Supporting Information

Saturation Transfer Difference NMR reveals functionally essential kinetic differences for a sugar binding repressor protein

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1. General methods

Proton nuclear magnetic resonance spectra (^{1}H NMR) were recorded on a Bruker AV400 (400 MHz), a Bruker DPX 400 (400MHz), or a Bruker AVII500 (500MHz) spectrometer. Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded on a Bruker AV400 (100.7 MHz) or a Bruker AVII500 (125.6 MHz) and are proton decoupled. Spectra were assigned using COSY, DEPT-135, HMQC, HSQC, and HMBC if required. Phosphorus nuclear magnetic resonance spectra (^{31}P NMR) were recorded on a Bruker AV400 (162 MHz). All chemical shifts are quoted on the δ scale in ppm using residual solvent as an internal standard.

Low resolution mass spectra were recorded on a Micromass Platform 1 spectrometer using electrospray ionization (ES), or on a Bruker Daltonics MicroTOF spectrometrer. High resolution mass spectra were recorded on a Bruker Daltonics MicroTOF spectrometer. m/z values are reported in Daltons. Infrared spectra (FT-IR) were recorded on a Bruker Tensor 27 Fourier Transform spectrophotometer using KBr discs. Absorption maxima are reported in wavenumbers (cm^{-1}). Only signals representing functional groups are reported; C-H absorptions and the fingerprint region are not listed. Optical rotations were measured on a Perkin-Elmer 241 polarimeter at 589 nm (Na D-line) with a path length of 1.0 dm. Concentrations are given in g/100 mL.

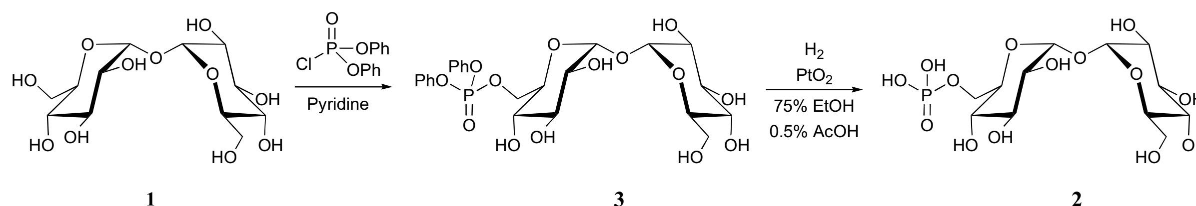
Thin Layer Chromatography (TLC) was carried out using Merck aluminium backed sheets coated with Kieselgel $60F_{254}$ silica gel. Visualization of the sheets was achieved using a UV lamp (λ_{max} = 254 or 365 nm) and/or ammonium molybdate (5% in 2M H_2SO_4), or sulfuric acid (0.2M in 1 MeOH : 1 H_2O). Silica gel chromatography was carried out using Fluka Kieselgel 60 220-240 mesh silica.

High Performance Liquid Chromatography was conducted on a Dionex UltiMate 3000 HPLC system at ambient temperature, with a Varian PLS400 Evaporative Light Scattering detector (ELSD) parallel to the main flow path.

Anhydrous pyridine was purchased from Fluka, and stored under argon over molecular sieves. All other solvents were used as supplied (analytical or HPLC grade). Anhydrous D-trehalose was purchased from Fisher Scientific.

2. Synthesis of trehalose-6-phosphate

Trehalose-6-phosphate (**2**) was synthesized in two steps starting from commercial trehalose (**1**) according to the following scheme:



Scheme S1.

Experimental

i) 6-O-(diphenoxyphosphoryl)-D –trehalose **(3)**

To a suspension of D-trehalose (7.50 g, 21.9 mmol, 1 eq) in anhydrous pyridine (100 mL) was added dropwise diphenylchlorophosphate (4.54 mL, 21.9 mmol, 1 eq). TLC (1 water : 4 isopropanol : 4 ethyl acetate) after 18 hours showed the presence of two products. The reaction was quenched with methanol (10 mL) and the reaction mixture concentrated *in vacuo*. The residue was co-evaporated with toluene to remove pyridine. Silica gel chromatography (1 water : 3 isopropanol : 13 ethyl acetate) allowed separation of the two products. Lyophilization yielded the desired compound **3** (3.02g, 24%) as a white amorphous solid.

R_f 0.68 (1 water : 4 isopropanol : 4 ethyl acetate), $[\alpha]_D^{22}$ +63.9 (c = 1.0, MeOH); ^{1}H NMR (500MHz, MeOD) δ ppm 3.34 (1H, t, $J_{H3'-H4'}$ 9.1Hz, $J_{H4'-H5'}$ 9.1Hz, H4'), 3.38 (1H, t, J_{H3-H4} 9.1Hz, J_{H4-H5} 9.1Hz, H4), 3.43 (1H, dd, $J_{H2'-H3'}$ 9.8Hz, $J_{H1'-H2'}$ 3.5Hz, H2'), 3.48 (1H, dd, J_{H2-H3} 9.8Hz, J_{H1-H2} 3.8Hz, H2), 3.70 (1H, dd, $J_{H6'a-H6'b}$ 12.0Hz, $J_{H5'-H6'b}$ 5.4Hz, H6'b), 3.80 (1H, t, $J_{H2'-H3'}$ 9.5Hz, $J_{H3'-H4'}$ 9.5Hz, H3'), 3.81 (1H, t, J_{H2-H3} 9.1Hz, J_{H3-H4} 9.1Hz, H3), 3.80-3.83 (1H, m, H6'a), 3.84 (1H, m, $J_{H5'-H6'b}$ 4.1Hz, $J_{H5'-H6'a}$ 2.2Hz, H5'), 4.09 (1H, dt, J_{H4-H5} 10.1Hz, J_{H5-H6a} 2.1Hz, J_{H5-H6b} 2.1Hz, H5), 4.48 (1H, ddd, $J_{H6a-H6b}$ 11.5Hz, $J_{H6b-31P}$ 7.1Hz, J_{H5-H6b} 3.5Hz, H6b), 4.55 (1H, ddd, $J_{H6a-H6b}$ 11.5Hz, $J_{H6a-31P}$ 6.8Hz, J_{H5-H6a} 1.9Hz, H6a), 5.09 (1H, d, $J_{H1'-H2'}$ 3.8Hz, H1'), 5.10 (1H, d, J_{H1-H2} 3.8Hz, H1), 7.21-7.31 (3H, m, ArH_{ortho}, ArH_{para}), 7.40-7.43 (2H, m, ArH_{meta}); ^{13}C NMR (126MHz, MeOD) δ ppm 62.6 (1C, C6'), 69.8 (1C, d, J_{C6-31P} 6.7Hz, C6), 71.2 (1C, C4'), 71.9 (1C, C4), 72.0 (1C, d, J_{C5-31P} 6.7Hz, C5), 73.0 (1C, C5'), 73.2 (1C, C2'), 73.9 (1C, C2), 74.4 (1C, C3'), 74.6 (1C, C3), 95.2 (1C, C1'), 95.3 (1C, C1), 121.4 (2C, d, J_{C-31P} 4.8Hz, ArC_{ortho}), 126.8 (1C, ArC_{para}), 131.1 (2C, ArC_{meta}), 151.9 (1C, d, J_{C-31P} 7.6Hz, ArC_{ipso}), 151.9 (1C, d, J_{C-31P} 7.6Hz, ArC_{ipso}); $^{31}P\{^1H\}$ NMR (162MHz, MeOD) δ ppm -12.0 (1P, $P(O)(OPh)_2$); FT-IR (KBr disc) υ 1287 (P=O), 3271 br (OH); HRMS m/z (ES^+) 596.1357 $[M + Na]^+$ required (596.1344).

ii) trehalose-6-phosphate **(2)**

A suspension of **3** (50 mg, 0.072 mmol, 1eq) and PtO_2 (5 mg, 0.02 mmol, 0.3 eq) in 75% aqueous ethanol (5 mL) with 0.5% glacial acetic acid (25 μL) was repeatedly degassed under high vacuum and the reaction vessel flushed with hydrogen. The reaction was maintained at room temperature with aggressive stirring under an atmospheric pressure of hydrogen for 5 hours after which TLC (1 water : 4 isopropanol : 4 ethyl acetate) showed the complete consumption of the starting material and the formation of a single product. The reaction mixture was filtered through Celite® and the solvent was removed *in vacuo*. The crude solid was taken up in water (30 mL) and washed with ethyl acetate (2 x 20 mL). The aqueous layer

was lyophilized and the compound purified using HPLC on an Applied Biosystems, Poros® HQ strongly basic anion exchange column (10 mm x 100 mm, 50 µm). A gradient from 0 mM to 500 mM aqueous NH_4HCO_3 was used as the mobile phase at a flow rate of 20 mL/min and eluants were detected with an Evaporative Light Scattering detector. Fractions containing the product were pooled and repeated lyophilization to removed residual NH_4HCO_3 afforded the title compound as a white amorphous solid (28.6 mg, 94%).

R_f 0.2 (5 ethanol : 3 NH_4OH : 1 water), $[\alpha]_D^{18}$ + 150.3 (c = 1.0, H_2O) lit.[1] $[\alpha]_D^{21}$ +151.2 (c = 0.8, H_2O); ^{1}H NMR (500MHz, D_2O) and ^{13}C NMR (126MHz, D_2O) see table S3. $^{31}P\{^1H\}$ NMR (202MHz, D_2O) δ ppm 3.6 (1P, $ROP(O)(OH)_2$); m/z (ES^-) 421.5 $[M - H]^-$.

3. Protein preparation

Recombinant TreR protein was expressed and purified as previously described[2]. After purification of the protein and concentration to 2.5 mg/ml in 20 mM TrisHCl buffer pH7.8 , the protein solution was buffer exchanged into deuterated 20mM sodium phosphate buffer at pH^* 7.5 using a PD10 (Amersham Biosciences) size exclusion column. Before each NMR sample preparation the protein concentration was checked using a nanodrop spectrophotometer (280 nm) and the theoretical molar extinction coefficient based on the protein amino acid sequence.

4. Preparation of the NMR samples

Samples of free sugars and protein were prepared in 20mM sodium phosphate buffer (pH^* 7.5) in D_2O at least 3 hours before measurement to ensure a complete deuterium exchange of all exchangeable sugar and protein protons. For the separate STD experiments with each ligand we employed 20 mM of carbohydrate and 23 µM (binding sites concentration) of TreR. For the competition experiments various volumes of a 9.06 mM trehalose-6-phosphate (**2**) solution were titrated to a 500 µl solution of 14.5mM trehalose (**1**) and 145µM (binding sites concentration) TreR.

Table S1: NMR assignment of trehalose (**1**) in D_2O.

	multiplicity	^{1}H (ppm)	^{13}C (ppm)	J_{HH} (Hz)
H-1	d	5.11	93.2	3.9
H-2	dd	3.56	71.1	3.9, 9.8
H-3	pt	3.76	72.5	9.7
H-4	pt	3.36	69.7	9.6
H-5	ddd	3.74	72.1	2.0, 5.1, 9.6
H-6a	dd	3.77	60.5	2.0, 11.6
H-6b	dd	3.68		5.1, 11.6

Table S2: NMR assignment of trehalose-6-phosphate (**2**) in D_2O.

	multiplicity	^{1}H (ppm)	^{13}C (ppm)	J_{HH} (Hz)
H-1	d	5.13	93.3	3.9
H-2	dd	3.60	71.2	3.9, 9.7
H-3	pt	3.77	72.1	9.6
H-4	pt	3.53	69.1	9.6
H-5		ca. 3.80	71.8	
H-6a	ddd	3.94	62.4	4.1, 7.6 (HP), 12.0
H-6b	ddd	3.85		2.0, 5.3 (HP), 12.0
H-1’	d	5.10	93.4	3.8
H-2’	dd	3.56	70.9	3.8, 9.9
H-3’	pt	3.76	72.4	9.7
H-4’	pt	3.36	69.7	9.6
H-5’	ddd	3.73	72.1	2.0, 5.1, 9.9
H-6a’	dd	3.77	60.5	
H-6b’	dd	3.67		5.1, 11.9

5. STD NMR Measurements

All STD experiments were recorded on a Bruker DRX500 instrument equipped with a 5-mm TBI probe. Acquisitions were performed at 298 K using the standard STD pulse sequence with a shaped Q5 pulse train (50 ms, 90°, 4µs delays between the pulses) for selective protein irradiation, and an alternation between on and off resonances[3]. Presaturation of the protein resonances was performed with an on-resonance irradiation at 0.82 ppm to saturate mainly Ile, Leu and Val; off resonance irradiation was applied at 50.0 ppm where no NMR resonances of ligand or protein are present. The STD build up curves were obtained by performing experiments with different saturation times (2s, 3s, 5s, 7s and 10s) and recorded with a recovery delay of 10 s to avoid incomplete relaxation to thermal equilibrium. Inversion recovery experiments for measuring T_1 times were performed immediately before or after the STD experiments on the same sample. For the competition experiments the saturation time, as well as the relaxation delay were set to 5s to allow fast recording.

6. Complete Relaxation and Conformational Exchange Matrix Calculations (CORCEMA-ST)

The published crystal structure of TreR in complex with trehalose-6-phosphate (PDB entry 1BYK) was used as basis for all calculations.[4] Because of a wrong stereochemistry of the ligand in this pdb-entry, the ligand was again fitted into the electron density with the right anomeric stereoconformation. All aminoacids within 15Å around the ligand were considered in the CORCEMA-ST calculations. Exchangeable protons in ligand and protein were removed and replaced with deuterium. The selection of the irradiated protons was done using a SHIFTX 1.1[5] prediction of the chemical shift and including every proton in the excitation width of the used selective pulse (0.68-0.92 ppm). The K_Ds previously reported by Horlacher and Boos (280 and 10 µM for **1** and **2** respectively)[2] were used for the calculation, the rest of the input parameters were exactly the same for both ligands. A protein correlation time of 15 ns and a relaxation leakage of 0.3 s^{-1} were found to optimal fit the measured STD factors of trehalose. Although we used a solution structure of trehalose previously reported by Poveda and co-workers as unbound ligand structure,[6] we could not achieve an alignment of the measured relaxation T_1 times with the T_1 times predicted by CORCEMA-ST. Therefore the reciprocal of the measured T_1 times was used as relaxation of the ligand in its unbound state. This procedure enhanced the NOE R-factor.

The k_{on} rate for trehalose was determined by calculating the STD factor at five different k_{on} rates (10^3, 10^4, 10^5, 10^6 and 10^9 M^{-1} s^{-1}) and comparing with the experimental buildup curve to

get the corresponding NOE R-factors in each case. The NOE R-factor was calculated for proton 2 (a well resolved resonance giving one of most intense STD effects) at all measured saturation times using the equation:

$$R = \sqrt{\frac{\sum W_k (STD^{\exp} - STD^{calc})^2}{\sum W_k (STD^{\exp})^2}}$$

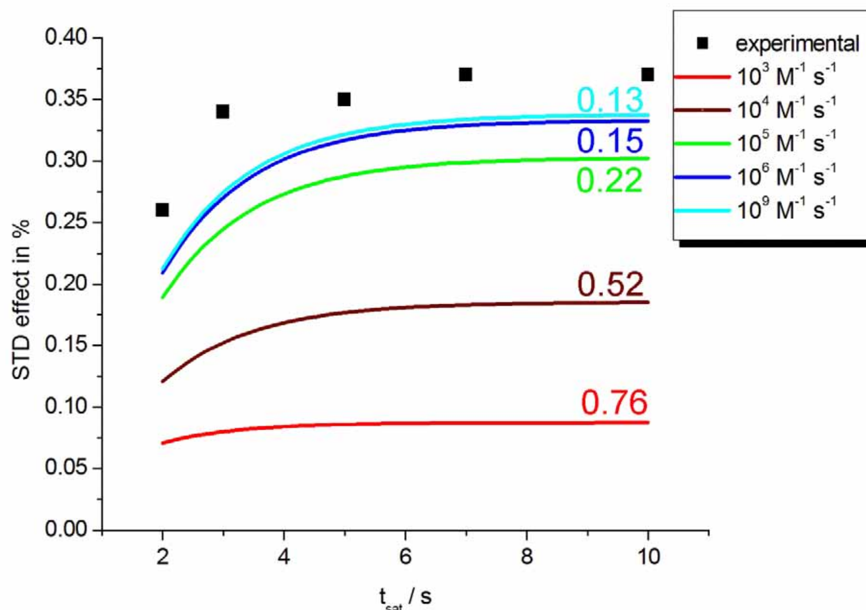


Figure S1: Experimental build up curve for the STD signal of proton 2 of trehalose (**1**) and the predicted STD curves from the CORCEMA-ST calculations for several k_{on} rates. Above the lines is given the R-factor for each on rate.

The minimum R-factor, which allows identification of the "true" on rate, is reached at k_{on} equal to 10^6 M^{-1} s^{-1} or higher. The precise k_{on} for trehalose was not determined since the predicted STD effects from the CORCEMA-ST calculations were insensitive to varying on rates above this lower limit (10^6 M^{-1} s^{-1}). This is in accordance with theoretical studies that predict STD factors become independent of K_D values when the exchange is fast on the relaxation rate scale and the ligand/protein ratio is high.[7]

7. Comparison of trehalose-6-phosphate conformation in solution and in the crystal structure of the complex

The torsional angles around the glycosidic bond for trehalose-6-phosphate in the crystal structure of the complex don't significantly differ from the reported ones for trehalose in

solution[6] (Fig. S2). It is reasonable to assume that the conformation (glycosidic bond torsional angles) of both disaccharides in solution is essentially equal, since the phosphate group is located at a primary hydroxyl well distant from the glycosidic bond.

To further prove this hypothesis selective 1D NOESY experiments were carried out with both sugars. Experiments were performed using the DPFGSE NOE sequence[8] with selective excitation of the anomeric protons (H-1 and H-1') and employing a range of mixing times from 50 to 800 ms (to get the corresponding buildup curves). In both cases the intraresidue H-1/H-2 (and H-1'/H-2') and the interesidue H-1/H-5' (and H-1'/H-5) NOEs were clearly seen (with similar intensities when comparing one sugar with the other) in agreement with the previously reported results for trehalose using NOE-difference[9] and 1D-selective ^{13}C edited NOESY[6] experiments. Thus, it was qualitatively proved that in solution both sugars display a similar conformation with interglycosidic angles ϕ / ψ ca. -50º / -50º. [6,9]

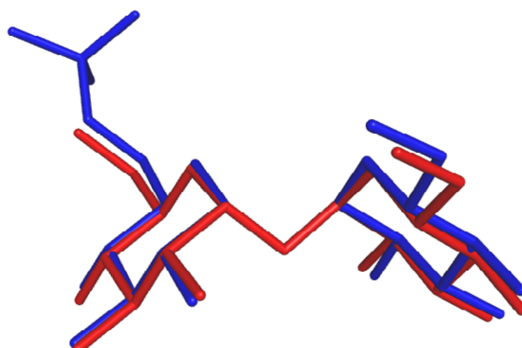


Figure S2: Overlay of trehalose-6-phosphate (blue) in the crystal structure of its complex with TreR and the reported conformation for trehalose (red) in solution. Hydrogens are omitted for clarity.

8. References

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