Saturation transfer difference NMR reveals functionally essential kinetic differences for a sugar-binding repressor protein†

Ignacio Pérez-Victoria,‡[a] Sebastian Kemper,‡[a] Mitul K. Patel,[a] John M. Edwards,[a] James C. Errey,[a] Lucia F. Primavesi,[b] Matthew J. Paul,[b] Timothy D. W. Claridge*[a] and Benjamin G. Davis*[a]

Received (in Cambridge, UK) 7th July 2009, Accepted 3rd August 2009
First published as an Advance Article on the web 19th August 2009
DOI: 10.1039/b913489a

The binding kinetics of disaccharides trehalose and trehalose-6-phosphate to repressor protein TreR have been determined using STD NMR and shed light on the contrasting biological roles of these two sugars.

Saturation transfer difference NMR (STD NMR) is a powerful and popular tool to identify and characterize the binding of ligands to protein receptors.[1,2] The technique is extremely well suited to the study of protein–carbohydrate interactions due to the broad range of binding affinities that may be studied (K_D from 100 nM to 10 mM;[2] K_Ds that are often typical of such interactions).[3]

We have employed STD NMR to study the binding of trehalose (**1**) and trehalose-6-phosphate (**2**) to the trehalose repressor protein (TreR) from *Escherichia coli*, the repressor which regulates the pathway of trehalose utilization in this bacteria.[4] TreR binds with **1** and **2** with similar affinities (trehalose-6-phosphate, K_D = 10 μM *cf.* trehalose, K_D = 280 μM), but it has long been known that only the binding and engagement of **2** results in a biologically relevant reduction of the repressor affinity for binding to the DNA operator site.[4] Despite this essential physiological functional difference, the crystal structures of the complexes of TreR with its inducer trehalose-6-phosphate (**2**) and the non-inducer trehalose (**1**) were found to be virtually identical.[5] This apparent similarity in such ground state measurements (structure, K_D) suggests that key mechanistic differences lie elsewhere.

Parent disaccharide trehalose **1** and its synthetic phosphate derivative **2** (see ESI† for synthesis) were surveyed for binding to TreR using a range of methods. Of these, the use of STD NMR was particularly noteworthy; unusually, under identical experimental conditions we observed a clear STD effect for **1** but none for **2**. Absence of an STD signal can be due to a number of mechanistic origins (including absence of binding). However, **2** is in fact a more potent ligand than **1**, critically confirmed by competition STD NMR experiments.[6] Titration of **2** into a solution of **1**·TreR complex (Fig. 1) confirmed that both ligands bound competitively to the same binding site and that trehalose-6-phosphate (**2**) is the stronger binder of the two: at a ratio of 1 : 4 : 100 TreR : **2** : **1** (X = 4) the STD effect for trehalose was essentially absent.

The absence of STD effect for the phosphate derivative of trehalose was intriguing since its dissociation constant (K_D = 10 μM) is well within the range of amenable binding affinities for the technique;[2] we reasoned that an unusually slow off (dissociation) rate k_{off} might explain this experimental observation since in this case transfer of saturation to ligand molecules into solution is not very efficient.[2] This striking kinetic contrast between **1** and **2** was probed further using comparative computational analysis employing the program CORCEMA-ST[7,8] to calculate theoretical STD effects *via* complete relaxation and exchange rate matrices based on the three-dimensional coordinates of TreR complexes, concentrations, equilibrium binding constants, and association and dissociation rate constants. Such an approach might allow

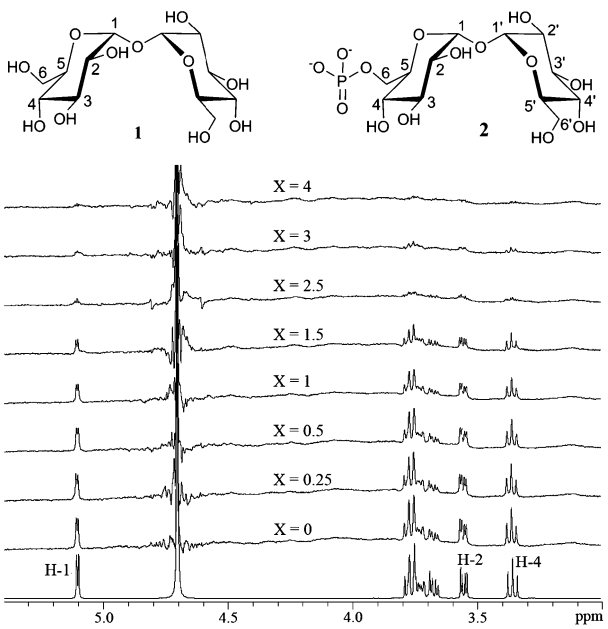

Fig. 1 Competition STD NMR experiment with increasing trehalose-6-phosphate (**2**). All STD spectra were acquired with a ratio 1 : X : 100 TreR : **2** : **1** as indicated. The lowest reference spectrum is for trehalose (**1**).

[a] *Department of Chemistry, University of Oxford, Chemistry Research Laboratory, Mansfield Road, Oxford, UK OX1 3TA. E-mail: Tim.Claridge@chem.ox.ac.uk, Ben.Davis@chem.ox.ac.uk; Fax: +44 (0)1865 285002; Tel: +44 (0)1865 285001*
[b] *Plant Science, Rothamsted Research, Harpenden, Herts, UK AL5 2JQ*

† Electronic supplementary information (ESI) available: Trehalose-6-phosphate synthesis, STD NMR measurements, CORCEMA-ST calculations and trehalose-6-phosphate conformation discussion. Links to view 3D visualisations of structures using FirstGlance. See DOI: 10.1039/b913489a

‡ Both of these authors contributed equally to this work.

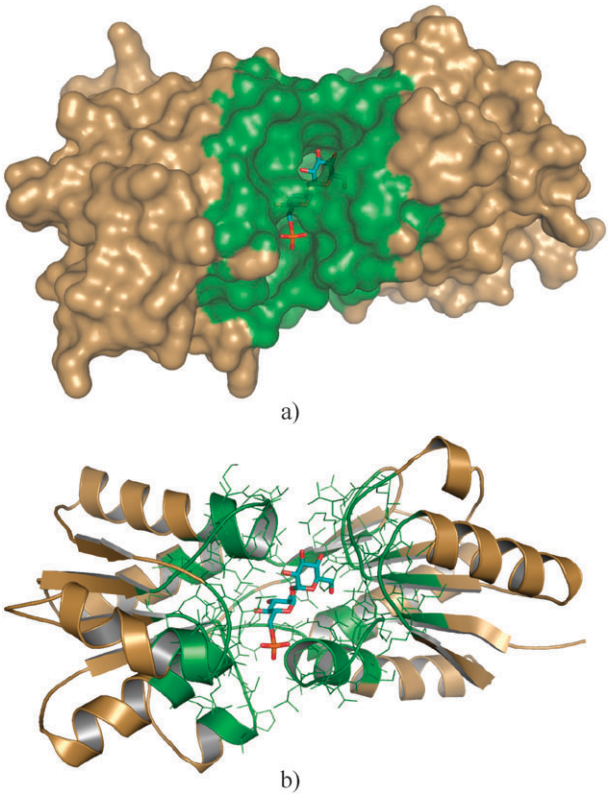

Fig. 2 Repressor protein TreR (PDB entry: 1BYK)[5] showing the ligand trehalose-6-phosphate (**2**, blue) and the residues (green) included within the CORCEMA-ST cutoff distance for calculations in both surface (a) and ribbon (b) representations generated using PyMOL.[12]

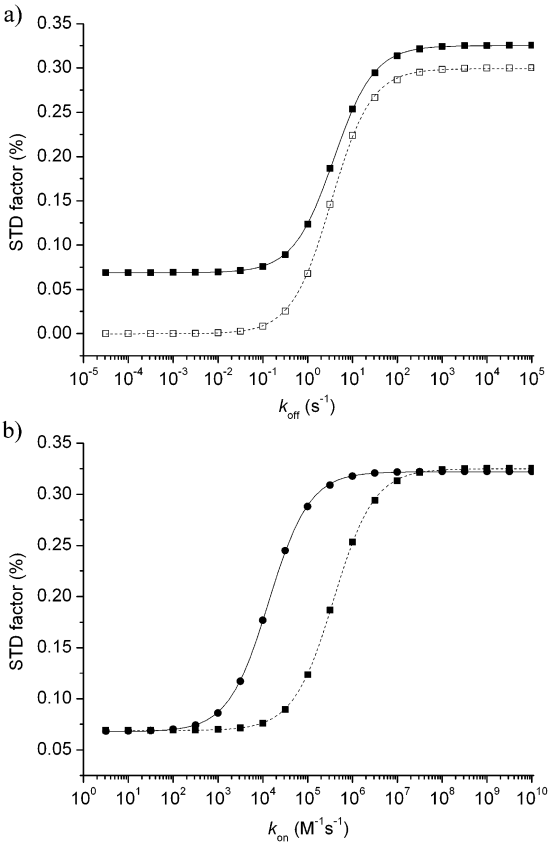

Fig. 3 (a) Dependence of predicted STD factor at 5 s saturation time (for H-2 in **2**) with off rate. Dashed line represents the calculated saturation for the unbound state. (b) Dependence of predicted STD factor at 5 s saturation time (for H-2 in both ligands) with the on rate (solid line ● **1**, dashed line ■ **2**).

the calculation of STD effects as a function of the dissociation rate (k_{off}).[9]

In this way the thresholds for the rates of binding of trehalose-6-phosphate to TreR could be probed. Using the crystal structure of trehalose-6-phosphate in complex with TreR (PDB entry: 1BYK),[5] residues within 15 Å of the bound ligand in the binding pocket (Fig. 2) were explicitly used to predict STD factors (using representative ligand protons) as a function of the dissociation rate (k_{off}) (Fig. 3). These revealed that a low STD factor is obtained for off rates $\leq 0.1\ s^{-1}$. The residual calculated STD factor is caused nearly exclusively by the ligand in its bound state; because of the slow kinetics only a negligible part will be released to the solution and the calculated unbound ligand saturation converges to zero at low off rates (Fig. 3a). The bound ligand saturation is hardly detectable since the ligand signals in its bound state have a similar linewidth to that of the protein and thus will merge with the protein background; therefore the unbound ligand saturation is more representative and allows the establishment of an upper limit value for k_{off}(**2**) = $0.1\ s^{-1}$, which implies a k_{on}(**2**) $\leq 10^4\ M^{-1}\ s^{-1}$ ($K_D = k_{off}/k_{on}$). Such a low on rate is typically observed in situations where there is a large conformational rearrangement of receptor or ligand upon binding.[2] Strong shifts in the frequency of the fluorescence emission maximum of TreR during binding of **2**[4] but an essential absence of difference in the conformation of bound or unbound ligand **2** (see ESI†) suggests that this is a protein associated conformational change. Such large changes in shape are often the mode of action of sugar-triggered repressors[10] and is here apparently caused by a subtle difference in ligand identity, the phosphate at C-6 of trehalose. Nonetheless, other mechanistic modes, including those utilizing through-protein electrostatic effects (such as is the case for "electric-genetic"[11] switches that utilize effector molecules like **2** that can contain only a single charged group), cannot be excluded.

Consistent with the difference in biological effect, use of the same novel 'kinetic STD' approach revealed key differences in on rate. Previous analysis of the STD effects of trehalose using a novel approach of group epitope mapping considering the relaxation of the ligand (GEM-CRL)[13] combined with CORCEMA-ST calculations has suggested that the reported structure by Hars and co-workers[5] is valid for the solution structure of the TreR–trehalose complex. Use of the GEM-CRL approach here revealed that the binding kinetics of trehalose (**1**) are in striking contrast to those of **2**; an off rate of $>100\ s^{-1}$ was required to properly reflect calculated values. Moreover, CORCEMA-ST calculations with different on rates resulted in a lower limit for the k_{on}(**1**) of $10^6\ M^{-1}\ s^{-1}$ ($k_{off} \geq 280\ s^{-1}$). This threshold value was determined by comparing the experimental STD build-up curves with those calculated in NOE *R*-factor determination.[7] The minimum *R* value, which allows identification of the "true" on rate, was

reached at $k_{on} \geq 10^6\ M^{-1}\ s^{-1}$ (see ESI†). At such high rates it cannot be discounted that binding of **1** to TreR is rapid, essentially diffusion controlled, and causes no functional change in TreR.

Taken together these results suggest that for TreR, although the ratio K_D(trehalose)/K_D(trehalose-6-phosphate) is small (only 28), the corresponding ratio of dissociation rates k_{off}(trehalose)/k_{off}(trehalose-6-phosphate) is ≥ 2800 and the ratio of association (k_{on}) rates $\geq 10^2$ (Fig. 3). This suggests that the different biological function of both sugars (trehalose-6-phosphate active, trehalose inactive) can be explained by considering kinetic ratios; these in turn suggest a large conformational rearrangement of TreR upon binding to trehalose-6-phosphate (the active inducer) or a strong electrostatic mechanism[11] which triggers an inability of the repressor to bind the operator site in the DNA sequence, thus allowing gene expression. Such a rearrangement does not occur upon trehalose binding, allowing the union of the repressor to the operator inhibiting gene expression.

In summary, taking advantage of the availability of structural protein data for TreR, calculations have allowed us to determine key kinetic data for an effector (**2**) and a non-effector ligand (**1**) (threshold dissociation and association rate values) that was probed experimentally using STD NMR. In this way insights into binding kinetics of both ligands with the repressor can be used to explain their very different biological roles and effects. To our knowledge, this is the first use of such STD kinetic analysis in the discovery of a functionally important kinetic difference in ligand–protein interactions. Such solution phase kinetic analysis we suggest might be generally useful to complement other biophysical techniques such as SPR[14] or QCM.[15] These other methods can also allow determination of kinetic values but require one component (typically ligand) to be bound to a solid phase (SPR chip or microbalance), which can bring with it a bias that may be less relevant to a solution phase ligand or interaction. The kinetic STD method therefore might valuably allow more general and relevant determinations free from such constraints.

Work in this communication was supported by BBSRC grant BB/D006112/1, the European Community (PIEF-GA-2008-221066, I. P-V.), the Deutscher Akademischer Austausch Dienst (S. K.) and the Kölner Gymnasial- und Stiftungsfonds (S. K.). Rothamsted Research receives grant-aided support from the Biotechnological and Biological Research Council (BBSRC) of the United Kingdom. We are grateful to Prof. Winfried Boos, University of Konstanz, Germany, for the gift of plasmid pCYTEXPtreR and to Prof. Rama Krishna, University of Alabama, USA, for making available to us the CORCEMA-ST program.

Notes and references

1 M. Mayer and B. Meyer, *Angew. Chem., Int. Ed.*, 1999, **38**, 1784–1788.
2 B. Meyer and T. Peters, *Angew. Chem., Int. Ed.*, 2003, **42**, 864–890.
3 J. Angulo, C. Rademacher, T. Biet, A. J. Benie, A. Blume, H. Peters, M. Palcic, F. Parra, T. Peters and F. Minoru, *Methods Enzymol.*, 2006, **416**, 12–30.
4 R. Horlacher and W. Boos, *J. Biol. Chem.*, 1997, **272**, 13026–13032.
5 U. Hars, R. Horlacher, W. Boos, W. Welte and K. Diederichs, *Protein Sci.*, 1998, **7**, 2511–2521.
6 Y.-S. Wang, D. Liu and D. F. Wyss, *Magn. Reson. Chem.*, 2004, **42**, 485–489.
7 V. Jayalakshmi and N. R. Krishna, *J. Magn. Reson.*, 2002, **155**, 106–118.
8 N. R. Krishna and V. Jayalakshmi, *Prog. Nucl. Magn. Reson. Spectrosc.*, 2006, **49**, 1–25.
9 J. Angulo, B. Langpap, A. Blume, T. Biet, B. Meyer, N. R. Krishna, H. Peters, M. M. Palcic and T. Peters, *J. Am. Chem. Soc.*, 2006, **128**, 13529–13538.
10 M. Taraban, H. Zhan, A. E. Whitten, D. B. Langley, K. S. Matthews, L. Swint-Kruse and J. Trewhella, *J. Mol. Biol.*, 2008, **376**, 466–481.
11 K. Phillips and S. E. V. Phillips, *Structure (London)*, 1994, **2**, 309–316.
12 W. L. DeLano, *The PyMOL Molecular Graphics System*, DeLano Scientific, Palo Alto, CA, USA, 2002.
13 S. Kemper, M. K. Patel, J. C. Errey, B. G. Davis, J. A. Jones and T. D. W. Claridge, submitted.
14 J. M. McDonnell, *Curr. Opin. Chem. Biol.*, 2001, **5**, 572–577.
15 T. Rudd, J. T. Gallagher, D. Ron, R. J. Nichols and D. G. Fernig, *Biochem. Soc. Trans.*, 2003, **31**, 349–351.

