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Complex carbohydrate recognition by proteins: Fundamental insights from bacteriophage cell adhesion systems[☆]

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Summary Protein—glycan interactions are ubiquitous in nature. Molecular description of complex formation and the underlying thermodynamics, however, are not well understood due to the lack of model systems. Bacteriophage tailspike proteins (TSP) possess binding sites for bacterial cell surfaces oligosaccharides. In this article we describe the analysis of TSP-oligosaccharide complexes. TSP provide large glycan interaction sites where affinity and specificity are guided by the protein surface solvation and the conformational space sampled by the respective glycan. Furthermore, we describe a computational approach to analyse the conformational space sampled by flexible glycans of bacterial origin, a prerequisite for a thorough understanding of TSP-oligosaccharide interactions.

Introduction

Carbohydrate—protein interactions are ubiquitous in nature and mediate the exchange of specific information (Gabius et al., 2011). The affinity of proteins towards sugars is tightly linked to the functional context of the respective interaction. It is therefore very important to study the driving forces for these recognition events and to understand in which way structural features of protein—carbohydrate complexes influence the thermodynamics and the mechanism of binding. Whereas energetics of protein—protein-interactions and protein folding have been studied in detail (Baldwin, 2007) and linked to structure, insight into thermodynamics of protein—carbohydrate interactions is far more incomplete. One important reason for this is the obvious lack of high resolution crystal structures of proteins in complex with oligosaccharides of more than two monosaccharide building blocks. Analysis of non-covalent protein—carbohydrate complexes in the Protein Data Bank showed that about 80% of the ligands were monosaccharides, 13% were disaccharides, and 4% tri- to pentasaccharides (Nakahara et al., 2008). The remaining 3% were mainly hexa-, hepta- or octasaccharides; however their majority mediates protein contacts only via small interfaces of one or two monosaccharide building blocks. This is in accordance with the fact that fundamental research about protein—carbohydrate complexes has mainly focused on lectins, proteins that particularly bind small saccharide units. Thermodynamics of complexes between carbohydrates and lectins have been intensively studied (Ambrosi et al., 2005; Dam and Brewer, 2002; Toone, 1994; Weis and Drickamer, 1996). A variety of methods has been used, like equilibrium dialysis, fluorescence spectroscopy, NMR, capillary electrophoresis or surface plasmon resonance (Lee and Lee, 2003). Global thermodynamic data analysis is achieved in isothermal titration calorimetry (ITC), where parameters like affinity, enthalpic and entropic contributions to the free enthalpy of binding as well as changes in heat capacity (ΔCp) can be obtained in a single experiment, or in experiments at different temperatures, respectively (Christensen and Toone, 2003). Lectins have weak affinities for monovalent sugars and broad specificities for oligosaccharides. Typical dissociation constants are in the millimolar range for monosaccharides and in the micromolar range for oligosaccharides (Ambrosi et al., 2005; Weis and Drickamer, 1996). Binding sites in lectins are largely preorganised shallow grooves and no major structural rearrangement of the proteins is observed upon complex formation. By contrast, in antibodies deeper binding pockets are found with high binding affinities that have been attributed to the hydrophobic effect. Therefore, small but significant negative ΔCp values (less than $0.5\,\mathrm{kJ\,mol^{-1}\,K^{-1}}$) are observed upon lectin—carbohydrate complexation (Toone, 1994). Consequently enthalpy—entropy compensation is observed; however, with a flatter slope of the enthalpy—entropy plot than found for proteins (Garcia-Hernandez et al., 2003).

High-resolution structural information on the interaction of proteins with oligosaccharides of more than five building blocks is sparse. Although some lectins have been found to bind longer oligosaccharides with higher affinities than smaller sugar units, structural information on the complexes is often lacking. Extended polymeric carbohydrate struc-
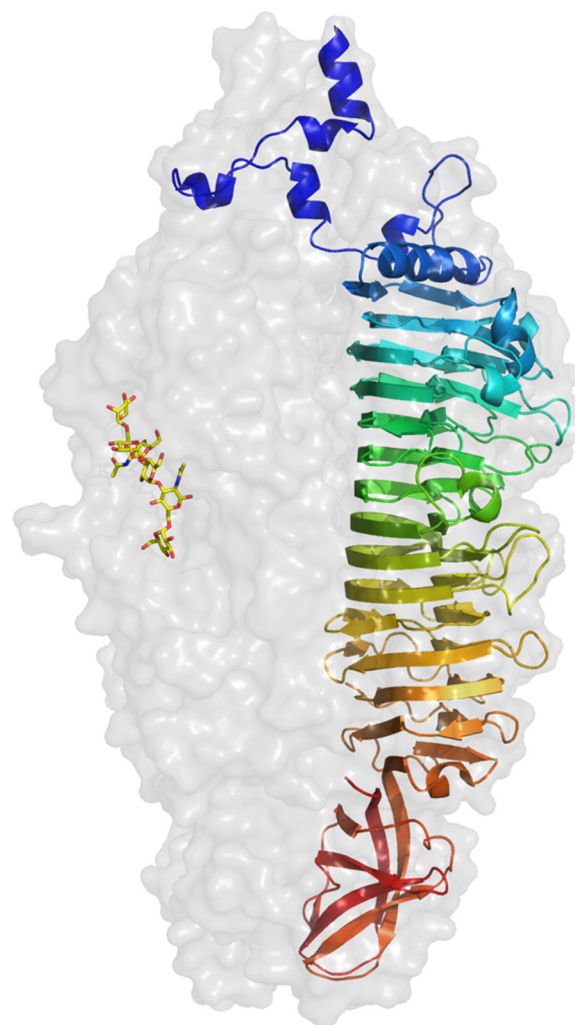
Figure 1 Structure of HK620TSP with oligosaccharide binding pocket. Structure of bacteriophage HK620 tailspike trimer lacking the N-terminal capsid binding domain. Side view of a surface representation with one subunit as a ribbon drawing gradually coloured from N-terminus (blue) to C-terminus (red). Each subunit has an O-antigen binding site; as an example an O-antigen hexasaccharide of *E. coli* serogroup O18A1 is shown in stick representation. Figure generated with PyMOL (SchroedingerLLC, 2015).

tures, often present as components of plant cell walls or storage polysaccharides are substrates for bacterial glycoside hydrolases that contain independent domains termed carbohydrate binding modules (CBMs) to target and concentrate the enzymes on their substrates (Abbott and Boraston, 2012). Crystal structures and affinity studies of CBMs in complex with oligosaccharides revealed characteristic elongated shallow binding sites with few hydrogen bonds and mainly aromatic side chains mediating the contact. Consequently, the quantity of stacking interactions was well described by accumulation of ΔCp-values predicted for desolvation of tryptophan or tyrosine in proteins (Zolotnitsky et al., 2004). Tailspike proteins (TSP) from bacteriophages are well studied model systems for protein folding, but also have been investigated for their role during the bacteriophage's infection process (Andres et al., 2010a, 2010b, 2012). TSP

Table 1 Dissociation constants and thermodynamics of TSP-oligosaccharide complex formation.

| TSP-oligosaccharide complex	$K_D/\mu M$	$\triangle G^a/kJ\,mol^{-1}$	$\triangle H^a/kJ\,mol^{-1}$	$-T\triangle S^a/kJ\,mol^{-1}$
HK620TSP E372Q-hexasaccharide O18A1[a]	0.079	−40.4	−23.6	−16.8
HK620TSP wild type-hexasaccharide O18A1[a]	129[b]	n.d.[c]	n.d.[c]	n.d.[c]
P22TSP—octasaccharide O2 (Paratyphi)[d,e]	51	−24.1	−44.1	20.0
P22TSP—octasaccharide O4 (Typhimurium)[d]	3.1	−31.1	−60.9	29.8
P22TSP—octasaccharide O9 (Enteritidis)[f]	4.5	−30.5	−61.4	30.9

[a] Broeker et al. (2013).
[b] From fluorescence titration.
[c] Not determined.
[d] Andres et al. (2013).
[e] Value for 20 °C.
[f] Baxa et al. (2001).

represent a class of carbohydrate binding proteins different from CBMs or lectins. In contrast to carbohydrate binding modules of other glycoside hydrolases TSP do not possess binding sites rich in aromatic side chains. Rather, carbohydrate interaction is mediated by lectin-type contacts, although TSP have large interaction sites that recognise oligosaccharides with high sequence and linkage specificity (Andres et al., 2013, 2012; Barbirz et al., 2008; Muller et al., 2008; Schwarzer et al., 2015; Steinbacher et al., 1996). Oligosaccharide binding sites are positioned on extended flat β-sheets on the TSP proteins (Fig. 1). TSP are thermostable and protease resistant proteins with parallel β-helix folds, with the peptide chains organised in native homotrimers of 150−200 kDa (Barbirz et al., 2009). TSP that bind and cleave the O-polysaccharide of Gram-negative bacteria serve to position the bacteriophage particle on the cell surface towards a secondary receptor during the infection process (Andres et al., 2010b). In the following we will describe analyses of structure—affinity relationships in glycan recognition processes obtained with the TSP model system.

Carbohydrate—protein interfaces: understanding how two amphiphilic partners interact in the presence of water

Water is the reason for the hydrophobic effect in biological association events. Accordingly, displacement of water occurs during ligand binding. The solvent arrangement on protein surfaces in and around binding sites therefore is an important prerequisite to prime the surface with both specificity and affinity. However, both kinetics and thermodynamics of solvent reorganisation upon binding is complex and not well understood (Dunitz, 1994; Kadirvelraj et al., 2008; Ladbury, 1996). In carbohydrate binding sites, energetic contributions to complex formation have been found to arise from hydrogen bond formation and desolvation of the partners, and, to a larger extent, from van der Waals interactions (Kadirvelraj et al., 2011). Accordingly, in lectin binding sites free energy contributions accumulate from clustered weak interactions at rather small specific sites that are further linked to the rest of the protein surface by water mediated networks. These networks can be disturbed by mutations far away from the direct binding site which cause long range effects on the binding mechanisms

without major changes in the observed binding affinities (Gupta et al., 1996). It is therefore difficult to dissect sites and assign affinity contributions to single interactions. In TSP-oligosaccharide binding sites the solvent also markedly influences the thermodynamic signature of complex formation. The TSP of bacteriophage HK620 recognises the O-antigen of its *Escherichia coli* host and produces hexasaccharides by enzymatic cleavage (Barbirz et al., 2008). In the HK620TSP-hexasaccharide complex a high number of solvent mediated contacts are found in the binding pocket (Broeker et al., 2013). Moreover, a substantial number of water molecules get displaced from the surface upon complex formation. In total, this results in small amounts of heat generated in the ITC experiment. The electrostatic interactions dominate HK620TSP-hexasaccharide complexes, resulting in an overall low affinity not amenable to an ITC experiment (Table 1) because desolvation energies are offset by the electrostatic contribution of ligand binding (Kadirvelraj et al., 2011). Water, however, can increase the affinity of an oligosaccharide binding site as shown for an HK620TSP mutant. Here, specific exchange of glutamate for glutamine formed a site for an additional water molecule (Fig. 2). Upon sugar binding, side chain rearrangements lead to desolvation. Together with additional hydrogen bonding this produced a thousand fold increase in hexasaccharide affinity corresponding to a $\triangle\triangle G$ of about −15 kJ mol^{-1} at 25 °C compared to the wild type (Table 1). In contrast, P22TSP has a more hydrophobic interface to form octasaccharide complexes via Van-der-Waals contacts (Baxa et al., 2001) (Table 1). Accordingly, P22TSP complex formation is comparable with what was observed for small lectin sites, i.e., enthalpically driven with an opposed entropy term. The HK620TSP hexasaccharide interaction site is an exception from this rule because now both enthalpy and entropy drive complex formation. However, it remains difficult to assign enthalpy and entropy contributions to single water molecules. The free energy needed to remove a bound water molecule from a protein is dependent on the specific surface structure at the water binding site and therefore contributes to the observed thermodynamics of ligand binding in a way difficult to predict (Fadda and Woods, 2011). As a consequence, surface-bound water can also be regarded as part of a protein surface and hence can shape binding sites and affect ligand affinities. This was shown exemplarily for
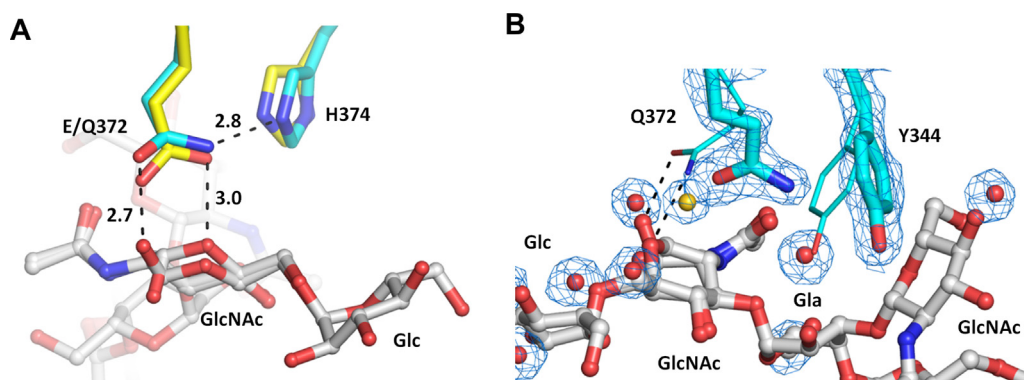
Figure 2 Rearrangements in the TSP-oligosaccharide binding pocket to generate a high affinity complex. (A) Amino acids in the ligand binding site of HK620TSP wild type (PDB: 2 × 85; yellow sticks) or E372Q mutant (PDB: 2 × 6w; cyan sticks) superimposed from two complex structures in the presence of O-antigen hexasaccharide of *E. coli* serogroup O18A1 (grey sticks). (B) The glutamine mutant has different side chain conformations in the unbound state (thick sticks). This leads to a different water network on the protein surface in the unbound state and an additional water site (gold). Upon binding, this water gets expelled because side chains rotate into place (thin sticks) and contributes about half of the additional free enthalpy difference. The rest of the 15 kJ/mol can be attributed to changes in hydrogen bonding networks on the protein when the side chains rotate.
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P22TSP-octasaccharide complexes. The TSP determines the host range of phage P22 which can infect *Salmonella enterica* subsp. *enterica* (*S.*) of serogroups O2, O4 and O9 (Eriksson et al., 1979) (Fig. 3). Structures of the O-polysaccharide repeat unit (RU) only differ in the 3-deoxyhexose epimer linked to the main chain mannose. The P22TSP binding site can bind 2RU corresponding to an octasaccharide (Andres et al., 2013; Steinbacher et al., 1996) (Fig. 3). ITC showed that P22TSP bind octasaccharides of serogroups O2 and O9 with higher affinity than of serogroup O2 (Table 1 and Fig. 3). Water molecules lining a hydrophobic pocket that accommodates the three different dideoxyhexoses of the first octasaccharide repeat unit force the serogroup O2-octasaccharide to bind as a conformer with a energetically less favoured torsion angle at the α-D-Man$p$-[1,4]-α-L-Rha$p$ glycosidic bond (Fig. 3D and E). As a consequence, the mannose bound galactose residue rotates away from the protein which reduces the overall affinity for the serogroup O2 ligand. Apparently, it is energetically more favourable to keep water on the surface and use it as hydrogen bonding partner than to meet the desolvation expenditure needed to bind the glycan low energy solution conformer.

Role of polysaccharide dynamics in protein binding

Carbohydrates naturally occur as oligomers and polymers of monosaccharide building blocks and restraints to accessible glycosidic torsion angles mostly dictate the sampling of the conformational space (Lütteke et al., 2005). It is a question of general interest either whether a preformed carbohydrate binding site selects a defined conformer from solution (conformational selection) or whether initial protein contact induces a conformational change on the carbohydrate (induced fit) (Weikl and von Deuster, 2009) and studies of binding kinetics may distinguish between the two mechanisms (Berger et al., 1999). Especially for bacterial polysaccharides it would be of great value

to be able to exactly describe these conformations to define protective epitopes for the development of vaccines (Jennison and Verma, 2004; Theillet et al., 2011; Vulliez-Le Normand et al., 2008). This is important because oligosaccharide vaccines often need to have specific hapten lengths (Johnson et al., 2012; Vulliez-Le Normand et al., 2008). Moreover, good conjugate vaccine epitopes that elicit an immune response must resemble the real bacterial surface carbohydrate structure. In this respect, regarding carbohydrate-antibody complexes does not always imply that the conformation found for the bound oligosaccharide can be used to deduce the correct structure for a good protective response as conformational selection or induced fit binding mechanisms might occur (Theillet et al., 2011). Studies on TSP revealed that sites could be created that can either bind solution or non-solution conformers of oligosaccharide binders. Whereas HK620TSP wild type binds the hexasaccharide conformer mostly populated in solution, mutations could alter the protein surface to bind a non-solution conformer (Broeker et al., 2013). By contrast, we could show for P22TSP that different oligosaccharide epimers bound as different conformers in an identical binding site (Andres et al., 2013). In both cases, binding of non-solution conformers could occur because the conformational space sampled by oligosaccharides does not contain high energy barriers. Description of the conformational space populated by long oligosaccharides or polysaccharides requires computational approaches due to the inherent chain dynamics (Woods and Tessier, 2010). Especially bacterial polysaccharides are important targets for analyses that explore alternative antibiotic or vaccine strategies. One example is the Gram-negative bacterium *Shigella flexneri* (*S. flexneri*) that causes bacillary dysentery mainly in developing countries. Its pathogenesis is related to production of the Shiga toxin. Accordingly, only live attenuated strains can be used as oral vaccines (Levine et al., 2007). However, they showed limited success rates, and the best protection against shigellosis remains wild type infection. Here,
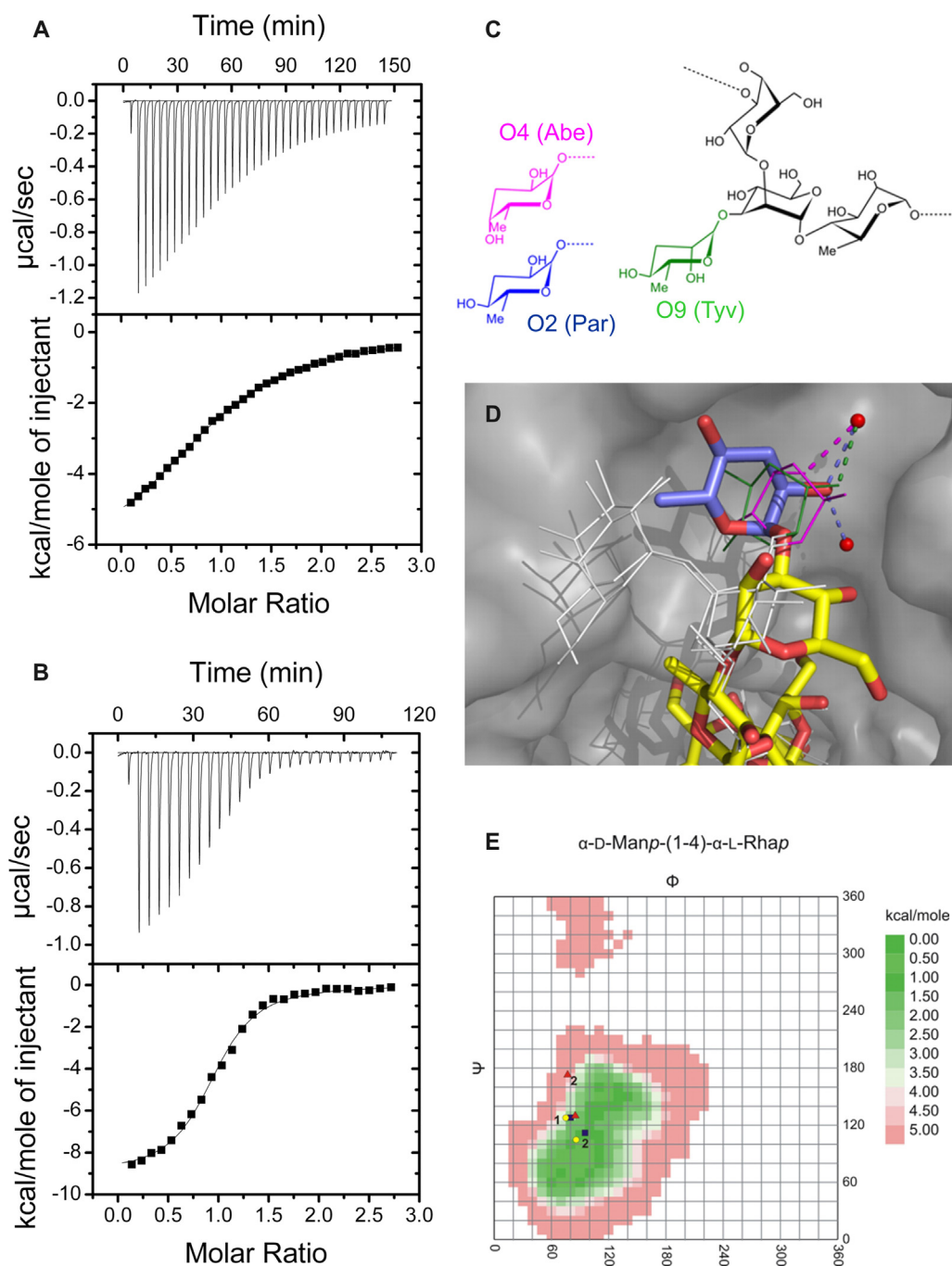
Figure 3   Representative calorimetric titration curves for TSP-*Salmonella (S.)* oligosaccharide affinity determinations. (A) Thermogram and binding isotherms for titration of P22TSP with *S.*Paratyphi A (serogroup O2) octasaccharide. At 10 °C a K_D of 26.3 μM was obtained. (B) Thermogram and binding isotherms for titration of P22TSP with *S.* Typhimurium (serogroup O4) octasaccharide. At 10 °C a K_D of 1.4 μM was obtained. (C) Structures of polysaccharide repeating units of *Salmonella* O-serogroups differing in the dideoxyhexose branch for *S.* Typhimurium (serogroup O4), *S.*Paratyphi (serogroup O2) and *S.*Enteritidis (serogroup O9) (Lüderitz et al., 1966). (D) Overlay of P22TSP-octasaccharide complex structures. The section shows the binding pocket for the different dideoxyhexoses (colour coded as in C) Octasaccharides of serogroups O4 (PDB: 1tyx) and O9 (PDB: 1tyu) are shown as thin grey sticks. The serogroup O2 octasaccharide (PDB: 3th0thick yellow sticks) is bound in a different conformation. As a result the non-reducing end galactose residue is rotated towards away from the protein towards the spectator and has been omitted for clarity. (E) Torsion angle positions of the α-D-Man*p*-[1,4]-α-L-Rha*p* glycosidic bonds occurring in the two octasaccharide repeat units (positions 1 and 2) of serogroups O2 (triangles), O4 (circles) or O9 (squares) bound to P22TSP plotted onto the phi-psi-energy diagram.
A, B and E reprinted with permission from Andres et al. (2013). Copyright 2013, Oxford University Press.
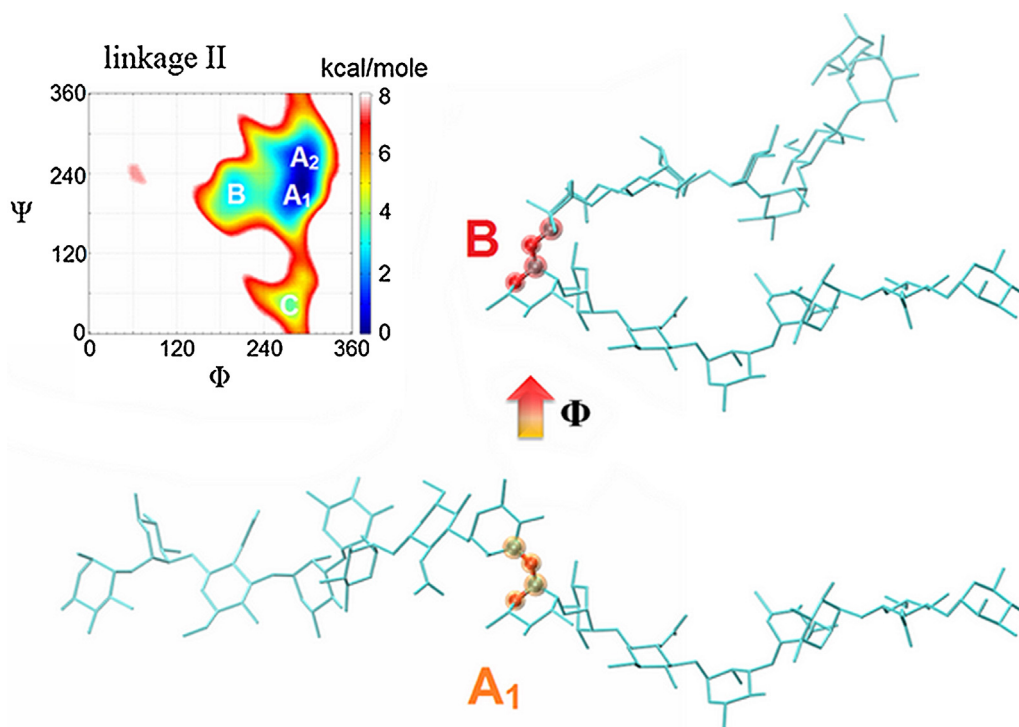
Figure 4 Computer simulations of end-to-end distances in *Shigella flexneri* O-polysaccharide fragments. Top left: Phi—psi free energy landscape of an α-L-Rha$p$- α-L-Rha$p$-OH disaccharide (''linkage II''). Bottom: Major solution conformer of *Shigella flexneri* O-serogroup Y docecasaccharide (3RU) obtained from MD simulation with linkage II in the A_1 state. Top right: About ten percent of the simulation time, a hairpin like conformer was observed resulting from rotation around phi and placing linkage II in the B state. Reprinted with permission from Kang et al. (2014). Copyright 2014, American Chemical Society.

strong reactions against O-antigen were observed, stressing its important role for immunisation. The *S. flexneri* O-antigen has a backbone with high (75%) rhamnose content and a growing body of evidence suggests that this specific rhamnose composition creates a polymer with highly dynamic glycosidic torsion angles (Jonsson et al., 2012). Several serotypes of this O-antigen exist that mainly vary in the substitutions of the backbone with acetylations or glucosylations (Foster et al., 2011). Especially the glucosylation pattern imposes constraints on the structures of extended O-antigen chains (Vulliez-Le Normand et al., 2008). During infection, *S. flexneri* can shield its toxin secretion system from the host's immune system by varying the thickness of the O-antigen layer via different glucosylation patterns (West et al., 2005). Accordingly, virulence of *S. flexneri* is intimately linked to its specific and dynamic O-antigen composition. A computational strategy was developed to describe *S. flexneri* O-serogroup Y chain dynamics (Kang et al., 2014) (Fig. 4). It showed that the conformational space sampled by a glycan can to a large extent be described at the level of independent glycosidic torsions the characteristics of which can be inferred from disaccharide fragments. The appearance of serotype Y O-Antigen is then largely that of a semiflexible polymer. However, the α-L-Rha$p$- α-L-Rha$p$-linkage assumes a special role in the sense that rotation about its phi angle into a state of higher energy can lead to extreme, hairpin-like conformations of the whole polysaccharide (Fig. 4). The possibility to assume these kink-like deformations may have some significance when long polysaccharide chains initially interact with bacteriophage TSP.

Conclusion

The analysis of TSP-glycan complexes shows the diverse features found to establish protein—carbohydrate interactions. Glycan dynamics are effectively described with computational approaches. Water in binding sites can tune affinity in carbohydrate complex formation and TSP complexes possess binding surfaces large enough to observe these effects experimentally. Moreover, carbohydrate conformational states are in balance with desolvation barriers. This opens the possibility to theoretically model desolvation enthalpies and compare with experiment.

Conflict of interest

The authors declare that there is no conflict of interest.

Acknowledgement

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