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Carbohydrate–Aromatic Interactions

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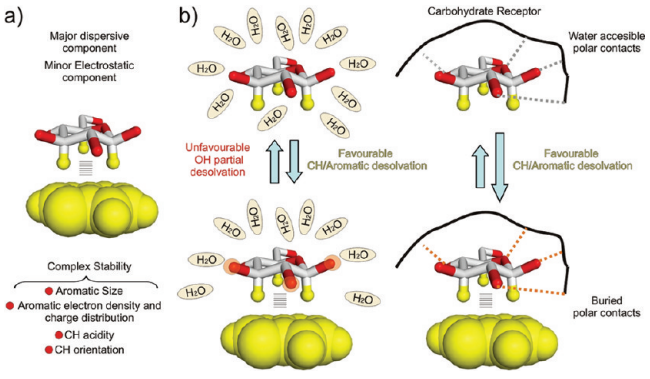
CONSPECTUS

The recognition of saccharides by proteins has far reaching implications in biology, technology, and drug design. Within the past two decades, researchers have directed considerable effort toward a detailed understanding of these processes. Early crystallographic studies revealed, not surprisingly, that hydrogen-bonding interactions are usually involved in carbohydrate recognition. But less expectedly, researchers observed that despite the highly hydrophilic character of most sugars, aromatic rings of the receptor often play an important role in carbohydrate recognition.



With further research, scientists now accept that noncovalent interactions mediated by aromatic rings are pivotal to sugar binding. For example, aromatic residues often stack against the faces of sugar pyranose rings in complexes between proteins and carbohydrates. Such contacts typically involve two or three CH groups of the pyranoses and the π electron density of the aromatic ring (called CH/π bonds), and these interactions can exhibit a variety of geometries, with either parallel or nonparallel arrangements of the aromatic and sugar units.

In this Account, we provide an overview of the structural and thermodynamic features of protein–carbohydrate interactions, theoretical and experimental efforts to understand stacking in these complexes, and the implications of this understanding for chemical biology. The interaction energy between different aromatic rings and simple monosaccharides based on quantum mechanical calculations in the gas phase ranges from 3 to 6 kcal/mol range. Experimental values measured in water are somewhat smaller, approximately 1.5 kcal/mol for each interaction between a monosaccharide and an aromatic ring. This difference illustrates the dependence of these intermolecular interactions on their context and shows that this stacking can be modulated by entropic and solvent effects. Despite their relatively modest influence on the stability of carbohydrate/protein complexes, the aromatic platforms play a major role in determining the specificity of the molecular recognition process.

The recognition of carbohydrate/aromatic interactions has prompted further analysis of the properties that influence them. Using a variety of experimental and theoretical methods, researchers have worked to quantify carbohydrate/aromatic stacking and identify the features that stabilize these complexes. Researchers have used site-directed mutagenesis, organic synthesis, or both to incorporate modifications in the receptor or ligand and then quantitatively analyzed the structural and thermodynamic features of these interactions. Researchers have also synthesized and characterized artificial receptors and simple model systems, employing a reductionistic chemistry-based strategy. Finally, using quantum mechanics calculations, researchers have examined the magnitude of each property's contribution to the interaction energy.

Introduction

The essential processes of life largely occur by specific interactions between biomolecules. Among them, carbohydrates are fundamental for cell–cell communications.[1] Carbohydrate–protein interactions are central to a variety of fundamental biological phenomena, including protein trafficking, cell adhesion, fertilization, infection, tumor metastasis, and different aspects of the immune response.[1,2]

Not surprisingly, early crystallographic studies revealed that hydrogen-bonding interactions are usually involved in carbohydrate recognition.[3] Somewhat less expected was the observation that, despite the highly hydrophilic nature of most sugars, aromatic rings of the receptor often play an important role in this process. The presence of aromatic amino acids in the carbohydrate binding sites of proteins was already observed in lysozyme–chitooligosaccharide

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complexes,[4] the first enzyme whose 3D structure was determined by X-ray crystallography. The importance of tryptophan residues for carbohydrate binding was further highlighted by nuclear magnetic resonance (NMR) strategies available at those times.[5,6] Later in 1986, Quiocho, after connecting the L-arabinose and D-galactose binding bacterial chemotactic proteins to other reported carbohydrate−protein complexes, proposed carbohydrate−aromatic stacking as a common feature for carbohydrate recognition.[6]

Indeed, data-mining tools[7,8] have highlighted the extraordinarily high frequency of aromatic amino acids, especially tryptophan, in the carbohydrate binding sites of proteins.

Interestingly, recent studies have revealed that carbohydrate/aromatic interactions are not restricted to protein complexes but also are present in carbohydrate-binding RNAs,[9] highlighting the relevance of the aromatic rings as key elements for carbohydrate recognition. Combined efforts of structural biologists, as well as biological and theoretical chemists have provided insights into the different contributions that stabilize carbohydrate/aromatic complexes.[10]

Experimental Evidence. The Different Architectures

Carbohydrate−aromatic stacking has been observed in most carbohydrate−protein complexes, with either enzymes or receptors, for a large variety of protein folds and functions. As examples, it is possible to mention many lectins, including hevein domains,[11] plant toxins, or animal galectins.[12] This structural feature is very frequent among those carbohydrate-binding modules (CBM) associated with glycosidases for polysaccharide metabolism.[13] Since there are significantly fewer glycosyl tranferase structures experimentally available, not many examples have been reported.[8,14] Carbohydrate−aromatic stacking is also frequently found in sugar-sensor/transport proteins, such as the chemotactic receptors,[6] and others.[15] In antibodies, the aromatic rings interact either with their own glycans in an intramolecular fashion[16] or intermolecularly with their polysaccharide antigens.[17]

The geometric features of the interaction are not strictly unique. From the point of view of the protein, different architectures of the binding sites can be delineated (Figure 1), depending on the number and relative location of aromatic residues. In many cases, such as galectins,[11,12] there is only one aromatic ring providing stacking with the sugar, defining one monosaccharide binding subsite. In other examples, spatially contiguous aromatic rings are grouped, forming an extended binding site with two (or more) aromatics, which define sequential subsites (n, $n + 1$, etc). Indeed, they are preorganized to stack with consecutive monomers in oligosaccharides, as in hevein domains.[18] There are also extended binding sites with even six subsites with aromatic residues located at every other subsite (n, $n + 2$, $n + 4$, etc). This presentation is observed in polysaccharide degrading enzymes and their associated CBMs.[19] Nevertheless, this organization may adopt different shapes, forming extended surfaces, grooves, or even tunnel-like motifs. Evidence of the importance of the presence of aromatic residues at the entrance of an active site tunnel to provide glycosidase activity has been presented by atomic force microscopy (AFM) techniques with native and mutant processive enzymes lacking one specific tryptophan residue.[20] Two aromatic residues may provide a double aromatic stacking over a monosaccharide, forming a sandwich-type arrangement, which can even give a more complex architecture, as in *Urtica dioica* lectin, in which two protein chains wrap around one oligosaccharide chain[21] (Figure 1).

The available structural information, with more than 90 nonredundant CBD 3D-structures showing carbohydrate−aromatic stacking, has allowed improvement of protein-modeling strategies by introducing a "hydrophilic aromatic residue" parameter as a restriction for structural modeling. This approach has been successfully employed to unravel cases where sequence homology was low.[22]

From the carbohydrate perspective, the stacking can take place in different manners. In principle, a pyranose presents two well-defined (α and β) faces (Figure 2), which could interact with the aromatic moieties. Experimental and theoretical evidence has shown that the interaction is favored for that face presenting several axially oriented C−H bonds and largely disfavored for those faces decorated with axial hydroxyl groups.[23] The interaction is strictly dependent on the sugar configuration. Pyranose−aromatic ring stacking has been documented for galacto- (or fuco)-type configurations, either for α or β anomers, but exclusively through its α-face (Figure 2c). This is also the case for β-mannoses, with exclusive stacking through the α-face (Figure 2b) and no stacking for the α-analogues. For gluco-type sugars, including xylose- or GlcNAc-containing oligosaccharides, the stacking can take place from both faces for β-glycosides (Figure 2d), even simultaneously, while for α-anomers, the aromatic moiety only sits on top of the β-face (Figure 2a).[23,24]

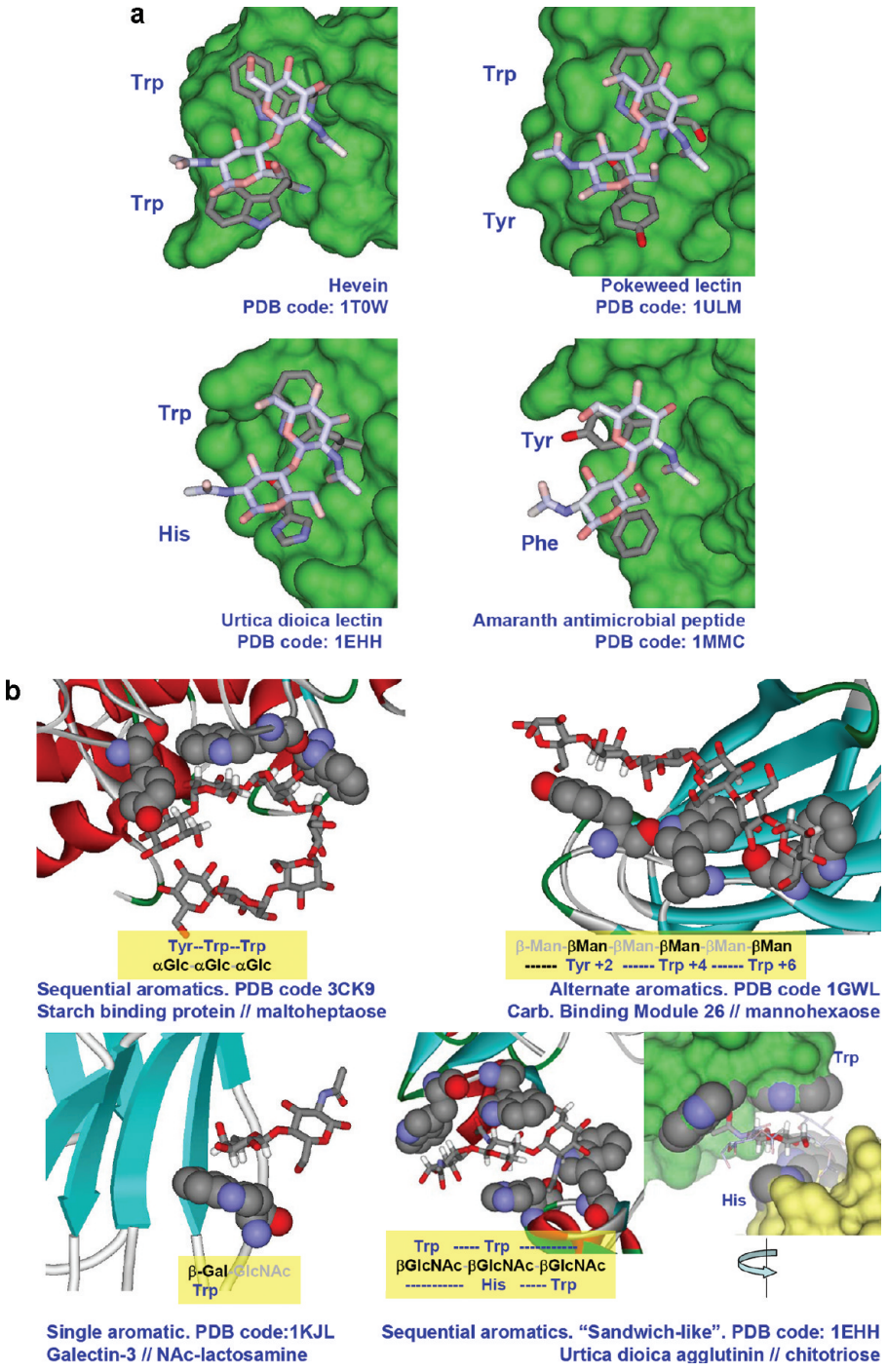

FIGURE 1. (a) Selected examples of carbohydrate binding sites, showing the presence of the four aromatic amino acids in homologous hevein domains when complexed with the chitin dimer. (b) Examples of the diversity of carbohydrate binding-site topologies and of the structures of carbohydrate ligands. The key hydrogens facing the aromatic rings are shown. In the case of the *Urtica dioica* agglutinin dimer (bottom right), the chitotriose entity is sandwiched between two different binding sites, each belonging to a different protein monomer.

However, additional geometrical orientations should also be considered. Sometimes, stacking interactions are not expressed through the exact parallel orientation of the pyranose chair with the aromatic ring. For galacto-type configurations, the sugar chair slides over the aromatic moiety and presents H3, H4, and H5 to the amino acid side chain (Figure 2c).[23−25] Indeed, a geometry analysis performed over an extended set of experimental sugar−protein complexes showed that the position of the center of the pyranose ring can take a large set of spatial orientations

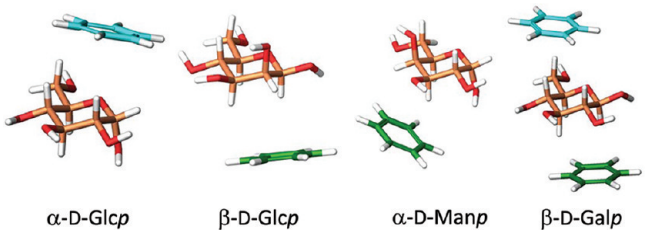

FIGURE 2. Examples of the different topologies of carbohydrate/aromatic stacking from the sugar point of view. Glcp stands for glucopyranose, Manp for mannopyranose, and Galp for galactopyranose. The α and β refer to the anomeric configuration. In the corresponding text, α- and β-faces refer to the spatial location where the corresponding α- and β-substituent is placed.

relative to the aromatic residue.[25] As it will be mentioned below, in the Theoretical Evidence section, there is a weak orientation dependence of the sugar–aromatic interactions. Therefore, these complexes display a high dynamic character.

There are very few reported cases for furanosides, although stacking interactions have been observed when the five-membered ring adopts the proper geometry for the favorable orientation of its CH bonds, as in the complex of an antibody with arabino-containing polysaccharides.[26] In any case, the thermodynamics of furanose–aromatic binding motif deserves further studies.

Aromatic stacking has been scarcely observed for protein complexes with charged saccharides. Indeed, for negatively charged sugars, as heparin glycosaminoglycans, the binding site is composed of complementary cationic amino acids, which establish electrostatic interactions and do not facilitate neighboring of aromatic chains.[27] For positively charged carbohydrates, there is not yet enough structural data available to generalize these interactions. Nevertheless, it has been shown in model systems that the interaction with the protein is very dependent on the protonation state of the interacting amino sugars.[28]

Affinity and Selectivity

From the protein perspective, the affinity and selectivity of the interaction depends on the nature of the aromatic residue. Using mutagenesis-based experiments, it has been shown that elimination of aromatic moieties drastically reduces the affinity,[29] while the exchange among aromatics permits the modulation of the receptor properties.

Theoretical calculations have highlighted the importance of stacking interactions, also in the context of enzymatic polysaccharide hydrolysis. It has been hypothesized that the efficiency of processive glycosidases is directly related to the existence of strategically positioned aromatic residues, since their removal in enzyme tunnels reduced the ligand binding free energy, and switched the enzyme function from processive to nonprocessive.[19]

In a parallel manner, the study of different GH10[8] xylanases with five conserved aromatic residues allowed estimating a favorable 0.5–1 kcal/mol contribution to the ΔG of binding for each subsite by isothermal titration calorimetry (ITC).[30] However, the geometrical positioning of the residues did not allow the simultaneous establishment of all possible carbohydrate–aromatic stacking interactions. The analysis of the thermodynamic parameters permitted guessing the potential of stacking interactions at the different subsites, relating the aromatic–carbohydrate contact surface area at each subsite to the corresponding changes in heat capacity (ΔC_p). In general, the exact contribution of stacking interactions in glycosidases cannot be generalized. The aromatic residues form a binding platform where stacking, solvation–desolvation of the exposed surfaces, conformational perturbations, and other interactions are differently balanced.

Residue exchange among the different aromatic amino acids has indicated that the affinity increases with the size of the aromatic ring. Nature has provided evidence for that: the four aromatic amino acids participate in carbohydrate–aromatic stacking in any of the four hevein domains of wheat germ agglutinin (WGA).[31] Using X-ray, fluorescence, NMR, and ITC experiments,[11] systematic studies of the importance of the type of the aromatic ring has been performed.[32,33] For the hevein fold, chemically synthesized mutants of the antimicrobial AcAMP peptide, with phenylalanine, tryptophan, or unnatural naphthylalanine and 4-fluorophenylalanine amino acids have been studied. The thermodynamic binding parameters were interpreted with the NMR-based 3D structures of the complexes. It was shown that increasing the size of the aromatic ring strongly favored binding, while electron withdrawal by fluorine significantly reduced the affinity.[33]

The knowledge of the key forces involved in sugar recognition has been also employed for protein engineering. For instance, specifically designed mutagenesis experiments have been elegantly employed for achieving galactose recognition, starting from a mannose-binding protein.[34]

The Substrate Point of View: Selectivity and Specificity

So far, few studies have quantitatively analyzed the influence that modifications in the sugar length or configuration

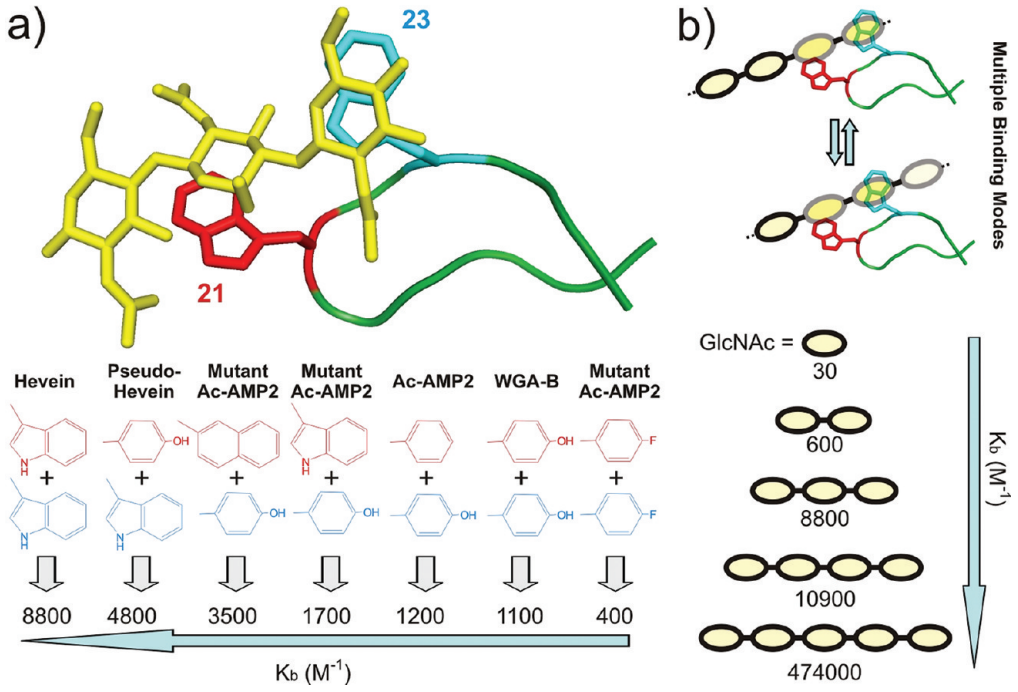

FIGURE 3. Hevein/chitooligosaccharide interactions: (a) Top, hevein domains display a double stacking of two aromatic residues with two contiguous GlcNAc moieties of the chitin chain. Hevein presents two tryptophan residues in the binding site. Bottom, changes in binding affinity depending on the chemical nature of the two aromatic residues.[11,18,35–40] (b) Top, the interaction of trisaccharide or larger GlcNAc oligosaccharides is dynamic, with exchange among distinct interaction modes. Bottom, the binding affinity increases with the oligosaccharide length, reflecting the importance of the existence of multiple binding modes along with the presence of multivalency. Above the pentasaccharide, two or more hevein domains interact with the same oligosaccharide chain.[35–40]

have on the stability of the carbohydrate/aromatic complexes. Using hevein domains,[11] a systematic analysis of the structural and energy features of the interaction of *N*-acetylglucosamine (GlcNAc) moieties with lectins has been presented (Figure 3).

The protocol implied the use of differently substituted mono- to hexa-saccharides, with different sugar stereochemistry, using NMR methods assisted by molecular modeling, ITC, and fluorescence techniques.[18,35–39] The obtained results were compared with those already published by other authors by employing X-ray crystallography and other procedures.[11] Combining the 3D structural perspective with affinity measurements, the enthalpy change associated with a single carbohydrate–aromatic interaction was estimated between 1.5 and 2 kcal/mol. All the observed variations in affinities were explained in structural terms, and key features of the molecular recognition process, including dynamic aspects, were unravelled. Thus, restriction to the motion of aromatic rings when passing from the free to bound states was detected, as well as the existence of complexes of different topology in chemical exchange.[35–39] For long oligosaccharides, the existence of multivalent processes in which a single oligosaccharide chain was bound to two protein domains was deduced.[35,40] The employment of sugar ligands with different stereochemistry at specific positions also permitted some to deduce that the stacking interactions were extremely sensitive to the glycoside shape. The receptor aromatic rings were major contributors to the selectivity and specificity of the molecular recognition process and disallowed binding of particular sugar epimers, through steric hindrance or by creating unfavorable nonpolar environments for axial OH groups.[18,39]

From Nature to the Bench: Carbohydrate–Aromatic Interactions in Simple Models, Chemical Systems, and Artificial Lectins

As frequently employed in chemistry-based approaches, reductionism has been used to study carbohydrate–aromatic stacking interactions. Using simple models, composed by just one monosaccharide and one aromatic ring, this interaction has been characterized using different methodologies.[41–44] The recognition process strongly depends on the nature of the sugar, and three CH groups must be on top of an aromatic ring to be an NMR-detectable interaction.[23] Calorimetric studies[45] established its enthalpic nature, while IR[46] has been essential to confirm its major dispersive character and also to detect OH–π hydrogen

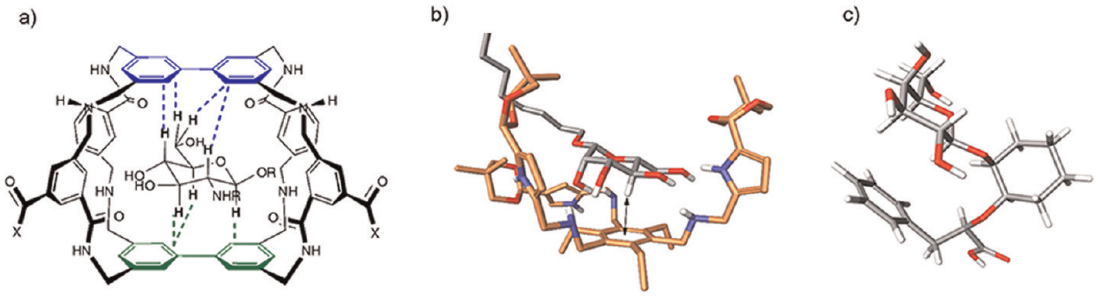

FIGURE 4. Different designs of artificial sugar receptors. (a) The design by Davis[57,58] is able to effectively recognize sugar molecules in water solution by hydrogen bonds and stacking interactions. (b) The open receptors developed by Roelens[59] recognize Man moieties in organic polar solvents. Again, stacking interactions and hydrogen bonds provide the driving force for recognition. (c) A glycomimetic of GM1 adopts the proper geometry to interact with cholera toxin thanks to intramolecular carbohydrate/aromatic stacking.[52,53]

bonding in the absence of water.[46] However, the existence of a certain hydrophobic component was deduced, since the interaction was not detected by NMR in other polar solvents, such as acetonitrile.[23,24] Interestingly, although the stacking interaction has been demonstrated in the gas phase by IR,[46] using more complex glycopeptide models, this weak interaction in the gas phase is not able to compete with classical hydrogen bonds, even intramolecularly.[47]

Stacking between aromatics and sugars has been observed and used in complex structures and further employed as platforms to design artificial systems,[48−51] to control the conformational behavior of glycomimetics,[52,53] or to control the formation of hydrogel-like supramolecular structures.[54]

The strength of a single carbohydrate–aromatic interaction has been studied in the context of the formation of β-hairpin in aqueous solution, employing model glycopeptides with diverse sugars and aromatics. It was shown that, in the absence of other noncovalent contacts, a single sugar–aromatic interaction may modulate protein folding with a magnitude of ca. −0.8 kcal/mol. Fittingly, replacement of the aromatic ring with an aliphatic group resulted in a decrease in the energy to −0.1 kcal/mol.[55,56] The importance of solvation/desolvation of the interacting groups was also highlighted.[55,56]

The growing knowledge of these interactions has been applied to design artificial carbohydrate receptors,[47,57] which employ a wise combination of hydrogen bonds and stacking interactions (Figure 4), as elegantly illustrated.[57−59] The use of differently substituted synthetic receptors has also highlighted the importance of hydration for effective sugar binding and proper stacking.[60]

Theoretical Evidence

From a theoretical perspective, initially it could be considered as analogous to a H-bond, although different in its physical origin.[61] It is well-known that, for interactions between aromatic surfaces and alkanes, experimental data in the gas phase compared well with those obtained from high-level *ab initio* calculations (CCSD(T)).[61,62] Following this reasoning, a first conclusion, derived from calculations on sugar–aromatic models, is that the dispersive component is dominant while the electrostatic contribution is minor.[61,62] Indeed, theoretical[23] and experimental studies[63] have confirmed the presence of electronic density between the sugar hydrogen atoms and the aromatic ring.[23] Also, the dominance of the dispersive contribution implies that the orientation dependence of the carbohydrate–aromatic interactions is weak, conferring to these complexes a dynamic character.

A systematic scan of the potential energy surface of carbohydrate–aromatic complexes, carried out using simple models,[64] showed that the dispersion interactions are highly distance dependent and not equally distributed around each carbohydrate atom. The energy for the dispersion interaction was beyond −5.0 kcal/mol, but only in small localized areas, for optimum interatomic distances. In principle, this number could suggest that the energies experimentally found correspond to dynamic systems in solution that could rise up to the theoretical value for rigid complexes, although other factors, such as solvation effects, should be contemplated (see below).

The cooperativity between multiple CH/π bonds has been investigated theoretically.[65] The structural information available showed that in most cases, two or three CH groups of the pyranose unit participate in CH/π contacts with the same aromatic system. When the additivity of these interactions was explored,[65] the calculations showed that bidentate complexes are weaker than the sum of two monodentate ones, this difference being larger for interactions with naphthalene than with benzene.

Consequences for Molecular Recognition

The interaction energy theoretically estimated for each sugar-aromatic stacking amounts to 3–6 kcal/mol.[23,64] These values are larger than the experimental ones in water: the contribution of every sugar–aromatic stacking in hevein complexes has been estimated at ca. 1.5–2.0 kcal/mol.[11] Also, the interaction energy between a single sugar and an aromatic amounted to 0.8 kcal/mol, as revealed by studies on glycopeptides.[55,56] The stacking of glycosides with DNA base-pairs contributes less than 0.5 kcal/mol to duplex stability.[66] These examples illustrate the context-dependent character of carbohydrate/aromatic stacking, modulated by entropic and solvent factors.

A soft nature and a low directionality seem to be essential features of the sugar/aromatic interaction. According to this view, the main role of the aromatic platforms in protein or nucleic acid receptors would be to provide a plastic contribution to the association energy that can be modulated by the local environment of the receptors to achieve both affinity and selectivity.

Theoretical analyses of stacking complexes have shown that dispersive forces play a dominant role. As a consequence, the interaction critically depends on the size and shape complementarity of the interacting surfaces. Regarding the electrostatic component, while relatively minor, it offers interesting opportunities to modulate the attractive forces between pyranoses and aromatic rings. In principle, the stability of the carbohydrate/aromatic complexes could be enhanced by incorporation of electron-donating substituents on the aromatic ring. Alternatively, the polar character of the interacting CH groups could be increased by substitutions on the pyranose. The potential of these simple strategies are yet to be explored. In addition, current research supports the notion that water plays an essential role in carbohydrate recognition.[25,58,59,63,67]

It should be noted that, despite their overall polar nature, saccharides include hydrophobic patches whose particular topology depends on the axial/equatorial orientation of the OH moieties. These acknowledgments led to the proposal that solvophobic effects represent a key stabilizing influence for the interaction. Studies on simple models have shown that replacement of the aromatic by simple aliphatic chains renders the interaction almost undetectable, suggesting that the hydrophobic component might be lower than originally suspected, even with no key role in the carbohydrate/aromatic interaction.[10] In our opinion, the safest assumption is that desolvation of the aromatic system and the pyranose CH groups constitutes a relevant driving force for complex formation. However, the precise contribution of this hydrophobic component to the net interaction energy remains an open question.

Alternatively, it has also been shown that solvent-dependent contributions to the interaction energy could also contribute to destabilize the stacked complexes.[55,56,60] Thus, depending on the topology of the complex, desolvation of the pyranose CH groups might be accompanied by partial desolvation of the polar substituents. This unfavorable effect seems to be especially relevant in charged glycosides and would also oppose molecular recognition of neutral ligands in a configuration-dependent manner. In agreement with this view, chemical modifications of the sugar, like O- or N-methylation, lead to an enhancement of the stacking.[29]

Despite this energy cost, proteins manage to recognize carbohydrates with moderate affinities and exquisite specificities. However, ligand desolvation does not rely exclusively on carbohydrate/aromatic contacts. In most cases, the hydroxyl moieties of the bound oligosaccharides are not free but are involved in extensive intermolecular hydrogen bonding. This observation suggests that Nature uses cooperativity to achieve affinity, with aromatics and hydrogen-bonding groups synergistically operating to desolvate and bind carbohydrates. By promoting desolvation of the hydroxyl groups, the receptor polar groups would favor the stacking of the pyranoses with aromatics. In turn, the hydrophobic environment provided by these aromatics might cooperatively enhance the strength of the receptor/ligand polar interactions.[55,56] These conclusions have important implications for the design of artificial carbohydrate receptors.

The future will bring further studies and applications of carbohydrate/aromatic stacking in different fields. Understanding the functional role of aromatic residues in glycosidases may be used for rational design of novel carbohydrate-active enzymes.[19,20] In particular cases, optimization of carbohydrate/aromatic contacts might lead to improved protein binders or enzyme inhibitors. This task would greatly benefit from a quantitative understanding of how dispersion, electrostatics, and solvent dependence contribute to the interaction energy. Additionally, stacking can be employed as a portable structural module. This concept has permitted stabilization of native states of glycoproteins up to −2.0 kcal/mol, thanks to the stacking provided by placing a phenylalanine residue two or three positions before a glycosylated asparagine in distinct reverse turns.[68] Additionally, technological applications of these interactions, including the solubilization of carbon

nanotubes[69,70] could also be envisaged. We are looking forward to future developments in this field.

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BIOGRAPHICAL INFORMATION

Juan Luis Asensio (Barcelona, Spain) received his Ph.D. degree (Chemistry) in 1995. After a postdoctoral period at the National Institute for Medical Research at Mill Hill, he moved back to the IQOG-CSIC in 1998, becoming a tenured scientist in 2000. He was promoted to senior research scientist in 2008 and has focused his research on molecular recognition studies of sugars, nucleic acids, and proteins.

Ana Ardá (Coruña, Spain) received her Ph.D. degree (Chemistry) in 2006. After a postdoctoral period at the Bijvoet centre in Utrecht working with NMR, in 2008, she moved to Jiménez-Barbero's group at CIB-CSIC, as Juan de la Cierva scientist, focusing her research on NMR and molecular recognition.

Francisco Javier Cañada (Bilbao, Spain) received his Ph.D. degree (Chemistry) in 1985. He spent a postdoctoral period with Prof. Vazquez at the Molecular Biology Centre in Madrid. Since then, his research interest has been at the interface between Chemistry and Biology. In 1988, he moved to Prof. Rando's group at Harvard Medical School. In 1991, he came back to Madrid (IQOG-CSIC) to work with Prof. Martín-Lomas. In 1992, he got a tenured scientist position, focusing his research on molecular recognition processes between carbohydrates and proteins. In 2002, he moved to CIB-CSIC (Madrid) to build up the NMR group in the Chemical & Physical Biology Department. In 2009, he was promoted to Full Professor.

Jesús Jiménez-Barbero (Madrid) got his Ph.D. (Chemistry) in 1987. He did postdoctoral studies at Zürich, at the National Institute for Medical Research at Mill Hill, and at Carnegie Mellon University (1988−1992). His main topic of work focuses on molecular recognition, especially on protein−ligand interactions, with particular emphasis on the application of NMR methods. He was promoted to CSIC Research Professor in 2002 and moved to CIB-CSIC (Madrid), where he is heading the Chemical & Physical Biology Department. Since 2012, he is also the President of the Royal Society of Chemistry of Spain.

FOOTNOTES

The authors declare no competing financial interest.

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