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Chapter 21

Origins of Flexibility in Complex Polysaccharides

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Abstract

Flexibility in complex bacterial polysaccharides and glycosaminoglycans is important both for their interactions with proteins and for such physical properties as gel formation and viscosity. Although the existence of high resolution NMR spectra for some polysaccharides with molecular weights over 100 kD implies some elements of flexibility, in fact molecular modeling shows the individual disaccharide linkages to be less flexible than those of peptides. Some small oligosaccharide epitopes adopt compact and rigid folded conformations as can be shown by NOE studies, molecular modeling and measurements of residual dipolar coupling in liquid crystal solutions. In polysaccharides, the rigid epitopes are connected by flexible hinges characterized by conformational exchange among a few local energy minima. ^{13}C scalar coupling measurements are an effective tool for characterizing the hinge residues.

The nature of polysaccharide flexibility

The complex polysaccharides of bacterial capsules and of glycosaminoglycans typically have high molecular weights, generally over 100 kD and some can be millions. Yet many of them give NMR spectra that are well

resolved with line widths of a few Hz. In contrast, globular proteins with molecular weights over 100 kD give generally poorly resolved spectra due to broad lines caused by slow tumbling. The short T_2 prevents coherence transfer so that many NMR experiments commonly used for assignment of spectra are ineffective. This is a serious limitation of the use of NMR spectroscopy for larger proteins. Clearly there are some very fundamental differences in the dynamic behavior of polysaccharides and globular proteins. If all the glycosidic linkages were completely rigid, the polysaccharide would tumble as a single extended unit and the polysaccharide would be expected to have very short T_2 and extensive line broadening. The existence of NMR spectra for polysaccharides argues that at least some of the glycosidic linkages must be partially flexible.

A possible conclusion from these observations is that the linkages of polysaccharides are more flexible than are those of polypeptides. But in fact, molecular modeling of the glycosidic linkages in disaccharides shows that, while the conformational space available is variable depending on details of the stereochemistry, disaccharides are generally somewhat less flexible than are dipeptides. When calculated with similar methods, the latter have a greater low energy area on the dipeptide map than is available in disaccharide maps. The conformational space available for linkages to the secondary hydroxyl groups for a typical disaccharide linkage is more restricted than that for a dipeptide map. This is illustrated in Figure 1 which compares the energy contour map for a typical β -linked (1 \rightarrow 3)-disaccharide with that for the alanine dipeptide calculated with the same algorithm and force field and plotted with the same energy range and contour interval. The greater restrictions on the disaccharide map result from stereochemical clashes between the bulky rings of the sugars. These restrictions are even more pronounced in the case of branched oligosaccharides or (1 \rightarrow 2)-linkages in which sugar substituents occur on adjacent positions, (1).

An important result of the flexibility of the peptide linkage is that if polar or nonpolar attractive interactions occur among the residues of a peptide, the flexibility of the chain allows it to fold into a compact globular form. While a polypeptide of random amino acid composition and sequence might not fold, the globular proteins which are generally studied by NMR spectroscopy are those which do express their inter residue interactions by adopting a folded conformation. In contrast, the less flexible linkages of a polysaccharide do not allow the polymer to fold into a globular conformation.

Since polysaccharides have polar groups as well as some hydrophobic fea-

tures, there can be inter residue interactions in a polysaccharide as well, but these attractions are expressed as interactions between different chains giving rise to such phenomena as crystallization which is prominently expressed in chitin and in cellulose. Other types of polysaccharides exhibit multistrand helix formation as in the case of scleroglucan whose tendency for triple helix formation leads to unusual physical properties. Still other types of polysaccharides can form cross links in a more random way leading to gel formation as in the case of pectin, agarose and alginates. Chain interactions in other polysaccharides such as hyaluronan, chondroitin or xanthan produce highly viscous solutions. These inter chain interactions give rise to some important biological properties which are characteristic of high molecular weight polysaccharides.

The highly varied physical properties of polysaccharides are of considerable interest both in technology and in biology. Likewise the NMR spectroscopic properties of different polysaccharides are highly varied. On the one hand, some polysaccharides, such as the antigenic cell wall polysaccharide of the common oral bacterium *Streptococcus mitis* J22 give very well resolved spectra with line widths of 1-2 Hz even in spectra recorded at room temperature (Figure 2). Heteronuclear relaxation experiments on this polysaccharide show that this is a typical dipolar relaxation phenomenon, (2). Oligosaccharides made from the polysaccharide show spectra which are not too widely different and detailed measurements of the relaxation data show the dynamics differ but only modestly (3).

Other high molecular weight polysaccharides, such as the capsule of *V. cholerae* O139, have modest line broadening and the line widths in room temperature spectra are quite large. But as shown in Figure 2, at temperatures of 60° C the line widths are reduced to approximately 5 Hz and the standard NMR coherence transfer experiments work reasonably well, (4). Since heteronuclear ¹³C NMR spectroscopy has become the predominant tool for obtaining information on the covalent structure of complex bacterial polysaccharides, the improvements in spectral line widths at elevated temperatures are important with NMR methods replacing many of the chemical techniques greatly simplifying complete structure determinations, (5).

But there are some serious complications in complete structure determination of polysaccharides by NMR. As illustrated in Figure 2C, some polysaccharides such as pneumococcal type 1 capsule give line widths so broad that they cannot be readily assigned by standard coherence transfer methods, (6). Even at elevated temperature the standard COSY and TOCSY

methods for coherence transfer fail and modern NMR methods for structure determination become useless. In the most extreme situation, there are certain polysaccharides which apparently dissolve well giving clear non-viscous solutions free from light scattering but which give NMR spectra so broad as to be essentially non-observable. Examples of this behavior are not generally discussed in the primary literature.

Both the wide variations in hydrodynamic properties and in the NMR spectroscopic properties of polysaccharides having different chemical structures must depend on details of the composition and linkages. Unlike polypeptides, the stereochemical possibilities for saccharide linkages are highly varied. The bond between the glycosidic carbon and oxygen atoms of a pyranoside residue can be either axial or equatorial as can be the configuration of the aglycone carbon atom providing four fundamentally different stereochemical schemes. An additional influence is the chemical functionality of substituents at positions adjacent to the linkage atoms. Commonly found in polysaccharides are amides and acetates, as well as additional glycosidic linkages and charged groups such as sulfate or phosphate. It is just such substituents which limit the conformational freedom of an individual glycosidic linkage.

Rigid linkages and internal motion of the first kind

At the extreme limiting case of the least flexibility are certain glycosidic linkages with very crowded stereochemistry and very little internal motion. The most well known of these relatively rigid oligosaccharide epitopes composed of three or four residues are the blood group oligosaccharides, especially the Lewis epitopes. They have been studied by workers in a number of different laboratories using different techniques over a span of many years. The results of NOE studies (7,8), molecular dynamics simulations (9,10) scalar coupling (11,12) x-ray crystallography (13,14) and most recently residual dipolar coupling measurements (15) generally agree on well defined single conformations for such oligosaccharides. Rather than considering such epitopes as completely rigid, we characterize them as having internal motion of the first kind. The pyranoside rings adopt well defined chair conformations which are readily characterized by ^1H - ^1H scalar coupling constants. The chair puckering motions are of a small amplitude and on a picosecond time scale. The glycosidic dihedral angles exhibit rapid fluctuations on the time scale of a few picoseconds and amplitudes on the order of 10 to 15°.

Flexibility and internal motion of the second kind

Obviously not all glycosidic linkages are so restricted and they may have much larger amplitude fluctuations which we will characterize as internal motions of the second kind. We will further describe this motion as being conformational exchange between local energy minima on the map of the Φ vs. Ψ surface such as Figure 1B. The energy minima differ by more than 30 to 50° and they are separated by an energy barrier. The height of such barriers is not generally well known but its magnitude clearly determines the kinetics of the conformational exchange involved in this type of internal motion. The detailed kinetics of this conformation exchange is also not known very well but NMR relaxation rates imply that the time scales must be more than a few nanoseconds and in some cases could be much longer (2,16,17).

The time scale is probably not longer than a few microseconds for if it were, the motions could be detected by well known methods for characterizing slow “chemical exchange” motions which range between 100 microsec and a few millisecc. Although such motions have been detected in a number of cases for proteins, they have not generally been seen in either polysaccharides or oligosaccharides. The fact that the broad NMR lines seen for some polysaccharides (Figure 2) are never seen in oligosaccharides argues against the wide occurrence of such slow chemical exchange broadening. Small oligosaccharides containing a few repeating units have been prepared from both the type 1 pneumococcal polysaccharide (6) and from the *V. cholerae* O139 polysaccharide (Stroop, Adeyeye and Bush, unpublished results) and in both cases these oligosaccharides show line widths of 1-2 Hz in room temperature spectra in contrast to the data of Figure 2. There remains considerable uncertainty about the kinetics of internal motion of the second kind in complex polysaccharides. The time range between 50 ns and 50 microsecs spans three orders of magnitude of unknown territory and unfortunately most NMR measurements are not very sensitive to motions in that time range.

Flexibility of high molecular weight polysaccharides

This classification of flexibility into distinct categories suggests that flexibility in complex polysaccharides can be described in terms of rigid units of several sugar residues exhibiting internal motion of the first kind which are connected by relatively more flexible hinges having internal motion of the second kind. Such a model has been shown to be applicable for the antigenic cell wall polysaccharide of *Streptococcus mitis* J22. (See Figure 3.) This polysaccharide, with seven sugar residues in the repeating subunit, is important in the coaggregation of oral bacteria in the early stages

of formation of dental plaque. It has, in addition to an antigenic epitope composed of four sugar residues, a lectin receptor epitope of two residues and has been studied by NMR techniques such as nuclear Overhauser effects and long-range C-H and C-C scalar coupling measurements on ^{13}C enriched samples of the polysaccharide (18). The isolated repeating subunit, a heptasaccharide, has been studied by similar methods and also by residual dipolar coupling data partially oriented in liquid crystalline media, (19). The data are interpreted with molecular modeling studies which show that the heptasaccharide contains a relatively rigid unit of four sugar residues which make up the antigenic site of the polysaccharide. A distinct disaccharide which serves as the lectin binding site in coaggregation with certain species of actinomyces is also shown to adopt a well defined conformation. Flexibility in this heptasaccharide occurs in the linkages of the β -galactofuranoside residue which joins these two more rigid structures by (1 \rightarrow 6)-linkages (Figure 3). In the polysaccharide, the heptasaccharide units are joined by phosphodiester linkages which confer additional flexibility to the high molecular weight polysaccharide. While the models for the rigid epitopes of this polysaccharide are reasonably well defined, the motions of the flexible hinge region are known with much less precision (18). Clearly some new experimental approaches will be needed to more fully characterize internal motions of the second kind.

In contrast to the polysaccharide of *S. mitis* J22, which has rather narrow ^1H NMR lines of just a few Hz when measured at room temperature (Figure 2A), the spectra of a number of polysaccharides of similar molecular weight exhibit greater line widths such as the capsular polysaccharide of *V. cholerae* O139 (Figure 2B). This high molecular weight polysaccharide has six residues in the repeating subunit with four residues in a tightly folded epitope which is a structural homologue of the Lewis^b blood group tetrasaccharide (Figure 3A, 3B). It has been shown by NOE and molecular modeling studies that this tetrasaccharide indeed adopts a conformation similar to the Lewis epitope (4). Presumably the remaining two residues, which have α -(1 \rightarrow 3)- and a β -(1 \rightarrow 6)-linkages provide the flexible hinge which confers some flexibility to this polysaccharide. At this point, no details on the internal motions of this hypothetical hinge region are available.

Methods for determining detailed models for polysaccharides

The remainder of this review will be devoted to some details of the NMR

methods which could be useful for testing and refining the model which we have proposed above for a flexible complex polysaccharide. Of the two components of this model, it is the more rigid parts that are most easily treated and many studies using simple molecular modeling and NOE experiments have been reported. Since these topics have been extensively reviewed, (20), we will focus our attention on a more recently introduced method involving residual dipolar coupling in weakly oriented solutions of oligosaccharides.

Residual dipolar coupling

Dipolar coupling, which occurs directly through space rather than through chemical bonds, should not be confused with scalar coupling which is generally more familiar to most chemists. Two structural parameters affect the strength of the dipolar coupling interaction between two nuclei. One is the distance r^3 separating the two nuclei in the molecule and the other is the angle Θ which defines the direction of the vector joining the two interacting magnetic dipoles with respect to the static magnetic field. The dipolar interaction is most commonly associated with solid state NMR where the fixed orientation of the internuclear vectors leads to the maximum dipolar interaction and causes it to dominate over the effect of chemical shift. In solution NMR the molecular motions result in a random and isotropic variation in time of the orientation of the dipole with respect to the magnetic field. In this situation the term, $\langle 3\cos^2\Theta - 1 \rangle$, which describes the angular dependence of the dipolar coupling averages to zero and no static contribution to the dipolar coupling interaction remains. Only the distance dependence can be indirectly observed by measuring NOE which depends on fluctuations in the dipolar coupling interaction. In the presence of certain mixtures that form liquid crystals which can be oriented in the magnetic field, a small degree of orientation of the oligosaccharide can occur since such an anisotropic medium imposes a preferred orientation to the molecule under study and the dipolar interaction does not average to zero.

Recently, it has been shown that orientation of molecules can be conveniently achieved in phospholipid bilayers known as bicelles (21) when the molecule under study is oriented by hydrodynamic interactions with the bicelles (22). The degree of orientation achieved by these weak hydrodynamic interactions is small, typically of the order of 1 molecule in 1000 being oriented. A scaled or reduced dipolar interaction occurs resulting in measurable dipolar couplings while the spectrum retains high resolution and the spectral simplicity of the regular isotropic phase. Using this technique several groups

have reported residual dipolar couplings in oligosaccharides (23,24,25) which can readily provide, at least for the case of relatively rigid models, highly accurate structural information which includes long range structural information not available from either NOE or scalar coupling data.

While several different liquid crystal orienting media have appeared in the literature for the measurement of residual dipolar couplings with carbohydrates, the first used was a binary mixture of long and short-chain phosphatidylcholines. It forms a well-oriented discotic nematic phase over a reasonably wide range of phospholipid composition and has been well characterized by NMR studies (21,26). The total lipid concentration affects the degree of alignment obtained and hence the magnitude of the residual dipolar couplings to be measured. This concentration can be adjusted in the range 5-40% (w/w) and the temperature can be used to modulate a phase transition (21, 26, 27) so that the system remains isotropic near 20° C while at about 35° C, the liquid crystal is formed and oriented in the NMR magnetic field.

Among the residual dipolar couplings that can be easily measured in carbohydrates, it is the one-bond ^{13}C - ^1H values which are most often used since natural abundance gives good results. The dipolar coupling, which is the difference between the total coupling in the oriented and in the isotropic phase, can be measured for CH and CH₃ groups in carbohydrates using a t_1 -coupled ^{13}C - ^1H HSQC experiment in which the proton 180° refocusing pulse in the middle of t_1 evolution is removed. Multiple bond ${}^nD_{HH}$ and ${}^nJ_{HH}$ can be determined using a homonuclear CT-COSY experiment in which the magnitude of ${}^nD_{HH}$ is determined quantitatively by the trigonometric dependence of the intensities in a series of experiments with different constant time delays (28,29). For complex carbohydrates for which ^{13}C chemical shift resolution is critical to avoid overlap, a heteronuclear ^1H - ^{13}C , ^1H - ^1H TOCSY experiment (30) can be applied. This experiment provides E.COSY type signals resolved in the ^1H and ^{13}C dimensions from which the splitting in the proton dimension can be used to determine the sign and magnitude of ${}^nD_{HH}$ (19).

Since orientation is essential for the observation of dipolar coupling, the precise orientation of the molecular model must be known for interpretation of the experimental data. The direction as well as the degree of orientation can be determined from the experimental data if at least five dipolar coupling values, each corresponding to a distinct direction in space, can be measured for an oligosaccharide fragment exhibiting internal motion of the first kind

(25). Of the five parameters which must be determined, three correspond to the Euler angles needed to orient the molecular model, one is the overall degree of order and one describes the departure from axial symmetry of the molecular model.

For rigid epitopes, measurement of the C-H bond vectors provides adequate data for calculation of the orientation and very accurate models of these epitopes can be constructed (31). For more flexible oligosaccharides having internal motion of the second kind, interpretation of dipolar coupling values is more difficult. One must treat the orientation of individual pyranoside rings which tumble as a rigid unit. Since in pyranosides all axial C-H bonds are essentially parallel, other types of dipolar coupling must be measured in order to gather five independent values (29). In this case, ^1H - ^1H couplings and long range ^{13}C - ^1H couplings must be acquired. A detailed interpretation of dipolar coupling for flexible oligosaccharides is difficult and no established method is available.

Methods for more flexible polysaccharides

The characterization of the motion of the flexible hinges in oligosaccharides and polysaccharides is a major challenge and requires some sort of simplifying framework. For this purpose, we propose a model in which motion is characterized by exchange among a small number of well defined energy minima. In support of this hypothesis, we argue that the bulky stereochemistry of the saccharides restricts the conformational space available to the linkage so that the allowed conformations are limited to a very small number of well defined energy minima, separated by significant energy barriers (Figure 1). Thus each minimum exhibits very restricted internal motion but there can be conformational exchange among them. Characterization requires we locate the energy minima and determine their statistical weights. If the number of significant minima is only two or perhaps three, we will propose schemes for determining the parameters describing such a conformational model for flexible polysaccharide hinges. The concept may be less useful for peptides, proteins or RNA for which motion may occur within much broader energy minima.

Molecular modeling

Although molecular dynamics simulations ought to be a suitable method for describing the internal motion in polysaccharides, this approach has some serious limitations. First, it has been shown that explicit inclusion of solvent in the simulation is necessary for quantitative accurate results for oligosac-

charides which are very polar with hydroxyl groups that bind water strongly. If water is left out of simulations, the polar hydroxyl groups interact with each other in an unrealistic way. In practice, solvent water breaks up these interactions with the formation of new hydrogen bonding patterns between the water and the sugar. Only by explicitly including water can the hydrogen bonding effects of these specific bridging water molecules be adequately described. (32, 33). MD simulations with an adjustment of the treatment of electrostatic effects or with an approximate bulk water treatment such as the GB-SA model minimizes the formation of artifactual intraresidue hydrogen bonds but accurate calculation of the contribution of specific hydrogen bonding to the energy minima is sacrificed. Thus it is possible to calculate the locations of most of the energy minima but without explicit water and a description of effect of solvent, the breaking of hydrogen bonds and competition with intra molecular hydrogen bonding is not accurately described. Therefore in vacuum MD simulations, the accuracy of relative energies of the minima is limited to a few kcal per mole which is the typical energy of a hydrogen bond. Without solvent, approximately correct location of the minima can be found but energies are not sufficiently accurate to get good statistical weights for them.

While explicit inclusion of water in the MD simulations considerably increases the size of the calculation, very accurate simulations have been reported for disaccharides which accurately reflect the specific effects of bridging waters, (33, 34). The disaccharides treated in these calculations have been generally flexible. But extensions of such rigorous calculations to larger complex oligosaccharides have not been reported. Simulations of the more flexible oligosaccharides having internal motion of the second kind have serious limitations on practical length of solvent MD trajectory. When explicit solvent water is included, it is difficult to simulate for times longer than about 10 ns. Since the time scale for conformation exchange between minima is at least 10 ns and might be considerably longer, it may not be possible to describe the kinetics of conformational transitions without resorting to more difficult techniques such as umbrella sampling or adiabatic mapping.

Scalar coupling

Given simple molecular modeling to locate positions of minimum energy wells, experimental data can be used to derive statistical weights. While NOE data are not a very satisfactory experimental method for this purpose because of difficulties in interpretation for flexible structures, scalar cou-

pling has been very successful for these purposes (3, 18). Scalar coupling has the advantage of giving local information on the value of a single dihedral angle not complicated with other conformational parameters. This greatly simplifies early stages of the molecular modeling search by allowing individual glycosidic linkages to be treated independently (18). Moreover, averaging the scalar coupling for two different conformers is a simple linear problem independent of the rates of conformational exchange. The main problem with interpretation of scalar coupling is in the details of the Karplus curve correlating the data with dihedral angles. Recent advances in *ab initio* methods for calculation of scalar coupling give us growing confidence in the reliability of these correlations (35, 36). Measurements of $^3J_{CH}$, $^3J_{CC}$ and $^2J_{CC}$ for the glycosidic linkages yields multiple coupling constants for a single glycosidic bond and provides an over determined system avoiding difficulties arising from the multivalued nature of the trigonometric correlation function. The data can also be used to determine whether a single value of a dihedral angle can fit all the data implying internal motion of the first kind. If multiple values of the dihedral angle are implied, statistical weights can be calculated for individual conformers.

For calculating the statistical weights of the low energy conformers, a procedure has been described by Martin-Pastor and Bush (18). Allowed regions of the dihedral angle space for the linkages are determined by molecular modeling and singular value decomposition is used to determine which combinations of those minima can be combined to reproduce the experimental $^3J_{CH}$, $^3J_{CC}$ and $^3J_{HH}$ coupling data.

The method described using ^{13}C scalar coupling measurements in complex oligosaccharides and polysaccharides has not been applied in many polysaccharide systems. While the principle of the method is sound and attractive, the experimental application is complicated by the requirement for isotope enrichment. While it is possible to measure $^3J_{CH}$ in natural abundance samples, more extensive and accurate data are available with ^{13}C enrichment and isotope enrichment is essential for $^3J_{CC}$ measurements. The scalar coupling values are small and the experiments are difficult. Dipolar coupling would be a very attractive alternative for studying the distribution of conformations in flexible oligosaccharides and a few reports have appeared in the literature (19, 29). But at this point there is no well established procedure for evaluating the data.

Conclusions

Some complex oligosaccharides contain rigid domains composed of three or four sugar residues which tumble together with a well defined 3-dimensional conformation. The existence of such rigid epitopes is strongly dependent on a particular stereochemical arrangement and other types of linkages may be more flexible. While experimental techniques exist for accurate determination of the conformation of the rigid domains, more flexible domains having internal motion of the second kind remain difficult to reliably characterize. The combination of these two types of domains in a complex polysaccharide produces a model which can be viewed as rigid domains connected by hinges. A detailed model of the flexible polysaccharide remains difficult and will require new experimental and computational methods for characterization of the flexible hinges.

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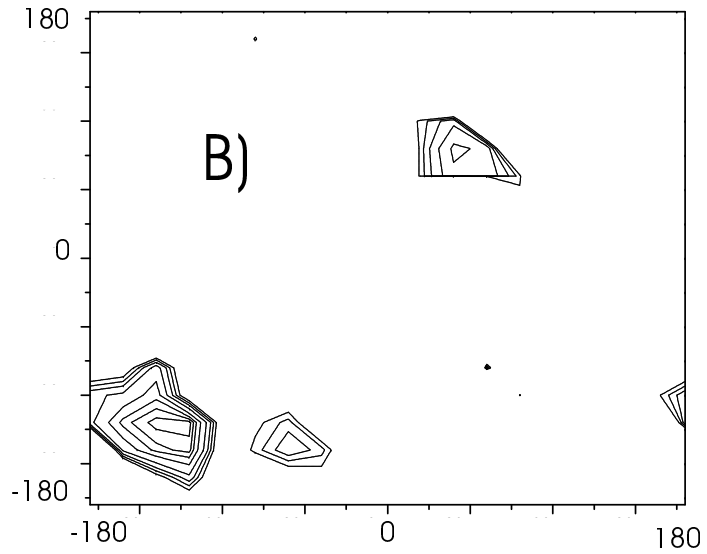
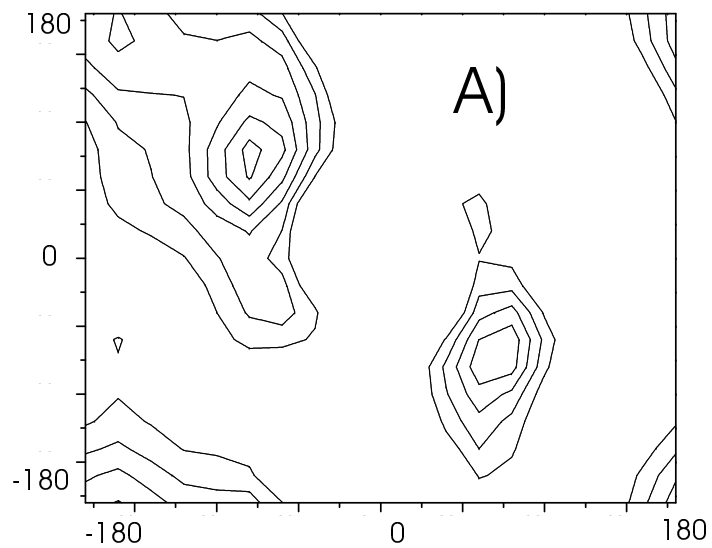


Figure 1. Comparison of relaxed maps made with Charmm software and plotted at contour intervals of 1 kcal/mole. A) Alanine dipeptide.
B) Disaccharide, Gal-(1-3)Gal

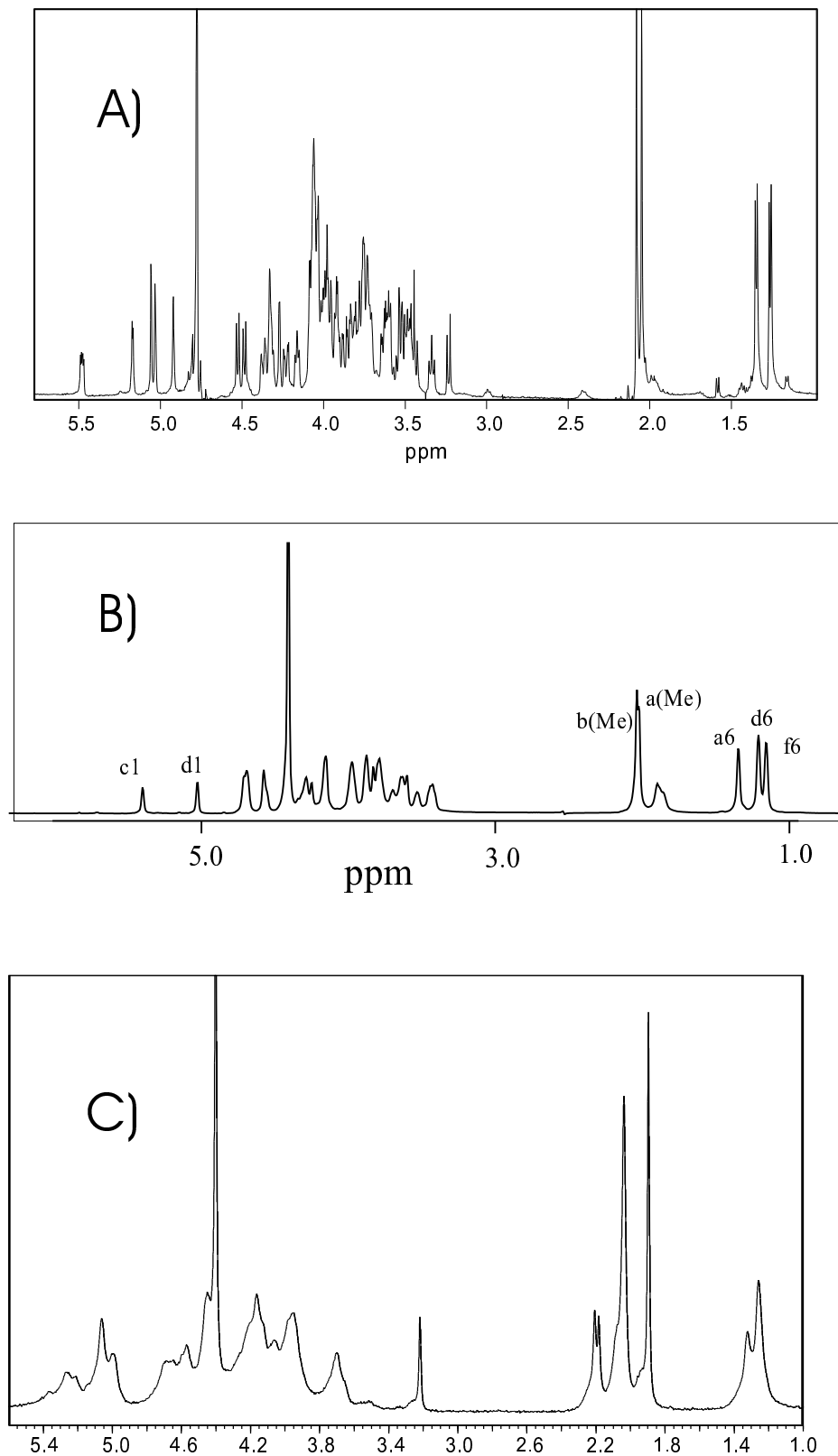


Figure 2. Comparison of ^1H NMR spectra recorded at 500 Mhz for three high molecular weight bacterial polysaccharides.
 A) Cell wall polysaccharide from *Streptococcus mitis* J22 at 23°C.
 B) Capsular polysaccharide of *Vibrio cholerae* O139 at 60°C
 C) Capsular polysaccharide from *Streptococcus pneumoniae* type 1 at 50°C.

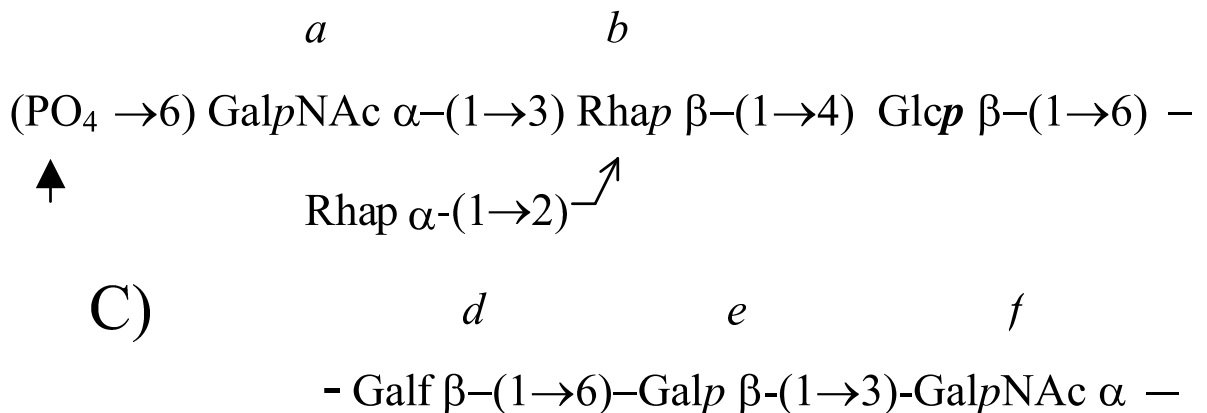
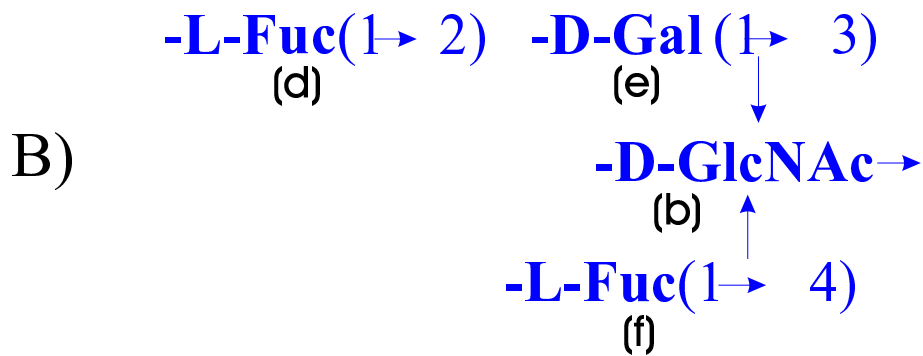
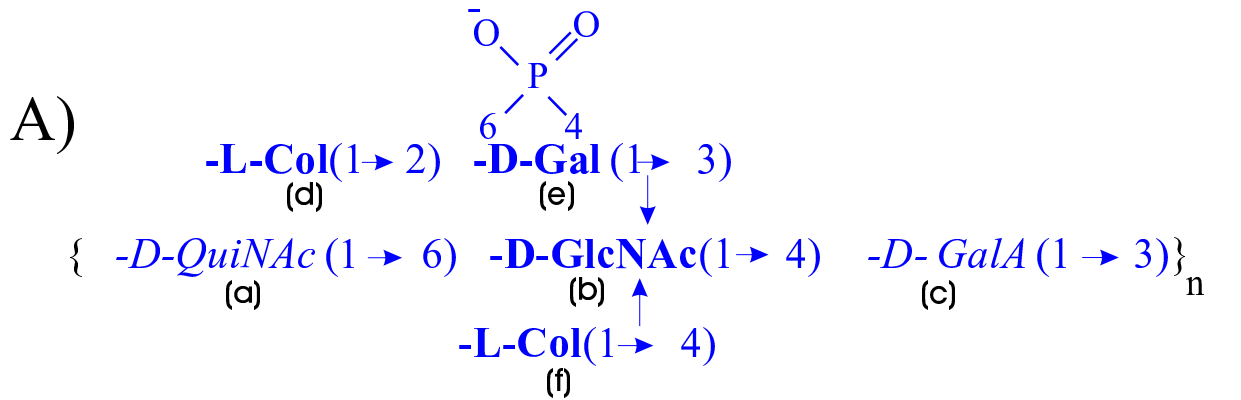


Figure 3. A) Repeating hexasaccharide of the capsular polysaccharide of *V. Cholerae* O139. Colitose is 3,6 di-deoxy-L-galactose. QuiNAc, N-Acetyl quinovosamine is 6-deoxy N-Acetylglucosamine.

B) The Lewis^b tetrasaccharide epitope C) The repeating heptasaccharide subunit of the cell wall polysaccharide of *S. Mitis* J22.