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Saturation–transfer–difference NMR to characterize substrate binding recognition and catalysis of two broadly specific glycoside hydrolases

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Abstract

Saturation–transfer–difference NMR spectroscopy (STD NMR) is used to delineate noncovalent enzyme–substrate interactions of β-glycosidases from *Pyrococcus furiosus* and *Aspergillus fumigatus* under binding-only conditions at low temperatures, and during catalysis. Glucopyranosyl and galactopyranosyl moieties display a distinct pattern of multiple contacts with each active site, revealing enzyme-specific elements of recognition and portraying the global binding effect caused by single-site modification of the substrate, at carbon 4. The glucopyranose leaving group of cellobiose or lactose shows small relative STD effects except for the anomeric carbon, particularly in the α-form. Its replacement in β-glucosides by an alcohol leaving group strongly affects sugar binding in the proximal enzyme subsite. A combination of STD effects of substrate and product, produced by the catalytic event or added exogenously, characterizes subsite binding during cellobiose hydrolysis.

Keywords: Enzyme–substrate interaction; Glycoside hydrolysis; β-Glycosidase; Noncovalent binding recognition; STD NMR

1. Introduction

Glycoside hydrolases (glycosidases) are widely used catalysts in carbohydrate biotechnology offering high chemo- and stereoselectivities and relaxed substrate specificity as a much desired combination of properties [1–6]. Although harnessed for numerous practical applications, the broad substrate acceptance of glycosidases often lacks a clear molecular interpretation, especially in the cases when crystal structures of enzyme–substrate complexes are unknown. Comprehensive mapping of enzyme–glycoside interactions, using a multitude of natural and artificial substrate analogues or point mutants of the enzyme, can clarify how specificity is achieved, but is laborious [4].

Saturation–transfer–difference NMR spectroscopy (STD NMR) is now introduced as a powerful and time-efficient method to characterize substrate binding recognition of glycosidases. This technique requires only small amounts (>100 nmol) of unlabeled protein and is independent of a high resolution protein structure. The principle of STD NMR is that small ligands bound to a comparably large protein exhibit a nuclear Overhauser effect (NOE), whereby magnetization is transferred from protons of the protein to spatially close protons of the ligand. The transferred magnetization can be measured after the ligand has dissociated from the protein [7]. Ligand protons, which chemically exchange with protons or deuterium atoms from the solvent cannot be detected. For that reason contributions of hydroxyl groups to binding cannot directly be observed.

The particular capability of STD NMR to detect small molecules in solution after their dissociation from a protein complex has recently been used in biomedicine to analyze signal transduction, cellular recognition, and drug–receptor interactions [7–9]. However, STD NMR has only rarely been applied in biocatalysis to examine binding of substrates, products, inhibitors, or cofactors to enzymes [10–15]. In this paper, the application of STD NMR is demonstrated using two broadly specific, configuration-retaining β-glycosidases from *Pyrococcus furiosus* (CelB) [16–17] and *Aspergillus fumigatus* BG1 (AfβGly) [18] as model carbohydrate-active enzymes. Both enzymes are fairly stable and possess mainly β-glucosidase [EC 3.2.1.21] but also, to some extent β-galactosidase [EC 3.2.1.23] activities.

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2. Experimental

2.1. Chemicals

All chemicals were purchased from Sigma–Aldrich Chemical Co., St. Louis, USA in the highest available purity and used without further purification.

2.2. NMR spectroscopy

Samples were prepared in 0.65 mL D_2O (99.95%) and contained ~7.5 μM of the respective β-glucosidase in a Tris solution (25 mM; pH 7.0). The narrow singulet from Tris overlapped only a negligible spectral region of the carbohydrate signals in ^{1}H NMR spectra and did not interfere with the interpretation of the STD spectra. In case of CelB, 7.5 mM of the respective saccharide were added, leading to a 1000-fold excess of the ligand. For measurements with AfβGly 3.3 mM of the respective saccharide were added to give a molar ligand to protein ratio of 440. These fairly high ligand excesses were necessary to keep decreasing substrate concentration distinctly above increasing product concentration, when substrates were transformed during the STD-measurements. The excess assured products not to compete seriously with substrates for binding to the enzymes. Reasonably high dissociation constants (K_D) of enzyme–ligand complexes furthermore allowed a magnetization of a large ligand excess during a saturation time of 2–3 s, which resulted in a shorter measurement time. Control experiments with significantly reduced excess of saccharides (50–200-fold) led to the same STD spectra with lower signal to noise (S/N) ratio and indicated that a 500–1000-fold ligand excess did not cause interfering artifacts.

The ^{1}H-spectra were measured on a Bruker DRX-600 AVANCE spectrometer at 600.13 MHz using a triple resonance 5 mm inverse probe. All spectra were recorded at 30 °C except otherwise stated. The chemical shifts were referenced to external acetone at 2.225 ppm. No water suppression was used to avoid influences to intensities of signals from anomeric protons, which were close to water signal. For recording STD spectra a selective saturation of the protein was achieved by a series of 2, 10, 20, 30, 40, and 60 Gaussian shaped pulses of 50 ms length, each separated by a 1 ms delay. The total irradiation times were 0.102, 0.51, 1.02, 1.53, 2.04, and 3.06 s, respectively. They all led to the same STD-spectra with, however, increasing S/N ratio, as reported earlier for an even distinctly larger variation of saturation times [19]. The intensity of the selective saturation Gaussian pulses was $\gamma B_1 = 68$ Hz. A 30 ms spin lock pulse was added after the $\pi/2$ pulse to eliminate protein frequencies. This spin lock did not lead to detectable spin diffusion in free ligands and did not influence the intensity of STD signals. Subtraction of the STD spectra was performed during the measurement via phase cycling and change of the irradiation frequency. The on resonance irradiation was performed at $\delta = -2.0$ ppm as irradiation at $\delta = 7.4$ ppm caused slight magnetization of α-anomeric protons ($\delta = \sim 5.2$ ppm). Off resonance irradiation was made at $\delta = 41.66$ ppm and $\delta = -41.66$ ppm. Control experiments with phase cycling "on–on" and "off–off" resonance as well as normal "on–off" resonance STD spectra without protein gave null spectra, except residues from overwhelming HDO and Tris signals. This indicated artifacts from subtraction of carbohydrate signals to be negligible. A total number of 512 scans was collected for each experiment using a spectral width of 5.0 Hz. In case of cellobiose transformation by AfβGly at 30 °C, a total of 256 scans were recorded. Reference ^{1}H NMR spectra were recorded with 256 and 128 scans, respectively, directly before and after the STD measurements to ensure a reasonably low substrate transformation.

2.3. Calculation of relative STD effects

For calculation of the STD effects intensities of all signals in the STD spectra were divided by the intensities of the respective signals in the reference spectra. Not all factors influencing intermolecular saturation transfer were assignable; namely K_D values, conformation of bound ligands, and protein structure of AfβGly. For this reason a complete relaxation and conformational exchange matrix (CORCEMA) analysis [20–23] was not sensibly applicable to calculate corrected absolute STD effects, which would have allowed a detailed comparison of the binding strength of different saccharides. Hence the largest STD effect in each sample was referenced to 100% and relative intensities were determined, as common for non-refined STD effects [7,8,12,13,15]. This allowed a well sufficient comparison of relative STD effects between the saccharides for an analysis of relative binding epitopes, but did not enable a comparison of absolute binding intensities.

3. Results and discussion

3.1. STD NMR in biocatalysis

The presented application of STD NMR allows to investigate binding of substrates and products to isolated enzymes using two slightly different approaches. Enzyme–product binding can be studied directly from an enzyme-containing product solution, when the dissociation rate constant (k_{off}) is in the range of 10^0–10^5 s^{-1} and the back reaction is negligible. The second approach is an investigation of enzyme–substrate binding, which requires a similar k_{off} rate of the substrate and a comparably rather small transformation rate. The latter condition can be fulfilled by suitable lowered temperature causing low enzymic catalytic center activity (k_{cat}) as well as a slight improvement of spectral quality as reported earlier [14,24]. These conditions allow the substrate to dissociate from the enzyme much faster than it reacts to products. An additional third approach requires a transformation of the substrate before it can release from the enzyme in larger amounts. This condition should enable STD NMR of products that passed through an intermediate state and leave the enzyme for the first time. Extensive transformation has to be avoided to keep the product concentration rather low. These three approaches provide STD NMR with the ability to illustrate, in global and site-specific fashion, how protein-derived noncovalent contacts with the substrate contribute to binding recognition and catalysis.

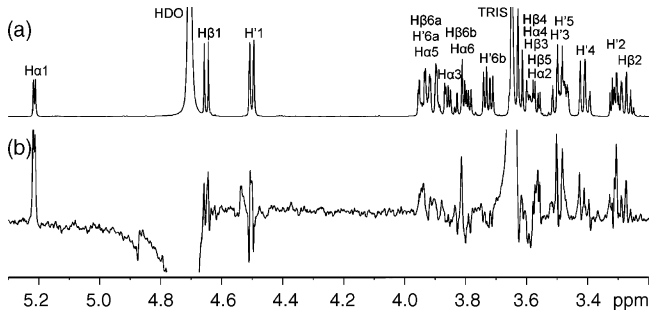

Fig. 1. STD spectrum of cellobiose in the presence of CelB (b) and the corresponding ^{1}H NMR spectrum of cellobiose (a). All signals of both anomeric forms are indicated.

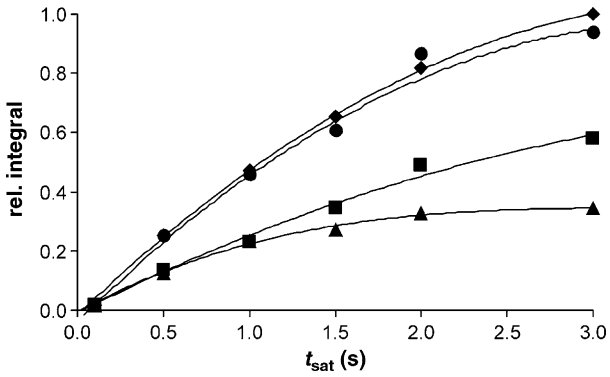

Fig. 3. Saturation time (t_{sat}) depending intensities of STD signals from glucose bound to CelB at 30 °C. Shown are the signal increment of H-1 and H-2 of β-glucose (rhomb and square, respectively), of H-2 of α-glucose (triangle), and summed H-6a and H-6b of both glucose anomeres (circle).

3.2. Binding studies for CelB

CelB is hyperthermophilic with an optimum temperature of >90 °C and thus well suited for STD NMR studies at 30 °C where it is barely active. Its catalytic efficiency for cellobiose hydrolysis is $9.4 \pm 1.5\ \mu M^{-1}\ s^{-1}$ at 30 °C, compared to $8.4 \pm 0.05\ mM^{-1}\ s^{-1}$ at 70 °C. Fig. 1 shows STD spectra of cellobiose in the presence of CelB at 30 °C together with the corresponding ^{1}H NMR spectrum as reference. In Fig. 2 the relative STD effects of the ligands investigated are presented. Fig. 3 shows the amplification of relative STD effects in dependence of increasing saturation time for four glucose protons, indicating that influences from relaxation and spin diffusion during the irradiation are rather small. These results indicate that no detectable transformation of magnetization between the protons occurs. The STD effects can therefore be definitely assigned to protons in each position of the saccharides.

The glucopyranoside moiety of cellobiose shows strong STD effects of most protons indicating a spatial closeness of these saccharide protons to enzyme (Fig. 2a). This proximity portends a noncovalent binding of their geminal or vicinal hydroxyl groups to functional groups of the protein. Although the binding strength is not directly proportional to the STD effects, the glucopyranoside is most probably bound tightly to subsite −1 of the enzyme. Sugar-binding subsites are numbered according to Davies et al. [25] where the catalytic site is located between

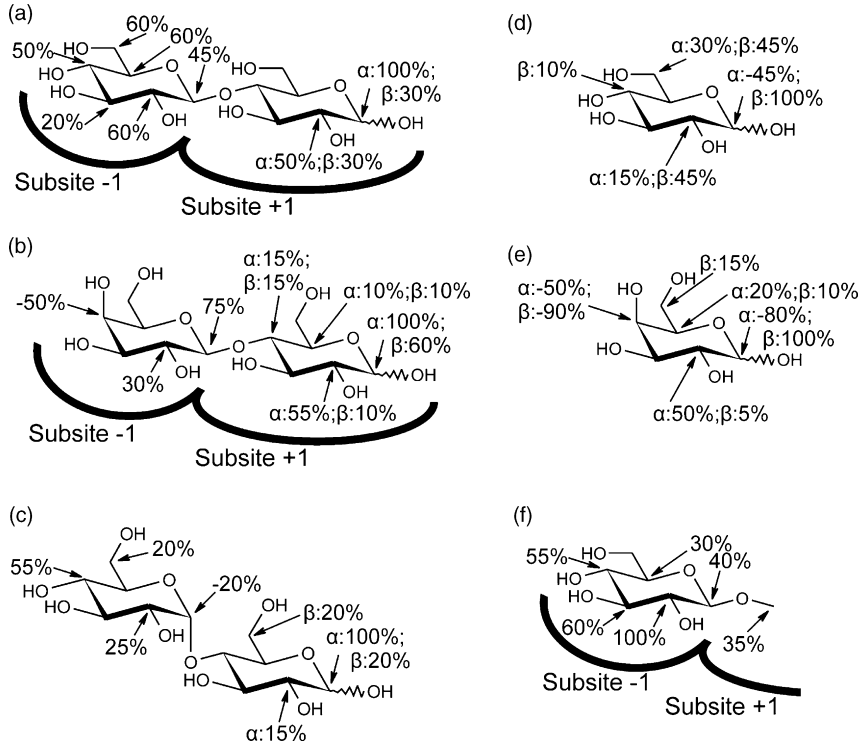

Fig. 2. Relative STD effects of cellobiose (a), lactose (b), maltose (c), glucose (d), galactose (e) and β-methyl glucopyranoside (f) bound to CelB at 30 °C, respectively. Positions not marked have effects <5%. For cellobiose, lactose, and methyl β-glucopyranoside the binding at subsites −1 and +1 are indicated.

subsite +1 and subsite −1. Subsite +1 binds the glycopyranose or aglycon leaving group and the glycopyranoside is bound in subsite −1. It is reasonable to assume that binding of enzymatically active β-glycosides will be mainly in the productive mode that spans both subsites −1 and +1.

The galactopyranoside moiety of lactose has a profoundly dissimilar overall binding pattern at subsite −1, reflected by altered relative STD effects of H-3 to H-6, compared to the STD effects at the same positions in the glucopyranoside of cellobiose (Fig. 2a and b). The graduated specificity of CelB to hydrolyze cellobiose and lactose [16,17] may thus result from globally (rather than locally) perturbed enzyme–glycon interactions caused by epimerisation of a single chiral center at C-4. However, proton H-4 in lactose, directly bound to the inverted stereo center shows a negative artifact in the STD spectrum. A similar effect can be detected inverting other chiral centers as shown in Fig. 2. An exact theory is presently lacking to explain this effect. Putatively, water (D_2O) is bound at the position vacated by the hydroxyl group and interferes with the ligand during saturation and spin lock time.

Relative STD effects of the glucopyranoside in maltose are entirely different from those in cellobiose (Fig. 2c), suggesting profound altered binding that is likely caused by steric conflicts of the α-1,4-disaccharide with the substrate binding site. This is consistent with the absence of measurable CelB activity on maltose at any temperature [16,17]. On subsite +1 bound glucopyranoses of all three disaccharides show notable STD effects only of H-1 and H-2. They are significantly larger in the α- than in the β-anomer, indicating a binding preference for CelB in this position. The small relative STD effects of all other protons in glucopyranoses suggest an unspecific binding at subsite +1 that is consistent with the generally broad acceptance of leaving group structures by CelB and of other β-glycosidases.

Methyl β-glucopyranoside shows reasonable STD effects of nearly all protons (Fig. 2f), although it is not hydrolyzed by CelB at 30 °C in noteworthy amounts due to kinetic hindrance. However, the replacement of the glucopyranose leaving group by a methanol leaving group leads to significant changes in the relative STD effects of the glucopyranoside moiety. Especially its proton H-2 shows a distinctly altered STD effect, indicating a different binding conformation in the subsite −1 close to the anomeric center. Therefore, the binding in the productive mode of substrate glycon and leaving groups is not independent of each other. Comparison of relative STD effects of H-2 for homologous *gluco*-configured substrates is of particular interest, because their enzymatic hydrolysis involves protein-induced changes in the ground state conformation of the bound glucopyranoside from relaxed chair ($^4C_1$) to skew-boat ($^1S_3$) [26,27], obviously involving position 2. Upon incubation of solely glucose or galactose with CelB, a large STD effect of the β-anomeric H-1 is observed, whereas the effects of all other protons are smaller (Fig. 2d and e). These binding patterns resemble that of the glucopyranose in cellobiose and suggest that STD effects of the free monosaccharides mainly reflect their interactions with subsite +1. Hence, binding of these monosaccharides to subsite −1 is quite weak or alternatively exceptionally tight ($k_{off} < 10\,s^{-1}$), both leading to low STD effects [7].

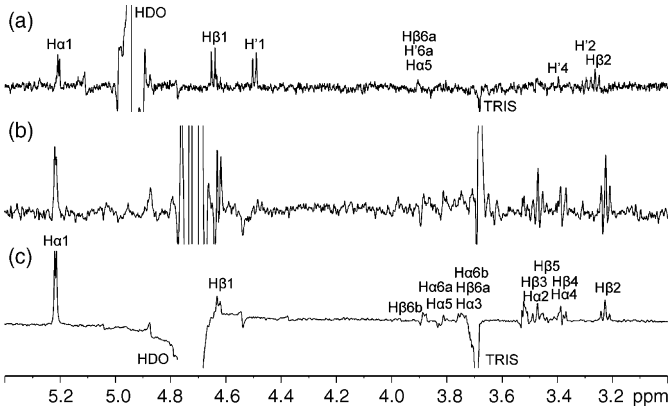

Fig. 4. STD spectra of cellobiose in presence of AfβGly at 10 °C (a) and at 30 °C (b). At 10 °C small STD effects of cellobiose are present. At 30 °C mainly STD effects of generated glucose are detectable. The STD pattern of free glucose at 30 °C is shown for comparison (c). All signals of glucose and all STD effect showing signals of cellobiose are indicated. Differences in the S/N ratio between (b) and (c) indicate distinct different K_D values between the two experimental approaches.

3.3. Binding studies for AfβGly

The catalytic efficiency of cellobiose hydrolysis by AfβGly at 30 °C is $0.91 \pm 0.1\,mM^{-1}\,s^{-1}$ exceeding the one of CelB by two orders of magnitude. At 10 °C, however, it is reduced about 15 times. All STD effects of cellobiose are rather small, suggesting the k_{off} rate to be different from the respective k_{off} rate of cellobiose bound to CelB at 30 °C. The strongest relative effects are observed in the anomeric positions of both glucose units, whereby the α- and β-protons of the glucopyranose show similar signal intensities (Figs. 4a and 5a). Results obtained at 30 °C suggest that cellobiose reacts faster to glucose before it can be released from AfβGly in larger amounts. STD effects are present for the glucose product but only slightly for cellobiose in spite of a much lower concentration of free glucose compared to the one of cellobiose (Fig. 4b). Under these conditions free glucose cannot compete with cellobiose for binding to the enzyme, implying that STD signals on glucose mostly reflect its noncovalent interactions present during the catalytic process. Relative STD effects of this released glucose molecules indicate pronounced interactions at positions 1 and 2, matching the binding pattern for each of the constituent sugar units in cellobiose at 10 °C. When solely free glucose is added to the AfβGly solution at 30 °C, the sugar displays a very similar relative binding pattern to the one observed in the conversion experiment (Fig. 4c). The S/N ratio is, however, distinctly increased and indicates a different binding kinetics.

Relative STD effects on the glucopyranose leaving group of lactose at 30 °C are comparable to those of the leaving group of cellobiose at 10 °C (Fig. 5a and b). These effects originate from interactions at subsite +1 and show a slightly higher STD-effect of the α-anomeric form bound to AfβGly. Furthermore comparison of STD effects of cellobiose (at 10 °C) and lactose (at 30 °C) points out 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

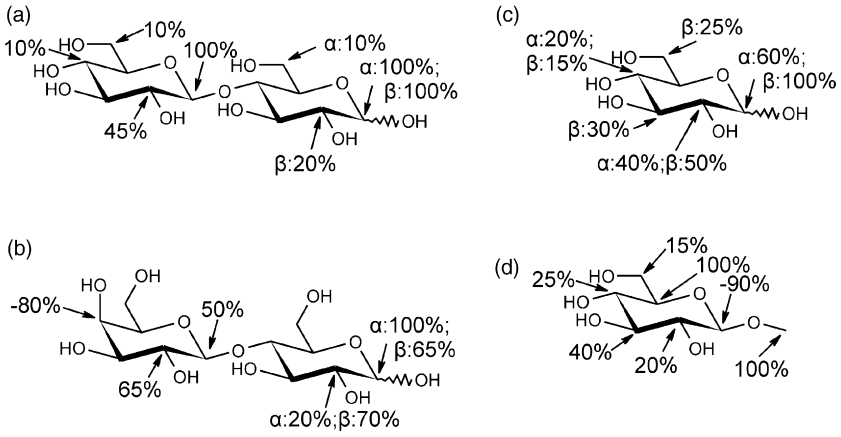

Fig. 5. Relative STD effects of cellobiose at 10 °C (a), lactose at 30 °C (b), glucose at 30 °C (c), and β-methyl glucopyranoside at 30 °C (d) bound to AfβGly, respectively. Positions not marked have effects <5%.

substrate. As found with CelB but more strongly so for AfβGly, the binding pattern at subsite −1 also depends strongly on the structure of the leaving group. Methyl β-glucopyranoside shows strongest STD effect on glucosyl H-5, while in cellobiose the glucopyranoside has most distinct contacts at H-1 (Fig. 5a and d).

Control STD experiments of all compounds tested have been performed in the presence of bovine serum albumin and show small STD effects for almost all protons, indicating a very weak as well as unspecific binding. Therefore, this result corroborates the notion that STD effects of substrates and products are the result of specific noncovalent contacts with the respective β-glycosidase in the productive mode. Comparison of the STD effects summarized in Figs. 2 and 5 indicates that β-glycosides and their constituent monosaccharides interact in a slightly different way with CelB and AfβGly, suggesting subtle differences in the substrate binding pockets of the two enzymes.

4. Conclusion

The application of STD NMR provides useful insights into how broadly specific β-glycosidases use noncovalent interactions to recognize and bind their substrates and products. A very important finding is that strictly local changes in the substrate structure cause various and significant proximal perturbations in contacts with the enzyme at both the reactive carbon and positions remote from it. The results also underscore a significant influence of subsite +1 bound glycone and aglycone parts to the binding of the glycopyranoside moiety and to the formation of the catalytically competent Michaelis complex. This application of STD NMR can be implemented to various other enzyme systems and broadens the general utilisability of this method to the field of biocatalysis.

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