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Studying non-covalent enzyme carbohydrate interactions by STD NMR

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Abstract—Saturation transfer difference NMR spectroscopy is used to study non-covalent interactions between four different glycostructure transforming enzymes and selected substrates and products. Resulting binding patterns represent a molecular basis of specific binding between ligands and biocatalysts. Substrate and product binding to *Aspergillus fumigatus* glycosidase and to *Candida tenuis* xylose reductase are determined under binding-only conditions. Measurement of STD effects in substrates and products over the course of enzymatic conversion provides additional information about ligand binding during reaction. Influences of co-substrates and co-enzymes in substrate binding are determined for *Schizophyllum commune* trehalose phosphorylase and *C. tenuis* xylose reductase, respectively. Differences between ligand binding to wild type enzyme and a corresponding mutant enzyme are shown for *Corynebacterium callunae* starch phosphorylase and its His-334→Gly mutant. The resulting binding patterns are discussed with respect to the possibility that ligands do not only bind in the productive mode.

Keywords: Binding-only conditions; Co-substrate binding; Glycostructures transforming enzyme; Point mutated enzyme; STD NMR

1. Introduction

Biocatalyzed transformations of glycostructures are well established in synthetic carbohydrate chemistry, glycobiotechnology, and glycobiology.[1–5] They find applications in research and development[1,2,6–8] and are widely used in industrial processes.[9,10] Glycosidases and glycosyltransferases find use in the synthesis of glycosides and oligosaccharides, whereas oxidoreductases, lyases, and ligases are basically applied to modify monosaccharides.[1,2,11,12] Such broad range of applications implies a general interest to understand molecular foundations of these diverse catalytic processes. For that purpose, it is necessary to get a detailed knowledge of substrate recognition to understand enzymatic effectiveness and to study roles of enzymes in cellular identification procedures. Classic methods analyzing recognition and binding of receptor proteins to ligands like enzyme-linked immunosorbent assays (ELISA)[13] and radioimmunoassays (RIA)[14] can, however, not be efficiently applied to investigate enzyme substrate binding as the bound substrate is transformed and dissociates from the enzyme as product.

During the last years saturation transfer difference NMR spectroscopy (STD NMR)[15,16] has been used as comprehensive and efficient method to investigate enzyme carbohydrate interactions in global as well as in site-specific fashion.[17–23] Only small amounts (>100 nmol) of non-isotope-labeled proteins are required as not the protein itself is analyzed, but rather magnetization is transferred from the protein to protons of a bound ligand and is detected after the ligand has dissociated from the enzyme.[15,16] The relative STD

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effects in the ligand enable determination of its non-covalent interactions in the enzyme binding pocket. Resulting binding pattern represents a molecular foundation of the specific ligand binding to the enzyme. We now present potentials and limitations of STD NMR to study the binding of carbohydrates to four different glycostructure transforming biocatalysts.

2. Experimental

2.1. Materials

All chemicals used were purchased from Sigma–Aldrich Chemical Co., St Louis, USA with the highest purity available and utilized without further purification. Materials for mutagenesis and protein purification were described elsewhere in more detail.[23]

2.2. Preparation of the biocatalysts

Procedures for protein production and purification have been described earlier in detail for the configuration retaining β-glucosidase from *Aspergillus fumigatus*,[21,24] *Candida tenuis* xylose reductase,[25] *Schizophyllum commune* trehalose phosphorylase,[26] and *Corynebacterium callunae* starch phosphorylase.[27,28] Mutagenesis, protein expression, and purification of the *Cc*StP His-334→Gly mutant have also been described earlier.[23]

2.3. NMR spectroscopy

Samples were prepared in 0.70 mL ^2H_2O (99.95%) and contained ~3.3–10.0 μM of the respective enzyme in phosphate, Tris, or MES buffer solution (10–50 mM) at a pH-value optimal for the respective enzyme catalyzed reaction. Prior to the addition to the NMR tube, the enzyme storage solution was gel-filtered using NAP 5 columns (GE Healthcare) equilibrated with the respective buffer in ^2H_2O. The narrow singulets from buffers did not interfere with the STD spectra. The respective saccharides were added in concentrations between 3.3 mM and 7.5 mM. The resulting 300–1000-fold excess of the ligands was necessary to keep decreasing substrate concentrations distinctly above the increasing product concentrations when transformations took place. Quite high dissociation constants of the studied enzyme–ligand complexes allowed magnetization of a large ligand excess during a saturation time of 2–3 s. Control experiments with significantly reduced excess of saccharides (50-fold) resulted in the same STD spectra with lower signal to noise ratio indicating that a higher ligand excess did not lead to interfering artifacts.

All ^1H and STD NMR spectra were measured on a Bruker (Rheinstetten, Germany) DRX-600 AVANCE spectrometer at 600.13 MHz using a triple resonance 5 mm inverse probe. Measurement temperature was 30 °C except otherwise stated and the recorded spectral width was 5.0 Hz. Chemical shifts were referenced to external acetone at 2.225 ppm and water suppression was not applied to avoid influences on signal intensities. STD spectra were recorded as described earlier.[21] A selective saturation of the protein was achieved by a series of 40 or 60 Gaussian shaped pulses of 50 ms length, each separated by a 1 ms delay leading to 2.04 s and 3.06 s measurement time. Intensity of the selective saturation Gaussian pulses was $\gamma B_1 = 68$ Hz and a 30 ms spin lock pulse was added after the π/2 pulse to eliminate protein frequencies. Subtraction of the STD spectra was performed via phase cycling and change of the irradiation frequency between on resonance ($\delta = -2.0$ ppm) and off resonance ($\delta = 41.66$ ppm or $\delta = -41.66$ ppm). Control experiments with 'on–on' and 'off–off' resonance as well as 'on–off' resonance STD spectra without protein gave null spectra, except residues from HDO and buffer signals, indicating artifacts from the subtraction of carbohydrate signals to be negligible. A total number between 48 scans and 512 scans were collected for each experiment depending on substrate transformation velocity and enzyme degeneration during the measurements. The resulting total measurement time was between 15 min and 3.0 h.

Intensities of all STD effects were calculated by division through integrals over the respective signals in ^1H NMR reference spectra. The largest STD effect in each sample was set to 100% and relative intensities were determined, as common for non-refined STD effects.[15,19,21,23] A complete relaxation and conformational exchange matrix (CORCEMA) analysis[29] was not sensibly applicable to calculate corrected absolute STD effects as not all factors influencing intermolecular saturation transfer were assignable. Namely the protein structures of the enzymes, K_D values, and conformations of bound ligands have not been available. Hence, sufficient comparisons of relative STD effects between the saccharides were possible, but absolute binding intensities could not be determined.

3. Results and discussion

STD NMR spectra were recorded under different measurement conditions to allow investigations of substrates and products bound under binding-only conditions as well as of products that pass through an intermediate state in the catalytic process and leave the enzyme for the first time. Influences of co-substrates and co-enzymes on substrate binding to the biocatalyst have also been studied. Additionally STD NMR has been applied to characterize structural disruption resulting from site-specific enzyme modification with respect to the caused effects. Figure 1 shows the different binding

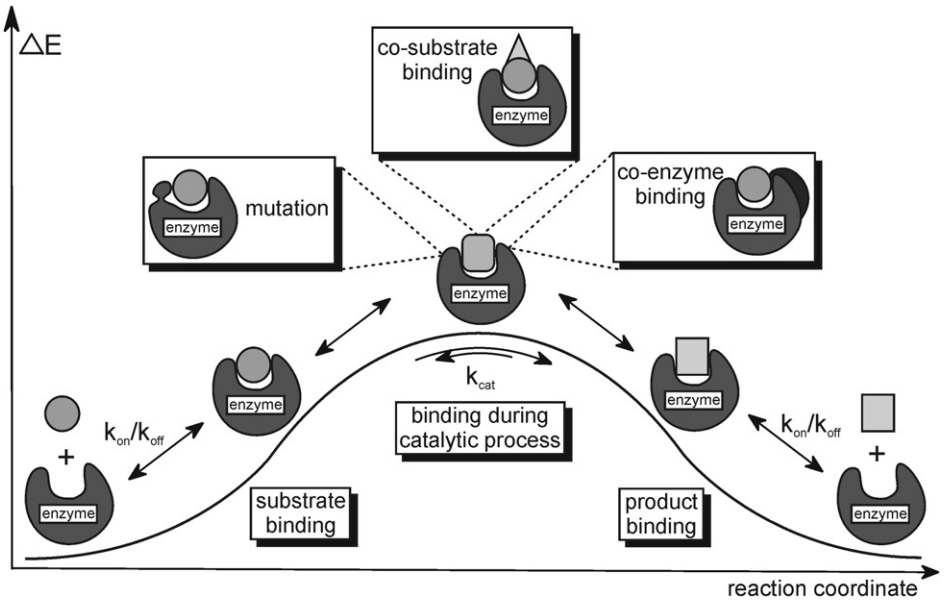


Figure 1. Analysis of binding situations during biocatalyzed reactions using STD NMR of the released substrates and products. Depending on measurement conditions a pure substrate or product binding as well as enzyme substrate interactions during the catalytic process can be monitored. Variations in the presence of co-substrates or co-enzymes allow studying differences between sole substrate binding and interactions in ternary complexes. Comparison of binding patterns between wild type enzymes and corresponding point mutants enables conclusions about variations of the active site.

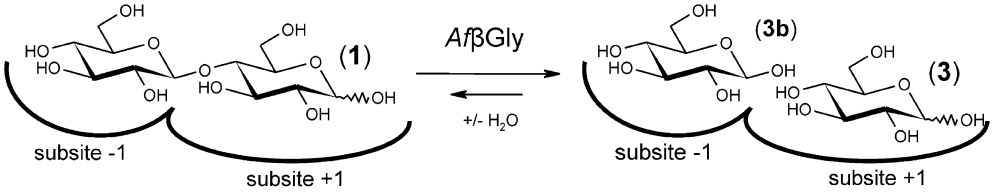
situations and the emerging questions in context of a reaction coordinate and highlights the investigated topics.

Enzyme–product binding is studied in a steady state with an equilibrium by far on the side of the products and an investigation of enzyme–substrate binding requires a low enzymic catalytic activity (k_{cat}) to keep the reaction beyond its thermodynamic equilibrium by kinetic hindrance. Furthermore, a high k_{on} of the substrate, a large k_{cat}, and a high k_{off} of the product enable STD NMR studies of compounds that are bound during catalytic process and leave the enzyme as product for the first time. The dissociation rate constants (k_{off}) of the detected ligands have to be in the range of 10^0–10^5 s^{-1}.[21]

3.1. Binding during catalytic processes

3.1.1. Configuration retaining β-glucosidase. Binding patterns are determined for disaccharide substrates and monosaccharide products interacting with a glycosidase from thermophilic *A. fumigatus* (*Af*βGly) possessing configuration retaining β-glucosidase (Scheme 1; [E.C. 3.2.1.21]) and β-galactosidase [E.C. 3.2.1.23] activities.[21,24] Studies of interactions between the enzyme and the substrate cellobiose (**1**) are performed at very moderate transformation rates reached at low temperature (10 °C). Comparison of STD NMR effects, which most likely originate from interactions at subsite +1, indicates the substrate cellobiose (**1**) and the substrate analogue lactose (**2**) to possess comparable relative STD effects on their glucopyranose leaving groups at 10 °C and 30 °C, respectively (Fig. 2). Rather weak effects of almost all glucopyranose protons of both compounds point out weak and unspecific binding in subsite +1, except for position 1. Further comparison of STD effects of cellobiose (**1**) and lactose (**2**) indicates different relative STD effects at H-1, H-3, and H-4 in their glycopyranoside moieties and reveals a slightly but globally altered binding of the *galacto*-configured substrate.[21]

The glucopyranoside moiety of cellobiose (**1**) shows reasonable STD effects indicating a distinct binding of



Scheme 1. *Af*βGly catalyzed hydrolysis of cellobiose (**1**) to glucose (**3**).[21,24] Binding sites of the enzyme are visualized and indicated according to the subsite nomenclature from Davies et al.[31]

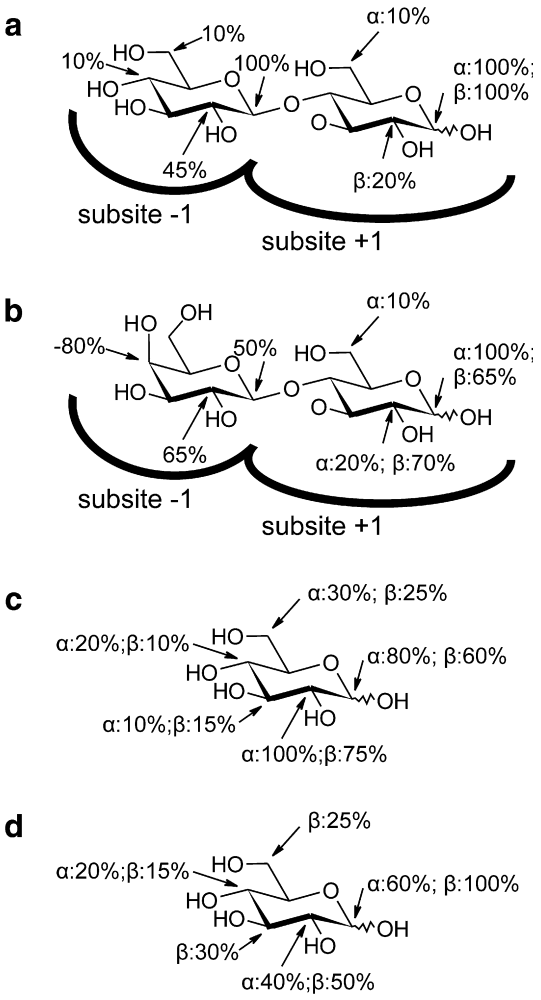


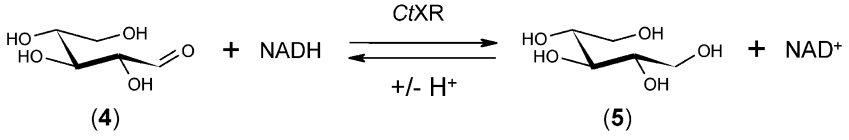
Figure 2. Relative STD effects of glucose (**3**), cellobiose (**1**), and lactose (**2**) bound to *Af*βGly. (a) Binding pattern of cellobiose (**1**) at 10 °C under binding-only conditions. (b) Binding pattern of lactose (**1**) at 30 °C under binding-only conditions. ('Negative' STD signal at H-4 is caused by an artifact.[21]) Interactions of glycopyranoside and glucopyranose moieties in subsites +1 and −1 are indicated for both the disaccharides. (c) STD effects of glucose (**3**) generated from cellobiose (**1**) at 30 °C reflect the non-covalent interactions present during the catalytic process. (d) Relative STD effects of free glucose (**3**) at 30 °C. Assignment of binding patterns to subsite +1 and subsite −1 in (c) and (d) is not possible, as glucose molecules are released from both subsites resulting in average STD effects.[21]

the non-reducing glucose unit in subsite −1. This site-specific binding pattern of the two glucose moieties is in good agreement with STD effects determined for the binding of other disaccharides to *Af*βGly as well as to *Pyroccoccus furiosus* β-glucosidase. All these binding patterns are also in good accordance with the wide glycopyranose and aglycon specificity as well as with the limited glycopyranoside acceptance of *Af*βGly and other β-glycosidases. The small STD effects in the glycopyranose unit point out weak and unspecific binding of this region, while the glycopyranoside shows strong interactions, which are likely a molecular basis for substrate selectivity of β-glycosidases. Binding of the product glucose (**3**) is investigated in the steady state of the reaction.[21,30]

At 30 °C, however, the binding pattern of cellobiose (**2**) to *Af*βGly cannot be determined. Only STD effects of glucose (**3**) were recorded, although the small amount of free glucose (**3**) cannot compete with cellobiose (**1**) for binding to the β-glucosidase at an early stage of the reaction. This indicates cellobiose (**1**) to be faster hydrolyzed to glucose (**3**) than it dissociates from the enzyme. Consequently, the generated two glucose molecules leave *Af*βGly and their binding pattern can be investigated by STD NMR (Fig. 2).[21] However, a differentiation between binding in the two subsites cannot distinctly be made, as glucose (**3**) dissociates from both the binding sites.

3.1.2. Xylose reductase. NADH consuming reduction of open chained xylose (**4**) to xylitol (**5**) is catalyzed with very high catalytic efficiency (680,000 $M^{-1} s^{-1}$) by NAD(P)H dependent *C. tenuis* xylose reductase (*Ct*XR; [E.C. 1.1.1.21]) as shown in Scheme 2.[32] Such high transformation rate leads to xylose (**4**) reduction before dissociation from the enzyme, as xylose (**4**) is a sticky substrate, implying that it is reduced faster than it dissociates from the active site.[25,33] At an early stage of xylose (**4**) transformation the small amount of generated free xylitol (**5**) is not able to rival with high quantities of xylose (**4**) for binding to *Ct*XR. Reasonably large xylitol (**4**) signals in the STD spectrum recorded during this period hence show a binding pattern of the formed product, which leaves the enzyme for the first time (Fig. 3b). The resulting xylitol (**5**) binding pattern provides an insight into non-covalent *Ct*XR-ligand interactions during the catalytic event. Simultaneously the binding pattern of NADH and newly formed NAD^+ can be determined. The latter is giving additional information about non-covalent interactions in the catalytically competent ternary complex (Fig. 3).

At later stages of the reaction a larger NAD^+ quantity is formed, which competes with NADH for binding to *Ct*XR and allows the formation of ternary complexes



Scheme 2. *Ct*XR catalyzed and NADH consuming reduction of xylose (**4**) to xylitol (**5**).[32]

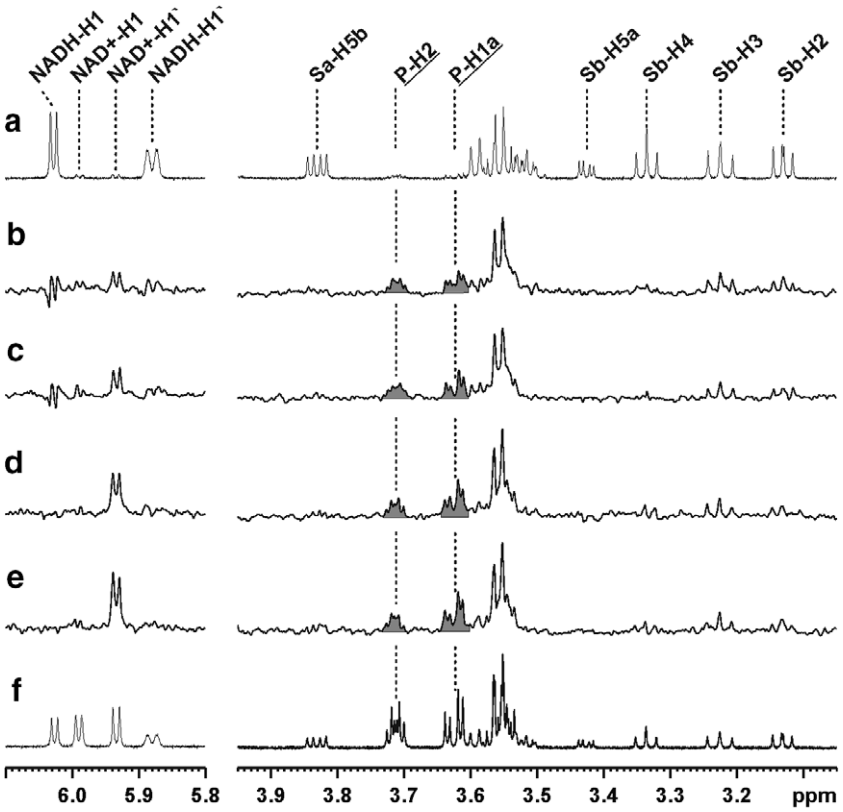
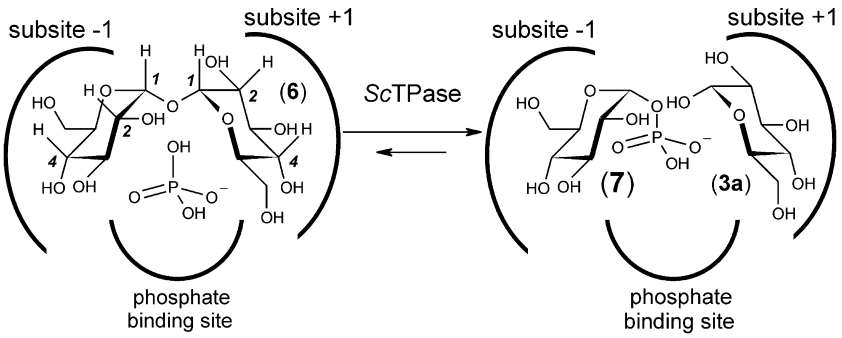


Figure 3. STD NMR spectra recorded during *Ct*XR (10 μM) catalyzed and NADH (10 mM) consuming reduction of xylose (10 mM, **4**) to xylitol (**5**). After a ^{1}H NMR spectrum recorded at the start (a) four STD NMR spectra were measured sequentially, each over 15 min (b–e) prior to measurement of a final ^{1}H NMR spectrum (f). Representative STD NMR signals of NADH are the anomeric protons (NADH-H1 and NADH-H1′), both showing quite low STD effects, which disappear during consumption of NADH within the reaction. Corresponding anomeric protons of NAD^{+} (NAD^{+}-H1 and NAD^{+}-H1′) possess STD effects with very different intensities both increasing during NAD^{+} production. STD effects of the open chained substrate can only be determined in form of rapidly generated α- and β-xylopyranose, represented by several signals, in particular Sa-H5b, Sb-H5a, Sb-H4, Sb-H3, and Sb-H2. These protons show relatively weak STD effects caused by effective reduction of xylose (**4**) before it is released from the enzyme in larger amounts. Their intensities are further reduced during the substrate consumption. Protons of the product xylitol (**5**), in particular P-H2 and P-H1a, show pronounced STD effects from the early beginning of the measurement (indicated in gray and by dashed lines). Such high STD effects are caused by newly formed xylitol (**5**), which is released from the enzyme for the first time. Different increases of P-H2 and P-H1a STD effects appearing during progress of the reaction are likely caused by additional STD effects of xylitol (**5**) forming ternary *Ct*XR–NADH–xylitol and *Ct*XR–NAD^{+}–xylitol complexes.

possessing different binding patterns. Therefore, the STD effects of xylitol (**5**) and xylose (**4**) as well as of NADH and NAD^{+} show changes during the progress of the transformation (Fig. 3b–e). These variations, however, cannot be assigned to a single ternary complex, as several different complexes are formed in parallel. Only STD effects of the independently generated catalytically incompetent *Ct*XR–NADH–xylitol and *Ct*XR–NAD^{+}–xylose complexes have been studied, which allow an insight to the co-enzyme dependent binding of substrates and products (data not shown).

3.2. Influence of co-substrates in binding

S. commune trehalose phosphorylase (*Sc*TPase; [E.C. 2.4.1.231]) is an α,α-trehalose (**6**) degrading enzyme, which forms α-glucose 1-phosphate (**7**) and α-glucose (**3a**). It catalyzes reversible glucosyl transfer from α,α-trehalose (**6**) to phosphate under retention of the anomeric configuration as shown in Scheme 3.[26,34] Despite intensive investigations, the mechanism of this transformation has not been univocally determined to be double displacement-like or S_Ni-like. Some recent binding studies show that α,α-trehalose (**6**) binding strongly depends on the presence of a phosphate ion in the active site and that the transformation only occurs from the resulting ternary complex. Such pronounced influence of phosphate on binding makes *Sc*TPase an interesting candidate for STD NMR studies of co-substrate binding influences. α,α-Trehalose (**6**) binding is hence investigated in presence and absence of vanadate ions, which substitute phosphate in the binding site and do not lead to the glucosyl transfer although binding in the productive mode is nearly reached.[26] Additional binding of the product glucose (**3**) is studied without and with phosphate and vanadate ions being present in the binding site.



Scheme 3. *Sc*TPase catalyzed reversible glucosyl transfer from α,α-trehalose (**6**) to phosphate yielding α-glucose 1-phosphate (**7**) and α-glucose (**3**).[26,34] Binding of substrates and products in the enzyme binding sites is indicated and protons in positions 1, 2, and 4 of α,α-trehalose are shown.

Site-specific α,α-trehalose interactions are difficult to determine as the two symmetrically arranged glucose units bind in different binding sites. Therefore, only average STD effects of protons in each position can be measured. However, a vanadate ion bound in the phosphate binding site leads to a quite pronounced decrease of all α,α-trehalose STD effects, likely caused by tight substrate binding.[15,26] Accompanied variations of STD effects relative to each other can only be determined for protons in positions 1, 2, and 4 (Table 1a). These protons are located opposed to the position of the vanadate (or phosphate) anion in the active site. Their pronounced STD effects indicate definitive non-covalent enzyme α,α-trehalose interaction in this region (Scheme 3).[26] The observation that vanadate influences the pattern of trehalose binding is in agreement with the notion that substrate binding in the productive mode of the ternary vanadate containing complex is different from the general enzyme α,α-trehalose binding. However, detailed site-specific differences of defined protons cannot be determined.

Co-substrate depending differences are even more pronounced in non-covalent glucose *Sc*TPase interactions. In the absence of any co-substrate the product α-glucose (**3a**) as well as β-glucose (**3b**) shows STD effects (Table 1b). Each anomer represents an average over the binding pattern in both the subsites. Binding patterns of α- and β-glucose (**3a**,**b**) can hence be determined separately, although a detailed picture of their site-specific interaction is not visible. Addition of phosphate or vanadate ions keeps β-glucose (**3b**) STD-effects by far unchanged indicating that the co-substrate does not alter its binding, which is anyway not productive. STD effects of the product α-glucose (**3a**), however, suffer an entire decrease likely caused by its very tight binding in subsite +1 in presence of the co-substrate, which has been reported earlier.[26]

Variations of STD effects between the presence of phosphate and vanadate are small and indicate that both anions cause quite similar binding situations, being in good agreement with previous results.[35] The inhibitory effect of vanadate ions is, therefore, not entirely gained by an influence in binding, which reinforces vanadate to be an excellent model for binding studies at *Sc*TPase. Conclusions about α-glucose (**3a**) binding pattern in subsite +1 while α-glucose 1-phosphate (**7**) is also present in the active site cannot be drawn as differences between phosphate and α-glucose 1-phosphate binding are quite pronounced. However, STD NMR indicates that the binding of α,α-trehalose (**6**) and α-glucose (**3a**)

Table 1. Relative STD effects of α,α-trehalose (**6**) and glucose (**3**) bound to *Sc*TPase in the presence and absence of vanadate and phosphate ions bound in the phosphate binding site

Compound		H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
(a)								
α,α-Trehalose (**6**)		90	30	30	100	20	40	nd[a]
α,α-Trehalose (**6**) and vanadate[b]		100	10	30	90	10	20	nd
(b)								
Glucose (**3**)	α	60	20	20	10	5	15	15
	β	60	100	60	30	5	30	nd
Glucose (**3**) and vanadate[b]	α	10	10	20	20	5	20	10
	β	80	100	70	40	5	20	nd
Glucose (**3**) and phosphate[b]	α	5	10	20	30	10	20	10
	β	100	80	100	60	5	70	nd

[a] nd: not determined due to overlap with artifacts caused by signals from the MES buffer.
[b] Signal/noise ratios of all STD signals in α,α-trehalose (**6**) and α-glucose (**3a**) are about four times weakened in the presence of vanadate or phosphate ions, likely caused by tight binding of the ligands (see main text).

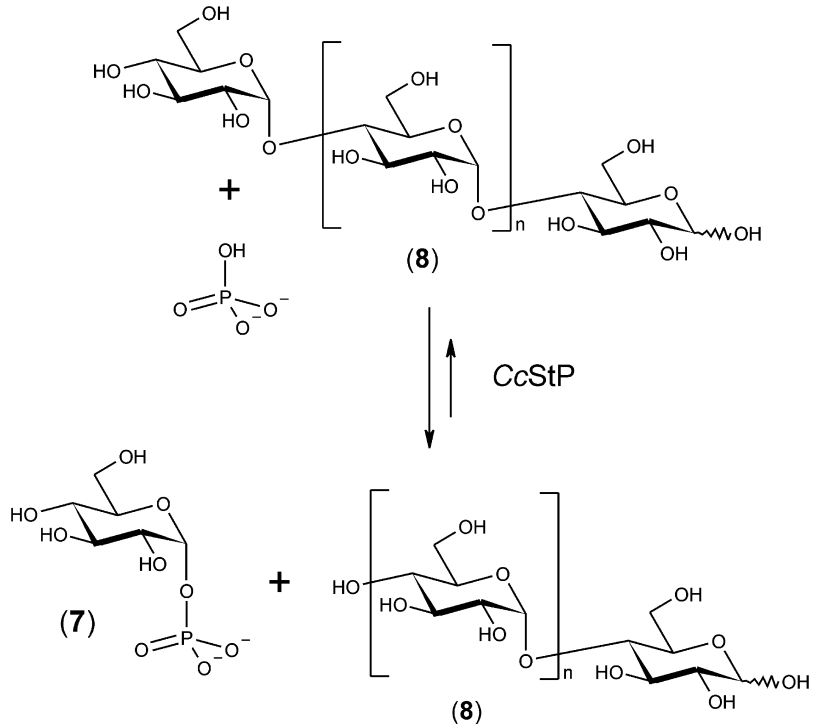
to *Sc*TPase depend on additional binding of a co-substrate. This result is very helpful to understand the enzyme catalyzed reaction in more detail.

3.3. Binding to a point mutated enzyme

Corynebacterium callunae starch phosphorylase (*Cc*StP) is a configuration retaining pyridoxal 5′-phosphate dependent glycosyltransferase [E.C. 2.4.1.1], which catalyzes the reversible α-glucose 1-phosphate (**7**) formation by phosphorolysis of maltodextrins or starch (**8**, Scheme 4).[27,28] Its amino acid His-334 is part of the binding pocket and inheres an important role in binding and stabilization of the transition state during the reaction as it forms a hydrogen bond between its imidazole ring and the hydroxy group at position 6 of the transferred glucosyl residue. A mutation by substitution of the His-334 by Gln or Asn causes an up to 150-fold reduction of the catalytic efficiency, while the His-334→Gly mutant was by far 2000-fold less active compared to the wild type enzyme.[23,28] Such remarkable loss of activity might be caused by the inserted Gly residue, which does not provide any functionality for substrate binding and gives additional space for substrate and product binding in the productive mode of the mutant enzyme. Differences in ligand binding between the wild type enzyme and the His-334→Gly mutant have hence been determined by STD NMR using α-glucose 1-phosphate (**7**) and α-xylose 1-phosphate (**9**) as exemplary ligands.

α-Glucose 1-phosphate (**7**) possesses quite similar binding patterns to the two enzymes. Largest effects are found for the proton at position 4 in both cases and STD effects of all protons in position 1, 2, 3, and 5 show only very minor differences between the enzymes. However, there are small but distinct differences for the two protons at position 6a and b, which possess a higher STD effect when bound to the wild type enzyme (Fig. 4).[23] Binding pattern of α-xylose 1-phosphate (**9**) shows a similar picture for the protons H-2, H-3, and H-4. Again the largest STD effect is in both cases at position 4 and variations at position 2 and 3 are quite low. However, the proton in position 1 as well as the 5eq-proton show larger STD effects when bound to the His-334→Gly mutant, while the STD effect of the 5ax-proton remains unaffected from the mutation.[23]

These differences clearly indicate changes in binding of the hydroxy group at position 6 in α-glucose 1-phosphate (**7**). Lack of the imidazole moiety at the position of the substituted His residue leads to modified binding at position 6 but does influence the binding at all other positions in negligible amounts. The increased STD effects at H-1 and H-5eq of α-xylose 1-phosphate (**9**) denote a slightly modified binding, likely caused by the presence of additional solvent molecules (^2H_2O) in the binding site. These findings show the His-334→Gly mutant to have sufficient space in the active site to bind the natural substrate. However, the missing interaction to the His-334 residue indicates binding in a non-productive



Scheme 4. *Cc*StP catalyzed phosphorolysis of the terminal maltodextrin saccharide unit to yield α-glucose 1-phosphate (**7**) and a one-unit shortened maltodextrin (**8**).[27,28]

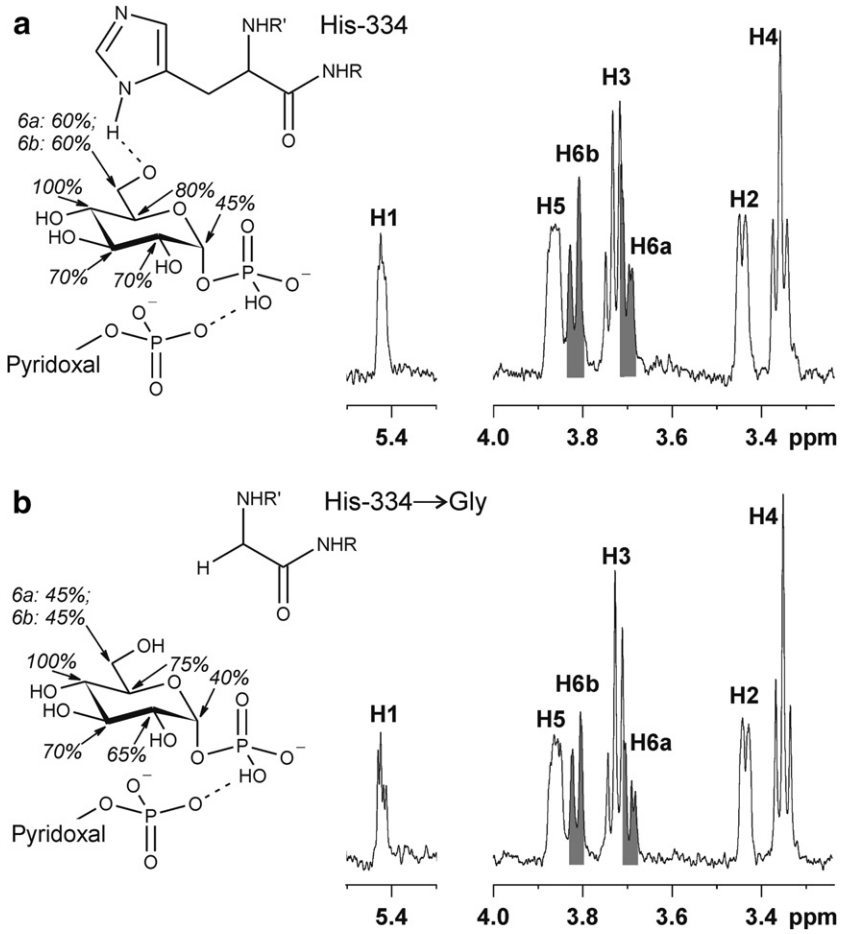


Figure 4. Schematic and simplified binding of α-glucose 1-phosphate (**7**) forming a ternary complex with pyridoxal 5′-phosphate and (a) *Cc*StP wild type enzyme or (b) the corresponding His-334→Gly mutant enzyme, respectively.[23,28] Relative STD effects are given for all protons of α-glucose 1-phosphate (**7**) in both complexes. Signals of H-6a and H-6b are indicated in the STD spectra as the only signals showing reasonable different STD effects caused by the His-334→Gly mutation.

mode, which very likely causes the entirely reduced catalytic activity of the His-334→Gly mutant.[23]

4. Conclusion

STD NMR owns a large potential as applicable method to investigate binding of substrates, products, and intermediates to glycostructure transforming enzymes under varying conditions. It enables access to numerous details of ligand binding states under binding-only conditions as well as during catalytic processes. The method is limited when potential ligands are bound in two different subsites or under different binding conditions and consequently a mixed binding pattern is provided. Furthermore, it does not give direct information about the enzyme, as only data of the dissociating ligands are applicable. STD NMR spectra should hence not be used as a single analytical method to analyze the complex binding procedure during a biocatalyzed reaction. However, the information can be achieved incorporating additional gained knowledge of the enzyme structure and dynamics. Concluding STD NMR finds its place as a valuable and powerful method in the canon of other techniques analyzing enzyme catalyzed reactions of saccharides and glycosides.

Acknowledgments

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