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Specificity of ligand binding to yeast hexokinase PII studied by STD-NMR

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Dedicated to Professor Hans Kamerling on the occasion of his 65th birthday

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ABSTRACT

Hexokinase catalyzes the phosphorylation of glucose and is the first enzyme in glycolysis. To investigate enzyme–ligand interactions in yeast hexokinase isoform PII under physiological conditions, we utilized the technique of Saturation Transfer Difference NMR (STD NMR) to monitor binding modes and binding affinities of different ligands at atomic resolution. These experiments clearly show that hexokinase tolerates several changes at C-2 of its main substrate glucose, whereas epimerization of C-4 significantly reduces ligand binding. Although both glucose anomers bind to yeast hexokinase, the α-form is the preferred form for the phosphorylation reaction. These findings allow mapping of tolerated and prohibited modification sites on the ligand. Furthermore, competitive titration experiments show that mannose has the highest binding affinity of all examined sugars. As several naturally occurring sugars in cells show binding affinities in a similar range, hexokinase may be considered as an 'emergency enzyme' in yeast cells. Taken together, our results represent a comprehensive analysis of ligand–enzyme interactions in hexokinase PII and provide a valuable basis for inhibitor design and metabolic engineering.

1. Introduction

Sugars are used by cells as a source of carbon or energy. The first step in sugar metabolism after transport into the cell is phosphorylation, which is catalyzed by specific sugar kinases. All metabolic sugars are phosphorylated to prepare them for further chemical reactions, either catabolic or anabolic. The main catabolic pathway is the glycolysis, with glucose as the key sugar. The other key sugar is ribose, essential in nucleotide biosynthesis.

Kinases that catalyze phosphorylation of sugars can be divided into at least three distinct non-homologous families.[1] The first is the hexokinase family, which contains many prokaryotic and eukaryotic sugar kinases with diverse specificities. The three-dimensional structure of hexokinase is the basis for models of functionally important regions of other kinases in this family. The second is the ribokinase family, comprising pro- and eukaryotic ribokinases and bacterial fructokinases amongst others. The third family contains several bacterial and yeast galactokinases and some other eukaryotic kinases. Each of the three families of sugar kinases appears to have a distinct three-dimensional fold, since conserved sequence patterns are strikingly different for the three families. Although the enzymes catalyze chemically equivalent reactions on similar or identical substrates, the enzymatic function of sugar phosphorylation appears to have evolved independently on the three distinct structural frameworks by convergent evolution.

Hexokinase (EC 2.7.1.1) is the first enzyme in the glycolytic pathway, catalyzing the transfer of the γ-phosphate group from ATP to the 6-hydroxyl group of D-glucose to form D-glucose-6-phosphate and ADP. Cloning of hexokinase genes from *Saccharomyces cerevisiae* has shown that two isoenzymes of hexokinases exist in yeast: PI and PII, with an overall amino acid sequence identity of around 76%.[2] The individual roles of these isoenzymes are still unclear. Hexokinase PII is the predominant hexose kinase in *S. cerevisiae* grown on glucose, while PI is the main hexose kinase when grown on other carbon sources[3] and is required for glucose-dependent catabolic repression of other genes.[4,5] Both yeast hexokinases are known to exist as phosphoproteins in vitro[6] and in vivo,[7] with the dimer–monomer equilibrium affected by phosphorylation. The crystallographic structure of yeast hexokinase PI complexed with glucose was solved at a resolution of 3.5 Å,[8] whereas the crystal structure of yeast hexokinase PII from *S. cerevisiae* has been determined at 2.2 Å resolution[9] without substrate or competitive inhibitor.

A detailed understanding of sugar kinases is particularly important in the context of many diseases related with sugar kinases. A

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; HSQC, heteronuclear single quantum coherence spectroscopy; GlcNAc, *N*-acetyl D-glucosamine; ManNAc, *N*-acetyl D-mannosamine; STD NMR, saturation transfer difference nuclear magnetic resonance; TOCSY, total correlation spectroscopy; Tris, *tris*-hydroxylethylaminoethane; UDP, uracil diphsophate.

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reduction of hexokinase activity causes illnesses in humans such as hemolytic anemia[10,11] and cardiomyopathy[12], whereas mutations are associated with early onset non-insulin-dependent diabetes mellitus.[13,14] Hexokinase has also been a target for the development of efficient inhibitors in the search for new drugs against diseases caused by trypanosomes.[15] A fundamental knowledge of the binding of the substrates to hexokinase PII in solution and the knowledge of mechanistic aspects of the enzymatic reaction are important to further our understanding of the enzyme and to obtain a more detailed insight into the action of other kinases of unknown structure. In this study, we characterize the interactions of yeast hexokinase PII with a number of different ligands at atomic resolution under physiological conditions, that is, in aqueous solution STD NMR. STD NMR allows the detection and characterization of ligand binding to large proteins by discriminating binding from non-binding ligands and by furnishing binding epitopes of the ligands at atomic resolution.[16,17] Most noteworthy, there is no size limit for the protein receptor, and an assignment of protein resonances is not required. Also, there is no requirement for isotope labeling of the protein, or the ligand. This method allows insights into so far not known mechanistic aspects of hexokinase, and the characterization of ligand binding epitopes. This is a potential basis for further design of inhibitors. Finally, the obtained data are compared to two human kinases, the *N*-acetyl glucosamine kinase (GlcNAc) and the *N*-acetyl mannosamine kinase (ManNAc).

2. Results

NMR experiments were performed with pure hexokinase dissolved in a deuterated phosphate buffer at physiological pH. Prior to conducting any STD NMR experiments with yeast hexokinase PII, non-deuterated substances have to be removed from the protein sample. For this purpose, different purification methods such as ultra filtration, dialysis, and size exclusion chromatography were tested. Only a combination of the tested methods was able to produce enzyme samples with the required purity and sufficient reduction of H_2O content. Dialysis over regenerated cellulose followed by gel filtration was shown to be suitable for hexokinase purification for STD NMR analysis. Between 80% and 90% of the initial amount of enzyme was recovered by this procedure.

2.1. NMR data reflect the binding of glucose and the activity of the enzyme

Glucose is the natural substrate of yeast hexokinase PII and is phosphorylated by the enzyme in presence of ATP to form glucose-6-phosphate. Significant STD effects were obtained for the ligand glucose in presence of hexokinase (Fig. 1), indicating that the enzyme binds glucose under the described conditions. STD K_D values in the range of ca. 1 nM to ca. 10 mM can be determined.[18,19] Since the STD experiments were performed in the absence of ATP, these results clearly show that the enzyme is able to bind glucose in absence of a nucleotide phosphate. The STD spectrum also unambiguously shows that the enzyme binds both the α- and β-anomer of D-glucose. By using glucose concentrations between 200 μM and 1 mM, an asymptotic correlation between the substrate concentration and the increase in the obtained STD signal intensity was observed. This strongly suggests that the binding of D-glucose to yeast hexokinase PII occurs to a defined site of the enzyme, and therefore glucose binding is specific.

To validate the activity of hexokinase PII under the experimental conditions, 1D NMR spectra of glucose in the presence of hexokinase and ATP were acquired. Formation of D-glucose-6-phosphate and ADP as products clearly indicated that hexokinase is active under the NMR conditions employed in this study. The ^{1}H NMR activity assay also revealed that the enzymatic conversion of glucose to D-glucose-6-phosphate predominately yields α-D-glucose-6-phosphate and that mainly α-D-glucose is removed from solution. The equilibrium ratio of 1:2 for α- to β-D-glucose-6-phosphate is reached after several hours via mutarotation.

To determine the binding epitope of each ligand, relative STD effects have been calculated from the STD amplification factors that were obtained from the STD spectra. These relative STD 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 closer contact with the protein surface than those with lower STD values. The resulting binding epitopes of glucose are summarized in Figure 2. The epitopes of α- and β-D-glucose are roughly the same, indicating that both sugars bind in a similar manner to the same binding site. In the α-anomer, the proton attached to C-1 shows the most intimate contact with the protein surface, whereas in the β-anomer, the proton at C-2 is closest to the protein surface. Not only the protons at C-1 and C-2 receive different amounts of saturation, but protons at C-4 and C-5 also differ.

2.2. Binding of glucose derivatives to hexokinase PII

In order to further characterize ligand binding to hexokinase PII, different glucose derivatives were investigated. First the sugars glucosamine and GlcNAc were studied. The insertion of an amino group or an acetamido group, respectively, at C-2 changes the binding epitope for the α-anomers drastically, whereas the β-anomers still have similar binding epitopes as β-D-glucose (Fig. 3). For the β-anomers, only the relative STD signal intensities of protons at the modified C-2 differ. For the α-anomer, the relative STD effects of all protons differ from the ones determined for α-D-glucose. Therefore, the insertion of an amino group or an acetamido group at C-2 changes the binding mode of the α-form, but not of the β-form.

Epimerization of the C-2 changes the binding mode of the α- and the β-anomer. When binding epitopes of glucose and its C-2-epimer mannose are compared, all protons differ except for the proton attached to C-3 in α-D-mannose (Fig. 3). Insertion of an additional amino group further changes the binding epitopes. β-D-Mannosamine shows no similarities to the epitopes of D-mannose, D-glucosamine, or D-glucose. The relative STD effects of α-D-mannosamine are different from those of D-mannose and D-glucose, except for the protons attached to the C-6 of D-mannose. But α-D-mannosamine shows a binding epitope similar to that of α-glucosamine, with the largest variation at the proton attached to C-4, suggesting a similar binding mode for these two molecules. For the C-4-epimer galactose, only very weak STD signals were observed, too weak to determine a binding epitope.

In further STD NMR experiments, products of the hexokinase PII reaction were analyzed: D-glucose-6-phosphate, D-glucosamine-6-phosphate, and D-mannose-6-phosphate. Only rather weak STD signals were obtained for D-glucose-6-phosphate, D-glucosamine-6-phosphate, and β-D-mannosamine-6-phosphate. Compared to α-D-mannose, the relative STD effects of α-D-mannose-6-phosphate are significantly different for all protons (Fig. 3), suggesting that the binding mode of the sugar changes drastically upon phosphorylation.

Lastly, binding of the methyl glycosides of α- and β-D-glucose was investigated. No quantifiable STD signals were observed for α-D-*O*-methyl glucoside, whereas the β form shows a binding epitope similar to that of β-D-glucose, with the protons attached to C-2 and C-6 varying the most (Fig. 3). This suggests that β-D-*O*-methyl glucoside and β-D-glucose bind to the same binding site, but with a slightly different binding mode. In addition, enzymatic activity was detected only toward β-D-*O*-methyl glucoside, whereas α-D-*O*-

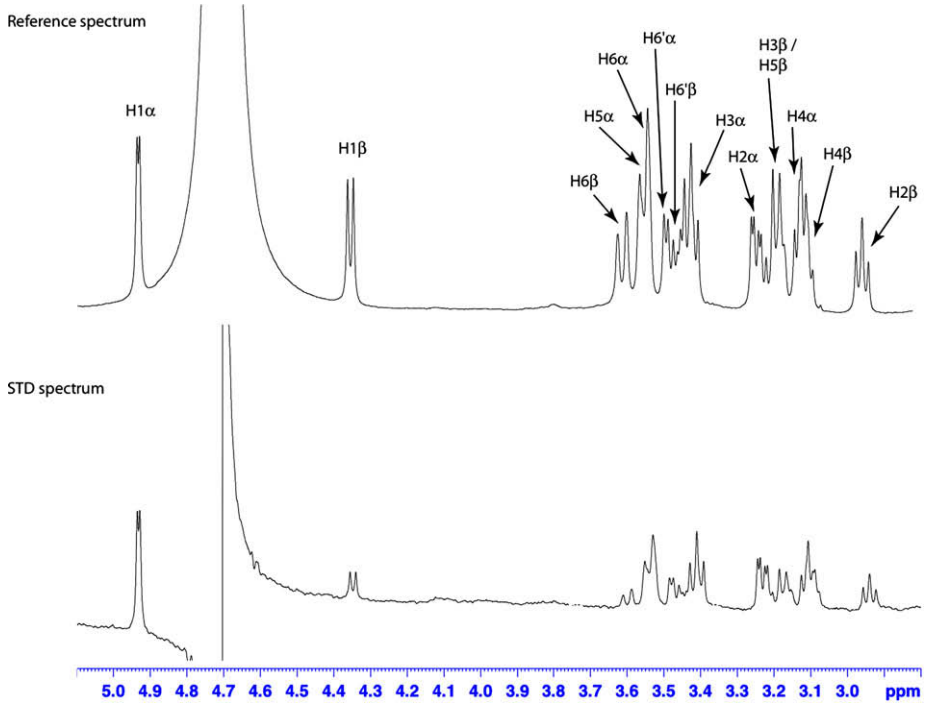

Figure 1. 1D reference (top) and STD NMR (bottom) spectra for D-glucose. The STD spectrum (bottom) shows signals from α and β D-glucose. The assignment is shown for all signals that can be clearly identified, α and β denote the anomeric forms of D-glucose.

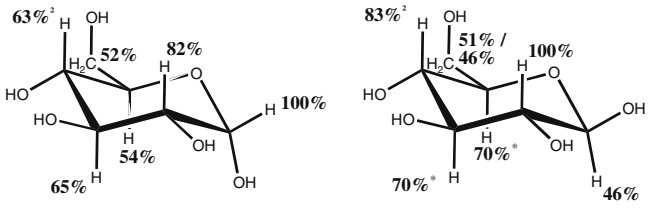

Figure 2. Binding epitopes as determined by ^{1}H STD NMR: α-D-glucose (left), β-D-glucose (right). The percentages denote the normalized extent of saturation transfer for a given molecule; larger percentages consequently denote a closer proximity to the protein surface. * denotes signal overlaps within one molecule and Δ denotes signal overlaps between α- and β-anomers. α- and β-anomers show slightly different binding epitopes.

methyl glucoside is not phosphorylated by hexokinase PII under the described conditions.

In order to obtain relative binding affinities for the different sugars, and to relate them to the natural donor substrate D-glucose, competitive STD titrations were performed.[18,19] A qualitative ranking of the binding affinities of the ligands is summarized in Figure 4. Between all ligands, competition in binding is observed. This indicates that all examined ligands bind to the same binding site. D-Mannose turned out to be the best binder of the ligands examined. Interestingly, D-mannose and GlcNAc have a higher binding affinity to hexokinase PII than the natural substrate D-glucose. The 6-phosphates were found to have a much lower affinity than the corresponding sugar substrates.

2.3. Dependence of the hexokinase activity on Mg^{2+}

For many kinases, it is known that Mg^{2+} is essential for their enzymatic reaction. Here we show for yeast hexokinase PII that enzyme activity persists even in absence of Mg^{2+}. The influence of Mg^{2+} on the enzymatic reaction of the hexokinase 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+} (Fig. 5). For example, for 50% conversion of D-glucose to D-glucose-6-phosphate in the absence of Mg^{2+}, about one hour was required, whereas in the presence of 0.1 mM Mg^{2+}, 50% conversion was reached after 4 min and the reaction was completed within 20 min. This demonstrates that the reaction rate is profoundly dependent on the Mg^{2+} concentration.

It is likely that Mg^{2+} influences the binding of ATP to hexokinase. Therefore, STD spectra for ATP binding to the kinase were recorded in absence and presence of Mg^{2+}. The resulting ATP epitopes show no change (Fig. 6), suggesting that Mg^{2+} has no significant influence on the binding mode of ATP. Moreover, these data show that ATP binds to hexokinase even in absence of glucose. Binding of ADP also occurs in the absence of a sugar ligand. No significant differences were observed for the binding epitopes of ADP and ATP, indicating that upon phosphorylation the binding mode of the nucleotide does not change. In order to prevent formation of insoluble magnesium phosphate, Tris buffer was used for all experiments. Repetition of the experiments in sodium phosphate buffer led to the same results, indicating that the buffer has no influence on the binding mode. Competitive titrations showed that in absence of Mg^{2+}, ADP binds slightly better to hexokinase than ATP. This preference is likely due to the lower negative charge of the diphosphate.

3. Discussion

Yeast hexokinase PII belongs to the family of hexokinases, which is part of the sugar kinase/heat shock protein 70/actin superfamily. High sequence identities show that all these proteins have a common ATPase domain.[20] The determined binding epitope of ATP bound to hexokinase PII revealed that the H-2 proton of the purine has the most intimate contact with the protein's proton surface. A similar binding epitope was found for ATP binding to ManNAc kinase[21] and GlcNAc kinase.[22] This indicates that the three-dimensional structure of the ATP binding site of these three en-

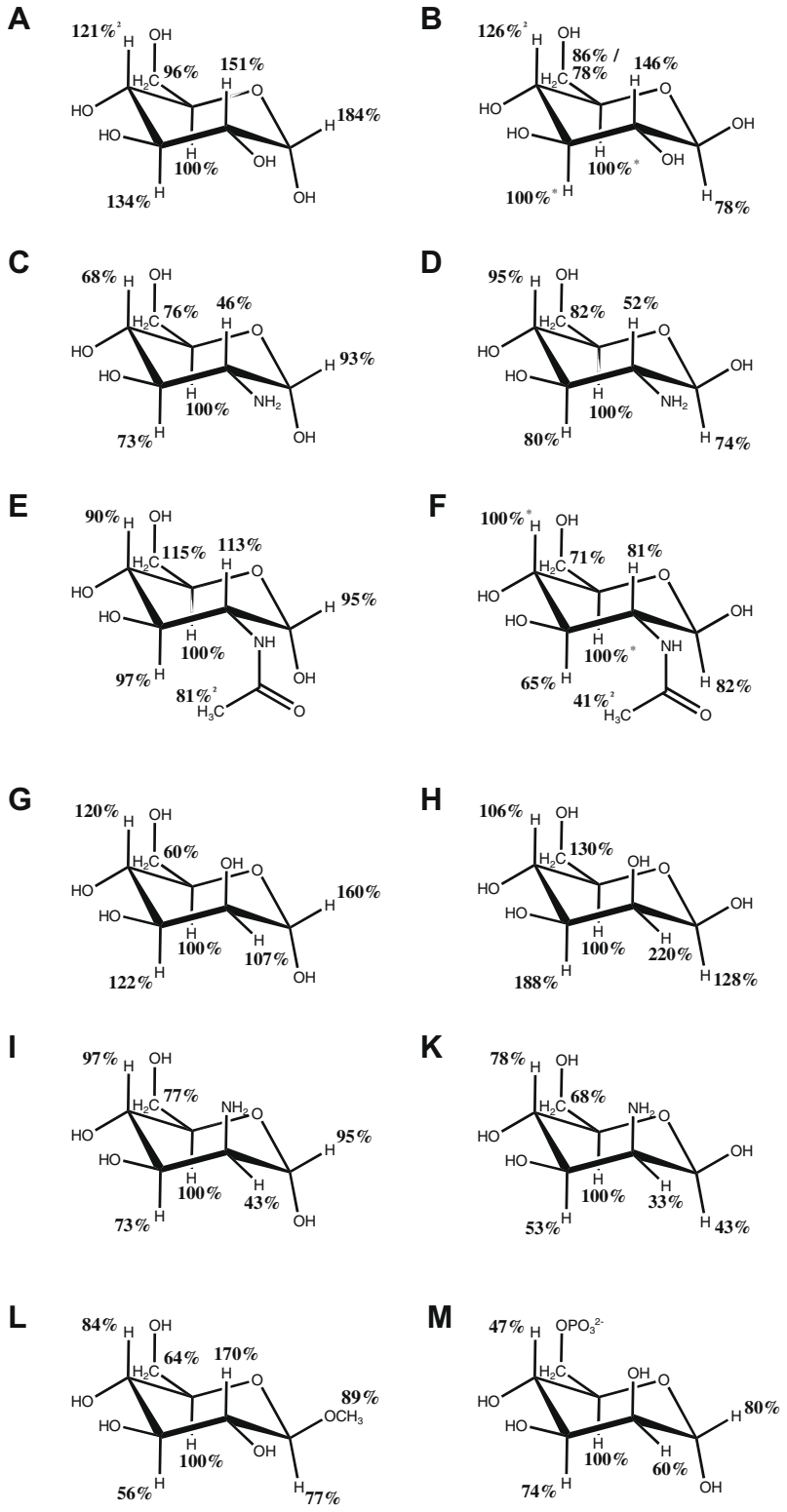

Figure 3. Binding epitopes of glucose derivatives as determined by ^{1}H STD NMR: α-D-glucose (A), β-D-glucose (B), α-D-glucosamine (C), β-D-glucosamine (D), α-D-GlcNAc (E), β-D-GlcNAc (F), α-D-mannose (G), β-D-mannose (H), α-D-mannosamine (I), β-D-mannosamine (K), β-D-*O*-methyl glucoside (L) and α-D-mannose-6-phosphate (M). 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 by rather against the STD effect observed for the H5 of each sugar. * denotes signal overlaps within one molecule and Δ denotes signal overlaps between α- and β-anomers. Signal intensities of α-D-*O*-methyl glucoside and β-D-mannose-6-phosphate were too weak for adequate quantification. Also signals from D-galactose, D-glucose-6-phosphate, and D-glucosamine-6-phosphate were too weak for quantification. Only the binding epitope of β-D-*O*-methyl glucoside is similar to that of β-D-glucose, and α-D-mannosamine is similar to α-D-glucosamine. Binding epitopes of all other ligands deviate.

zymes is very similar. Therefore these data show that within the family of sugar kinases, there is not only a high sequence identity for the ATPase domain, but the enzymes also have a common three-dimensional structure for the ATP binding site. On

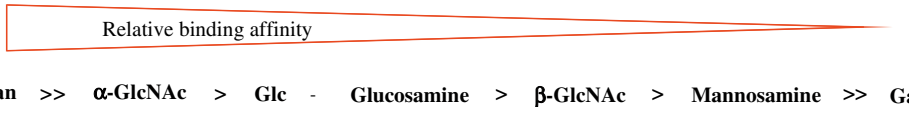

Figure 4. Schematic representation of relative binding affinities of D-glucose and derivatives. Competitive titrations show that all these ligands compete with each other and therefore bind to the same binding site. D-Mannose and α-D-GlcNAc have higher binding affinities than D-glucose, the natural substrate of hexokinase PII. '>>' denotes an STD signal intensity decrease of >90% for the ligand with lower affinity, '=' denotes an STD signal intensity decrease of 40-50%, indicating no strong affinity difference between the competing ligands.

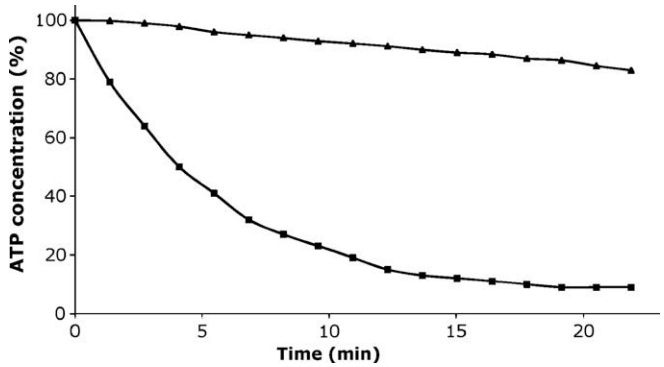

Figure 5. Intensity change of the ATP signals in the presence of glucose and yeast hexokinase PII with time. The filled triangles denote data obtained in the absence of Mg^{2+} and the squares in the presence of 0.1 mM Mg^{2+}. The data show that the enzyme is also active in the absence of Mg^{2+}, but the presence of Mg^{2+} increases the phosphorylation rate of hexokinase PII by about 13-fold.

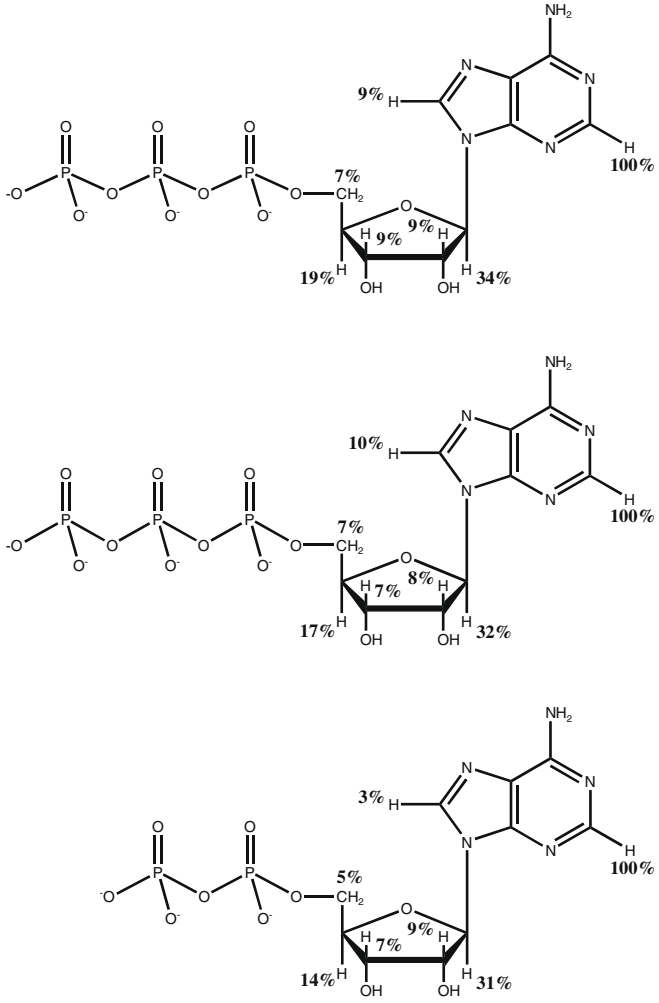

Figure 6. Binding epitopes as determined by ^1H STD NMR: ATP in absence of Mg^{2+} (top), ATP in presence of Mg^{2+} (middle), ADP in absence of Mg^{2+} (bottom). In the presence of Mg^{2+} the binding mode of ATP does not change significantly. Upon phosphorylation, the binding mode does not change, as the epitopes of ATP and ADP are similar.

the other hand, a comparison of the sugar binding epitopes, for example, of D-glucose bound to hexokinase PII, D-GlcNAc bound to GlcNAc kinase[22] and D-ManNAc bound to ManNAc kinase[21] revealed that the epitopes of D-glucose and D-GlcNAc show high similarities, whereas D-ManNAc has a completely different binding mode. These similarities highlight that the three-dimensional structure of the sugar binding site of hexokinase and GlcNAc kinase is similar, while ManNAc kinase has a different sugar binding site.

A comparison of the binding epitopes of the educt ATP and the product of the catalyzed phosphorylation reaction, ADP, bound to hexokinase, shows that the binding modes of these two ligands are the same. Competitive titrations reveal that in absence of Mg^{2+}, only slight differences in binding affinity for ATD and ADP can be detected. This indicates that during phosphorylation, the nucleotide binding site does not undergo any structural changes that affect nucleotide binding. In the absence of a sugar, hexokinase is in its open conformation. In this state, the ATPase domain on the large domain of hexokinase PII binds the adenosine ring, the ribose, and the α-phosphate moiety of the nucleotide.[9] When a sugar is present, the small domain closes the binding pocket, enabling hexokinase to bind the β- and γ-phosphate with the *phosphate 2* motif in the small domain. Hence, the enzyme cannot distinguish between ATP and ADP when no sugar is present, and therefore the binding mode and binding affinity of the nucleotides are the same. Similar observations were made with GlcNAc kinase.[23] For ManNAc kinase, on the other hand, changes in the nucleotide binding mode and binding affinity were observed during phosphorylation,[21] suggesting structural changes in this binding site during the enzymatic reaction.

Several kinases require Mg^{2+} as an essential cation for their enzymatic activity.[24] Here it is shown that yeast hexokinase PII has residual activity even in the absence of Mg^{2+}. Nevertheless, the addition of small amounts of Mg^{2+} to the buffer results in a 13-fold increase in enzyme activity. Interestingly, in contrast to other sugar kinases,[21] no change in the binding epitope of ATP was detected for the ribose protons close to the phosphates upon addition of Mg^{2+}. This corresponds well with trNOESY results obtained by Maity et al., which show that the conformation of the bound nucleotide does not significantly change when Mg^{2+} or glucose are added.[25] X-ray structures suggest that the activity increase may be due to the complexation of the triphosphate moiety with Mg^{2+}, which facilitates the nucleophilic substitution reaction. Pollard-Knight et al. have suggested that Mg^{2+} shields the negative charges of the β- and γ-phosphate groups of ATP and thus facilitates the nucleophilic attack of the hexose C-6 hydroxyl group on the γ-phosphate of ATP.[26] In contrast to the other studied kinases, no changes in the binding epitope of ATP were detected upon addition of Mg^{2+}, although the mechanism of

increasing the activity may be the same for all kinases as the enzyme activity is always increased 10- to 15-fold.

In contrast to the nucleotide, a comparison of the non-phosphorylated and phosphorylated sugars reveals that the binding epitopes are changed and that the binding affinity is drastically reduced for the phosphorylated form. Phosphorylation therefore changes the binding mode and the binding affinity of the sugar. This is consistent with the fact that during phosphorylation, some structural changes occur in the enzyme's sugar binding site.[9] Kuser et al. have suggested that product release of hexokinase PII is due to repulsive forces between the γ-phosphate transferred to the sugar and the β-phosphate of ADP.[9] Our data indicate that in addition to such repulsive forces, movements within the enzyme control product release, as the binding affinity of the 6-phosphate is drastically reduced and the binding mode is completely changed. Comparison of the binding epitopes of D-mannose and D-mannose-6-phosphate suggests that phosphorylation of the ligand prevents domain closure: In absence of a sugar substrate, yeast hexokinase is in its open conformation. The crystal structure shows that in the open form of the enzyme, a sugar substrate can be bound by the enzyme's large domain, which binds the sugar from below.[9] This is in agreement with relatively large STD effects observed for protons below the pyranose ring. If the bound sugar is not phosphorylated, it induces movements of the small domain to form the binding pocket. Inside the closed form of the enzyme, the upper side of the pyranose ring also gets into close contact with the protein surface, resulting in high STD effects of the protons above the sugar ring (Fig. 2). The phosphorylated sugar, for example, D-mannose-6-phosphate, shows relatively low STD effects for protons above the pyranose ring, indicating that it is not able to induce domain closure. This preference of the 6-phosphates for the open form of the enzyme and their very low binding affinities toward the enzyme are the driving forces for product release. Thus, the reduced binding affinity and the changed binding mode of the phosphorylated sugar are dictating the direction of the enzyme reaction, forcing the enzyme into an open form after phosphorylation has occurred. Furthermore, in the 'area' of the C-6, very low STD effects were observed, in the open as well as in the closed form, indicating a spacious binding pocket around this position, which is needed for phosphorylation of the 6-hydroxyl group of the sugar.

STD NMR spectra of nearly all ligands show that hexokinase PII binds the α- and β-anomers of the sugars, although with slightly different binding modes. Following the enzyme reaction, it is unambiguously clear that the enzyme predominantly produces the α-form of phosphorylated product and that mainly α-D-glucose is removed from solution. Interestingly, and in contrast to this only the β-D-O-methyl glucose is bound and phosphorylated by yeast hexokinase PII, whereas no binding has been detected for the α-form. Taken together, these results indicate that β-D-glucose has a higher binding affinity toward yeast hexokinase than the α-anomer. But due to steric hindrance or an energetically unfavorable orientation of β-D-glucose in the binding pocket, the α-D-glucose binding mode promotes phosphate transfer better than that of β-D-glucose, resulting in a higher phosphorylation rate of α-D-glucose. Although these experiments cannot absolutely clarify whether the enzyme exclusively converts the α-form, they clearly show the α-anomer is the preferred species for the enzymatic conversion. This confirms results from earlier studies, which have suggested a higher affinity for β-D-glucose and a higher reaction rate for α-D-glucose.[27,28] For the ManNAc kinase, it was found that the enzyme binds only the α-anomer and exclusively phosphorylates the α-form.[21] For the ManNAc kinase, which is part of the bifunctional UDP-GlcNAc 2-epimerase/ManNAc kinase, there is a strong evolutionary pressure for such an α-preference, since the epimerase function only produces α-ManNAc.[29] For hexokinase PII, however, there is no such pressure toward the α-form. It can be hypothesized that therefore hexokinase PII binds both anomers, but as α-D-glucose should be the favored anomer under cellular conditions, this could in turn be the preferred form to be phosphorylated by yeast hexokinase PII.

Interestingly, methylation of the anomeric hydroxyl group results only in the binding and phosphorylation of the β-anomer. α-D-*O*-Methyl glucoside is neither bound by the enzyme to a detectable degree nor phosphorylated. Taking into account the high α-preference of the enzyme, loss of α-D-*O*-methyl glucoside binding ability shows that the binding pocket is not very spacious in the area of the anomeric center, so that α-D-*O*-methyl glucoside cannot bind to the enzyme. On the other hand, the binding pocket has enough space to bind β-D-*O*-methyl glucoside and to phosphorylate it. It may also be possible that the proposed substrate recognition from below the pyranose ring is impossible in case of α-D-*O*-methyl glucoside. In the crystal structure of hexokinase PI in complex with glucose, residue Glu302 comes into close proximity of the area of the axial hydroxyl group and has been proposed to limit the space available for axial substitutions on C-1 of the sugar ligand.[30] Since Glu302 is conserved in hexokinase PII,[9] it can be speculated that similar steric clashes prevent binding of α-D-*O*-methyl glucoside to hexokinase PII. However, so far no crystal structure information is available for the active site of hexokinase PII in complex with a sugar ligand.

For further characterization of the sugar binding pocket, other naturally occurring sugars in a cell such as D-glucosamine, GlcNAc, D-mannose, and D-mannosamine were studied for their binding to yeast hexokinase PII. An insertion of an amino group or acetamido group at the C-2 changes the binding epitope of the α-form significantly, whereas the β-form binds only slightly differently. Epimerization and additional insertion of an amino group at the C-2 also changes the binding epitope significantly. Interestingly, although the binding modes of all these ligands have changed drastically, their binding affinities are still similar to that of the natural substrate D-glucose or even higher, except in case of D-mannosamine, where the affinity is reduced. Therefore, yeast hexokinase PII accepts a variety of modifications at C-2. On the other hand, the enzyme is much more stringent at C-4, as binding of the C-4-epimer galactose is hardly detected. Such a high selectivity at the C-4 was also found for related kinases, for example, ManNAc kinase.[21] This indicates a selection criterion for these kinases to differentiate their natural substrates from galactose-based sugars.

The STD NMR experiments show that yeast hexokinase PII has a very strong preference for D-mannose; at equimolar concentration, D-glucose is almost completely displaced by D-mannose. This is also reflected in a 50% lower K_m value for D-mannose compared to the natural substrate D-glucose and an in vitro phosphorylation rate of 80% (Roche AG, Germany). The strong D-mannose preference may be explained by energetically favorable interactions between the axial C-2-hydroxyl group and the small domain of hexokinase. Yeast is known to use mannose as a building block in its cell wall, although it is a rare sugar in its habitat. As mannose phosphorylation is very important for survival of the yeast cells, hexokinase may act as an 'emergency enzyme' in case of a phosphomannose isomerase loss, which is the enzyme that epimerizes glucose-6-phosphate into mannose-6-phosphate for cell wall biosynthesis. The determined D-mannose preference confirms results obtained from studies with thermolabile phosphomannose isomerase yeast mutants, in which cell wall synthesis was inactivated by loss of phosphomannose isomerase function, but reactivated again through addition of mannose, which indicates that other enzymes are able to perform mannose phosphorylation.[31] A hexokinase was postulated to be responsible for mannose phosphorylation. Due to its very high D-mannose affinity, it seems very likely that hexokinase PII phosphorylates D-mannose in this case.

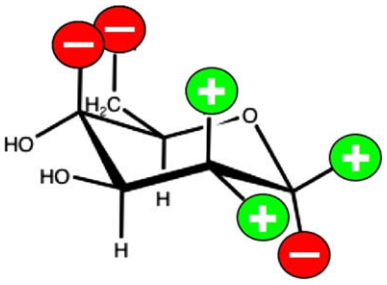

Figure 7. Allowed and prohibited modification sites on hexokinase PII sugar ligands. Introduction of a methyl group in the equatorial position at C-1 or an amino- or *N*-acetyl group introduced in axial or equatorial position at C-2 do not abolish detectable enzyme–ligand interactions (plus signs). Introduction of an axial hydroxyl group at C-1 or C-4, and introduction of a phosphate group at C-6 abolish ligand binding to hexokinase PII (minus signs).

A comparison of the crystal structures of hexokinase[9] and GlcNAc kinase[23] reveals a similar enzyme structure and ligand recognition mechanism. Both kinases consist of a small and a large domain, and the active site is formed by domain closure upon substrate recognition. Sugar substrate recognition occurs through the large domain, which forms hydrogen bonds with the sugar's C-1, C-2, and C-4 in case of GlcNAc kinase, and with C-1, C-3, and C-4 in case of hexokinase from below the pyranose ring. Initial ligand recognition is then followed by closure of the cleft between the large and small domain. Weihofen et al. showed that the substituent of the C-2 of the sugar substrate is important for domain movement and catalytic activity in GlcNAc kinase.[23] In case of hexokinase, we observed a strong preference for the C-2-epimer of its main substrate D-glucose, showing that in hexokinase, too, the C-2 of the sugar substrate affects ligand binding. These findings further support the hypothesis that both enzymes have very similar ligand binding mechanisms and might have overlapping catalytic functions in vivo.

To summarize, this study delivers information on structure-activity and structure-binding relationships of yeast hexokinase PII with its natural substrate D-glucose and its derivatives. For example, it was observed that yeast hexokinase PII shows high binding affinities to several other sugars, and therefore might act as a kind of 'emergency enzyme' in cells. Especially at the C-2 a number of variations are accepted, whereas modifications at the C-4 are not tolerated, being in agreement with the crystal structure. On the basis of this work it will be possible to design glucose analogues for metabolic engineering or inhibitors for the hexokinase, based on allowed and prohibited modification sites on its sugar ligands, as illustrated in Figure 7.

4. Experimental

4.1. Materials

Yeast hexokinase PII was purchased from Roche Diagnostics (Germany) as lyophilized powder. All ligands were obtained from Sigma (Germany) or Fluka (Germany).

4.2. Enzyme purification for NMR

For ^{1}H STD NMR experiments, deuterated buffers are required. Therefore 4.3 mg lyophilisate was resolved in 500 μL D_2O and dialyzed twice against D_2O for one hour to remove non-deuterated substances and solvents. The dialyzed protein was then applied to a PD-10 column (Pharmacia) and eluted using 5 mL of 50 mM sodium phosphate, 100 mM sodium chloride in D_2O, pH* 7.5, (uncorrected reading for the presence of $^2H^+$). Fractions of 5 drops were collected and protein concentration was determined using the Bradford assay. When binding modes of ligands were compared 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.

4.3. NMR experiments

STD NMR spectra were obtained at 5 °C using a Bruker DRX 500 spectrometer equipped with a triple resonance probe and *z*-gradients. Samples contained 15 μM protein and a ligand concentration of 1 mM. 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 6144 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 32,768 data points. Reference spectra were acquired using the same conditions, but with only 3072 transients. All ligand proton resonances were assigned under the same conditions as the STD NMR spectra through the use of ^{1}H–^{1}H TOCSY, ^{1}H–^{13}C HSQC, and ^{1}H–^{13}C HMBC spectra.

In order to determine the magnitude of the STD effect, the observed signal intensities were compared to the corresponding proton signal in the reference spectrum. An STD effect of 100% is therefore defined as the signals in both spectra having the same intensity. For group epitope mapping, the STD signals from all protons of one ligand were normalized against the strongest observed ligand proton STD signal. The strongest signal was assigned 100%; the others were normalized to this signal.

Titrations to determine relative binding affinities were performed under the conditions described above, by recording STD spectra in absence and then presence of a second ligand at a molar ratio of 1:1 with respect to the first ligand. Here, STD NMR spectra were acquired only with a total of 2048 transients and reference spectra with only 1024 transients. The observed STD signals were then plotted against the molar ratio of the ligands in order to determine which of the ligands had the higher affinity.

For the investigation of the enzymatic activities, samples with 1.3 μM yeast hexokinase PII and 5 mM glucose and 5 mM ATP were measured in the presence and the absence of 0.1 mM $MgCl_2$. Each time point consists of a 1D 'pulse and acquire' NMR experiment with eight scans for acquisition and eight 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 and glucose signals and product formation.

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References

1. Bork, P.; Sander, C.; Valencia, A. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7290–7294.
2. Kopetzki, E.; Entian, K.; Mecke, D. *Gene (Amst.)* **1985**, *39*, 95–102.
3. Gancedo, J. M.; Clifton, D.; Fraenkel, D. G. *J. Biol. Chem.* **1977**, *252*, 4443–4444.
4. Dynesen, J.; Smits, H. P.; Olsson, L.; Nielsen, J. *Appl. Microbiol. Biotechnol.* **1998**, *50*, 579–582.
5. Rodriguez, A.; de la Cera, T.; Herrero, P.; Moreno, F. *Biochem. J.* **2001**, *355*, 625–631.
6. Fernandez, R.; Herrero, P.; Fernandez, M. T.; Moreno, F. *J. Gen. Microbiol.* **1985**, *131*, 3467–3472.
7. Vojtek, A. B.; Fraenkel, D. G. *Eur. J. Biochem.* **1990**, *190*, 371–375.
8. Steitz, T. A.; Fletterick, R. J.; Anderson, W. F.; Anderson, C. M. *J. Mol. Biol.* **1976**, *104*, 197–222.

9. Kuser, P.; Krauchenco, S.; Antunes, O.; Polikarpov, I. *J. Biol. Chem.* **2000**, *275*, 20814–20821.
10. Valentine, W. N.; Oski, F. A.; Paglia, D. E.; Baughan, M. A.; Schneider, A. S.; Naiman, J. L. *N. Engl. J. Med.* **1967**, *276*, 1–11.
11. Magnani, M.; Stocchi, V.; Cucchiarini, L.; Novelli, G.; Lodi, S.; Isa, L.; Fornaini, G. *Blood* **1985**, *66*, 690–697.
12. Barrie, S. E.; Saad, E. A.; Ubatuba, S.; Da Silva Lacaz, P.; Harris, P. *Res. Commun. Chem. Pathol. Pharmacol.* **1979**, *23*, 375–381.
13. Vionnet, N.; Stoffel, M.; Takeda, J.; Yasuda, K.; Bell, G. I.; Zouali, H.; Lesage, S.; Velho, G.; Iris, F.; Passa, P.; Froguel, P.; Cohen, D. *Nature* **1992**, *356*, 721–722.
14. Gupta, B. L.; Nehal, M.; Baquer, N. Z. *Indian J. Exp. Biol.* **1997**, *35*, 792–795.
15. Willson, M.; Alric, I.; Perie, J.; Sanejouand, Y. H. *J. Enzyme Inhib.* **1997**, *21*, 101–121.
16. Vogtherr, M.; Peters, T. *J. Am. Chem. Soc.* **2000**, *122*, 6093–6099.
17. Mayer, M.; Meyer, B. *Angew. Chem., Int. Ed.* **1999**, *38*, 1784–1788.
18. Mayer, M.; Meyer, B. *J. Am. Chem. Soc.* **2001**, *123*, 6108–6117.
19. Meyer, B.; Peters, T. *Angew. Chem., Int. Ed.* **2003**, *38*, 1784–1788.
20. Bork, P.; Sander, C.; Valencia, A. *Protein Sci.* **1993**, *2*, 31–40.
21. Benie, A. J.; Blume, A.; Schmidt, R. R.; Reutter, W.; Hinderlich, S.; Peters, T. *J. Biol. Chem.* **2004**, *279*, 55722–55727.
22. Blume, A.; Berger, M.; Benie, A.J.; Peters, T.; Hinderlich, S. *Biochemistry* (*Nov. 12, Epub ahead of print*), **2008**.
23. Weihofen, W. A.; Berger, M.; Chen, H.; Saenger, W.; Hinderlich, S. *J. Mol. Biol.* **2006**, *364*, 388–399.
24. Kosow, D. P.; Rose, I. A. *J. Biol. Chem.* **1971**, *246*, 2618–2625.
25. Maity, H.; Jarori, G. K. *Eur. J. Biochem.* **1997**, *250*, 539–548.
26. Pollard-Knight, D.; Potter, B. V.; Cullis, P. M.; Lowe, G.; Cornish-Bowden, A. *Biochem. J.* **1982**, *201*, 421–423.
27. Bailey, J. M.; Fishman, P. H.; Pentchev, P. G. *J. Biol. Chem.* **1968**, *243*, 4827–4831.
28. Wurster, B.; Hess, B. *Eur. J. Biochem.* **1973**, *36*, 68–71.
29. Blume, A.; Benie, A. J.; Stolz, F.; Schmidt, R. R.; Reutter, W.; Hinderlich, S.; Peters, T. *J. Biol. Chem.* **2004**, *279*, 55715–55721.
30. Kuser, P.; Cupri, F.; Bleicher, L.; Polikarpov, I. *Proteins* **2008**, *72*, 731–740.
31. Payton, M. A.; Rheinnecker, M.; Klig, L. S.; DeTiani, M.; Bowden, E. *J. Bacteriol.* **1991**, *173*, 2006–2010.