors obtained from the STD spectra, relative STD effects have been calculated as described in Materials and Methods. These values reflect the relative amount of saturation transferred from the protein onto the ligand. Therefore protons with a high STD value are assumed to be in more intimate contact with the protein surface than those with lower STD values. The binding epitopes of the substrate GlcNAc and the product GlcNAc-6-phosphate are summarized in Figure 2a. As already shown for the substrate GlcNAc, also for the product GlcNAc-6-phosphate binding of both anomers was detected. In the α-anomer of GlcNAc

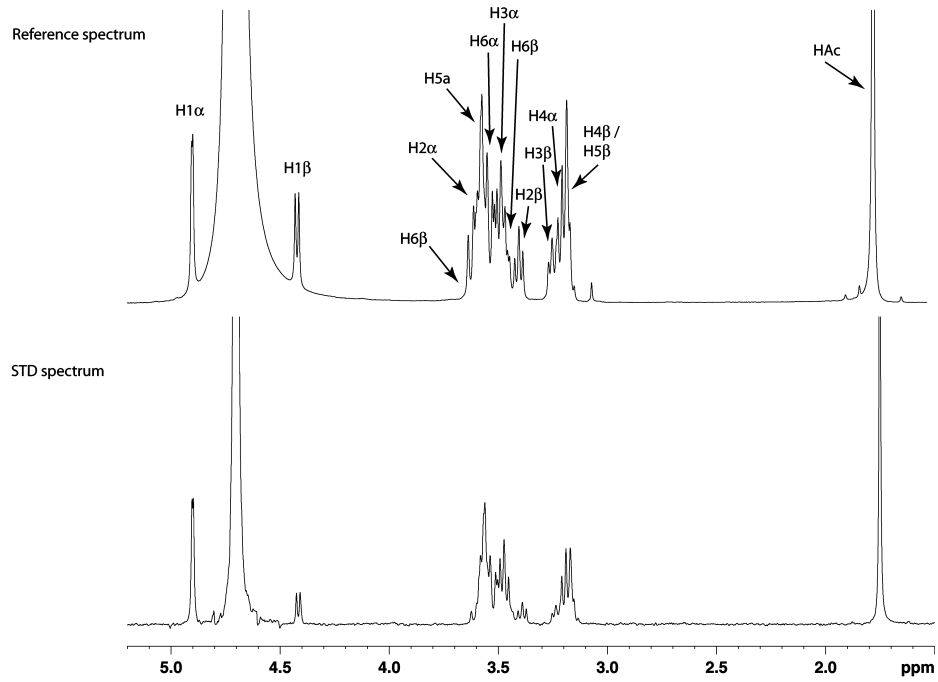

FIGURE 1: 1D reference (top) and STD NMR (bottom) spectra for GlcNAc. The STD spectrum (bottom) shows signals from α and β GlcNAc. The assignment is shown for all signals that can be clearly identified; α and β denote the anomeric forms of the GlcNAc.

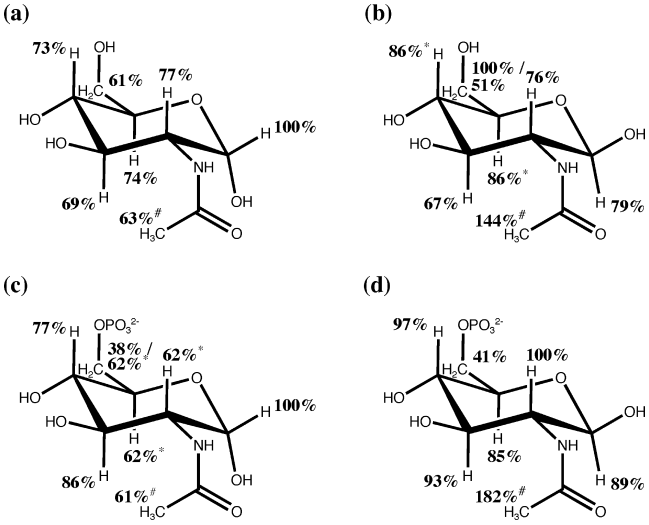

FIGURE 2: Binding epitopes as determined by 1H STD NMR: α-GlcNAc (a), β-GlcNAc (b), α-GlcNAc-6-phosphate (c), β-GlcNAc-6-phosphate (d). The percentages denote the normalized extent of saturation transfer for a given molecule; larger percentages consequently show a closer proximity to the protein surface. * denote signal overlaps within one molecule and # denote signal overlaps between α- and β-anomers. α- and β-anomers as well as the substrate and product epitopes show small differences, indicating that they bind to the same binding site, but with a different binding mode. STD spectra were acquired with a ligand concentration of 1 mM.

and GlcNAc-6-phosphate, the proton attached to C1 shows the most intimate contact with the protein surface, whereas in the β-anomers, the protons at C6 and C2 respectively are closest to the protein surface.

When comparing binding epitopes, it is important to focus on the overall pattern and not on the absolute values of each proton. In this light, there are no significant differences between the α- and β-anomers of GlcNAc other than at C4 and C5, and therefore it can be suggested that both anomers bind to the same site, albeit with differing binding modes. Due to the longer relaxation of the acetyl groups resulting in spectral artifacts, these groups were not taken into account when comparing the binding epitopes.

Upon phosphorylation of GlcNAc only slight changes in the binding mode are detected. This suggests similar binding modes for GlcNAc and GlcNAc-6-phosphate. Competitive STD titrations with GlcNAc and GlcNAc-6-phosphate clearly show that they bind to the same binding site as they compete with each other. Moreover, these titration experiments suggest that GlcNAc-6-phosphate binds more weakly to GlcNAc kinase than the substrate GlcNAc. Thus, upon phosphorylation the binding affinity changes along with only slight changes in the binding mode.

In further experiments binding epitopes for the substrate analogues *N*-acetylmannosamine (ManNAc), *N*-acetylgalactosamine (GalNAc), glucosamine and glucose were determined (Figure 3A). Signal intensities with 1 mM ligand were too weak to determine an accurate binding epitope, therefore spectra were measured with 5 mM ligand instead. As the relaxation of the ligand is to an extent concentration dependent, it is difficult to compare the binding epitopes obtained at 1 mM and 5 mM ligand concentration. In order to facilitate the comparison between the sugar analogues and GlcNAc the latter was also measured at 5 mM.

For all ligands binding of α- and β-anomers was detected, but in the case of β-GalNAc and β-glucosamine the β-signals were too weak for quantification. Compared to α-GlcNAc, α-ManNAc shows nearly the same binding epitope with the largest variation at the proton attached to C3 (except for the site of epimerization, C2). In case of β-ManNAc the largest changes were observed for the protons at C4 and C5. Therefore ManNAc is suggested to bind to the same binding site as GlcNAc but with a slightly different binding mode. On the other hand for α-GalNAc the relative STD effects show significant differences except for the proton attached to C5 and the *N*-acetyl group.

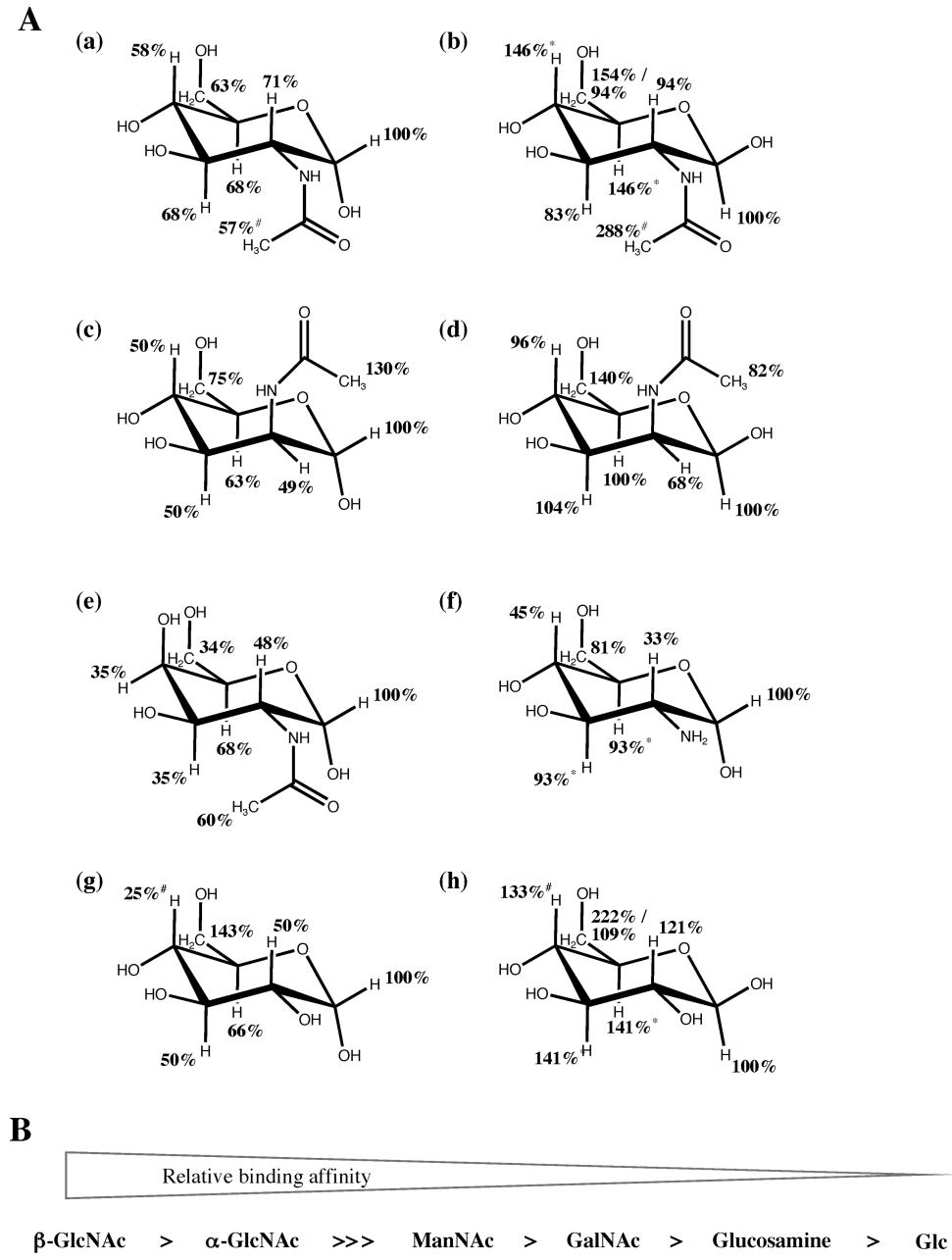

FIGURE 3: (A) Binding epitopes as determined by 1H STD NMR: α-GlcNAc (a), *β*-GlcNAc (b), α-ManNAc (c), *β*-ManNAc (d), α-GalNAc (e), α-glucosamine (f), α-glucose (g) and *β*-glucose (h). To simplify the direct comparison of the epitopes, the group epitope mapping was in this case performed not by normalizing with respect to the largest STD effect but rather against the STD effect observed for the H1 of each sugar. * denote signal overlaps within one molecule and # denote signal overlaps between α- and *β*-anomers. Signal intensities of *β*-GalNAc and *β*-glucosamine were too weak for adequate quantification. Binding epitopes of ManNAc and glucosamine are roughly similar to that of GlcNAc, whereas the epitopes of GalNAc and glucose show larger differences. STD spectra were acquired with a ligand concentration of 5 mM as the ligand binding is too weak to detect STD signals with the routineously used1 mM ligand concentrations. (B) Schematic representation of relative binding affinities of GlcNAc and natural derivates. Competitive titrations show that all these ligands compete with each other and therefore bind to the same binding site. Compared to the natural substrate GlcNAc all ligands show a drastically reduced affinity.

Although the relative STD effects of α-glucosamine differ from those of α-GlcNAc the overall binding epitope is conserved with differences only at the proton attached to C2. Therefore, glucosamine has the same binding mode as GlcNAc whereas GalNAc has a completely different binding mode. The binding epitopes determined for glucose differ substantially from those of GlcNAc, which suggests a different binding mode for glucose compared to the natural substrate GlcNAc.

In order to obtain relative binding affinities of the different sugars and relate them to the natural donor substrate GlcNAc competitive STD titrations were performed. A qualitative ranking of the binding affinities of the ligands is summarized in Figure 3B. Between all ligands competition in binding is observed. This, in turn, indicates that all examined ligands bind to the same binding site. GlcNAc turned out to be the best binder of the examined ligands. The affinity of the GlcNAc kinase for ManNAc, GalNAc, glucosamine and glucose is much lower than for the natural substrate GlcNAc. Interestingly, the enzyme displays a higher affinity for *β*-GlcNAc than for α-GlcNAc. This was also observed for ManNAc (data not shown).

Binding of Synthetic GlcNAc Derivatives to GlcNAc Kinase. For the design of inhibitors or for metabolic engineering of glycoconjugates it is necessary to know which positions of the GlcNAc molecule are very important for binding and therefore should not be modified and which positions can be modified. Therefore a panel of synthetic GlcNAc derivatives was studied. The binding epitopes determined for methyl-α-GlcNAc, methyl-β-GlcNAc, *N*-butyl-glucosamine (GlcNBut), 3-methyl-GlcNAc and 3,4-methyl-GlcNAc are shown in Figure 4A. In case of a α-/β-mixture again signals for both anomers were obtained.

The binding epitope of 1-OMe-α-GlcNAc is rather similar to that of α-GlcNAc with a slight change for the proton attached to C3. On the other hand the binding epitope of 1-OMe-β-GlcNAc deviates from β-GlcNAc for all protons. Therefore 1-OMe-α-GlcNAc seems to have the same binding mode as α-GlcNAc and therefore the methyl group has no significant influence on the binding mode. In case of 1-OMe-β-GlcNAc the insertion of a methyl group at the 1 position drastically changes the binding mode. A modification of the *N*-acetyl group to a *N*-butyl group has no influence on the binding mode, as compared to GlcNAc no significant changes in the binding epitopes were detected. The methylation of the 3 position only changes the binding epitope of the α-anomer. In 3-OMe-α-GlcNAc the protons attached to C2 and C4 receive a different amount of saturation whereas in 3-OMe-β-GlcNAc the amount of saturation stays the same. An additional insertion of a methyl group at the 4 position does not change the binding epitope of the β-anomer whereas the epitope of the α-anomer differs mainly at H4 from that of 3-OMe-α-GlcNAc. Compared to α- and β-GlcNAc the binding epitope of α−3,4-OMe-GlcNAc only differs for the proton attached at the C2 position whereas the epitop for the β-anomer is the same.

Competitive titration experiments show that all ligands studied bind to the same binding site as they all compete with each other. Furthermore, an assignment of relative binding affinities to the different ligands was obtained and is summarized in Figure 4B. Although all examined modifications of the GlcNAc molecule result in a reduction of binding affinity the methylation of the 1 position causes the strongest reduction.

In order to differentiate between an inhibitor and an alternative substrate for GlcNAc kinase conversion rates of the examined ligands were determined (Figure 5). Within 12 h there was no phosphorylation of 1-OMe-α-GlcNAc and 3-OMe-GlcNAc detectable. The phosphorylation rate of 1-OMe-β-GlcNAc and 3,4-OMe-GlcNAc is similar, 35% of the ligands are phosphorylated within 12 h corresponding to a specific GlcNAc kinase activity for these ligands of 4 mU/mg under these conditions. 35% of GlcNBut are already phosphorylated within 4 h and 20 min, corresponding to a specific activity of 11 mU/mg. Therefore GlcNBut is phosphorylated about 3 times faster than 1-OMe-β-GlcNAc and 3,4-OMe-GlcNAc. Interestingly, it is also phosphorylated 1.5 times faster than the natural substrate GlcNAc as 35% of this ligand are phosphorylated after 6 h and 40 min. But taking into account the much lower binding constant of GlcNBut, at lower substrate concentrations the natural substrate GlcNAc should be much better phosphorylated.

Finally, we investigated the binding of GlcNAc kinase to GlcNAc β-linked to a threonine residue within the artificial c-myc peptide, which is also *O*-GlcNAcylated *in vivo*. With this experiment the hypothesis that GlcNAc kinase may act on *O*-GlcNAcylated proteins in cells should be proven. However, no significant STD signals were detected, indicating a binding constant out of the range of 1 nM to 10 mM. Therefore, next we investigated the phosphorylation of the *O*-GlcNAc moiety by GlcNAc kinase. But even after incubation of the peptide with ATP and GlcNAc kinase for 5 days at 37 °C no altered signals were observed in the ^{1}H NMR.

Dependence of the GlcNAc Kinase Activity on Mg^{2+}. For many kinases it is known that Mg^{2+} is essential for the enzyme reaction to proceed. Here we show for the GlcNAc kinase that enzyme activity persists even in the virtual absence of Mg^{2+}. The influence of Mg^{2+} on the enzyme reaction of the GlcNAc kinase was examined by ^{1}H NMR experiments. The presence of 0.1 mM $MgCl_2$ in the sample caused a significant increase in enzyme activity as compared to the absence of Mg^{2+} (Figure 6). For example, for 50% conversion of GlcNAc to GlcNAc-6-phosphate in the absence of Mg^{2+} 10 h were required whereas in the presence of 0.1 mM Mg^{2+} 50% conversion were detected within 60 min. In the presence of 10 mM Mg^{2+} 50% of the GlcNAc molecules are phosphorylated in 30 min. Therefore, Mg^{2+} cannot be considered as being essential for the GlcNAc kinase activity, however, the reaction rate is profoundly dependent on the Mg^{2+} concentration. Interestingly, in the presence of Mg^{2+} no preference for the synthesis of β-GlcNAc-6-phosphate was detected. The fact that the α/β ratio of GlcNAc-6-phosphate during the first few minutes of the reaction is equal to that of GlcNAc and only afterward adjusts to that of GlcNAc-6-phosphate suggests that the presence of Mg^{2+} lowers the activation energy of the enzyme reaction so that both anomers can be phosphorylated equally well in the presence of Mg^{2+}.

Hence, it is likely that Mg^{2+} influences the binding of ATP to GlcNAc kinase. Therefore STD spectra for ATP and ADP binding to the kinase were conducted in the presence and absence of Mg^{2+}. These data show that ATP and ADP bind to the GlcNAc kinase in the absence of a sugar and that the spectra of ATP and ADP are rather similar. Hence, upon phosphorylation the binding epitope and therefore the binding mode does not change. Furthermore, competitive titrations show that ATP and ADP have about the same binding affinity. The binding epitopes of the nucleotide phosphates in the absence and presence of Mg^{2+} show changes mainly in the STD effects of the ribose protons closest to the tri- and diphosphate (Figure 7). Furthermore, the chemical shift of most of the signals changes. Therefore the divalent cation Mg^{2+} changes the binding mode of the nucleotide tri-/diphosphate.

DISCUSSION

Several studies have utilized STD NMR experiments to detect and characterize the binding of ligands to receptor proteins (*16*). For GlcNAc kinase currently little is known regarding the atomic details of ligand binding to the active site of the enzyme in aqueous solution. This report describes the application of STD NMR approaches to the GlcNAc kinase and the binding of several different ligands to the enzyme at atomic resolution.

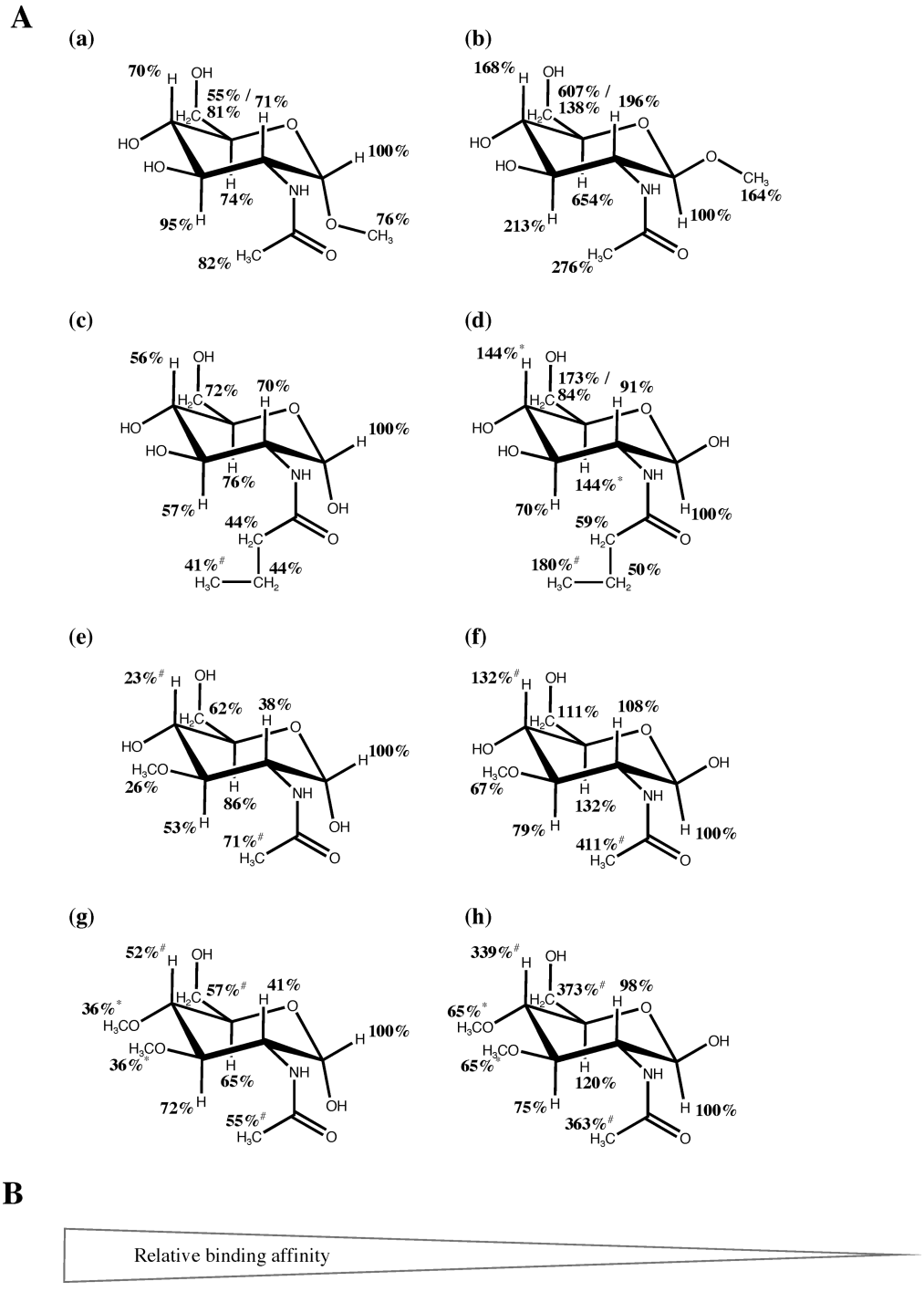

FIGURE 4: (A) Binding epitopes as determined by ^{1}H STD NMR: 1-OMe-α-GlcNAc (a), 1-OMe-β-GlcNAc (b), α-GlcNBut (c) and β-GlcNBut (d), 3-OMe-α-GlcNAc (e), 3-OMe-β-GlcNAc (f), 3,4-OMe-α-GlcNAc (g) and 3,4-OMe-β-GlcNAc (h). To simplify the direct comparison of the epitopes, the group epitope mapping was in this case performed not by normalizing with respect to the largest STD effect but rather against the STD effect observed for the H1 of each sugar. * denote signal overlaps within one molecule and # denote signal overlaps between α- and β-anomers. The metylation of the hydroxyl group at C1 changes the binding mode of the β-anomer, whereas the methylation of the hydroxyl group at C3 or C3 and C4 only changes the binding mode of the α-anomer. Replacing the acetyl group by a butyl group has no significant influence on the binding mode. STD spectra were acquired with a ligand concentration of 5 mM. (B) Schematic representation of relative binding affinities of synthetic GlcNAc derivates. Competitive titrations show that all these ligands compete with each other and therefore bind to the same binding site. Modifications at the 1- and 3-position as well as replacing the acetyl group by a butyl group reduce the binding affinity.

GlcNAc kinase belongs to the sugar kinase/heat shock protein 70/actin superfamily. Sequence homologies show that all these proteins have a common ATPase domain (*22*). The determined binding epitope of ATP bound to GlcNAc kinase revealed that the H2 proton of the purine has the most intimate contact with the protein. A similar binding epitope was found for ATP binding to ManNAc kinase functionality of the bifunctional enzyme UDP-GlcNAc 2-epimerase/ManNAc kinase (*23*) and to yeast hexokinase (*24*). This indicates that the three-dimensional structure of the ATP

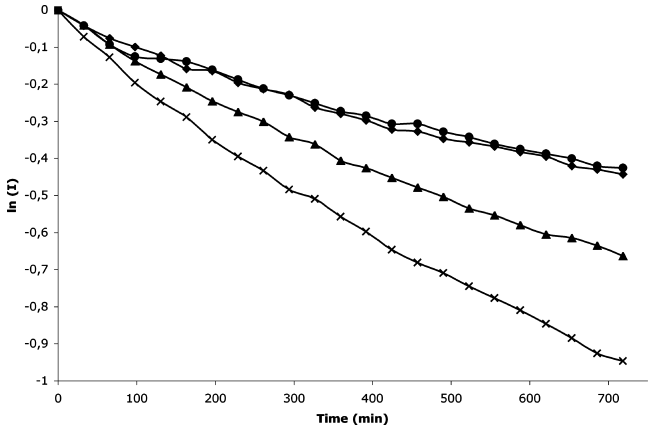

FIGURE 5: Enzymatic conversion rates of synthetic GlcNAc derivatives. The filled circles denote data obtained for the phosphorylation of 1-OMe-β-GlcNAc, the squares for 3,4-OMe-GlcNAc, the triangles for GlcNAc and the asterisks for GlcNBut. Although for all ligands STD effects were observed 3-OMe-GlcNAc and 1-OMe-α-GlcNAc are not phosphorylated by GlcNAc kinase. GlcNBut shows the best phosphorylation rate, 3 times higher than 1-OMe-β-GlcNAc and 3,4-OMe-GlcNAc and 1.5 times higher than the natural substrate GlcNAc.

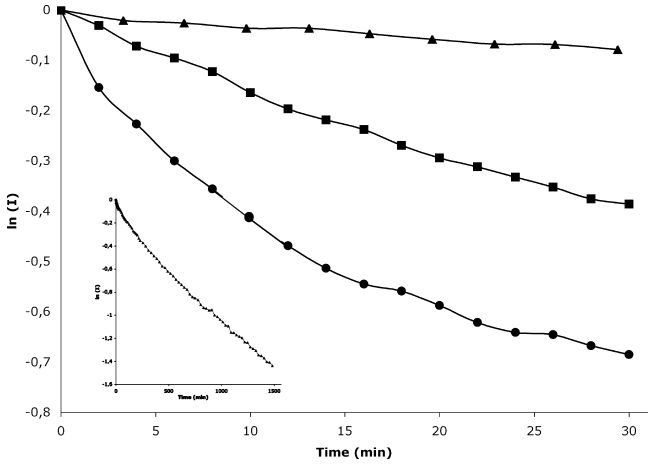

FIGURE 6: Intensity change of the ATP signals in the presence of GlcNAc and GlcNAc kinase with time. The filled triangles denote data obtained in the virtual absence of Mg^{2+}, the squares in the presence of 0.1 mM Mg^{2+} and the circles in the presence of 10 mM Mg^{2+}. The inset shows an extension of the data obtained in the absence of Mg^{2+} in 25 h, showing that the enzyme is active under these conditions.

binding site of the three enzymes is similar. Therefore these data show that within the family of sugar kinases there is not only a high sequence homology for the ATPase domain but the enzymes have a common three-dimensional structure for the ATP binding site. On the other hand a comparison of the sugar binding epitopes, e.g. of GlcNAc bound to GlcNAc kinase and ManNAc bound to ManNAc kinase, revealed that the epitopes are completely different. Therefore it is highly probable that the three-dimensional structure of the sugar binding site is different. Until today there is no study available that describes a common sugar binding motif of sugar binding enzymes, most likely due to the different structures of the sugars.

A comparison of the binding epitopes of the substrate ATP and the product of the catalyzed phosphorylation reaction ADP shows that the binding modes of these two ligands are the same. Furthermore, competitive titrations reveal that ATP and ADP have similar binding affinities. Therefore upon phosphorylation neither the binding mode nor the binding affinity changes. This indicates that little or no change in the structure of the ATP binding site occurs during the reaction. Interestingly, for ManNAc kinase ATP and ADP have different binding modes and affinities (*23*), indicating structural changes during the enzymatic process, affecting the binding affinity and the binding mode of the nucleotide. A similar binding affinity and binding mode of ATP and ADP in case of GlcNAc kinase is in agreement with the observation that ADP is a strong competitive inhibitor of GlcNAc kinase and that GlcNAc kinase activity is regulated by the ATP/ADP ratio in cells (*11*).

Almost all kinases require Mg^{2+} as an essential cation for their enzymatic activity (*22*). Here it is shown that the GlcNAc kinase is also active in the virtual absence of Mg^{2+}. Furthermore, the crystal structure of GlcNAc kinase in complex with ADP was obtained without addition of Mg^{2+} (*14*), confirming that divalent cations are not essential for ligand binding. Nevertheless, the addition of μM amounts of Mg^{2+} to the buffer results in a 10-fold increase of enzyme activity. Also, upon addition of Mg^{2+} a slight change in the binding epitope of ATP and ADP was detected for the ribose protons close to the phosphates. A similar observation concerning the changes in the binding mode and the increase of enzyme activity after addition of Mg^{2+} was made for the ManNAc kinase (*23*). X-ray structures from related sugar kinases (*25, 26*) and a model of the active site of GlcNAc kinase in complex with ATP-Mg^{2+} (*14*) suggest that this may be due to the complexation of the triphosphate moiety with Mg^{2+}, whereby the cation shields the negative charges of the β- and γ-phosphate groups of ATP and thus facilitates the nucleophilic attack of the hexose C6 hydroxyl group on the γ-phosphate of ATP.

An inspection of the binding epitopes of the natural substrate GlcNAc and the product of the enzyme reaction GlcNAc-6-phosphate revealed that upon phosphorylation only slight changes in the binding mode occur. But at the same time the binding affinity of the product is reduced compared to the substrate, favoring the direction of the enzyme reaction, the kinase above the phosphatase activity. The same binding mode and the fact that GlcNAc competes with GlcNAc-6-phosphate in competitive titrations suggests that substrate and product bind to the same binding site. This was also observed for the ManNAc kinase, but contrasts with the hexokinase I, where different binding pockets for the products and substrates have been described (*27*). In the case of the hexokinase I, product binding is associated with an allosteric inhibition of the enzyme. Our data and the fact that until today no allosteric product inhibition was observed for GlcNAc kinase, indicate that a regulatory domain similar to that in hexokinase I is not likely to exist in GlcNAc kinase.

STD NMR spectra of nearly all ligands show that the GlcNAc kinase binds the α- and β-anomer of the sugars. Although in the crystal structure GlcNAc has α configuration, modeling approaches show that the β-anomer can also bind, supporting the hypothesis that GlcNAc kinase has no anomeric preference with respect to substrate binding. But interestingly, following the enzyme reaction it is unambiguously clear that the enzyme predominantly produces the β form of phosphorylated product. Another fact substantiating the preference of the enzyme for the β-anomer is that from 1-OMe-GlcNAc only the β form is phosphorylated. Although

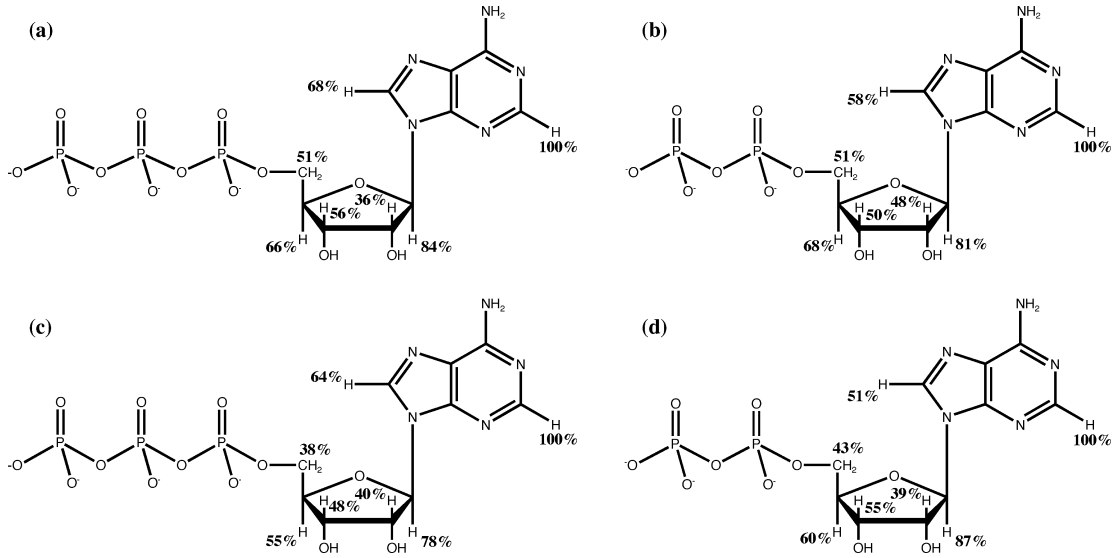

FIGURE 7: Binding epitopes as determined by ^{1}H STD NMR: ATP in absence of Mg^{2+} (a), ADP in absence of Mg^{2+} (b), ATP in presence of Mg^{2+} (c) and ADP in presence of Mg^{2+} (d). Binding epitopes of ATP and ADP are similar. In the presence of Mg^{2+} the binding mode changes slightly mostly seen at the protons attached to C5 of ribose. STD spectra were acquired with a ligand concentration of 200 μM and 10 mM $MgCl_2$.

these experiments cannot clarify whether the enzyme exclusively converts the β form they clearly show the β-anomer is the preferred anomer of the enzyme reaction. In contrast, hexokinase has a slight preference for the α-anomer in the phosphorylation reaction (*28*) and the ManNAc kinase only binds and exclusively phosphorylates the α-anomer (*23*). Whereas for the ManNAc kinase as part of the bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase there is a strong evolutionary pressure for such a α preference as the epimerase function only produces α-ManNAc, there is no such pressure for the GlcNAc kinase toward the α form as both anomers are available in cells.

The strong β preference of GlcNAc kinase in addition with the drastically reduced binding affinity toward other sugars than GlcNAc may also suggest an involvement of the enzyme in phosphorylation of protein bound *O*-GlcNAc, as this form of GlcNAc has always the β configuration. Although it was found that GlcNAc kinase is able to phosphorylate *O*-GlcNAc modified proteins *in vitro* (M. Berger, unpublished observation), no phosphorylation of the c-myc Thr58-*O*-GlcNAc peptide was observed in the NMR experiments. However, in this experiment the two spacious prolins surrounding the threonine with the bound *O*-GlcNAc may hinder the binding of the peptide to GlcNAc kinase.

Other naturally occurring sugars in a cell like ManNAc, GalNAc, glucosamine and glucose were studied for their binding to the GlcNAc kinase. All these sugars show a slightly changed binding epitope, except for the 4 epimer GalNAc and the hexose glucose, where the binding mode deviates drastically from that of GlcNAc. These results show that the 4 position and the *N*-acetyl group of the natural substrate GlcNAc are very important for binding to GlcNAc kinase. As the binding mode for glucosamine only shows slight changes compared to the natural substrate but glucose has a drastically changed epitope, the amino function of the *N*-acetyl group of GlcNAc seems to be more important for binding than the acetyl moirty. This is in agreement with the observation that extension of the *N*-acyl group by a butanoyl moiety does not change the binding mode. Competitive titrations with these naturally occurring sugars show that the binding affinity of all these sugars is drastically reduced compared to the natural substrate GlcNAc. Therefore, these results suggest that in a cell the GlcNAc kinase will predominantly, if not exclusively phosphorylate GlcNAc. Other sugars do not play a significant role for phosphorylation. This is also reflected in the K_m value of ManNAc, the sugar with the highest affinity after GlcNAc to the GlcNAc kinase, with 5 mM being about 20 times larger than that for GlcNAc with 250 μM (*12*).

For further characterization of the sugar-binding pocket synthetic GlcNAc derivates were examined for binding to GlcNAc kinase. A methylation of the hydroxyl group at C1 does not change the binding mode of the resulting α ligand. Also a *N*-butyl instead of a *N*-acetyl group does not influence the binding mode. These results suggest that in the area of the 1 and 2 positions of the natural substrate the binding pocket is more spacious. This is underlined by a nearly unchanged binding epitope of the 2 epimer ManNAc. Despite these unchanged binding modes of the ligands the binding affinity is reduced for all of them. Although 1-OMe-α-GlcNAc has the same binding mode as GlcNAc it is not phosphorylated, whereas 1-OMe-β-GlcNAc, which has a different binding mode, is phosphorylated by the enzyme. Therefore 1-OMe-β-GlcNAc is a less efficient substrate for GlcNAc kinase, whereas 1-OMe-α-GlcNAc is an inhibitor. Due to the reduced binding affinity 1-OMe-α-GlcNAc will be a weak competitive inhibitor.

Methylation of the hydroxyl group at C3 or at C3 and C4 changes the binding epitopes of the α-anomers slightly with the largest variation at the proton attached to C2. In 3-OMe-α-GlcNAc an additional change for the proton at C4 was observed. Therefore the binding epitope of 3,4-OMe-GlcNAc is much more similar to that of GlcNAc than that of 3-OMe-GlcNAc. This is also reflected in the relative binding affinities as 3,4-OMe-GlcNAc has a higher affinity than 3-OMe-GlcNAc and in the phosphorylation rates as 3,4-OMe-GlcNAc is phosphorylated by GlcNAc kinase, whereas 3-OMe-GlcNAc is not. Therefore 3-OMe-GlcNAc is a competitive inhibitor of GlcNAc kinase with a lower binding

affinity than the natural substrate. The inhibitory effect of 3-OMe-GlcNAc toward GlcNAc kinase was already observed earlier using rat liver homogenates (*21*). Methylation of the 3 hydroxyl group changes the binding mode, decreases the binding affinity and prevents phosphorylation of the ligand, whereas an additional methylation of the 4 hydroxyl group adjusts the binding epitope again to that of GlcNAc, increases the binding affinity again and causes phosphorylation again. These data suggest that 3-OMe-GlcNAc and 3,4-OMe-GlcNAc have different conformations at which the conformation of 3,4-OMe-GlcNAc is more similar to GlcNAc or more flexible than that of 3-OMe-GlcNAc. Therefore for 3,4-OMe-GlcNAc it is possible to bind to GlcNAc kinase in the right mode and thus to get phosphorylated, whereas for 3-OMe-GlcNAc this is not possible.

To summarize, this study for the first time delivers information on structure−activity and structure−binding relationships of natural and unnatural ligands of GlcNAc kinase. It is clearly shown that the enzyme, although it binds both anomers, has a significant preference toward phosphorylation of the β-anomer. This fact in addition with the drastically reduced binding affinity toward other sugars than the natural substrate GlcNAc suggests a highly specific role of GlcNAc kinase in cells. Furthermore, on the basis of this work it will be possible to design novel GlcNAc analogues for metabolic engineering that may lead to novel therapeutic applications as well as inhibitors for the GlcNAc kinase.

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SUPPORTING INFORMATION AVAILABLE

Chemical shift assignments. This material is available free of charge via the Internet at http://pubs.acs.org.

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