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STRUCTURE AND CONFORMATION OF COMPLEX CARBOHYDRATES OF GLYCOPROTEINS, GLYCOLIPIDS, AND BACTERIAL POLYSACCHARIDES

C. Allen Bush and Manuel Martin-Pastor
Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, Maryland 21250; e-mail: bush@umbc.edu, mmartin@chem.umbc.edu

Anne Imberty
Centre de Recherches sur les Macromolécules Végétales, CNRS, BP 53, F-38041 Grenoble cedex 9, France; e-mail: imberty@cermav.cnrs.fr

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ABSTRACT

For nuclear magnetic resonance determinations of the conformation of oligosaccharides in solution, simple molecular mechanics calculations and nuclear Overhauser enhancement measurements are adequate for small oligosaccharides that adopt single, relatively rigid conformations. Polysaccharides and larger or more flexible oligosaccharides generally require additional types of data, such as scalar and dipolar coupling constants, which are most conveniently measured in ^{13}C-enriched samples. Nuclear magnetic resonance relaxation data provide information on the dynamics of oligosaccharides, which involves several different types of internal motion. Oligosaccharides complexed with lectins and antibodies have been successfully studied both by X-ray crystallography and by nuclear magnetic resonance spectroscopy. The complexes have been shown to be stabilized by a combination of polar hydrogen bonding interactions and van der Waals attractions. Although theoretical calculations of the conformation and stability of free oligosaccharides and of complexes with proteins can be carried out by

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molecular mechanics methods, the role of solvent water for these highly polar molecules continues to present computational problems.

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INTRODUCTION

Although the structures of proteins that bind complex carbohydrates have been described, the conformation of the oligosaccharide ligands has not been previously reviewed in this series (120). The best general source is a series of short reviews that appeared in *Current Opinions in Structural Biology* (22, 26, 60, 104, 119, 136).

The oligosaccharides that are the subject of this review function primarily in cell signaling rather than in energy metabolism. Composed of such monosaccharides as galactose, mannose, amino sugars, and deoxy sugars, with glucose occurring only rarely, the complex carbohydrates are made by biosynthetic pathways that are distinct from those of energy metabolism. Complex carbohydrates are found most commonly at the cell surface, where they are positioned to interact with extracellular receptors such as soluble lectins and antibodies or receptors on the surface of other cells. Although some particular functions are documented for complex carbohydrates, no general integrated overview of function is available.

Among the known functions is leukocyte adhesion to epithelial cells by selectin binding to the sialyl Lewis^x tetrasaccharide, which initiates rolling (46, 137). Glycosphingolipids act as receptors for such bacterial toxins as cholera or tetanus toxin (83). In infectious disease, complex carbohydrates play a role as receptors for such viruses as the influenza virus, which binds to the residues of the 9 carbon atom amino sugar, sialic acid (42, 129). The highly varied polysaccharide structures of the bacterial capsule interact with the immune system of the host, provoking such responses as antibody formation, inflammation, or abscess formation. (115, 135). Although most functions of complex oligosaccharides are associated with the cell surface, protein *N*-glycosylation

begins at the ribosomal level in the endoplasmic reticulum. Transfer of the *N*-glycosyl chain occurs during translation and it functions in protein folding (52).

CONFORMATION OF COMPLEX OLIGOSACCHARIDES

Like polypeptides and polynucleotides, complex oligosaccharides may adopt three-dimensional (3D) conformations that are relatively fixed, or they may exist as several conformations in equilibrium, thereby leading to a flexible model. The monosaccharides in all mammalian glycoproteins and glycolipids, as well as almost all the bacterial polysaccharides, are composed of pyranosides. Homonuclear nuclear magnetic resonance (NMR) coupling constant data show that for the great majority of cases, these six-membered rings adopt fixed chair conformations, a $^4C_1$ chair for D sugars and a $^1C_4$ chair for L sugars (1). For fixed pyranoside ring conformations, the two dihedral angles, ϕ and φ, about the glycosidic linkage are the main internal coordinates specifying the oligosaccharide conformation. Although this parallels the polypeptide case, the pyranoside rings are larger and bulkier, introducing greater stereochemical restriction, which leads to disaccharide maps with smaller minimum energy space than occurs in dipeptide maps. The furanosides found in nucleic acids are much more flexible.

Although our understanding of the conformation of complex carbohydrates remains primitive by comparison with the structures of proteins and nucleic acids, there is no obvious reason why the spectacular success of X-ray crystallography, NMR spectroscopy, and computer molecular modeling cannot be duplicated for this challenging problem. We propose that appropriate application of these methods will yield progress toward the understanding of carbohydrate conformation and interactions.

X-Ray Crystallography

The methodology used for extracting structural data from crystalline carbohydrates depends on the size of the molecule and its ability to form crystals. For compounds as small as disaccharides or oligosaccharides, crystallization is the main difficulty, although the cause is not obvious. It could result in part from the high polarity of the hydroxyl groups, from the flexibility of the carbohydrate linkage, or simply from the lack of pure samples in sufficient quantity and quality. As a result, only about 50 structures of disaccharides are available in the Cambridge Structural Database. For larger oligosaccharides, fewer than 20 structures have been solved (excluding cyclodextrins), most of them sucrose-containing molecules. In fact, only two trisaccharides that are part of complex

glycans have been crystallized, i.e. the αMan$(1 \rightarrow 3)\beta$Man$(1 \rightarrow 4)$GlcNAc (138) and the Lewis^x histo—blood group antigen (102). The information that can be extracted from crystal structures of di- and oligosaccharides includes not only the conformational features but also the intermolecular interaction. For example, networks of hydrogen bonding that involve one or two water molecules have been extensively studied by Jeffrey & Saenger (67). In other cases, the analysis of the crystal packing can be directly compared with biological data. In the Lewis^x crystal structure (102), some interactions observed between adjacent rows of trisaccharide can provide the basis for the previously postulated involvement of Le^x-Le^x interaction in cell-cell adhesion (41).

Although oligosaccharides are difficult to crystallize in their native state, they can be "trapped" in protein crystals, either covalently linked to the peptide chain (glycoproteins) or as ligands to the protein. More than 100 of the protein structures available from the Protein Data Bank (10) are *N*-glycosylated. The conformational features of the GlcNAc-Asparagine linkage have been analyzed from the structures solved at high resolution (63). Because the glycan moiety is often poorly resolved, only one or two carbohydrate residues can generally be seen in glycoprotein crystal structures. More information can be obtained from complexes between protein and carbohydrate ligands, as is discussed below.

Because it is impossible to obtain single crystals from polysaccharides suitable for X-ray analysis, different strategies have been used for diffractometry. One possibility is to stretch the polysaccharide into an oriented fiber. Since the beginning of X-ray fiber diffraction, in the early 1950s, more than 200 biological and synthetic polymer helices have been successfully investigated, among them over 50 polysaccharides (28). This includes the major plant polysaccharides, amylose and cellulose, but also many macromolecules from animal and bacterial origin. An alternate strategy is to use electron diffraction for studying very small crystals, needles, or platelets that can be obtained from polysaccharides (100). In both X-ray fiber diffraction and electron diffraction, the amount of data collected is small, and building a model by molecular mechanics is necessary in the resolution of the 3D structure.

NMR Spectroscopy

The early studies of Lemieux et al (74, 75, 131) focused on a simple molecular modeling method known as hard sphere exo-anomeric (HSEA) using a rigid geometry model. They proposed that complex oligosaccharides of the blood group type do not have random or flexible conformations but that they adopt single, defined conformations. Their conclusions, which were supported by qualitative interpretations of nuclear Overhauser effects (NOE), have subsequently been tested by more sophisticated modeling and NOE simulation methods and found to be generally correct (22, 24, 56). Simulations of steady

state NOE for testing conformational models of oligosaccharides were first developed by Brisson & Carver (19) to account for three spin relayed NOE and spin diffusion effects, which complicate the interpretation of long-range NOE between residues that are not directly bonded. The treatment has been extended to 2D NOESY (23, 25, 150) and rotating frame NOESY (ROESY) (8, 73).

The first studies on asparagine N-linked oligosaccharides of glycoproteins were interpreted as implying single conformations (19). However, subsequent studies have shown that these structures in solution are more flexible (33). Extensive molecular dynamics simulations as well as fluorescence energy transfer experiments have also shown these oligosaccharides to have multiple conformations in exchange (118, 121). Although the consensus of these studies on N-linked glycopeptides is that they may have a modest number of discrete conformations in exchange, there are other oligosaccharides that appear to be more flexible and may have many conformations in exchange (143, 146, 147).

The flexibility of a complex oligosaccharide depends strongly on the stereochemistry of the linkages. In some cases, such as the Lewis type blood groups, the stereochemistry leads to crowding and only a single conformation is allowed (71, 86). The stereochemistry of other blood group oligosaccharides, such as sialylated Lewis^x and blood group A, have limited flexibility (60).

NOE METHODS Most of the conformational data on complex carbohydrates has come from NOE data. There are, however, a number of technical details that are unique to oligosaccharides (136). For medium-sized oligosaccharides such as tri- or tetraccharides, which commonly have intermediate molecular tumbling between approximately 0.7 and 2 ns, nearly null intensity of NOE can be expected for some experimental conditions (92). This problem can be avoided by (*a*) changing the temperature (116), (*b*) using a different magnetic field, or (*c*) using rotating frame NOE (ROESY) (7).

Recently, the T-ROESY experiment (59) was applied to the study of carbohydrates (112). In this experiment, spurious TOCSY cross peaks mediated by scalar coupling that complicate the analysis in ROESY are effectively eliminated from the spectra. Another experiment recently applied to medium-sized carbohydrates is the off-resonance ROESY experiment, which differs from ROESY in that the spin locking frequency is set far outside the spectral window (38). Under these conditions, proton magnetization is not aligned with the transverse plane but forms a certain effective angle θ, depending on the offset. Spurious TOCSY peaks are efficiently eliminated with this technique, and cross relaxation rates, measured at different mixing times and at different values of the effective angle θ, have been used to estimate proton-proton interresidue distances and effective correlation times of medium-size oligosaccharides (11) and cyclic glucans (78, 79).

Several NMR methods (55) have been used to increase the amount of conformational information and to overcome spectral overlap, which arises from the generally poor chemical shift dispersion that is characteristic of carbohydrates. Such 3D homonuclear experiments as TOCSY-NOESY (20) have been used to accurately measure certain cross peaks critical to conformational analysis.

Because the chemical shift dispersion in ^{13}C spectra is much greater than in the ^{1}H dimension, 3D heteronuclear methods are most promising. Although oligosaccharide spectra have been reported for natural abundance samples (39), ^{13}C enrichment has been found necessary for most applications, such as HMQC-NOESY (143, 146) or ROESY-HSQC (51, 87).

Another strategy for increasing the potential information from NOE restrictions involves the resonances of the exchangeable hydroxyl protons of carbohydrates. To observe these NOEs, it is necessary to reduce the chemical exchange rate of the hydroxyl groups with the solvent. Exchange in dimethyl sulfoxide is slow, allowing the measurement of the NOE of hydroxyl protons (35). In aqueous solution, solvent suppression methods are required along with reduction of the temperature below $0^{\circ}C$ using mixed solvents (water/acetone or water/methanol) (106) or by using water in supercooling conditions (-15 to $-20^{\circ}C$) (51, 110, 123).

The recent application of 3D heteronuclear experiments using gradient techniques such as gradient (gd)-HOHAHA-HSQC, gd-ROESY-HSQC, and gd-NOESY-HSQC has allowed measurement of up to 35 NOE involving OH and NH groups in a ^{13}C-labeled disaccharide (51). Although the techniques for reducing hydroxyl exchange are cumbersome, the method has enormous potential for the conformational analysis of carbohydrates in solution.

SCALAR COUPLING CONSTANTS One feature that distinguishes the structural analysis of oligosaccharides by NMR from biomolecules such as proteins is that, in general, the number and distribution of the NOEs obtained from conventional experiments is not sufficient to fully characterize the solution conformation with reasonable certainty (53), especially when flexibility is present among the glycosidic linkages (143). If the interpretation of interresidue NOE data in terms of an unique structure is impossible, a combination of conformations or a virtual conformation is required (33). For these situations, NOE data generally must be combined with other sources of structural constraints, such as those obtained from scalar coupling constants. In flexible structures, scalar coupling constants have the advantage that calculation of average values over an ensemble of conformations is simpler than for NOE. In addition to the $1/r^6$ dependence on distance, NOE has a complex and poorly understood time dependence, in which the overall molecular tumbling may interact with kinetics of the conformational exchange involved in the internal motion. The scalar coupling values

for a flexible structure are simple linear averages over the ensemble of individual conformers.

Recent developments in multidimensional NMR have made possible a number of new methods for measurement of the required heteronuclear coupling constants. The poor sensitivity characteristic of ^{13}C detection methods in natural abundance can be improved by such ^{1}H detection methods as heteronuclear multiple bond correlation (HMBC) (107, 108). Recently, the access to ^{13}C-enriched carbohydrates (69) has allowed the use of new 2D and 3D NMR experiments for the determination of long-range H-C coupling constants such as HMQC-NOESY (147) and HMQC-TOCSY (47). With highly enriched oligosaccharides and polysaccharides, it also possible to measure long-range C-C coupling constants that provide additional information on the glycosidic dihedral angles (87, 145).

The homonuclear $^{3}J_{HH}$ coupling constants characterize the disposition of axial and equatorial sustituents in a pyranose and furanose residue. In addition, the homonuclear $^{3}J_{H5\text{-}H6proR}$ and $^{3}J_{H5\text{-}H6proS}$ couplings are related to the ω torsion angle (defined by atoms O5-C5-C6-O6) of the hydroxymethyl group in pyranose rings. These couplings are usually interpreted in terms of a combination of the three possible staggered conformations, gauche-gauche ($\omega = -60^{\circ}$), gauche-*trans* ($\omega = 60^{\circ}$), and *trans*-gauche ($\omega = 180^{\circ}$). The recent use of experiments such as HCCH-COSY or HCCH-TOCSY in ^{13}C-enriched carbohydrates allows accurate measurements of these homonuclear coupling constants, which are significant to the overall conformation of oligosaccharides that have a $1 \rightarrow 6$ linkage (136).

The interpretation of heteronuclear coupling constants $^{3}J_{COCH}$ across a glycosidic linkage requires a Karplus relationship to correlate the data with the ϕ and ψ glycosidic torsion angles. The correlation Equation 1 (134) is similar to one proposed by Mulloy et al (90),

$$^{3}J_{COCH}(\theta) = 5.7\cos^{2}(\theta) - 0.6\cos(\theta) + 0.5. \qquad 1.$$

Correlation of the coupling constants $^{3}J_{COCC}$ across the glycosidic linkage to deduce structural constraints is a more complex problem because of the great variety of carbohydrate stereoisomers and the possible linkages among them. Nevertheless, important restraints for the ϕ and ψ torsion angles can be deduced from the the vicinal coupling constants, $^{3}J_{C2C1O1Cx}$, $^{3}J_{C1O1CxCx-1}$, and $^{3}J_{C1O1CxCx+1}$, and a considerable effort has recently been dedicated to this topic (87). Recently, Bose et al (12) made a systematic study of a wide variety of ^{13}C-labeled carbohydrates containing C-O-C-C coupling pathways with different structures and dihedral angles. A combination of experimental and computational data was used to identify the structural factors that influence $^{3}J_{COCC}$ coupling constants in carbohydrates. They proposed a Karplus type equation

general enough to deal with most common carbohydrates and linkages types:

$$^{3}J_{COCC}(\theta) = 3.49\cos^{2}(\theta) + 0.16. \quad 2.$$

Recently, a "projection resultant" method has been reported that correlates the magnitude and sign of the geminal coupling constant, $^{2}J_{COC}$, with the glycosidic dihedral angle, ϕ (29). The general features of this empirical method have been confirmed by determinations of $^{2}J_{COC}$ signs from COSY-45 spectra using triply ^{13}C-labeled compounds.

DIPOLAR COUPLING IN PARTIALLY ORDERED SYSTEMS In NMR spectra of ordered molecules, as in solid state NMR, dipolar coupling is not completely averaged, and the coupling values are related to the direction of the vector joining the coupled nuclei. This feature has been employed in studies of the conformation and dynamics of oligosaccharides, especially in lipids in partially ordered membrane layers (66).

Glycolipids may also be partially ordered in a magnetic field when complexed with ordered layers of a liquid crystal. This fact has been exploited by Prestegard and coworkers for determining the conformation of complex oligosaccharides of gangliosides (5, 122). Tjandra & Bax (132) recently showed that in the absence of complex formation, the degree of anisotropy can be conveniently adjusted, which suggests that this method will be especially well suited for complex oligosacharides.

RELAXATION AND DYNAMICS The question of the dynamics of carbohydrates and especially the flexibility across the ϕ and ψ glycosidic linkages and around the ω torsion in $1 \rightarrow 6$ linked carbohydrates is a topic of considerable interest (56) because of its relationship with biological function. In recent years, the access to inverse detection experiments and to ^{13}C-enriched carbohydrates has allowed a significant improvement in the sensitivity of relaxation measurements (144). New pulse sequences that remove cross-correlation effects between dipolar, and chemical shift anisotropy relaxations has been applied to measure ^{1}H-^{13}C–labeled heteronuclear T_1, T_2, $T_{1\rho}$ relaxation times and ^{1}H-^{13}C–labeled NOE in carbohydrates with higher precision (84). The acquisition of these data at different magnetic fields strengths can be of use in the analysis of the slowest internal motions in solution (57, 72).

The measurement of homonuclear cross relaxation rates σ_{NOESY}, σ_{ROESY}, $\sigma_{T\text{-}ROESY}$ estimated from the slope of the build-up intensities at short mixing times (109) was recently applied to the study of the dynamics of a tetrassaccharide analogue of Le^{x} (113) and a manan polysaccharide (114). Effective correlation times can be determined from the ratios $\sigma_{NOESY}/\sigma_{ROESY}$, $\sigma_{T\text{-}ROESY}/\sigma_{NOESY}$, and $\sigma_{ROESY}/\sigma_{T\text{-}ROESY}$, which are independent of the interproton distance (37).

The information obtained in the homonuclear experiments is complementary to the heteronuclear measurements, as it provides information on the dynamics on differing timescales.

It is important in the interpretation of the relaxation rates, as well as of computer simulations, to distinguish between two types of flexibility. The first type is due to small fluctuations within a low-energy region of the conformational space in which there are different but closely related minima connected by small energy barriers. The second type of flexibility involves large conformational changes usually crossing high-potential energy barriers to other regions of the conformational space.

Under the usual conditions of solvent and temperature, the first kind of flexibility seems to be present in practically all carbohydrates, as deduced by dipolar relaxation measurements and molecular modeling studies. Small-amplitude internal motions on a fast timescale around the glycosidic linkages are commonly observed in molecular dynamics simulations both in vacuo or using explicit solvent molecules (20). This kind of internal motion could correspond to the vibrational motions experimentally deduced from ^{1}H-^{13}C relaxation measurements, which have correlation times ranging between 10 and 150 ps (84, 112, 113). It is important to notice that this kind of flexibility can be present even when NOE and coupling constant data are compatible with a single rigid conformation.

The second type of flexibility is more complicated, and it appears to be mixed with the first type. Its detection typically involves the observation of interresidue NOE or interglycosidic coupling constants that are not compatible with a single conformation (33). This second kind of flexibility has been found in many oligosaccharides (45, 50, 84, 146) and polysaccharides (17, 144). A combination of experimental and molecular modeling calculations is needed to interpret the data in terms of reasonable conformations. It is difficult to describe these conformational changes in detail by molecular dynamics simulations because these transitions are rarely observed in the timescale (hundreds of picoseconds to a few nanoseconds) used in the simulations. The data suggest that these motions are probably found over a wide timescale, from several nanoseconds up to milliseconds. The interpretation of the relaxation measurements using the model-free approach (31, 77) detects motions in the range of nanoseconds, but the assignment to a particular transition is not clear, because other motions, such as chair interconversions, global motions, and segmental motions (see below), could be on the same timescale.

Although the applicability of the model-free approach to the interpretation of the relaxation data for small, rigid oligosaccharides can be justified, the application to more flexible cases of oligo- or polysaccharides has recently been questioned (17, 144). Measurements of ^{13}C relaxation for a series of homologous oligosaccharides have shown that the motion of some polysaccharides

can be viewed in terms of a "persistence length," or segmental motions of some 10–15 sugar units, as discussed by Brant et al (17). The assumption of a single, global correlation time, or global anisotropy for the whole molecule, may not be justified for these cases in which the experimental data reflect only the dynamics of the persistence length unit. It is possible that there could be internal motions on a longer timescale (microseconds to milliseconds) that are not readily detected (17). A recent study of the dynamics of a cyclic glucan (78) has suggested that slow chemical exchange on the millisecond timescale is required to explain the observation of a temperature dependence in the HSQC intensities and discrepancies observed between the T_2 experimental values and the correlation times deduced by off-resonance ROESY.

The dynamics of small and medium oligosaccharides is considerably different from that of polysaccharides. Global and internal motions may have a similar magnitude and be coupled (6, 43, 56). Under these circumstances, the model-free approach is not applicable. A recent NMR study of the disaccharide α,α-trehalose (6) has reported the effect of temperature on global and internal motions and showed that although the global structure of this disaccharide was not affected over the range of temperatures used, the importance of internal motions is drastically reduced over the range of 0°–30°C, so that the distinction between global and internal motions is possible. The dynamics of the oligo- and polysaccharides is far from being well understood and further advances are to be expected in the future.

Molecular Modeling

For the energy refinement of carbohydrates and of carbohydrate-protein complexes, many of the most widely used classical force fields have been extended to deal with carbohydrate moieties. Generally these modifications consist in the incorporation of parameters or energy functions to deal with the anomeric (76) and exo-anomeric (70) effects, which affect the geometry and energetic properties of the R-O-C-O-R′ patterns characteristic of carbohydrates. Some of the force fields that have incorporated these modifications are CHARMM (49, 117), AMBER (54, 141), Macromodel-AMBER* (128), GROMOS (98), OPLS (36), CVFF (58), TRIPOS (101), and MM3 (3).

Several molecular mechanics–based strategies, such as relaxed energy maps, molecular dynamics, Monte Carlo simulations (103), and simulated annealing (91), have been applied to the conformational analysis of carbohydrates and their complexes (60).

The most complete theoretical description of the structure and dynamics of molecules in solution can be obtained from molecular dynamics simulations with explicit solvent molecules (s-MD). These simulations take into account two of the main driving forces that affect the structure and dynamics of carbohydrates, hydrogen bonding, and van der Waals' interactions.

The dynamic formation and breaking of intermolecular carbohydrate-water and intramolecular hydrogen bonds has been studied for small oligosaccharides using s-MD (16, 44, 81, 99). These studies have revealed that there is an anisotropic distribution of solvent adjacent to the carbohydrate in which solvent molecules preferentially occupy specific sites relative to the carbohydrate functional groups (81). This disposition differs from that of the bulk water and can play an important role in the establishment of these nonbonded interactions, which affect the conformation. The importance of intramolecular hydrogen bonding is drastically diminished because solvent is present (16, 81, 99). It has been observed that intramolecular hydrogen bonds found in the crystal structure (43, 81) or in minima from in vacuo calculations can easily be broken once water molecules are included (99), in some cases causing a conformational shift in the molecule (16). Solvent molecules in s-MD simulations also introduce a frictional damping of the high-frequency motions of the solute compared with in vacuo simulations. This damping seems to be important to reproduce the fast internal motions in carbohydrates, as deduced from relaxation measurements (43, 113).

INTERACTION BETWEEN OLIGOSACCHARIDES AND PROTEINS

The biological activities of most complex oligosaccharides are determined by binding to specific multivalent proteins known as lectins. Although the binding of single sugar residues is generally specific, the dissociation constants, typically $\approx 10^{-4}$ M, indicate weak binding (120). Thus, the binding strength responsible for most of the observed biological phenomena comes from cooperativity. Not only is the interaction of lectin and sugar multivalent, there is also evidence for specific and cooperative interaction between lectin molecules (13, 96). Such interactions suggest that cell surface signaling could be initiated by multivalent complexes between several different types of sugars and lectins, analogous to the transcription complexes formed between DNA, regulatory proteins, and RNA polymerases.

X-Ray Crystallographic Studies of Protein/Carbohydrate Complexes

THE BIOLOGICAL DIVERSITY OF COMPLEXES Among the many proteins that interact with sugars, only those that specifically bind complex oligosaccharides and that do not display enzyme activity are considered in this review. This includes lectins and antibodies but ignores all glycosylhydrolases or other enzymes, as well as bacterial transport proteins for monosaccharides and growth factors that bind polyanionic polysaccharide fragments. All the crystal

Table 1 Three-dimensional structures of lectin and antibodies available in the Protein Data Bank

Species	Protein	Native	Complexes	Total
Plant lectins	Legume	24	36	60
	β-Trefoil lectin	2	2	4
	Agglutinin with hevein domain	3	5	8
	Monocot	2	6	8
	β-Prism	0	2	2
Animal lectins	S-lectins	0	6	6
	C-lectins	8	12	20
	Pentraxins	2	1	3
	I-type lectin	0	1	1
	P-type lectin	0	1	1
Bacterial lectins	AB5 toxins	6	5	11
	Tailspike protein	2	3	5
	Others	0	2	2
Virus lectins	Hemagglutinins	5	8	13
	Coat protein	1	3	4
Antibodies		3	7	10
Total		58	100	158

structures of lectins and antibodies available in the July 1998 version of the Protein Data Bank (10) and from the literature have been gathered in Table 1. A large majority of the structures are from lectins and, more particularly, from plant legume lectins (82), which are easily extracted from seeds and can be purified in large quantity. The classification in Table 1 is based on the fold of the protein illustrating the many schemes that nature can use to build a sugar binding entity (for review see 80). From the plethora of structures available, 100 exist as a complex between the carbohydrate and the protein, therefore giving access to a immense source of information on the atomic basis of the interaction. Among these structures, 20 complexes contain disaccharides, 14 contain a trisaccharide, and 24 contain a tetrasasaccharide or a longer oligosaccharide. As for the antibodies, only seven structures are available from the Protein Data Bank[1] (10): human and murine (code 1CLY and 1CLZ) Fab fragments complexed with the Le^Y tetrasaccharide (68), complexes between three murine Fab fragments (code 1MFE, 1MFC, and 1MFD) (21, 34), and one engineered Fv fragment (code 1MFA) (149) complexed with several *Salmonella* polysaccharide fragments. The last structure is a complex between the Fab fragment (code 2H1P) of an antibody directed against *Cryptococcus neoformans* polysaccharide and a peptide from a phage library (148), therefore giving insight into the mode of action of peptide mimics for oligosaccharide.

[1]Protein Data Bank: http://pdb.pdb.bnl.gov/

A selection of protein/oligosaccharide complexes is presented in Figure 1 (see color figure at end of volume) that shows the multivalent and multimeric aspects of these carbohydrate-binding proteins. Bovine galectin is a dimer (13), whereas rat mannose-binding protein (93) and viral hemagglutinin (124) are trimers. Concanavalin A (ConA) (88) and snow-drop agglutinin (142) are tetramers, whereas cholera toxin (85) and polyomavirus coat protein (130) are pentamers. The distances between the binding site resulting from the multimeric arrangement is not random but rather corresponds to the distance between the terminal part of the oligosaccharide ligands when carried by glycoproteins or by patches of glycolipids at the cell surface.

ATOMIC FEATURES OF PROTEIN/CARBOHYDRATE INTERACTION Some basic features are common to all sugar binding sites in lectins (40). Both hydrophobic and polar interactions contribute to the stabilization of the sugar-lectin interaction. The polar interaction can involve either hydrogen bonds between the many hydroxyl groups of the carbohydrate and the amino acid residues, or metal coordination bonds between the same hydroxyl groups and divalent cations present in the binding site of some animal lectins. It therefore appears that an important feature for the selectivity of the recognition is the orientation of some key hydroxyl groups. More particularly, the orientation of the O-4 hydroxyl group is critical because it is always involved in the binding. Indeed, some lectins, such as C-type mannose binding protein and mannose-specific legume lectins, interact only with monosaccharides of the gluco or manno series by hydrogen bonding with the O-3 and O-4 hydroxyls, whereas others, such as galectins and galactose-specific legume lectins will interact only with galacto type of residues through hydrogen bonds with O-4 and O-6.

It should not be forgotten that van der Waals interactions contribute strongly to the stabilization of the complexes. For example, the methyl moiety of the N-acetyl group is often surrounded by two or three aromatic amino acids, thereby creating a strong hydrophobic cluster. Aromatic residues often interact with the hydrophobic face of monosaccharides. This parallel interaction, often referred to as stacking, plays an important role in the specificity because its strict steric complementarity does not allow any unwanted substitution or epimerization.

The role of solvent is difficult to evaluate. Whereas the most buried carbohydrates tend to satisfy their hydrogen bonding potential with the amino acids of the protein, the more exposed ones establish hydrogen bonds with water molecules that are already bound to the protein, or they bond with the bulk solvent.

CONFORMATION OF THE BOUND OLIGOSACCHARIDE Previous studies of protein-carbohydrate interaction (61) demonstrated that, in most cases, the

lectin recognizes and binds the conformation of the oligosaccharide that corresponds to one already existing in solution. From the large database that is now available for lectin/oligosaccharide complexes, there is no example of a glycosidic bond conformation that lies outside the low-energy region of the conformational map calculated for a free disaccharide. Nevertheless, in many cases, the bound conformation does not correspond to the global energy minimum of the free oligosaccharide but to a secondary energy minimum. This fact can be illustrated by examination of the conformations observed for oligosaccharides in the lectin-bound state on the energy map of their constituent disaccharides calculated in vacuo.

Figure 2 shows the many conformations observed for αMan(1 $\rightarrow$ 3)Man, βGlcNAc(1 $\rightarrow$ 2)Man, and αNeuAc(2 $\rightarrow$ 3)Gal in crystalline complexes, with lectins plotted on the energy maps of each linkage, as calculated with the MM3 program (2). For the αMan(1 $\rightarrow$ 3)Man linkage, all conformations but one are distributed in the low-energy region extending from $\Psi = 90^\circ$–180°. The occurrence of conformer A or B in solution has been the subject of much controversy, but in the complexed state, it appears that the whole area is equally populated. Conformer C was predicted nearly 10 years ago from a combination of NMR and calculations (65). This conformer is indeed observed in the complex between *Lathyrus ochrus* isolectin I and a biantennary oligosaccharide (15).

In the βGlcNAc(1 $\rightarrow$ 2)Man energy map, most of the observed conformations occur in the main energy minimum. However, conformer B or C is found in several of the structures. Conformer B differs from A by about 120° in Φ and therefore does not correspond to the conformation predicted by the exoanomeric effect (74, 75). However, upon binding, this special conformation can be induced or selected by the lectin. It should be noted that such secondary minima are observed for the glycosidic linkage of a disaccharide when one of the constituent monosaccharides is involved in the primary binding site, i.e. in the region where the interaction with the protein surface is maximum. Conformer B of βGlcNAc(1 $\rightarrow$ 2)Man has been observed in the complexes between *Lathyrus ochrus* lectin and several oligosaccharides (14, 15), whereas conformer C is found in the ConA/pentasaccharide complex (88). In all these cases, the mannose is located in the lectin monosaccharide binding site, and because of steric interaction, there is no possibility for the GlcNAc linkage to adopt the lower-energy A conformation. This feature is illustrated in Figure 3*a* and 3*b* (see color figures at end of volume), where the pentasaccharide complexed with ConA and part of the octasaccharide complexed with LOL1 are displayed with the same orientation of the αMan residue in the primary binding site of the legume lectins.

The αNeuAc(2 $\rightarrow$ 3)Gal disaccharide has been shown to have a flexible behavior. The MM3 energy map (64) predicts the occurrence of three main

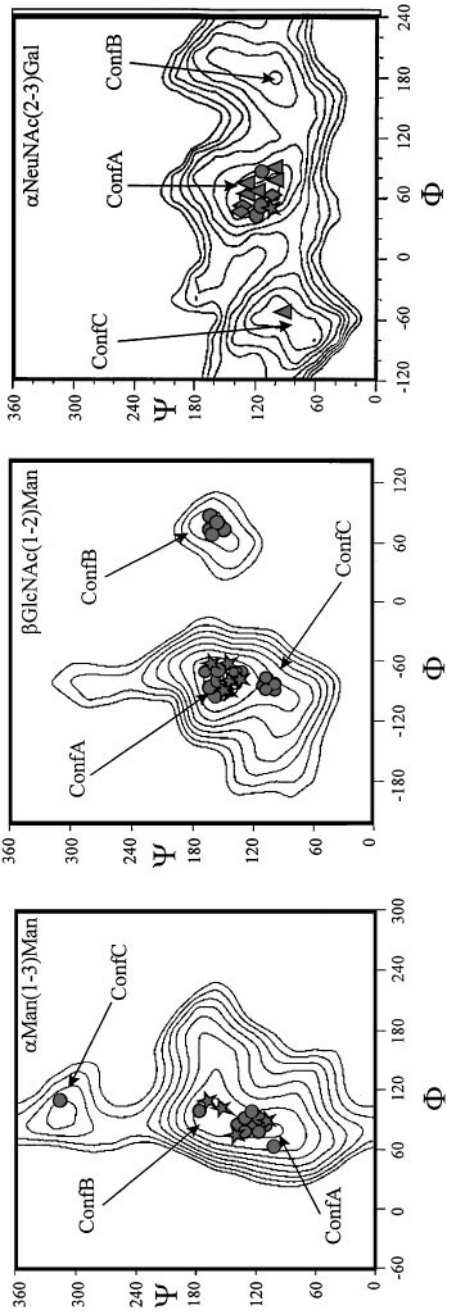

Figure 2 Superposition of the conformation observed for three types of linkages in complexes with proteins on the corresponding MM3 energy map. Iso-energy contours are drawn at intervals of 1 kcal/mol above the minimum. The conformation observed in crystal structure is for plant (*circle*), animal (*star*), and microbial (*triangle*) lectin complexes. (*Diamonds*) Conformation bound in solution with E-selectin. Glycosidic dihedral angles are defined following the standard heavy atom convention.

minima with distinct Φ values, and NMR spectroscopy showed that the conformations A and C are adopted in solution (18). Many pathogens bind to host sialic acid receptors, and therefore, complexes of several lectins with the disaccharide αNeuAc(2 → 3)Gal have been crystallized. Indeed, conformation A is observed in all cases, with the exception of cholera toxin, where this disaccharide adopts conformation B.

Two observations can be made from the above examples: (*a*) As stated above, the interaction with the lectin surface does not yield any new conformations that were not present on the energy map of the isolated disaccharide; and (*b*) upon binding, the lectins may select secondary minima remote from the global one.

It can be hypothesized that the lectins induce such conformations because the population of these secondary minima may be very small in solution. However, these minima are always present among those predicted from a thorough conformational analysis of the isolated disaccharides.

NMR Study of the Conformation of the Oligosaccharide Ligand

Transferred NOE (trNOE) experiments (30) are an efficient tool for studying protein-ligand interactions (94). When a carbohydrate ligand is bound to a large protein in fast exchange mode, the relaxation is governed by the slow protein tumbling time, resulting in strong negative NOEs that reflect the bound conformation of the carbohydrate. The conformation of the protein cannot be studied because its molecular weight is generally outside the limits of NMR spectroscopy analysis. Measurements of trNOEs in the rotating frame (trROEs) are generally required in order to identify false trNOEs that arise from spin diffusion effects (4). As in other NMR studies, the limits of such methods are the solubility of the protein and the amount of oligosaccharide needed.

Because the interaction between protein and carbohydrate is generally in the millimolar to the micromolar affinity range, several complexes involving lectins and antibodies have been investigated with the use of trNOEs (for reviews see 60, 104, 111). The quantitative analysis of trNOE and trROE data is best accomplished with an extended full relaxation matrix treatment that includes the effect of chemical exchange (89). In many cases, the NMR data show that the protein binds the conformation with lowest energy in solution. In other cases, the protein selects only one defined conformation of the oligosaccharide from several that are predicted to occur in solution. This has been observed for the binding of blood group A trisaccharide by the lectin of *Dolichos biflorus*, (27) and also for the binding of a branched but flexible trisaccharide from *Streptoccoccus* by the monoclonal antibody strep 9 (139). In the most extreme case, the protein induces a conformational change of the carbohydrate ligand;

NMR experiments, in agreement with X-ray data, indicate that a branched trisaccharide fragment from *Salmonella* polysaccharide undergoes an antibody-induced conformational change about one of its glycosidic linkages (21). The resulting conformation does not correspond to that observed in solution.

Because of the biological significance of the interaction between the sialyl Lewis^x tetrasaccharide (sLe^x) and E-selectin, several attempts have been made to obtain the bioactive conformation by means of trNOEs (32, 105, 126). A large number of interglycosidic trNOE were measured (Figure 4), and the use of 3D NMR experiments allowed determination of the extent of spin diffusion effects (125). Clear differences appeared between the NOE fingerprints for the free and E-selectin bound tetrasaccharide, and particularly the interglycosidic trNOE between H8 of sialic acid and H3 of galactose indicated that the αNeuAc(2 $\rightarrow$ 3)Gal linkage corresponds to the energy minimum in the energy map of Figure 2 (*ConfA*). The Le^x moiety is more rigid, but a slight reorientation of the fucose residue also occurs on binding. On the basis of the crystal structures of E-selectin, and the related mannose-binding protein complexed with oligosaccharide, there have been some attempts to model the interaction between sLe^x and the E-selectin (133). The agreement between this model and the recent crystallographic complex of the selectin-like mutant of MBP with sLe^x is poor. This exemplifies the difficulties and pitfalls in modeling protein-carbohydrate interactions.

Molecular Modeling of Protein/Carbohydrate Complexes

When no crystal structure is available for a given protein/oligosaccharide complex, it is possible to model the structure if enough 3D information is known for each partner. The aim of the work is then to predict the best possible interaction between the protein and ligand while taking into account the flexibility of the oligosaccharide and of the amino acid side chains. Such a procedure implies two steps. The first is a docking procedure defining all the possible modes of interaction, and the second is to maximize the interaction between the two partners by an energy optimization. For the docking step, a wide range of methods is available, including hand docking, a systematic search for the ligand position (a six-dimensional procedure that is time-consuming) (62, 127), or a Monte Carlo simulation. A simple and effective procedure involves mapping of the best interaction sites on the protein for the polar and hydrophobic groups on the ligand (GRID program) (48).

The molecular modeling methods have yielded models of several protein carbohydrate complexes. A system of medical importance is the complex between bacterial toxins and their glycolipid acceptors, which could be useful in the design of antiadhesion drugs. A model of verotoxin from *Escherichia coli* was proposed as a cleft-site complex with Gb3 globotriaosyl-ceramide (95).

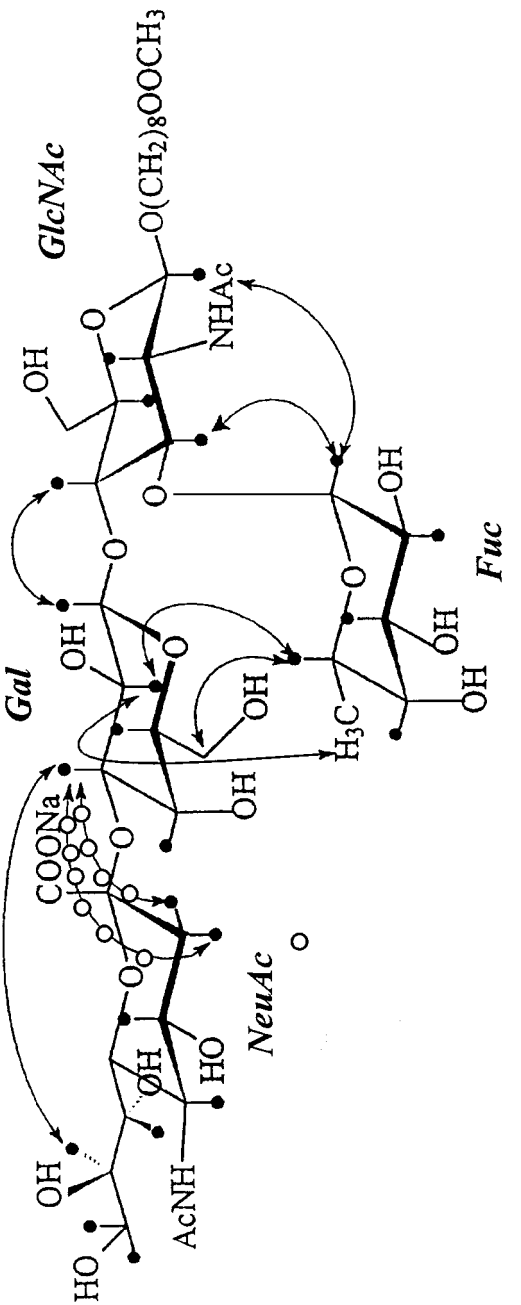

Figure 4 Schematic representation of sialyl Lewis^x derivative showing the observed interglycosidic NOEs. (*Arrows*) Negative constraints because these particular NOEs are not observed when SLex is bound to E-selectin. [From Scheffler et al (125), with permission from Kluwer Academic Publishers.]

The interaction of GM1 ganglioside with *E. coli* heat-labile enterotoxin, which is related to cholera toxin, has also been recently simulated (9). Complexes between oligosaccharides and plant legume lectins have been the subject of many modeling studies. Because these proteins belong to a family of homologous sequences with many known crystal structures, but with different specificities, it has been possible to build some proteins that could not be crystallized and to simulate their interactions with oligosaccharide ligands. For example, the *Dolichos biflorus* seed lectin and the lima bean lectin were modeled in complex with blood group A trisaccharide. The same homology modeling approach was used to propose a model for the complex between the *Brucella abortus* O-chain polysaccharide and a specific monoclonal antibody (97).

Although the above-mentioned computational studies produced models of good structural quality, it is at the moment difficult to predict the affinity constants for a protein and a carbohydrate ligand. The difficulties in accurate calculation of the interaction energies include the significant role of water in the interactions of these highly polar ligands and the flexibility of the oligosaccharide. Some recent attempts employing molecular dynamics simulation and free-energy perturbation to compute the relative binding affinities for the well-characterized oligosaccharide-protein complex between the O-antigen of *Salmonella paratyphi* B and its monoclonal antibody (140) and the computed $\Delta\Delta G$ are in reasonable agreement with the experimental data.

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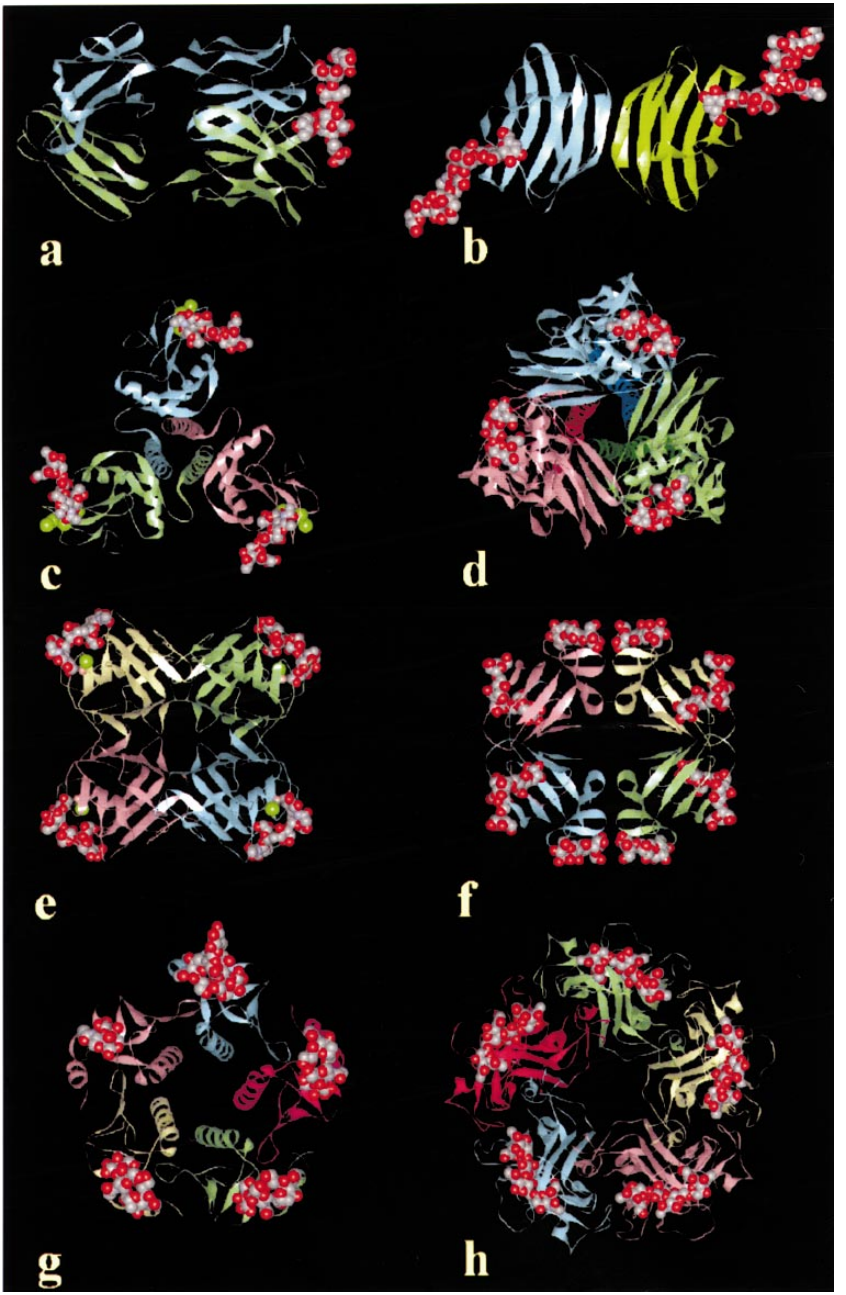

Figure legend appears on the following page.

Figure 1 Graphics representation of several protein/oligosaccharide complexes from crystal structures from the Protein Data Bank. The protein chains are represented by colored ribbon and the carbohydrate atoms by balls. The different complexes are *(a)* mouse Fab fragment with a heptasaccharide from *Salmonella* polysaccharide (1MFB), *(b)* bovine galectin with biantennary heptasaccharide (1SLC), *(c)* mutant of rat mannose binding protein with 3'-sialy-Lewis X (2KMB), *(d)* influenza virus hemagglutinin with α-2, 3-sialyllactose (1HGG), *(e)* concanavalin A with pentasaccharide (1TEI), *(f)* snowdrop lectin with oligomannose (1JPC), *(g)* cholera toxin with GM1 oligosaccharide (1CHB), and *(h)* polyomavirus coat protein with disialylated hexasaccharide (1VPS).

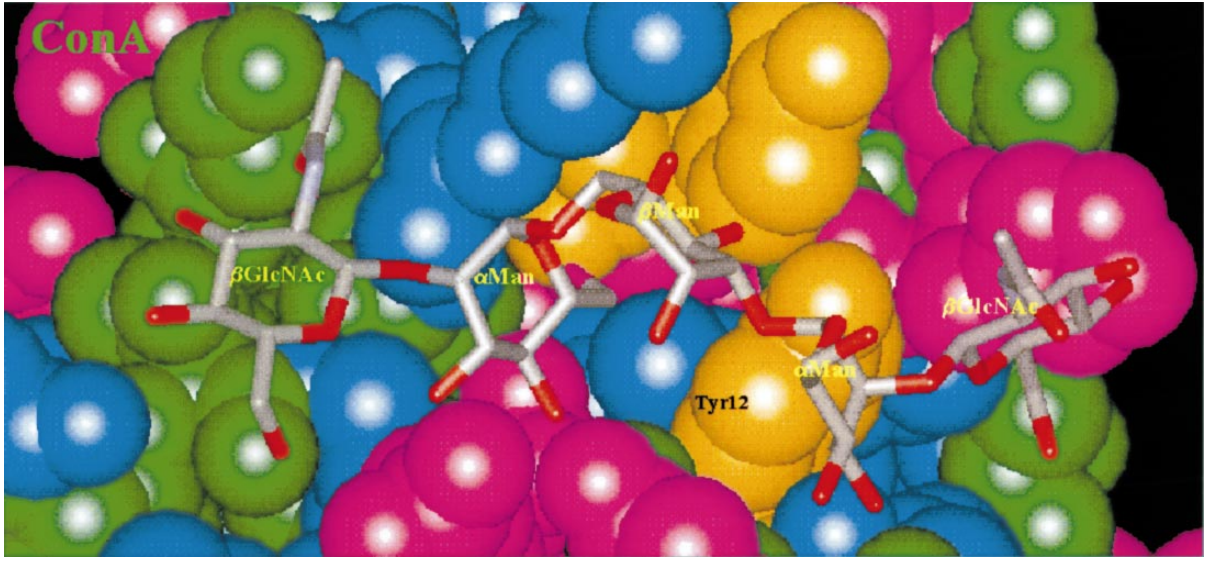

Figure 3a

Figures 3a, b (next page) Comparison of the binding sites of *Lathyrus ochrus* isolectin I and concanavalin A with their bound oligosaccharides.

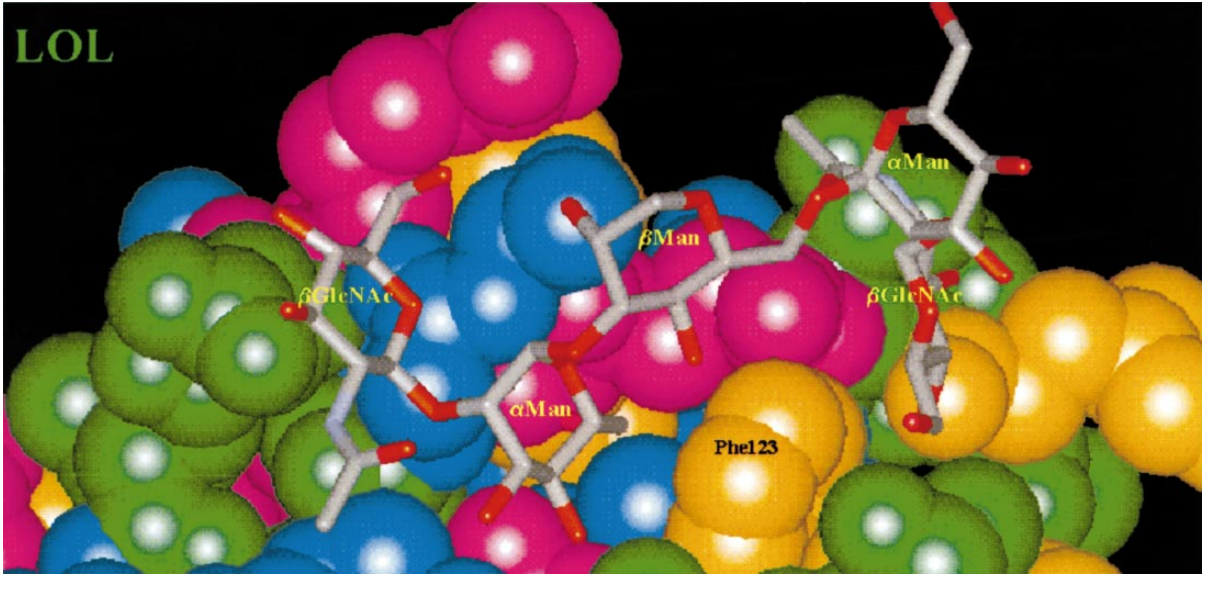

Figure 3b