Human (α2→6) and Avian (α2→3) Sialylated Receptors of Influenza A Virus Show Distinct Conformations and Dynamics in Solution

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S *Supporting Information*

ABSTRACT: Differential interactions between influenza A virus protein hemagglutinin (HA) and α2→3 (avian) or α2→6 (human) sialylated glycan receptors play an important role in governing host specificity and adaptation of the virus. Previous analysis of HA–glycan interactions with trisaccharides showed that, in addition to the terminal sialic acid linkage, the conformation and topology of the glycans, while they are bound to HA, are key factors in regulating these interactions. Here, the solution conformation and dynamics of two representative avian and human glycan pentasaccharide receptors [LSTa, Neu5Ac-α(2→3)-Gal-β(1→3)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc; LSTc, (Neu5Ac-α(2→6)-Gal-β(1→4)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc] have been explored using nuclear magnetic resonance and molecular dynamics simulation. Analyses demonstrate that, in solution, human and avian receptors sample distinct conformations, topologies, and dynamics. These unique features of avian and human receptors in solution could represent distinct molecular characteristics for recognition by HA, thereby providing the HA–glycan interaction specificity in influenza.

LSTa-H1 Solution Dynamics of Influenza ligands LSTc-H1
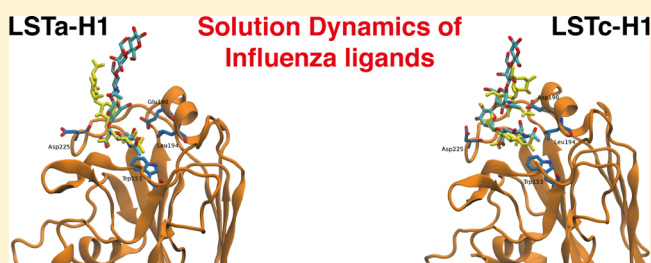
The interaction between the influenza A virus surface protein hemagglutinin (HA) and its cell surface glycan receptors is one of the critical steps governing virus selectivity. Several crystal structures of different HAs have been determined, both native and cocrystallized with glycan receptors.[1−5] These studies have provided insight into the ability of HA to interact with receptor glycans. Human-adapted influenza viruses preferentially bind to sialylated glycan receptors terminated by N-acetylneuraminic acid (Neu5Ac) α2→6 linked to galactose (Gal) (human receptors), which are predominantly expressed on the apical surface of the upper respiratory epithelia in humans.[6−8] In contrast, avian-adapted influenza viruses preferentially bind to sialylated glycans terminated by Neu5Ac α2→3 linked to Gal, which are expressed predominantly in the gut and respiratory tract of birds and, importantly, in the deep lung (alveolar region) of humans.[7] Therefore, one of the characteristics of human adaptation of influenza A viruses is the change in glycan receptor binding preference of HA from avian to human receptors.[9−14]

Recent studies based on analysis of HA–glycan cocrystal structures[15,16] have highlighted the importance of glycan conformation and topology in governing the receptor specificity of avian- and human-adapted HAs. In the case of avian receptors, the majority of contacts between HA and glycan involve the Neu5Ac residue at the nonreducing end. Chandrasekaran et al.[16] proposed that, when the Neu5Ac is fixed in the glycan-binding pocket of HA, the other residues in this trisaccharide motif sample different torsional angles at their glycosidic linkages, such that the space occupied by the whole glycan resembles a cone with Neu5Ac at the vertex, characterized by a topological angle θ of >110°. The topological angle θ is defined by the C2, C1, and C1 atoms of the residues Neu5Ac, Gal, and GlcNAc (N-acetylglucosamine), respectively, in going from the nonreducing end to the reducing end. They also proposed for the case of human receptors that a longer glycan motif, comprising at least a tetrasaccharide Neu5Ac-α(2→6)-Gal-β(1→4)-GlcNAc-β(1→3)-Gal- at the nonreducing end, spans the glycan binding surface on HA. The additional C5−C6 bond of the Neu5Ac-α(2→6)-Gal glycosidic linkage provides added mobility to its glycosidic junction and the remaining backbone, allowing it to explore conformations

similar to an umbrella progressing from a fully open form to a fully closed form (defined as having a θ of <110°).[16] It is clear that the cone and umbrella topologies are generated by their motions while they are bound to the receptor, but when in their free states, they adopt conformations that are dependent on their respective nonreducing end connectivity, $\alpha(2\rightarrow3)$ or $\alpha(2\rightarrow6)$. The role of glycan shape and topology in regulating binding specificity for HA has also been investigated using molecular dynamics simulations,[17] indicating that there are distinct differences in the conformational sampling of avian and human receptors from the different subtypes (H3, H5, and H9) upon binding to HA.

The previously mentioned studies highlighted differences in glycan receptor conformation and structural details when the receptor cocrystallized bound to HA, illustrating the structural specificity of HA−glycan interactions. Consequently, it is reasonable to suppose that receptor dynamic properties, strictly related to its structure, could play a role in the recognition and binding by the HA protein. To characterize human and avian sialylated glycan receptors in terms of conformation and topology in solution, detailed nuclear magnetic resonance (NMR) analyses, MD simulations, and subsequent numerical analysis of the data generated were performed on the two sialylated pentasaccharides: LSTc (NeuSAc-$\alpha(2\rightarrow6)$-Gal-$\beta(1\rightarrow4)$-GlcNAc-$\beta(1\rightarrow3)$-Gal-$\beta(1\rightarrow4)$-Glc), representing the human receptor, and LSTa (NeuSAc-$\alpha(2\rightarrow3)$-Gal-$\beta(1\rightarrow3)$-GlcNAc-$\beta(1\rightarrow3)$-Gal-$\beta(1\rightarrow4)$-Glc), representing the avian receptor (Figure 1 of the Supporting Information). We refer to Gal1 and Gal2 as labels for the first and second Gal residues encountered in the sequence of LSTc and LSTa, respectively, from the nonreducing to the reducing end. NOESY, ^{13}C NMR relaxation measurements, and analysis of the glycan MD trajectories revealed that LSTa and LSTc have distinct conformational dynamics, topologies, and intramolecular hydrogen bond networks in solution. Specifically, LSTc preferentially adopts a bent shape in solution, which is consistent with the "umbrella"-like conformation, whereas LSTa adopts a bow shape in solution, which agrees with a "cone"-like structure. These observations suggest that the different species-adapted HAs could recognize specific shapes, conformations, and dynamic behaviors of glycan in solution.

■ EXPERIMENTAL PROCEDURES

NMR Chemical Shift Mapping. NMR measurements were taken using Bruker 500, 600, and 900 MHz AVANCE series NMR spectrometers (Bruker GmbH, Silberstreifen, Germany) with a high-sensitivity 5 mm TCI cryoprobe. Human milk lactosyl sialyl-*N*-tetraoses, namely, LSTa (PROzyme, Hayward, CA) [NeuSAc-$\alpha(2\rightarrow3)$-Gal(Gal1)-$\beta(1\rightarrow3)$-GlcNAc-$\beta(1\rightarrow3)$-Gal(Gal2)-$\beta(1\rightarrow4)$-Glc)] and LSTc (PROzyme) [NeuSAc-$\alpha(2\rightarrow6)$-Gal(Gal1)-$\beta(1\rightarrow4)$-GlcNAc-$\beta(1\rightarrow3)$-Gal(Gal2)-$\beta(1\rightarrow4)$-Glc], were dissolved in D_2O (99.9%) or a buffered solution [150 mM sodium chloride, 100 mM sodium phosphate, and 10% D_2O (pH 7.2)] according to the NMR experiment. Chemical shifts (^{1}H and ^{13}C) of the oligosaccharides were measured at 295 K in D_2O (99.9%, 2 mg/0.2 mL) using a 3 mm NMR tube. Two-dimensional (2D) NMR experiments were conducted using edited HSQC, HSQC-TOCSY, HMBC, TOCSY, and NOESY (using different mixing times between 100 and 1000 ms), recorded for quadrature detection in the indirect dimension and acquired using 16−48 scans per series of 1024 × 320 data points, with zero filling in

F1 (4096) prior to Fourier transformation. Figures of spectra were created using R[18] and the libraries rNMR[19] and lattice.[20]

Estimation of Intra- and Interproton Distances. Experimental distances were obtained by selective one-dimensional (1D) and 2D NOESY experiments conducted with mixing times between 100 and 800 ms (295 K) and used to construct the kinetic NOEs. Selected distances were estimated on the basis of the (two-spin model approximation) time-dependent NOE measurements extrapolated to short mixing times, considering the initial buildup enhancement up to 300 ms.[21] The extrapolated NOEs signals were converted to proton distances considering the NeuSAc distance ($H3_{ax}$−$H3_{eq}$) as a reference (1.78 Å).

^{13}C NMR Relaxation Data. NMR relaxation times were obtained at 295 K using a 14.0 T magnetic field. [13]C longitudinal relaxation (T_1) and transverse relaxation (T_2) were measured via 2D double INEPT-based inverse detection methods optimized to suppress the cross correlation between the chemical shift anisotropy (CSA) and the dipolar relaxation.[22−24] The 2D spectra were recorded using 1024 × 192 data points, recording 24−32 scans at eight different relaxation delays arranged randomly to avoid systematic errors in every experiment. T_1 relaxation delays were 0, 0.1, 0.2, 0.3, 0.5, 0.8, 1.0, and 1.4 s. T_2 transverse relaxation times were obtained by the CPMG pulse sequences differing in spin echo delays (22.4, 44.8, 67.2, 89.6, 134.4, 179.2, 224.0, and 313.6 ms). The volumes of the cross-peaks were integrated using Topspin 2.1, and relaxation times were obtained by best nonlinear and linear fitting of normalized data of the evolution of the peak volumes as a function of the inversion recovery delays. All the values and estimated errors (2−10%) were calculated and plotted in Origin 8.0 (OriginLab Corp., Northampton, MA).

Determination of the $^nJ_{C−H}$ Heteronuclear Coupling Constants. Long-range $J_{C−H}$ couplings were measured using the J-HMBC pulse sequence (295 K), with a delta delay corresponding to a $^nJ_{X−H}$ of 2 Hz [LSTc (0.7 mg) and LSTa (1 mg) were dissolved in 220 μL of D_2O]. Data matrices of 1024 × 320 points covering 2003.2 × 9054.9 Hz were expanded to 2048 × 1024 points using linear prediction and subsequently zero-filled to 8192 × 2048 points prior to echo/antiecho Fourier transformation. The traditional sequence was applied for LSTa, while the non uniform sampling (NUS) variant was used for LSTc (Bruker Topspin version 3.1).

Determination of Temperature Coefficients and Chemical Shift Deviations. Temperature coefficients were recorded for 0.5 mg/0.2 mL oligosaccharide solutions in buffered D_2O or in a 90:10 H_2O/D_2O mixture at pH 7.2. All temperature gradients were measured by differences observed in spectra recorded at 3−9 K intervals between 278 and 313 K (^{1}H). Temperature coefficients are expressed in units of parts per billion per kelvin, with a negative sign indicating an upfield shift upon warming; chemical shifts were measured using ^{1}H and HSQC experiments.

Molecular Dynamics Simulations. The starting geometries of both oligosaccharides LSTa and LSTc were generated using the carbohydrate biomolecule builder;[25] following the primary structure and the glycosidic constraints, both structures were minimized by the online energy minimizer.[25] The energy description of both oligosaccharides included the amber/glycam06 force field.[25,26] The atomic partial charges required by the oligosaccharide force field were calculated using the semiempirical bond-corrected AM1-BCC

scheme.[27] The MD simulations were conducted in explicit solvent using the TIP3P water model.[28] Oligosaccharide partial charge management and topology files were constructed with AmberTools version 1.4.[29] The MD trajectories were generated using the NAMD MD engine.[30] Nonbonded interactions, i.e., electrostatic and van der Waals, are described using the cutoff approach set to 12.0 Å with a switching function beginning at 6.0 Å, and the time step for the integration algorithm is 1 fs. The simulation cell, including the oligosaccharide, was built enveloping the solute molecule in a layer of water 20.0 Å wide in each direction; the result is a cubic cell of approximately 60 Å × 67 Å × 59 Å. The oligosaccharide models that were compared to the experimental data were generated by the following procedure; the simulation cell was first optimized applying 10000 minimization steps of the NAMD minimizer algorithm, restraining each atom of the carbohydrate to the initial conformation by a harmonic restraint with a scaling constant of 500 kcal/mol. The next step was a MD simulation reproducing the constant-temperature and pressure statistical ensemble (NPT) applying a pressure on the cell walls of 1.01325 bar (atmospheric pressure). The simulation temperature was set at 295 K and was controlled by a Langevin thermostat as implemented in NAMD. The Nosé-Hoover Langevin piston algorithm controlled the pressure applied on the cell walls, with periodic boundary conditions being applied. The whole length of the simulation was 104 ns, where the first 4 ns was conducted restraining the carbohydrate atoms (solute) by a harmonic potential with a scaling constant of 50 kcal/mol to initially equilibrate the cell density without changing the solute conformation significantly, while the remaining simulation time was undertaken with the solute free to move. The cell densities calculated for LSTa and LSTc after equilibration (~4 ns) were in both cases 1.04 g/cm^3. The MD trajectory consisted of the last 100 ns, with a single geometry being sampled every 10 ps. During the whole dynamic, the conformation of the residues remained in $^2C_5$ for Neu5Ac and $^4C_1$ for the others, as set from the beginning for both LSTa and LSTc, and in agreement with the three-bond proton−proton coupling constants ($^3J_{H−H}$).

The progression of the MD simulation was observed by determining the root-mean-square displacement (rmsd) of the trajectories, performed in R using the *rmsd* function from the Bio3D library,[31] with the starting conformation as the reference. The rmsd distances were determined using eq 1:

$$\text{rmsd}(t) = \sqrt{n^{-1} \sum_{i=1}^{n} [D_i(t) - D^{\text{ref}}_i]^2} \tag{1}$$

where D^{ref}_i is the position of the ith atom of the reference structure ($t = 0$), $D_i(t)$ is the position of atom i at time t, and n is the number of atoms.

Glycosidic Torsion Angles. The torsional angles (ϕ and ψ) are defined in this paper by the following pairs of dihedral angles, from the nonreducing termini: ϕ_1 and ψ_1, ϕ_2 and ψ_2, ϕ_3 and ψ_3, and ϕ_4 and ψ_4. For LSTa, ϕ_1 is the C1−C2−O3−C3 angle and ψ_1 is the C2−O3−C3−H3 angle, while for LSTc, ϕ_1 is the C1−C2−O6−C6 angle and ψ_1 is the C2−O6−C6−C5 angle, in agreement with Xu et al.[17] The successive pairs are defined as ϕ_i and ψ_i [H1−C1−O4′−C4′ and C1−O4′−C4′−H4′ (1→4 linkage) or H1−C1−O3′−C3′ and C1−O3′−C3′−H3′ (1→3 linkage)], as conventionally defined for sugars. Atoms labeled with a prime belong to the monosaccharide on the reducing side of the glycosidic linkage, while atoms without

a prime are on the nonreducing side of the glycosidic linkage. To be consistent with the work of Chandrasekaran et al.[16] and Xu et al.,[17] ϕ and ψ were illustrated in the range of −120° to 240°.

Topological θ Angle. The θ angle is defined by the C2, C1, and C1 atoms of residues Neu5Ac, Gal, and GlcNAc (*N*-acetylglucosamine), respectively, going from the nonreducing end to the reducing end

Comparison of the MD Results to the Experimental Data. The rmsd$_{\text{exp}}$ values (eq 2) were used to compare the MD trajectories with the experimental data, i.e., the estimated inter-residue proton−proton distances for both LSTc and LSTa using the two-spin model approximation, reported for LSTc (H5 Neu5Ac−CH$_3$ GlcNAc, H1 GlcNAc−H3 Gal2, H1 GlcNAc−H4 Gal2, H1 Gal2−H4 Glc, and H1 Gal1−H4 GlcNAc) and LSTa (H3$_{ax}$ Neu5Ac−H3 Gal1, H3$_{eq}$ Neu5Ac−H3 Gal1, H1 Gal1−H3 GlcNAc, H1 Gal1−CH$_3$ GlcNac, H1 GlcNAc−H3 Gal2, H1 GlcNAc−H4 Gal2, H1 Gal2−H4 Glc, and H1 Gal2−H5 Glc). In eq 2, $D_{\text{exp},i}$ is the experimental ith inter-residue proton−proton distance and $D_{\text{MD},i}$ is the corresponding distance for a single MD-generated conformation. The number of the available inter-residue proton−proton distances is n.

$$\text{rmsd}_{\text{exp}} = \sqrt{n^{-1} \sum_{i=1}^{n} (D_{\text{exp},i} - D_{\text{MD},i})^2} \tag{2}$$

2D NOESY Spectral Simulation (multispin model relaxation). The 2D NOESY spectra were simulated using two different sets of 40 conformations each from the MD trajectory: 40 with the smallest rmsd (rmsd$_{\text{exp}}$ min) and highest (rmsd$_{\text{exp}}$ max). The 2D NOESY simulation was undertaken assuming isotropic motion with a single correlation time τ_c estimated to be 400 ps. The τ_c estimation was conducted on the smallest rmsd set of conformations (rmsd$_{\text{exp}}$ min) and simulating the intraresidue H1−H3 and H1−H5 2D NOE signals for the selected residues: GlcNAc for LSTc and Gal1, GlcNAc, and Gal2 for LSTa. The τ_c parameter was adjusted by trial and error until the NOE kinetic enhancement from 0 to 500 ms mixing time reproduced qualitatively the corresponding experimental values. The software for 2D NOESY simulation was NOEPROM.[32]

Principal Component Analysis of the MD Trajectory. The MD trajectories for LSTc and LSTa were exported as *XYZ* coordinates via VMD,[33] allowing the data to be imported directly into R.[18] Principal component analysis (PCA) was performed using the bio3d library, the *pca.xyz* function.[31] The data were referred to the first conformer of the MD trajectory, removing translational and rotational effects from the analysis, and then mean centered before PCA was performed. The calculated eigenvalues (scree plot) provide a weighting for the importance of the eigenvectors (also known as loadings) (illustrated as porcupine plots), and the component scores (score plot) illustrate the relationship between the determined eigenvector (independent molecular motion) and the conformers that constitute the MD trajectory. Porcupine plots were produced by constructing a trajectory along the desired principal component, imposing the molecular motion extracted by that component onto the average geometry of the conformers.[31] This was then visualized using VMD and the porcupine plot created using a VMD script.[33,34]

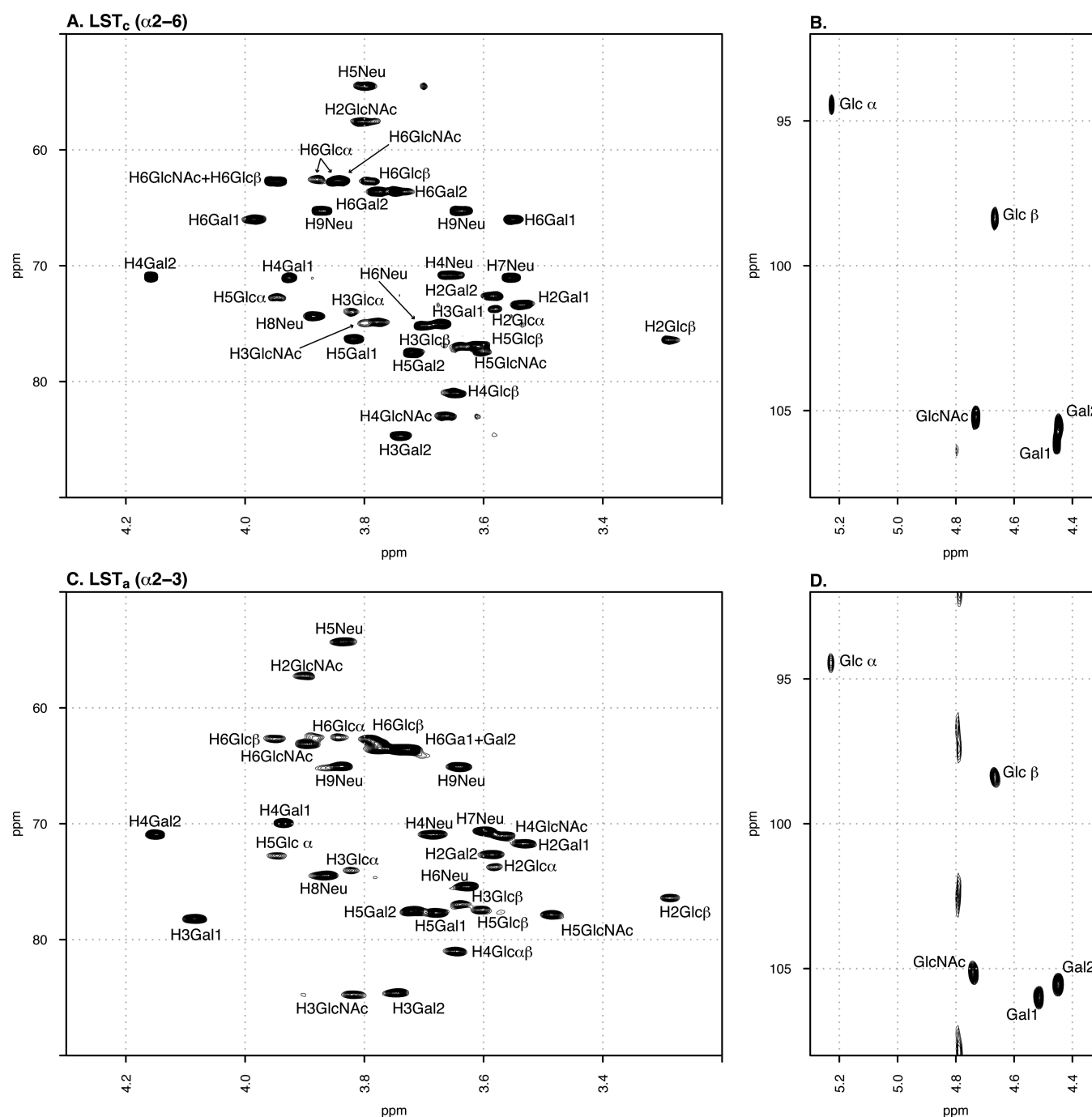The convergence of the glycan PCA was observed by determining the root-mean-square internal product (rmsip) of

Figure 1. $^1\text{H}-^{13}\text{C}$ HSQC NMR spectra of LSTc (A and B) and LSTa (C and D).

the first 10 eigenvectors. If rmsip is equal to 1, then there is complete convergence between the sets of eigenvectors; a value of ≥0.70 shows that the eigenvectors are consistent with each other.[35] In this circumstance, rmsip was used to see if the MD simulation was tending toward a stationary state. PCA was performed on the trajectory at different times, and this was compared to the eigenvectors derived for the trajectory at 100 ns. The rmsip was determined using R and the library Bio3d;[31] the *rmsip* function was utilized. The rmsip is defined as

$$\text{rmsip} = \sqrt{10^{-1} \sum_{i=1}^{10} \sum_{j=1}^{10} (\eta_i \cdot \nu_j)^2}$$

(3)

where η_i is the *i*th eigenvector of trajectory η and ν_i is the *i*th eigenvector of trajectory ν.

■ RESULTS

NMR Analyses of LSTa and LSTc. NMR characterization of sialyl-lacto-*N*-tetraoses LSTa and LSTc (Figure 1 of the Supporting Information) were conducted using homonuclear (COSY, TOCSY, and NOESY), heteronuclear (edited HSQC and HSQC-TOCSY), and heteronuclear multiple-bond correlation HMBC ($^1\text{H}/^{13}\text{C}$) experiments. This permitted the complete assignment of the hydrogen and carbon resonances for both LSTc and LSTa (Figure 1 and Table 1 of the Supporting Information). TOCSY analysis allowed the

connectivity of the pyranosyl rings to be observed, with H−H axial correlations confirming the monosaccharide identities. The chemical shifts of the acetamide methyl groups of GlcNAc and Neu5Ac for both LSTa and LSTc were determined by 2D TOCSY (buffered system, 10% D_2O), providing NH correlations with some of the hydrogens of each ring and with those from acetamide (Figure 2 of the Supporting Information). However, the strong peak superimposition did not permit the complete hydrogen assignment by homonuclear experiments; these were complemented by HSQC-TOCSY and by the COSY effect generated by homonuclear coupling in sensitivity-enhanced HSQC. These correlation peaks, usually considered as artifacts, are very useful in the assignment of signals in crowded spin systems when performing sensitivity-enhanced HSQC experiments[36] (Figure 3 of the Supporting Information). Long-range correlations ($^1H/^{13}C$) of the quaternary C1 and C2 atoms from the sialyl residues were observed between the H3$_{ax}$ Neu5Ac atom at 1.782 ppm and the corresponding carbon signals at 176.7 and 102.3 ppm (C1 and C2, respectively). C2 correlated with H3 Gal1 at 4.082 and 78.2 ppm of LSTa, confirming the presence of the Neu5Ac $\alpha2\rightarrow3$ Gal linkage. Instead, LSTc correlated H3$_{ax}$ Neu5Ac at 1.711 ppm with carbon signals at 176.2 (C1) and 102.7 (C2) ppm, with the latter correlating with H6α/β Gal1 at 3.983α/3.544β/66.0 ppm, confirming that C2 Neu5Ac is O-linked to H6α/β Gal1. Anomeric proton correlations from LSTa were observed at 4.513/84.7 ppm (H1 Gal1/C3 GlcNAc), 4.739/84.5 ppm (H1 GlcNAc/C3 Gal2), and 4.446/81 ppm (H1 Gal2/C4 Glc). LSTc exhibited cross-peaks at 4.451/82.9 ppm (H1 Gal1/C4 GlcNAc), 4.73/84.6 ppm (H1 GlcNAc/C3 Gal2), and 4.445/80.9 ppm (H1 Gal2/C4 Glc). These measurements were confirmed by 1D selective and 2D NOESY experiments.

Three-bond proton−proton coupling constants, $^3J_{H−H}$, were measured for both pentasaccharides, which indicated that all Hex$p$ residues assumed the $^4C_1$ conformation, because $^3J_{H1−H2}$ values vary from 7.9 to 8.2 Hz and $^3J_{H3−H4}$ values for Gal residues are ∼3.5 Hz; Glc and GlcNAc had values of 9.0 and 9.8 Hz, respectively. The Neu5Ac residue had $^3J_{H−H}$ values compatible with those of a pyranosyl ring in the $^2C_5$ conformation [$^3J_{H3−H4}$ = 4.8$_{(eq)}$ Hz/12.1$_{(ax)}$ Hz, and $^3J_{H4−H5}$ = 10.1 Hz], thus suggesting no significant differences in the ring conformation between the pentasaccharides.

As in proteins, NH temperature coefficients can reflect the extent to which N-acetyl-amide protons are protected from exchange through hydrogen bonding. Evaluation of NH temperature coefficients showed different behavior between GlcNAc and Neu5Ac. The first had a value of −8.28 ppb/K for LSTc and −8.00 ppb/K for LSTa, while the NH group for Neu5Ac exhibited lower temperature coefficients with values of −6.77 and −6.60 ppb/K for LSTc and LSTa, respectively. This behavior suggested that the amide group from GlcNAc could be involved in an intramolecular hydrogen bond. This result is supported by a study of the half-height line widths ($\Delta\nu_{1/2}$) extracted from each NH acetamide after a Lorentzian deconvolution and plotted at different temperatures. The NH data from the Neu5Ac N-acetyl group broadened before those of GlcNAc, suggesting that the NH group of GlcNAc is involved in intramolecular hydrogen bonds (Figure 4 of the Supporting Information).

^{13}C NMR Relaxation Data. NMR relaxation studies have been utilized to interrogate the internal motion of the glycans, providing dynamic properties that can be qualitatively or quantitatively assessed.[23,24,37,38] T_1 and T_2 relaxation times and

heteronuclear NOEs were measured at 14.0 T (Table 2 of the Supporting Information). T_1 varied from 315 to 412 ms for LSTa and from 328 to 421 ms for LSTc. T_2 varied from 229 to 355 ms and from 232 to 387 ms for LSTa and LSTc, respectively. To qualitatively observe the main differences in the motional properties between LSTc and LSTa, the average values of T_1 and T_2 of each CH ring were used (Figure 2). The
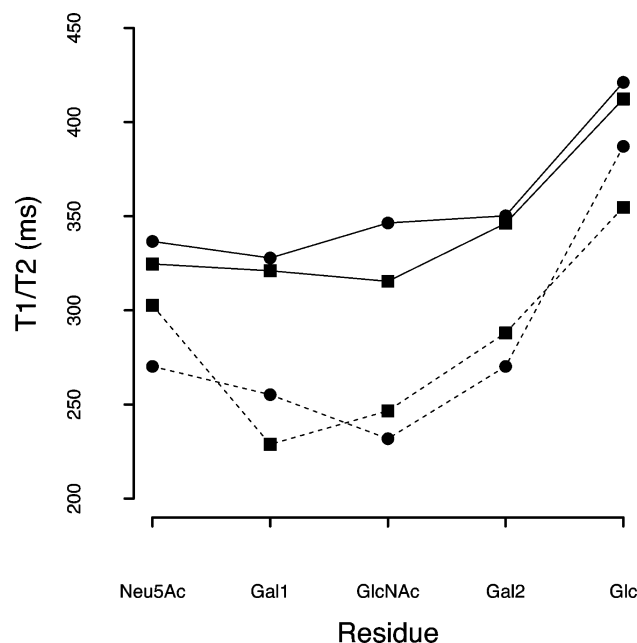

Figure 2. Average ^{13}C relaxation times of CH group carbons for each monosaccharide unit from LSTc (●) and LSTa (■). The solid line represents the average T_1 of each monosaccharide, while the dashed line represents the average T_2 of each monosaccharide.

numerical values of T_1 for the different residues suggest an anisotropically tumbling molecule, because the Neu5Ac-Gal1-GlcNAc trisaccharide of both glycans gave rise to T_1 values ranging from 315 to 346 ms, whereas values of the reducing Glc residue varied from 410 to 421 ms. This finding indicates a higher degree of motion for the reducing lactosyl disaccharide than for the nonreducing end of the oligosaccharide. The heteronuclear NOEs corroborate the T_1 values, because larger heteronuclear NOEs are observed for the terminal units. Using the same approach, significantly lower T_2 values were detected for both LSTa and LSTc, T_2 values of the reducing Glc residue of both glycans (355 and 387 ms for LSTc and LSTa, respectively) confirmed the higher degree of motion of this residue than of the internal Gal1, and GlcNAc residues showed lower T_2 values between 228 and 255 ms for both glycans. The main differences between LSTa and LSTc were the values observed for T_2 of the Neu5Ac residues, being 303 and 270 ms, respectively; such a difference, larger than the experimental error, accounts for the significantly higher rigidity of the nonreducing end of LSTc. Contrary to that of the nonreducing side, the higher mobility of the reducing end residues has little biological relevance, because this part of the molecule *in vivo* is part of a greater structure.

Inter- and Intra-NOESY Experimental Distances. The inter- and intraresidue proton−proton distances were measured by NOESY enhancement (see Experimental Procedures for details). Intraresidue distances (H1−H3 of Hex$p$ units and H3−H5 of the Neu5Ac residues) were measured between 2.0

and 2.6 Å for all the residues (Table 3 of the Supporting Information). These values were in agreement with the expected $^4C_1$ and $^2C_5$ ring chair conformations, supporting the observed $^3J_{H-H}$ coupling constants. Inter-residue proton–proton distances were obtained mainly from O-glycosidic bonds (Table 1). LSTa exhibited NOE H3$_{eq}$ and H3$_{ax}$ Neu–H3

Table 1. Selected Interglycosidic Proton–Proton Distances (angstroms) for LSTa and LSTc Derived from NOE Measurements

	LSTc (Å)	LSTa (Å)
H3$_{ax}$ Neu5Ac–H3 Gal1	–	2.76
H3$_{eq}$ Neu5Ac–H3 Gal1	–	4.70
H5 Neu5Ac–CH$_3$ GlcNAc	4.24	–
H1 Gal1–H3 GlcNAc	–	2.25
H1 Gal1–CH$_3$ GlcNAc	–	4.00
H1 GlcNAc–H3 Gal2	1.94	1.95
H1 GlcNAc–H4 Gal2	3.05	2.84
H1 Gal2–H4 Glc	1.96	2.20
H1 Gal2–H5 Glc	–	2.80

Gal1 contacts, providing distances of 4.7 and 2.8 Å, respectively, while LSTc exhibited an inter-residue NOE between H5 Neu5Ac and CH$_3$ GlcNAc corresponding to an estimated distance of 4.2 Å (Figure 5 of the Supporting Information). This contact between GlcNAc and Neu5Ac residues, observed only in the NOESY spectra of LSTc, can be considered the experimental proof of a bent conformation that LSTc assumes compared to LSTa, which does not exhibit this signal at all.

Molecular Dynamics Simulations of LSTa and LSTc. To complement the NMR experiments, MD simulations were performed to compare the dynamics of the glycans in solution. The MD simulations were allowed to run for 100 ns, sampling conformers every 0.01 ns, therefore producing a comprehensive data set, with the simulation length considerably greater than the estimated correlation time (isotropic motion) of either glycan, of ∼400 ps. After 70% of the simulation time had elapsed, several molecular parameters exhibited stationary behavior without significant drift; these include the end-to-end distance of the sugars, which is defined as the distance between C5 of Neu5Ac (nonreducing end) and C1 of Glc (reducing end). This property is one of the slowest-evolving degrees of freedom for an oligomer (Figures 7 and 8 of the Supporting Information). Stationary behavior is also observed for the θ angle of the glycans, which is characteristic for each glycan (Figures 9 and 10 of the Supporting Information) and the ω torsional angle of Gal1, which is defined as the H5–C5–C6–O6 angle (Figure 11 of the Supporting Information). Similar behavior was seen over the same time scale for the other four pairs of glycosidic torsional angles (ϕ_i and ψ_i) (see Experimental Procedures), the angles that define the backbone conformation of the two oligomers.

During the MD simulation, the residues comprising LSTc and LSTa adopted the $^2C_5$ (Neu5Ac) and $^4C_1$ (Gal, Glc, and GlcNAc) conformations, respectively, with only small deviations from the ideal chair of cyclohexane; the intraresidue proton–proton distances (H1–H3, H1–H5, and H3–H5) have values ranging between 2.5 and 2.7 Å for the five sugars, compared to the ideal value of 2.64 Å for cyclohexane. The same distances are estimated by NOE enhancement using the two-spin approximation (see Experimental Procedures); the

values obtained ranged between 2.0 and 2.6 Å, in good agreement considering the strong coupling effects (Table 3 of the Supporting Information).

The intersugar unit conformations were defined using four pairs of inter-residue glycosidic dihedral angles, running from the nonreducing end to the reducing end of the glycans (ϕ_i and ψ_i) (see Experimental Procedures). The torsional angle values explored during the whole MD simulation are reported in Figure 3, and the torsional angles are displayed in the range of −120 to 240°. Visited torsional states are reported as cluster centroids with their relative populations in Table 2. The principal difference between LSTa and LSTc is located at the first glycosidic linkage (ϕ_1 and ψ_1), which is in agreement with those obtained by molecular modeling,[39] and is related to the different connectivity at the nonreducing end [Neu5Ac-α(2→3)-Gal for LSTa and Neu5Ac-α(2→6)-Gal for LSTc]. For LSTa, four ϕ_1 and ψ_1 torsional states are observed, a principal state is located at −62° and −8° (89.8%), respectively, while the most significant member of the minor states is located at −83° and −55° (7.7%), respectively (Figure 3A, α and β, respectively). LSTc possesses two ϕ_1 and ψ_1 torsional states; the cluster with the greater population (96.2%) is located at −57° and 190°, respectively, and the minor population at 56° and 174°, respectively (Figure 3E, ξ and o, respectively).

To fully describe the first glycosidic linkage of LSTc [Neu5Ac-α(2→6)-Gal], the ω torsional angle (see Experimental Procedures for the definition) needed to be considered. This degree of freedom is absent for LSTa. LSTc has a single state characterized by an average ω value of −50°, which is in agreement with the prediction of Xu et al.[17] (Figure 11 of the Supporting Information). The ω torsional angle of the Neu5Ac-α(2→6)-Gal-β disaccharide has previously been discussed by Sabesan et al.[40] for similar di- or trisaccharides. They propose that two states are allowed for ω torsional angles, located approximately at −50° and 180°, corresponding to antiperiplanar (gt conformer) and synclinal (tg) for H5–H6(R) (Gal1), respectively. These states have characteristic $J_{H5-H6(R)}$ couplings, ∼10 Hz for the former and a smaller value of ∼3.5 Hz for the latter. We observed a coupling constant of 10 Hz for $J_{H5-H6(R)}$ in LSTc, which according to Sabesan et al.[40] corresponds to the antiperiplanar H5–H6(R) Gal1 conformer, which supports the values determined by the MD simulation.

As we move along the glycans, from the nonreducing end to the reducing end, the glycosidic linkages exhibit progressively decreasing disparities between the glycosidic torsional state positions and populations (Figure 3). The ϕ_2 and ψ_2 distribution for LSTc and LSTa (Figure 3 and Table 2) qualitatively agree with those found previously by Sabesan et al.[40] for disaccharides Gal-β(1→3)-GlcNAc and Gal-β(1→4)-GlcNAc, which are model compounds for LSTa and LSTc, respectively.

The torsional states determined for LSTa broadly agree with those described by Xu et al.,[17] while the torsional angles determined for LSTc are less consistent with the data of the aforementioned work. Xu et al.[17] found the following additional LSTc torsional states: ∼180° and ∼180° for ϕ_1 and ψ_1, ∼60° and ∼180° for ϕ_2 and ψ_2, ∼30° and ∼160° for ϕ_3 and ψ_3, and ∼50° and ∼180° for ϕ_4 and ψ_4, respectively. We predict a state in the first glycosidic linkage of LSTc located at 56° and 174°, which was not observed by Xu et al.,[17] but this state is seen in the conformational map obtained at GlycoMapsDB for Neu5Ac-α(2→6)-Gal.[41] This discrepancy is possibly related to the use of a different starting conformation for LSTc during
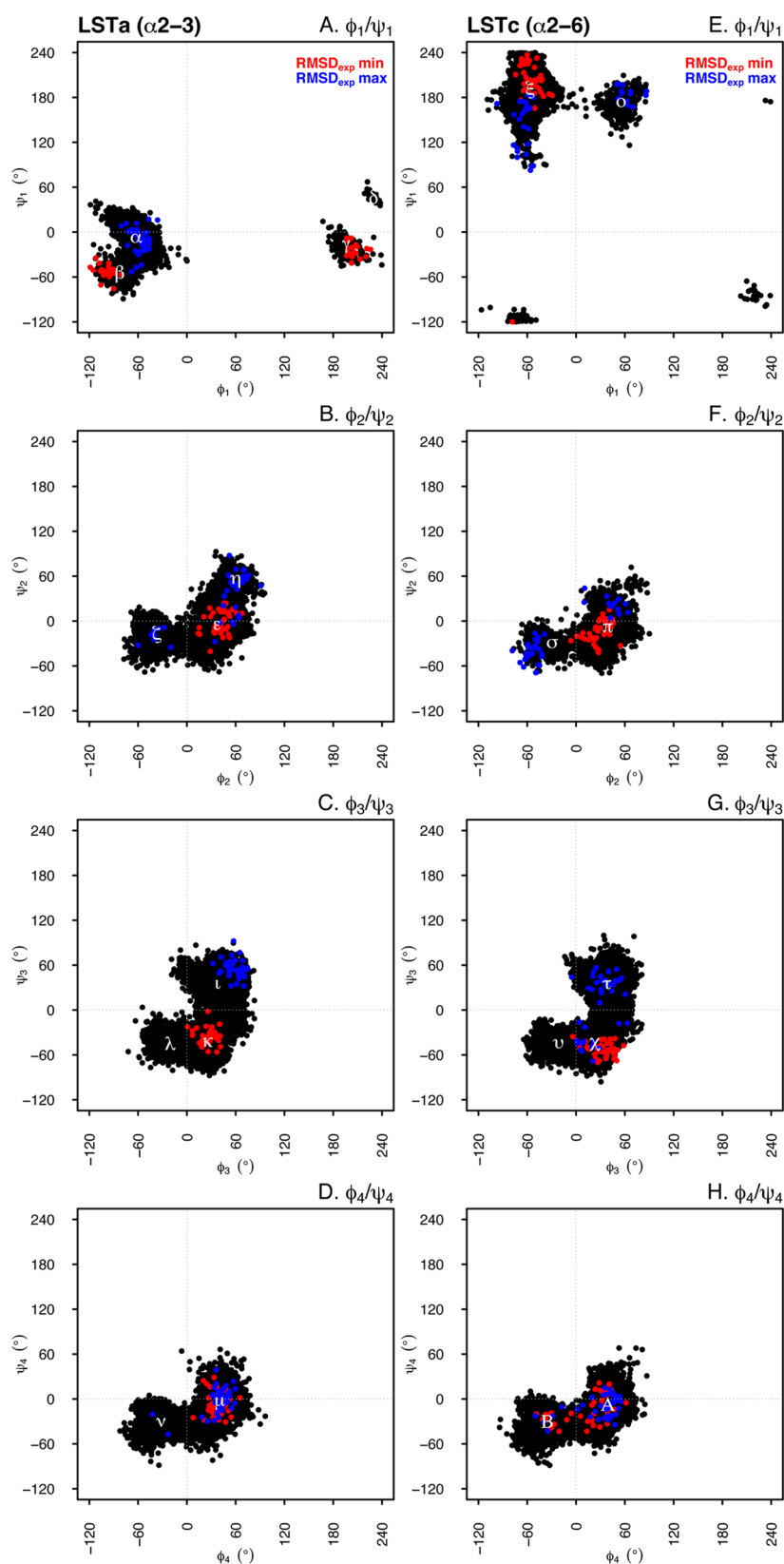
Figure 3. Torsional states visited during the 100 ns simulation time for LSTa (left) and LSTc (right). For each glycan, the dihedral angle pairs [ϕ_1 and ψ_1 (A and E), ϕ_2 and ψ_2 (B and F), ϕ_3 and ψ_3 (C and G), and ϕ_4 and ψ_4 (D and H)] are shown. The red dots represent conformers that better fit the experimental NMR NOESY data (rmsd$_{exp}$ min set of conformations), while blue dots correspond to the conformers that exhibit poor agreement with the experimental constraints (rmsd$_{exp}$ max). The positions and populations of the torsional states are listed in Table 2.

the MD simulation, which prevents an ensemble of conformations from being visited as a consequence of the

potential energy walls restricting exploration of the conformational space. To partially address this limitation, shorter MD

Table 2. Torsional States of LSTa and LSTc as Determined by Molecular Dynamics

linkage		ϕ (deg)	ψ (deg)	no.	%
		LSTa ($\alpha2\rightarrow3$) Torsional angles			
1	α	−62	−8	8982	89.8
	β	−83	−55	770	7.7
	γ	196	−19	227	2.3
	δ	230	47	21	0.2
2	ε	37	−5	6908	69.1
	ζ	−37	−18	2402	24.0
	η	60	54	690	6.9
3	ι	38	35	5534	55.3
	κ	26	−43	3134	31.3
	λ	−21	−44	1332	13.3
4	μ	40	−-6	7732	77.3
	ν	−32	−31	2268	22.7
		LSTc ($\alpha2\rightarrow6$) Torsional angles			
1	ξ	−57	190	9622	96.2
	o	56	174	378	3.8
2	π	40	−9	9024	90.2
	σ	−30	−30	976	9.8
3	τ	39	34	4108	41.1
	χ	23	−46	4118	41.2
	υ	−21	−45	1774	17.7
4	A	39	−7	5840	58.4
	B	−36	−30	4160	41.6

simulations (20 ns) were performed at higher temperatures (395 and 495 K) using the same starting conformation at each temperature (Figures 12 and 13 of the Supporting Information). At these higher temperatures, the missing LSTc torsional states in linkages ϕ_1 and ψ_1 and ϕ_4 and ψ_4 started to be populated (Figure 12 of the Supporting Information). Interestingly, for both LSTc and LSTa, the first glycosidic torsional state (ϕ_1 and ψ_1) reproduces qualitatively in position and shape the torsional angle map of the Neu5Ac-α(2→6/3)-Gal glycosidic junction shown in GlycoMapsDB.[41]

A parameter used to monitor the evolution of a MD simulation is the root-mean-square displacement (rmsd) of the atoms in a molecule (determined using the conformation at time zero as a reference), which illustrates the different conformational states that the molecule assumes during the simulation, as compared to the starting state. Unsurprisingly, the conformational states assumed by LSTc and LSTa are related to the torsional angle states of their glycosidic linkages. During the simulation, LSTc moves between two conformational states, rmsd states located at ∼2.10 and ∼3.50 Å (Figure 14 of the Supporting Information), while LSTa assumes a single broad distribution of related conformational states (Figure 15 of the Supporting Information) centered at ∼2.8 Å. The conformers that populate the different rmsd states observed for LSTc, located at rmsd values of ∼2.10 and ∼3.50 Å, have stark differences in the third glycosidic linkage (ϕ_3 and ψ_3), GlcNAc-β(1→3)-Gal-β (Figure 16 of the Supporting Information). This correlation is also true for LSTa; a subset of conformers located at rmsd values of 2.65 and 3.80 Å (±10 Å) also exhibit significant differences in the third glycosidic linkage (Figure 17 of the Supporting Information). This result is interesting for the case of LSTc, as it suggests that the third glycosidic linkage is the most conformationally mobile junction and not the first, extended linkage Neu5Ac-α(2→6)-Gal, as might be assumed. This could be due to the first residue

forming hydrogen bonds with the rest of the molecule, the result being that the first linkage is static compared to the third.

NMR Data and MD Simulation Comparison. To compare the conformers derived by MD simulation and the experimental NMR data, an rmsd$_{exp}$ was derived from five experimentally estimated inter-residue proton−proton distances (see eq 2 and Table 1) and the corresponding MD predicted distances for each structure in the MD trajectories (see Experimental Procedures). The smaller the rmsd$_{exp}$ value, the more closely the conformer matches the NMR data.

The MD-generated conformers of LSTc have two distinct rmsd$_{exp}$ states. The first state located at 0.64 Å is very narrow, and these conformers match the NMR data well; this population ends at 1.05 Å, and thereafter, there is a very broad declining population of conformers with increasing disparity between the NMR-estimated inter-residue proton−proton distances and MD-predicted distances (Figure 18 of the Supporting Information). The conformers that populate these states have distinct glycosidic torsional states, with the conformers that more closely match the NMR data, the population with the smaller rmsd$_{exp}$ values (<1.05 Å), belonging to the following glycosidic torsional angle states: −57° and 190° for ϕ_1 and ψ_1 and 39° and −9° for ϕ_2 and ψ_2, respectively (clusters ξ and π, respectively, Figure 3E,F and Figure 19 of the Supporting Information). LSTa also has two rmsd$_{exp}$ populations, located at 0.75 and 0.97 Å (Figure 20 of the Supporting Information); again the conformers that comprise these different populations belong to differing torsional angle states. In this circumstance, the differentiating linkage is closer to the reducing end of the glycan, ϕ_3 and ψ_3; the MD-generated conformers that are in closer agreement to the NMR data are members of the torsional angle states located at −21° and −45° and 23° and −46° for ϕ_3 and ψ_3, respectively (clusters λ and κ, respectively, Figure 3C and Figure 21 of the Supporting Information). It is interesting to note that on the whole the MD conformers of LSTa more closely match the NMR data, with the whole population falling within the rmsd$_{exp}$ range of 0−1.5 Å, whereas the LSTc conformers have rmsd$_{exp}$ values spanning the range of 0−5 Å.

To further compare the experimental NMR data with the MD-determined structures, two subsets of conformers were chosen for each glycan, a subset containing the conformers with the 40 smallest rmsd$_{exp}$ values (rmsd$_{exp}$ min) (0.24−0.33 Å for LSTc and 0.12−0.43 Å for LSTa) and a subset comprising the structures with the 40 largest rmsd$_{exp}$ values (rmsd$_{exp}$ max) (1.26−1.41 Å for LSTa and 3.83−4.40 Å for LSTc). The 2D NOESY spectra were then simulated for these selected structures (see Experimental Procedures), with the calculated intra- and inter-residue proton−proton NOE signals then being compared (Table 4 of the Supporting Information). The H1−H3, H1−H5, and H3−H5 intraresidue NOEs signals are qualitatively reproduced for LSTc residues Neu5Ac, Gal1, GlcNAc, and Gal2, while for LSTa, the signals are reproduced for only Gal1, GlcNAc, and Gal2. Interestingly, the agreement between the simulated and experimental intraresidue 2D NOE signals is similar for both rmsd$_{exp}$ min and rmsd$_{exp}$ max conformer subsets, because the rmsd$_{exp}$ values were determined using inter-residue distances and not intraresidue distances. In fact, the H1 Gal1−H4 GlcNAc, H1 GlcNAc−H3 Gal2, H1 Gal2−H4 Glc, and CH$_3$ GlcNAc−H5 Neu5Ac inter-residue distances compare well for the LSTc rmsd$_{exp}$ min conformer subset, while the rmsd$_{exp}$ max conformers reproduce only the H1 Gal2−H4 Glc distance. This trend is also true for LSTa,

with the rmsd_{exp} min set of structures reproducing the H3 Gal1−H3$_{\text{ax}}$ Neu5Ac, H1 Gal1−H3 GlcNAc, H1 GlcNAc−H3 Gal2, and H1 Gal2−H4 Glc inter-residue distances well and the rmsd_{exp} max conformers replicating adequately only the experimentally determined H1 Gal2−H4 Glc distance (Table 4 of the Supporting Information). This illustrates the ability of the rmsd_{exp} minimization procedure to select conformers from the MD simulation trajectory, providing a means of determining that quality of the MD simulation and allowing the structures that more closely match the experimental data to be extracted. To make a broader comparison of the modeled and measured data, with MD conformers not being chosen by experimental constraints, the last 60 geometries of the MD simulation for LSTa and LSTc were used to generate NOE magnitudes (Table 4 of the Supporting Information). For both glycans, the agreement between the NOE magnitudes derived from MD and experimental data is qualitatively good, but the NOE magnitudes for the rmsd_{exp} min MD conformer subset more closely match the experimental data than the NOE magnitudes derived from the MD structures, which were chosen without experimental constraints. It appears that the first glycosidic linkage, Neu5Ac-α(2→6/3)-Gal, for both glycans is the least well described by the MD simulation, while the remaining interglycosidic NOEs are better modeled in LSTa. This could be due to the isotropic motion approximation introduced by NOEPROM to generate the theoretical NOE magnitudes, with LSTc exhibiting stronger motional anisotropy than LSTa, which is observed in the NMR relaxation measurements.

The torsional angles for the rmsd_{exp} min and rmsd_{exp} max conformer subsets are shown in Figure 3 for LSTa and LSTc, respectively (Table 5 of the Supporting Information). rmsd_{exp} min structures are colored red, while the rmsd_{exp} max conformers are colored blue. Both of the conformer subsets for LSTc and LSTa show significant differences in their torsional angle states; the ϕ_4 and ψ_4 linkage for both glycans is the only linkage where there is little divergence. Interestingly, the LSTa rmsd_{exp} min conformers have two ϕ_1 and ψ_1 states, the linkage between Neu5Ac and Gal1, which are located at −100° and −53° and 207° and −24°, corresponding to clusters β and γ, respectively. The conformers in the state centered at −100° and −53° are in better agreement with the NOE values for the H3$_{\text{ax}}$ Neu5Ac−H3 Gal1 distance (Table 4 of the Supporting Information). Sabesan et al. also observed a similar NOE, between H3$_{\text{ax}}$ Neu5Ac and H3 Gal1, for the di- and trisaccharide structures.[40] The glycosidic torsional angles of the conformers that more closely fit the NMR data are as follows: −55° and 197° for ϕ_1 and ψ_1, 28° and −15° for ϕ_2 and ψ_2, 34° and 54° for ϕ_3 and ψ_3, and 11° and 15° for ϕ_4 and ψ_4, respectively, for LSTc and 207° and −24° for ϕ_1 and ψ_1, 41° and −1° for ϕ_2 and ψ_2, 27° and 34° for ϕ_3 and ψ_3, and 38° and −5° for ϕ_4 and ψ_4, respectively, for LSTa. To corroborate this result, selected long-range coupling constants, $^3J_{\text{CH}}$, across the glycosidic linkages ($J\phi$ for H1−C1−Ox−Cx and $J\psi$ for C1−Ox−Cx−Hx) were measured; this allowed an indirect estimation of the glycosidic torsional angles for both glycans. The measured coupling constants (in hertz) starting from the first torsion (ϕ_1 and ψ_1) are as follows: $J\psi_1$ = 4.4, $J\phi_2$ = 2.6, $J\psi_2$ = 5.4, $J\phi_3$ = 4.2, $J\psi_3$ = 4.5, and $J\phi_4$ = 4.1 for LSTa and $J\psi_1$ = 2.6, $J\phi_2$ = 3.6, $J\phi_3$ = 4.1, $J\psi_3$ = 4.4, and $J\phi_4$ = 3.4 for LSTc, respectively. Calculated pairs of couplings ($J\phi$ and $J\psi$), determined using the rmsd_{exp} min subset of conformers, in accordance to the Karplus-type equation,[42] are reported in

Table 7 of the Supporting Information. The only measured long-range coupling that does not agree with the rmsd_{exp} min selected subset of conformers is the ϕ_1 and ψ_1 coupling for LSTa; the measured coupling ($J^{\text{exp}}\psi_1$ = 4.4 Hz) is in better agreement with the glycosidic torsional angles ϕ_1 and ψ_1 of −99° and −53°, respectively, and not ϕ_1 and ψ_1 values of 207° and −24°, respectively, which were determined by the NMR-selected MD conformers. J-HMBC spectra of LSTa and LSTc, overlaid with HSQC spectra, can be found in Figure 6 of the Supporting Information.

Analyses of the Shape and Topology of LSTc and LSTa. The global shape of the two oligosaccharides is described by the topological θ angle, as defined by Xu et al.[17] (see Experimental Procedures). Chandrasekaran et al. previously proposed that the θ angle of LSTc and LSTa defines their conformation, with LSTa adopting a linear form, coined a cone, which is defined as having a θ angle >110°, while LSTc, with its extra degree of freedom at the nonreducing end, Neu5Ac-α(2→6)-Gal-β(1→4)-, forms a bent conformation, termed the umbrella form, defined as having a θ angle of <110°.[16]

The LSTc conformers determined by MD have two θ angle populations; the much larger population is located at 87°, and the second smaller population starts above 109° (Figure 9 of the Supporting Information). These differences in θ values also correspond to differences in the torsional angle states for the linkage between Gal1 and GlcNAc; the subset of conformers with a θ angle centered at 87° correlates with the torsional angle state with values for ϕ_2 and ψ_2 of 39° and −9°, respectively, while the structures with a θ angle of >88° populate torsional states with values for ϕ_2 and ψ_2 of 39° and −9° and −30° and −30°, respectively (Figure 22 of the Supporting Information). This indicates that the second glycosidic linkage is important in the "opening and closing" of the umbrella conformation proposed for LSTc and not the first glycosidic linkage, which might be assumed because of the α2→6 linkage between Neu5Ac and Gal1.

LSTa also has two θ angle populations, with the more populated state centered at 153° and the less populated state located at 119° (Figure 10 of the Supporting Information). As for LSTc, the LSTa conformers that belong to the different θ angle populations correlate strongly with the torsional states of the second glycosidic linkage, ϕ_2 and ψ_2 (Figure 23 of the Supporting Information). The θ angle population centered at 153° correlates with the torsional state with values for ϕ_2 and ψ_2 of 37° and −5°, respectively, while the population located at 119° correlates with the torsional state with values for ϕ_2 and ψ_2 of −37° and 18°, respectively. These results highlight that the conformational and subsequent dynamic properties of both LSTc and LSTa are linked strongly to the second glycosidic linkage.

The LSTc conformers that more closely fit the NMR data (rmsd_{exp} min structures) have an average θ angle of 87° (standard deviation of 4°), while the corresponding LSTa conformers have an average θ angle of 159° (standard deviation of 5°). These values, for the NMR-selected MD-generated conformers, agree with the results published by Xu et al.[17] and the hypothesis proposed by Chandrasekaran et al.[16]

Principal Component Analysis of the LSTa and LSTc MD Trajectories. Principal component analysis (PCA) has been applied on the MD-simulated trajectory to extract the independent mode of motion of both oligosaccharides, allowing an easier correlation with its structural properties. PCA has been a powerful tool for the analysis of protein[35,43] and
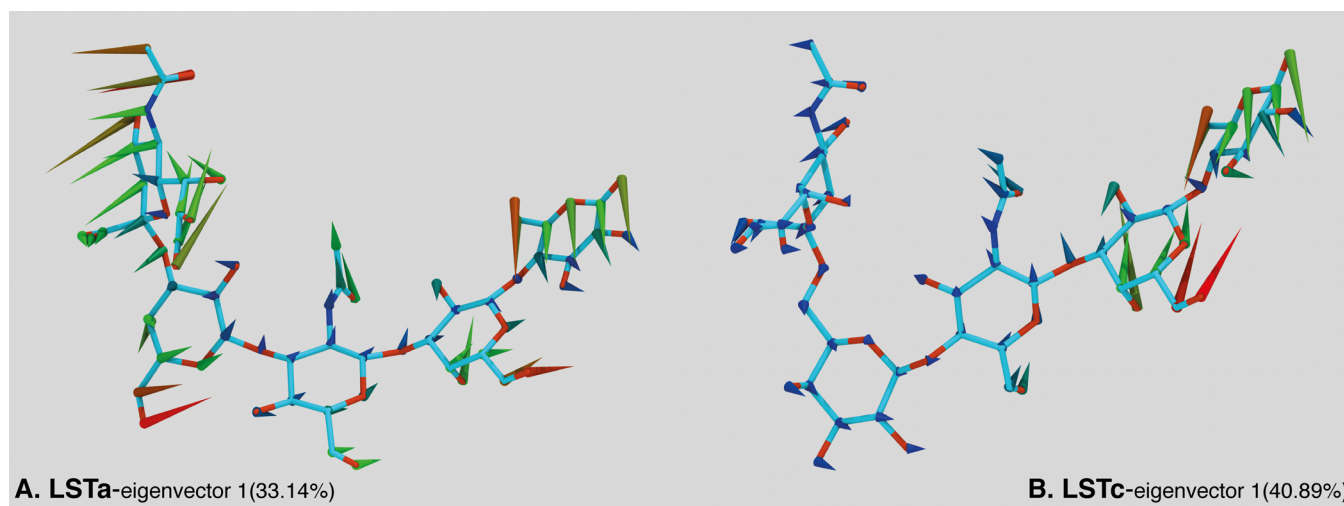
Figure 4. Porcupine plot representations of the first eigenvectors for LSTa (A) and LSTc (B). The direction, size, and color of the spines are proportional to the direction and magnitude of the motion of the atoms. The illustration shows LSTa moving in a bowlike motion while LSTc is moving in two independent sections: Neu5Ac synchronized with the GlcNAc and the reducing-end disaccharide as a single unit. In LSTc, Gal1 is the least mobile residue.

glycan[44] molecular dynamics. The convergence of the distinct ensembles of motion was observed by determining the rmsip of the MD trajectory eigenvectors, comparing the eigenvectors generated by PCA of the whole MD tracjectory (100 ns) to the PCA of the trajectory at different time points (increasing steps of 1 ns). The rmsip provides a measure of the similarity of the "essential modes" extracted; if the value is zero, then they are dissimilar, and if the value is one, the subspaces have converged. The rmsip for both LSTc and LSTa is ≥0.9 for at least 50% of the trajectory; Amadei et al.[35] opined that values of >0.7 provide evidence of good convergence (Figure 24 of the Supporting Information).

Here, only the first four components generated by PCA for LSTa and LSTc are considered. They explain 73.8 and 66.2% of the total variance, respectively. The PCA scree, loading, and normalized eigenvector plots can be found in Figures 25−31 of the Supporting Information. The normalized eigenvector plots indicate the contribution each atom makes to the extracted components, i.e., whether an atom, or a group of atoms, is involved in the independent dynamic motions isolated by PCA.

The independent modes of motion described by component one for LSTc have the nonreducing end of the glycan close to stationary compared to the other residues, while the reducing end (Gal2-Glc) rotates around their glycoside linkages, moving as a single unit. This is in agreement with the rmsd analysis of the LSTc MD trajectory. Figure 4 provides an illustration of this mode of motion, "porcupine" representations of the first eigenvector for LSTc and LSTa [supplementary data contain graphical representations of all four components (Figures 30 and 31 of the Supporting Information)]. The three other distinct modes of motion extracted for LSTc show both the nonreducing and reducing ends of the molecule are in motion, with the reducing end disaccharide (Gal2-Glc) moving as one unit and the sialyl group of Neu5Ac moving toward the N-acetyl group of GlcNAc. The least mobile residue is Gal1, which exhibits the smallest variation of the four components examined, which is illustrated in the normalized eigvector figure for LSTc (Figure 28 of the Supporting Information). These results are consistent with a small θ angle and a hydrogen bonding network between GlcNAc and Neu5Ac of LSTc, while

the fact that the reducing end of the molecule moves in unison is indicative of a cellobiose-like hydrogen bonding network between Glc and Gal2. This dynamic description agrees with the results of the T_1 and T_2 relaxation measurements.

The independent modes of motion extracted for LSTc correlate with structural parameters that have been previously discussed; the motion described by component 1 correlates with the rmsd of the conformers and is therefore related to the different torsional states in the third glycosidic linkage, GlcNAc-β(1→3)-Gal-β (Figures 16 and 32 of the Supporting Information). The molecular motion illustrated by the second component correlates with the structures that belong to the different θ angle populations, which are strongly tied to differences in the second glycosidic linkage (Gal-β(1→4)-GlcNAc) (Figures 22 and 34 of the Supporting Information). Finally, the dynamic that distinguishes whether a conformer agrees well with the experimental NMR data, rmsd$_{exp}$, correlates with component 3, which is related to the different torsional states at glycosidic linkages 1 and 2 (Figures 19 and 36 of the Supporting Information).

The distinct modes of motion isolated for LSTa are very different from those of LSTc. LSTa has "bowlike" dynamics for the four components examined, the whole molecule moving in unison; this is particularly true of the motion isolated for component 1 (Figure 29 of the Supporting Information). Again, the modes of motion isolated by PCA correlate with previously discussed structural parameters. A mixture of components distinguishes the conformers with different rmsd values, for example, the mixture of components 1 and 2 and also components 1 and 4. These dynamics, which are described by the mixtures of components, are generated by specific states in the second and third glycosidic linkages (Figures 17 and 33 of the Supporting Information). Component 1 correlates strongly with the θ angle of the conformers, which in turn is related to the torsional state of ϕ_2 and ψ_2 (Figures 23 and 35 of the Supporting Information). Component 2 is the independent motion that discriminates the structures that fit well with the experimental NMR data from those that do not; high or low rmsd$_{exp}$ values are associated with differences in the torsional
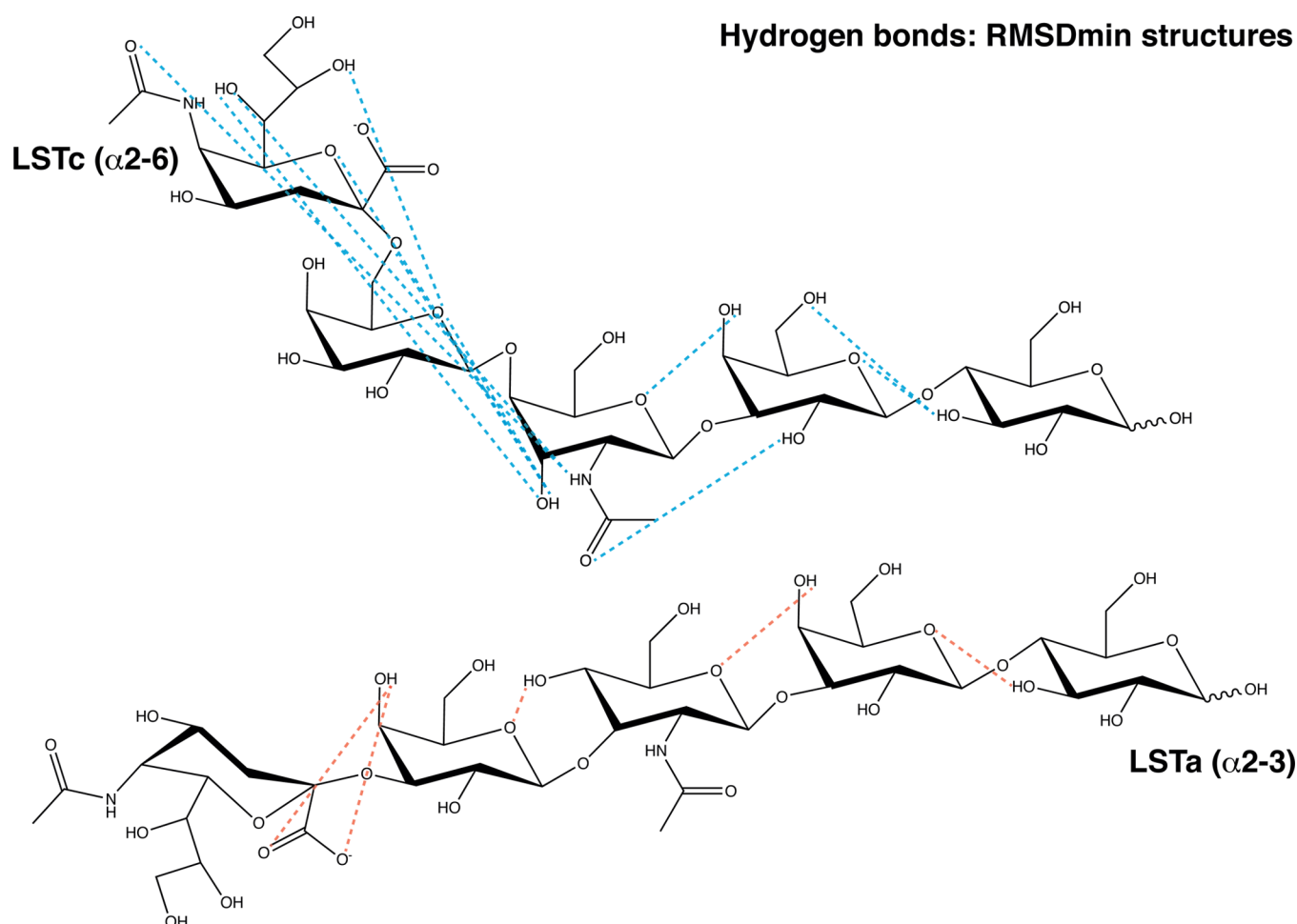
Figure 5. Proposed hydrogen bond network for LSTc and LSTa rmsd$_{exp}$ min conformers. The hydrogen bond network at the nonreducing end of each glycan is strongly affected by the different linkage between Neu5Ac and Gal1 [$\alpha(2\rightarrow3)$ for LSTa and $\alpha(2\rightarrow6)$ for LSTc]. The acceptor and donor atoms lie within 2.6 Å of each other, and the X—H···Y (X is the donor and Y the acceptor) angle was greater than 110°, tending toward 180°. Predicted hydrogen bonds for both LSTa and LSTc appear in Table 6 of the Supporting Information.

states of ϕ_3 and ψ_3 (Figures 20 and 37 of the Supporting Information).

Inter-Residue Hydrogen Bond Analysis. To further understand the consequences and driving forces behind the different conformations assumed by LSTc and LSTa, the possible inter-residue hydrogen bonds were determined for the rmsd$_{exp}$ min and rmsd$_{exp}$ max conformer subsets. The analysis employed distance and angular restrictions; the distance between the donated hydrogen and the acceptor was ≤2.6 Å, and the X—H···Y (X is the donor and Y the acceptor) angle was greater than 110°, tending toward 180°. Here we will discuss the proposed hydrogen bonds for the rmsd$_{exp}$ min subset of conformers, the structures that more accurately represent the experimental data; the hydrogen bond analysis for the rmsd$_{exp}$ max conformers, for comparison, is shown in Table 6 and Figure 38 of the Supporting Information.

For both glycans, several possible hydrogen bonds were determined for the rmsd$_{exp}$ min conformers, 5 for LSTa and 11 for LSTc, illustrated in Figure 5, while the populations of each are reported in Table 6 of the Supporting Information. Both LSTc and LSTa have potential hydrogen bonds that offer an explanation for the tandem motion of the reducing end residues Glc and Gal2 isolated by PCA: H3O Glc—O5 Gal2 (percentage of conformers, 62.5% for LSTc and 80% for LSTa) (cellobiose-like), H4O Gal2—O5 GlcNAc (82.5% for LSTc and 22.5% for

LSTa), H2O Gal2—O2N GlcNAc (42.5% for LSTc), and H3O Glc—O6 Gal2 (12.5% for LSTc). The cellobiose-like hydrogen bond between H3O of Glc and O5 of Gal2 has previously been observed in a structurally related molecule, Neu5Ac-$\alpha(2\rightarrow6)$-Gal-$\beta(1\rightarrow4)$-Glc, sialyl-$\alpha(2\rightarrow6)$-lactose (S6L).[45]

The hydrogen bond networks present at the nonreducing end of either glycan are a direct consequence of their θ angles. The nonreducing end hydrogen bond network for LSTc, which has a bent form in solution ($\theta = 86°$ for the selected conformers), originates from GlcNAc, with two predicted bonds to Gal1 [H3O GlcNAc—O5 Gal1 (100%) and H3O GlcNAc—O6 Gal1 (25.0%)] and five others spanning Gal1 to Neu5Ac [O3 GlcNAc—H7O Neu5Ac (55.0%), O5 Gal1—H8O Neu5Ac (20.0%), H2N GlcNAc—O5N Neu5Ac (15.0%), H3O GlcNAc—O6 Neu5Ac (12.5%), and H2N GlcNAc—O7 Neu (12.5%)] (Figure 5 and Table 6 of the Supporting Information). A hydrogen bond similar to the O3 GlcNAc—H7O Neu5Ac hydrogen bond is observed in S6L, between O3 of Glc and H7O of Neu5Ac.[45] Because of the bowlike backbone conformation of LSTa, its hydrogen bonds can be formed only between directly adjacent residues: H4O GlcNAc—O5 Gal1 (92.5%), H4O Gal1—O1A/O1B (acid group) Neu5Ac (52.5%), H4O Gal2—GlcNAc (22.5%), and H3O Glc—O5 Gal2 (80.0%) (Figure 5 and Table 6 of the Supporting Information). All of the proposed hydrogen bonds are listed in
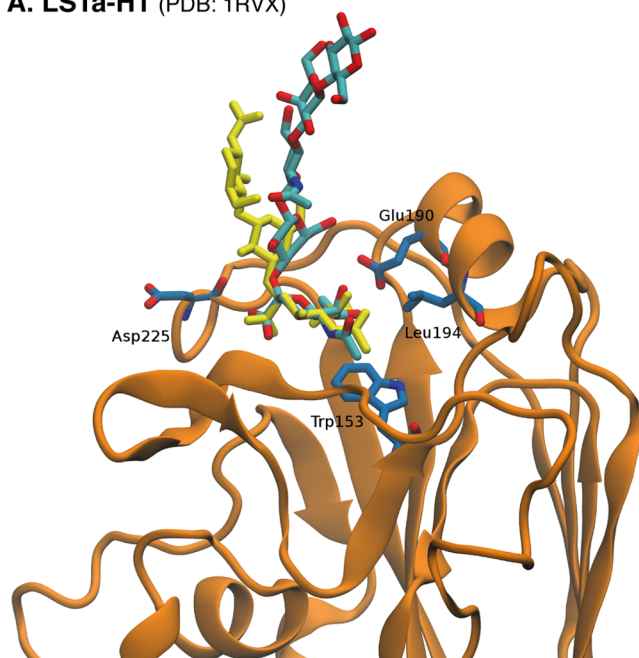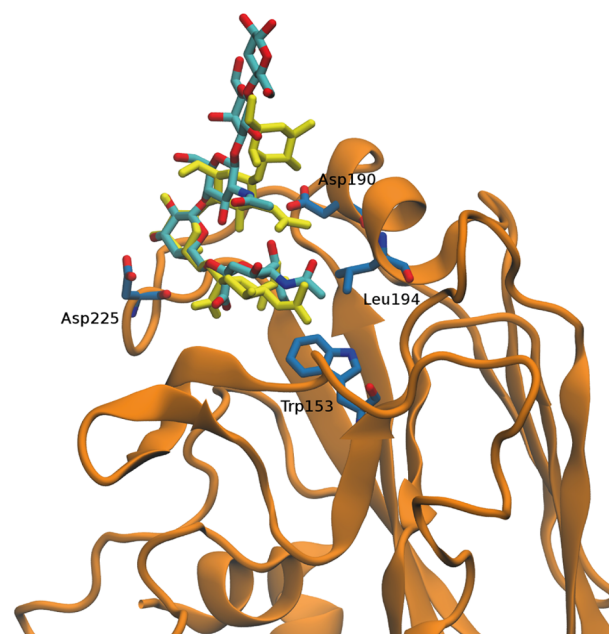
A. LSTa-H1 (PDB: 1RVX)   **B. LSTc-H1** (PDB: 2WRG)

Figure 6. Comparison of the MD -generated LSTa (A) and LSTc (B) conformers (structures with the smallest rmsd$_{exp}$ value, rmsd$_{exp}$ min) and crystal structures of LSTa and LSTc bound to hemagglutinin from H1. The conformers generated via MD simulations are colored cyan (C atoms) and red (O atoms), while the conformers determined by X-ray crystallography are colored yellow. The rmsd values between the MD simulation-generated structures and the cocrystallized glycans are 6.2 and 6.5 Å for LSTa and LSTc, respectively.

Table 6 of the Supporting Information, while Figure 5 is a schematic showing the major proposed inter-residue hydrogen bonds.

DISCUSSION

This study has identified the distinct conformational, topological, and dynamic properties of the glycan receptors [two pentasaccharides, LSTc (Neu5Ac-α(2→6)-Gal-β(1→4)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc) and LSTa (Neu5Ac-α(2→3)-Gal-β(1→3)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc] of influenza A viruses in solution, i.e., without its receptor. The study involved utilizing extensive NMR measurements, including the complete ^{1}H and ^{13}C assignment for each glycan, MD simulations, and the numerical analysis of the data generated, to provide not only information concerning the conformation in solution but also, for the first time, details of its overall dynamic properties that could be related to the receptor binding ability.

Conformers from the MD simulations were selected using a rmsd$_{exp}$ function, comparing NOE enhancement-derived inter-residue proton−proton distances and the equivalent predicted distances. Using this approach, two subsets of conformers were selected, in good (rmsd$_{exp}$ min) or poor (rmsd$_{exp}$ max) agreement with the experimental data. In particular, the conformers that were in better agreement with the experimental restraints, rmsd$_{exp}$ min, generated 2D NOESY spectra, using the multispin approximation (NOEPROM), in good agreement with the values measured at 600 and 900 MHz. These results were subsequently corroborated by experimental $^nJ_{C-H}$ heteronuclear coupling constants, which were measured across the glycosidic linkages for LSTc and LSTa; these results supported the concept of using NMR experimental constraints to select conformers from MD simulation trajectories. LSTc exhibits a bent shape characterized by a topological angle θ of

87°, whereas LSTa has a more linear form and therefore a greater θ angle of 159°, a structural difference in agreement with the previous work of Chandrasekaran[16] and Xu et al.[17] that concurs with the glycan ligand cocrystallized with HA.[15]

As a consequence of these structural differences, the nonreducing end terminal Neu5Ac of LSTc could form an intricate hydrogen bond network with GlcNAc, which is not the case for its isomer, LSTa, because hydrogen bond analysis revealed interactions with only directly adjacent residues. PCA of the glycan trajectories extracted the independent modes of motion that differentiate the structures, with LSTa moving like a flexing bow and the reducing and nonreducing disaccharides moving around the GlcNAc fulcrum, whereas the dynamics of LSTc are different from those of LSTa, the sialyl group of Neu5Ac moving in tandem with the N-acetyl group of GlcNAc, which moves in a manner that is independent of the reducing end disaccharide.

In Figure 6, the structures of LSTc and LSTa extracted from the respective rmsd$_{exp}$ min sets are superimposed on the Neu5Ac and Gal1 residues in the cocrystallized glycan−HA complexes [PDB entries 2WRG[46] (panel B) and 1RVX[2] (panel A)]. The rmsd values between the superimposed structures and the cocrystallized ligand, considering the three nonreducing end residues (i.e., Neu5Ac, Gal1, and GlcNAc), are 6.5 and 6.2 Å for LSTa and LSTc, respectively. These results illustrate how the NMR-selected conformation for LSTc and LSTa in solution, i.e., determined without HA, matches qualitatively the cocrystallized ligand bound to HA and also suggests that the respective sugar conformation does not significantly change upon interaction with HA. This observation supports the hypothesis that HA recognizes the solution conformation of the glycan, instead of the ligand and receptor undergoing a "bind and fit" process. This study contributes to an improved

understanding of the structural basis for the glycan receptor specificity of HA in influenza.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Supporting NMR data and analyses of the MD simulations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

HA, hemagglutinin; LSTa, Neu5Ac-α(2→3)-Gal-β(1→3)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc; LSTc, Neu5Ac-α(2→6)-Gal-β(1→4)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc; MD, molecular dynamics; Gal, galactose; Neu5Ac, N-acetylneuraminic acid; GlcNAc, N-acetylglucosamine; Glc, glucose; rmsip, root-mean-square internal product; rmsd, root-mean-square displacement; $rmsd_{exp}$, comparison of root-mean-square displacements between experimental and theoretical data; PCA, principal component analysis.

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