On the role of aromatic–sugar interactions in the molecular recognition of carbohydrates: A 3D view by using NMR*

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Abstract: This revision describes an up-to-date review of our efforts to investigate the inter-action of carbohydrates with aromatic moieties at different levels of complexity. Protein–sugar interactions have been studied using NMR experiments on a variety of hevein/chitooligosaccharide systems. In addition, NMR and computational methods have also been used to evaluate the interaction of simple aromatic entities with simple mono-saccharides. In between, the stacking features of aromatic-containing glycomolecules have also been described by using an analogous experimental–theoretical approach.

Keywords: NMR; molecular recognition; interactions; carbohydrates; aromatics.

INTRODUCTION

Many of the biologically relevant recognition processes are mediated by interactions between proteins and the carbohydrates that are present on the external surface of cell membranes [1]. The selectivity and specificity of the carbohydrate–protein interaction depend on a variety of factors. Indeed, because of the amphipathic character of sugars, different types of forces may be involved in their recognition by macromolecular receptors. It has become clear that apart from hydrogen-bonding and solvation effects, the presence of aromatic rings in the binding sites of lectins is essential for recognizing neutral sugars, especially of the Gal/GalNAc and Glc/GlcNAc families. In particular, the structure and conformation of the carbohydrate [2] as well as the nature and orientation of the aromatic rings are of paramount importance. Furthermore, each single sugar–protein interaction is weak in nature and multivalency is required for molecular recognition processes to take place [3]. While polar hydroxyl groups participate in intermolecular hydrogen bonds to both water and the side chains of polar amino acids [4], the hydrophobic face of saccharides, composed by many nonpolar C–H groups, interacts with the aromatic residues of protein side chains [5]. Although the latter is much weaker, its existence has been sufficiently proven by different means. Experimental data and theoretical models [6] have demonstrated that CH/π interactions play an important role in stabilizing carbohydrate–protein complexes [7]. Depending on the stereochemistry of the monomer constituents of the oligosaccharide chain, the presence of a number of rather apolar C–H groups in fact constitutes regions for which hydrogen bonds are not re-

sponsible for binding. When an apolar face of a monosaccharide shows three or more C–H groups close in space, it has been found that the corresponding surface can come into contact with an aromatic ring of an amino acid side chain, and additional affinity-enhancing factors can be envisaged. It may be proposed that the mutual shielding of the nonpolar surfaces from bulk water by ligand contact is entropically favorable, and that the electrostatic interaction between the positive net charge of the C–H groups and the quadrupole created by the π-system of the aromatic ring makes a favorable enthalpic contribution. Probably, the polarizability of the aromatic electrons and the polarizing nature of the C–H vector leads to an attractive force. Very recently, this type of interaction has also been shown to occur in gas phase [8].

RESULTS AND DISCUSSION

Sugar–aromatic interactions in protein receptors (lectins)

In Nature, sugar–aromatic interactions have been found in a number of lectin-sugar complexes, from which hevein domains have been extensively studied by us and other groups [9]. Hevein domains display a common structural motif of 30–43 residues rich in glycine and cysteine residues in highly conserved positions and organized around a three-disulfide core [10]. It is present in several lectins, as in hevein itself and its natural variant, pseudohevein, in the *Urtica dioica agglutinin (UDA), wheat-germ agglutinin (WGA),* and *Ac-AMP* antimicrobial peptides. The structures of these proteins and several of their domains have been solved by NMR and/or X-ray methods [11–15]. It has been demostrated that hevein domains are able to bind to chitin, a β-(1-4)-linked *N*-acetylglucosamine (GlcNAc) polysaccharide.

The binding affinities and thermodynamic parameters for chitooligosaccharide binding to hevein domains have been determined by several methods [16]. As typically observed for lectin–saccharide interactions, the processes are enthalpy-driven, while entropy opposes binding [17]. Apolar and polar interactions contribute to the complexation process, stabilizing the orientation of the sugar rings through formation of hydrogen bonds and by stacking interactions with aromatic side chains. The structural view obtained in solution agrees perfectly with the insights generated from the equilibrium thermodynamic parameters.

Variations in binding constants may be explained in structural terms: the minimum sugar size that can be bound by hevein is *N*-acetyl-D-glucosamine (GlcNAc), the parent monosaccharide, whose binding is stabilized by nonpolar forces involving Trp23 and Tyr30 and by hydrogen bonds involving Ser19 and the hydroxyl group of Tyr30 [18]. The binding constant of chitobiose is higher than that of GlcNAc. The use of methyl β-chitobioside enhances the binding. The presence of additional nonpolar interactions between the second sugar residue as well as the *O*-methyl group and the extended surface of Trp21 are probably the key factors in this instance. The binding constant found for chitotriose is even higher, probably as a result of the stronger van der Waals contacts established between the rather large surface area of the Trp21 indole ring and the pyranose chair. Two clear sugar–aromatic interactions are observed of the CH/π type, one between the nonreducing sugar and Trp23 and the second between the central moiety and Trp21 (Fig. 1B). Nevertheless, a second complex is also possible, arising through the shift of one sugar unit, the reducing and the central GlcNAc residues making the contacts with the aromatic amino acids implicated in binding (Fig. 1C).

As an additional example among these hevein domains, the interaction between Ac-AMP2, a lectin-like small protein isolated from *Amaranthus caudatus* of only 30 amino acids presenting three disulfide bridges and having antimicrobial and antifungal activity, and *N,N',N''*-triacetylchitotriose has been studied by NMR (protein database, pdb code 1MMC) [19,20]. The most pronounced NMR shifts in the protein resonances were observed mainly in the C-terminal half of the sequence, and involved the aromatic residues Phe18, Tyr20, and Tyr27. From the 3D structural viewpoint, the NMR-derived solution structure of Ac-AMP2 shows a structure which is very similar to the equivalent regions of hevein
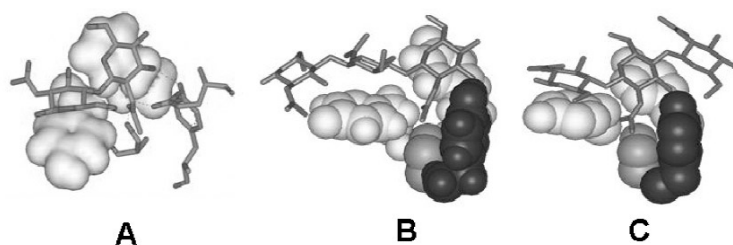
Fig. 1 The typical interactions in the complexes of hevein domains with chitooligosaccharides. A. Two stackings between two aromatic rings with two sugar residues, flanked by two hydrogen bonds (Ser19 to the carbonyl group of the acetamide sugar moiety and the hydroxyl group of Tyr30, in dark black, to a sugar hydroxyl group) are the key interactions. B and C. Two possible complexes are evidenced for trisaccharide binding to hevein domains (see text).

domains [20]. This molecule has served as a valuable scaffold for verifying the importance of CH/π interactions in the molecular recognition of carbohydrates by protein receptors [21,22]. Thus, the free-solution structure complexes of Ac-AMP2 mutants, in which Phe18 has been changed to either Trp, naphthylalanine, and 4-fluorophenylalanine was studied by NMR and molecular dynamics (MD) simulations as well as their binding ability to the same chitin fragment (the chitotrisaccharide) [22]. The polypeptide structure is very similar between all mutants and is also basically identical to that of wild-type Ac-AMP2 [20], with backbone rsmd values smaller than 1 Å. The NMR-derived 3D structure of wild-type Ac-AMP2 and the complexes of the different mutants with chitotriose shows the features described above for hevein, with two key aromatic–sugar interactions. The thermodynamic parameters of the interaction of Ac-AMP2 with triacetylchitotriose were determined by a van't Hoff analysis of the binding constants measured at different temperatures. Although the van't Hoff analysis of the data may only yield a fair estimate, as deduced for the other hevein domains, the process is enthalpy-driven, while entropy opposes binding. The association constant at 300 K amounts to 1200 M^{-1}, while the binding enthalpy is of the order of –50.1 kJ mol^{-1}. Mutations of Phe18 of Ac-AMP2 to residues having larger aromatic rings, namely Trp, β-(1-naphthyl)alanine, or, β-(2-naphthyl)alanine, enhanced the affinity [21,22]. Deactivation of the aromatic cloud by a fluorine atom, through transforming Phe to 4-fluoro-phenylalanine, also provided a 2-fold decrease in the binding affinity to chitotriose [22]. Thus, the affinity of a hevein domain for chitooligosaccharide binding depends on the chemical nature of the aromatic residues of the receptor and might be enhanced by adjusting the size and chemical nature of the aromatic residues involved in the interaction. The single replacement of any aromatic residue of Ac-AMP2 by Ala resulted in a significant diminution in affinity, suggesting the importance of the complete set of three aromatic residues in the ligand-binding site.

Sugar–aromatic interactions in model systems

These aromatic–sugar interactions also occur in simpler molecules and have been detected both experimentally and theoretically, in solution and in the solid state. Carbohydrate–arene interactions have also been shown to direct the conformational equilibrium of glycophanes [23], oligosaccharide mimics [24] and DNA-based glyco-oligoamide moieties [25] in water solution, and aromatic-protected sugar derivatives in less polar solvents [26]. It is interesting to emphasize the existence of intramolecular CH/π interactions. Within a collaboration of our group with those of Profs. Bernardi and Baldridge [27], it has been recently suggested that aromatic–sugar interactions may find application in the design of glycomimetics, since *intramolecular* sugar–aromatic interactions can help to stabilize bioactive conformations of small-molecule oligosaccharide mimics. In particular, NMR and computational studies of a functional mimic of the GM1 oligosaccharide (Fig. 2), strongly suggest that one of the factors determining its micromolar affinity for the cholera toxin (CT) is the presence of a stacking interaction be-
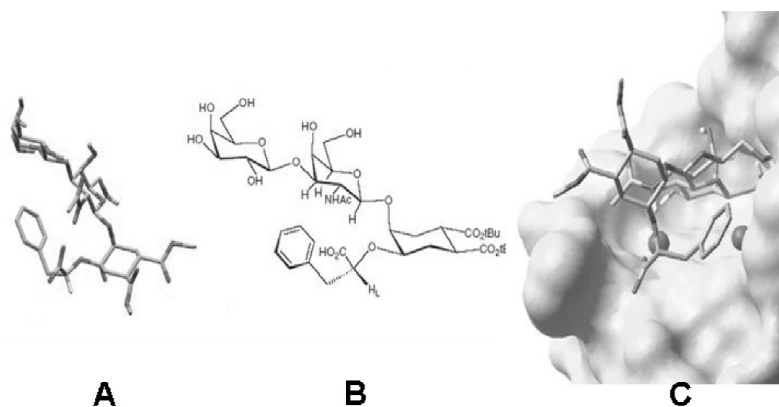
Fig. 2 Intramolecular carbohydrate–aromatic interaction for a GM1-glycomimetic (B). NMR experiments unequivocally shows the presence of a high percentage of the stacked conformation depicted on the left-hand side (A). Moreover, NMR experiments also show that this is the bound conformation to the lectin part of CT, as also confirmed by docking simulations (C). The preorganization allows for optimal interaction of the binding determinants to the binding region of the toxin with μM affinity [28].

tween the phenyl ring and the GalNAc residue (Fig. 2) [24]. Such an interaction biases the conformational behavior of this molecule and further analogs [27] by restricting the conformational freedom of the ether side chain. As a result, the side chain of the glycomimetic is "preorganized" in a suitable spatial orientation that allows optimal interaction of the binding determinants to the binding region of CT (Fig. 2) [28].

The existence of three C–H vectors pointing toward the same spatial orientation seem to be a key factor to define the stabilizing intramolecular carbohydrate–aromatic interaction that gives rise to the existing conformation. Nevertheless, preliminary results obtained from nuclear Overhauser enhancement spectroscopy (NOESY) spectra of some of these molecules in CD_3OD solution seem to indicate a weakening of the NOE contacts and possibly suggests a role for hydrophobic packing in defining the molecule conformation. Simple receptors based on aromatic entities have also recently been employed to recognize simple monosaccharides. However, in these cases, the driving force for the molecular recognition process is not completely clear [29].

Simple aromatics interact with certain carbohydrates: NMR-based evidences
A simple methodology for exploring carbohydrate–aromatic interactions from the structural viewpoint has also been presented by using NMR. Since chemical shift values are very sensitive to environmental changes concerning molecules in solution (e.g., solvent, temperature, neighbor molecules), the observation of chemical shift variations has been demonstrated to be crucial for identification of protein–ligand interactions [30], determination of binding constants [31], and protein secondary structure elucidation [32]. In the field of CH/π interactions, it has been reported by our group that specific ^{1}H NMR resonances of β-methyl galactoside dissolved in an aqueous solution undergo upfield shifting upon addition of phenol [33]. This behavior has been taken as a direct probe of the existence of CH/π interactions. We and other groups have recently applied this observation to other systems [34].

When molecules in which only one favored CH/π arrangement (α-methyl galactoside) are compared with others for which several favored CH/π arrangements might be present (α-methyl galactoside), the perturbation of the chemical shift is more evident (see Figs. 3 and 4 for details). In fact, the chemical shift variations observed for α-methyl galactoside (Fig. 3, only hydrogens 3,4,5 point toward the same spatial orientation) are larger than those observed for its β-methyl analog (Fig. 4, which presents two putative arrangements, those provided by hydrogens 3,4,5 and by hydrogens 1,3,5) in the presence of an aromatic moiety (phenol or any aromatic amino acid: Trp, Tyr, Phe) [33,34]. If only two CH
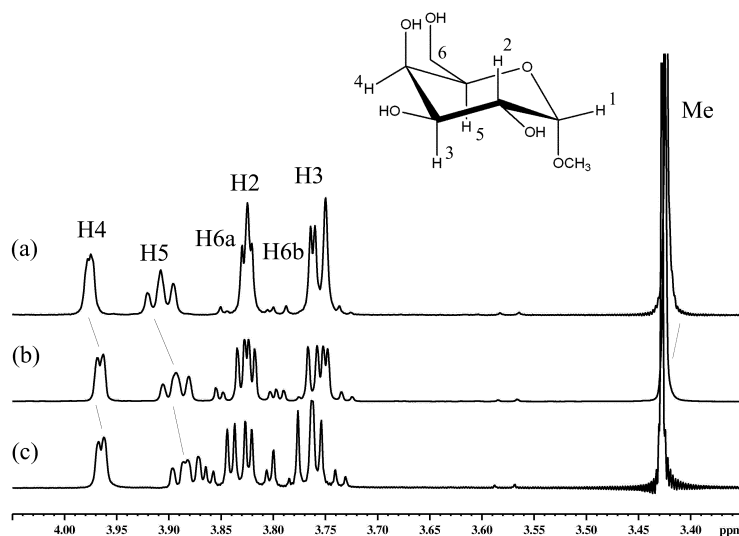
Fig. 3 500 MHz ^{1}H NMR spectra for α-methyl galactoside at 298 K in (a) D$_2$O and upon addition of phenol until sugar:phenol molar ratios of (b) 1:0.75 and (c) 1: 6.5 were reached.
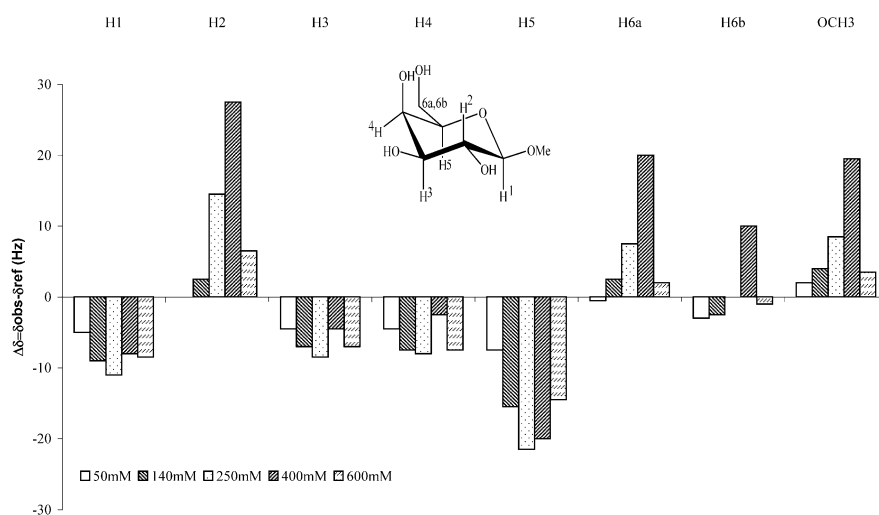

Fig. 4 Variation of chemical shift of the signals of β-methyl galactoside after addition of different amounts of phenol. The observed chemical shift (δ_{obs}) is subtracted from the chemical shift of the sugar in water (δ_{ref}) taken as reference.

vectors point on the same orientation (i.e., α-methylglucoside or mannoside) no measurable perturbation is evidenced in the NMR spectrum of the sugar. In summary, for carbohydrates bearing a single CH/π favoring arrangement (or 1,3,5- or 3,4,5-type), it is possible to observed systematic variations of specific chemical shifts as a consequence of the CH/π interaction with aromatic rings.

A 3D model: Theoretical calculations
As an additional support to verify the existence of stabilizing CH/π interactions on simple sugar–aromatic systems, calculations at different levels of theory have been performed [7,33,34]. Thus, the fucose/benzene complex (Fig. 5) has been carefully analyzed [33], as well as other complexes of fucose
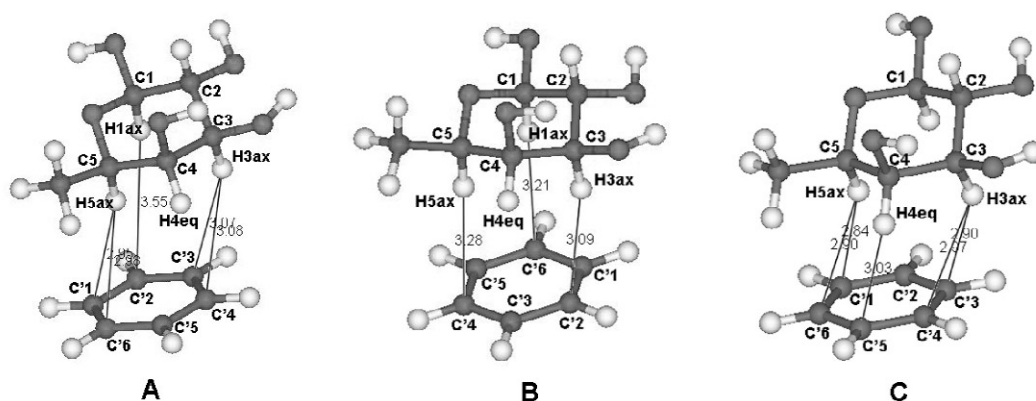
Fig. 5 Stationary states of the model system compose by fucose and benzene. A. The MP2/6-31G(d,p) with CP correction geometry. B. The B3LYP/6-31G(d,p) geometry. C. The AMBER-based structure. The stacking between both entities is evident at each level of theory. Remarkably, the calculated by molecular mechanics is close to that corresponding at MP2 level of theory, the one that has taken into account the correlation between electrons and the "counterpoise" correction.

with simple models of tryptophane and tyrosine. Different calculation methods, from basic molecular mechanics [35] to "ab initio" calculations using Gaussian program [36] at both, the density functional method (B3LYP/6-31G(d,p)) [37] and the computationally expensive MP2/6-31G(d,p) level [38] have been employed. Nevertheless, in all cases, the computed complexes were stable, except when fluorinated amino acids were employed as the aromatic entity [34]. Other authors have also approached this problem in a similar manner [7] using simple monosaccharide or disaccharide models with simple aromatic rings [7,34], or using an aromatic-containing glycomimetic [24–28].

It has been proven that the geometry of experimentally based galactose-lectin complexes [39] was properly accounted for by using the MP2/6-31G(d,p) level of theory, including the basis set superposition error correction [40] during optimization. It is important to point out that the use of simple molecular mechanics with the AMBER (assisted model building and energy refinement) force field also allows us to describe this geometry due to this kind of long-distance interactions can be studied by means of classical mechanics.

The stabilizing interaction energy of the fucose–benzene complex amounts to 2.4 kcal/mol [33], close to the experimentally available information of 1.5–2.0 kcal/mol obtained from the hevein/chitooligosaccharide cases in solution (see above) [22]. However, entropy factors and the role of water may influence the actual value for the aromatic–sugar interaction [17].

The theoretical results obtained indicated that the carbohydrate–aromatic interactions have a stabilizing character, as indicated by the minima in the potential energy curves calculated at the different levels of theory. Moreover, it was shown that inclusion of the correlation energy (as considered by the MP2 theory), that is, the dispersion interaction between uncorrelated monomers, was a key factor in producing a well-determined minimum in the interaction energy curve. The obtained results indeed established the importance of considering the dispersion energy for evaluating the energy for complex formation involved in aromatic–sugar interactions. The interaction between the sugar and aromatic molecules was also studied, using Bader's topological theory of atoms in molecules (AIM) [41]. The analysis of the electronic density generated by the geometry obtained at the MP2/6-31G(d,p) level (which is very similar to that proposed by molecular mechanics, as shown in Fig. 5C), showed the presence of bond, ring, and cage critical points that supports the existence of three maxima in the electronic density between the two moeities, in a 3,4,5-type arrangement (Fig. 5A). This sugar–aromatic geometry is a recurrent arrangement for galactose in many carbohydrate–protein complexes deposited in the Protein Data Bank (PDB) [39].

Although it is well known that the hybrid method, based on the density functional theory, B3LYP is not fully adequate to study long-distance interactions, due to the fact that its consideration of the dispersion term is somehow deficient, calculations were also performed using density functional theory to estimate the effect of the absence of this term in the determination of the geometry of the supramolecular complex. The calculated minimum at the B3LYP/6-31G(d,p) level features a substantial change in the molecular geometry. Arrangement between the two entities is now 1,3,5-type with much longer C–H distances between the sugar and the aromatic moieties. Each fucose hydrogen atom is oriented toward a specific carbon on the aromatic rings, with distances above 3.20 Å between both entities, much longer than on the AMBER or MP2-based geometries, which are well below 3.0 Å. The absence of the dispersion term possibly increases the repulsion, and the monomers are more separated than in the previous complex (Fig. 5B).

CONCLUSIONS

Sugar–aromatic interactions are key forces for molecular recognition of carbohydrates in Nature, as evidenced from the study of a variety of lectin–sugar systems, as the hevein–quitin pair described herein. In addition, depending on its chemical nature and relative stereochemistries at their chiral centers, certain sugars show an intrinsic tendency to interact with simple aromatic residues in water solution, provided that at least three C–H vectors point toward the same spatial direction. This fact has been demonstrated experimentally by using NMR [42]. The perturbation of chemical shifts is a fairly reliable indicator for the existence of CH/π interactions between carbohydrates and aromatic compounds under neutral conditions in water solution. Finally, a theoretical study using calculations at different levels of theory have provided a 3D picture of the formed complexes. The use of molecular mechanics calculations provides a fair description of the supramolecule, which indeed shows a remarkably similar geometry to that obtained through the computationally expensive MP2/6-31G(d,p) level of theory.

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