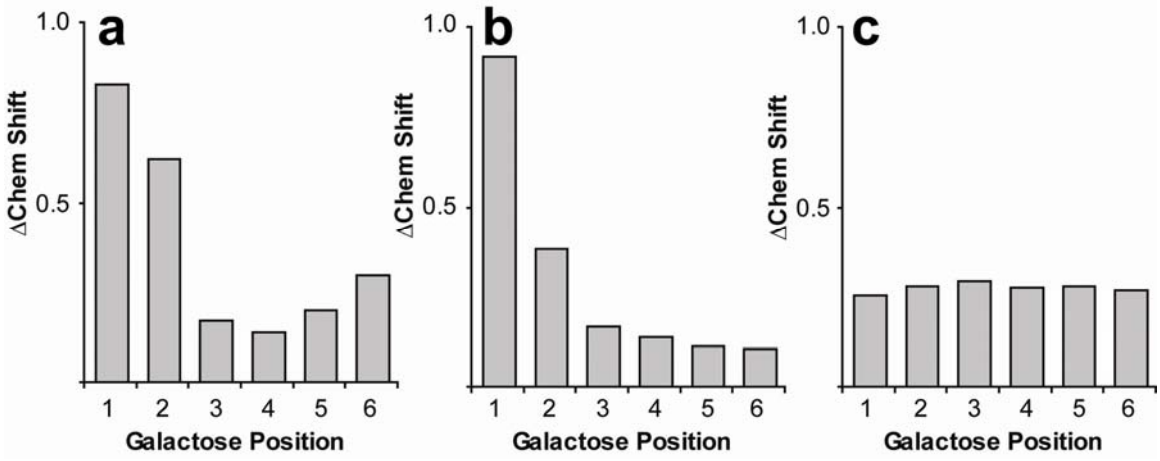
Supplement for:

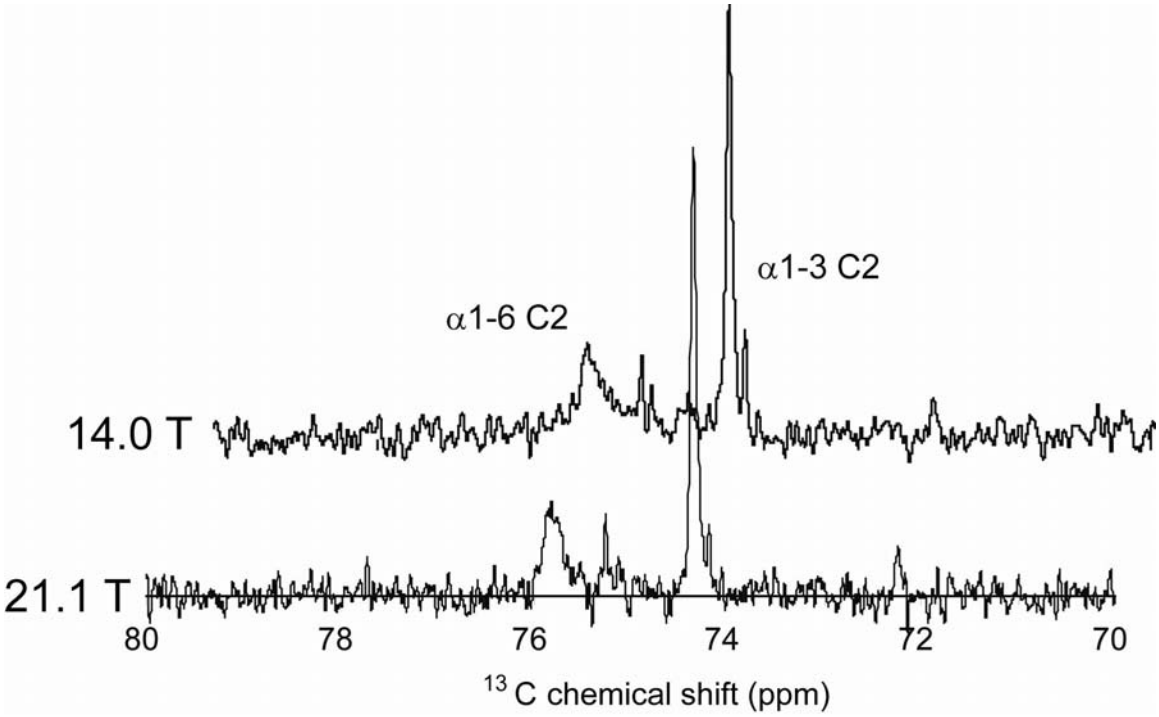
NMR Analysis Demonstrates Immunoglobulin G *N*-glycans are Accessible and Dynamic

Adam W. Barb and James H. Prestegard

Supplementary Results



Supplementary Figure **1**. Chemical shift differences (in ppm) between the (**a**) α1-6Man branch galactose and free glycan and (**b**) the α1-6Man and α1-3Man branch galactose residues are most dissimilar for the anomeric and $^{13}C_2$ resonances. (**c**) Chemical shift differences between the α1-3Man branch galactose and free glycan resonances are less pronounced. These difference were calculated using $\sqrt{[(\Delta\delta_H{}^2 + \Delta\delta_C{}^2/4)/2]}$.



Supplementary Figure **2.** ^{13}C NMR spectra of $^{13}C_2$-Gal labeled Fc fragment at 150.812 (top) and 226.257 (bottom) MHz without line broadening. The top spectrum resulted from 18,384 scans on an instrument equipped with a probe designed for a 3 mm tube and the bottom spectrum was the summation of 4,096 scans collected on a 5 mm probe. In both cases the sample contained 0.45 mM Fc fragment. The top spectrum is slightly offset to prevent peak overlap. At 21.1T, the α1-6Man $^{13}C_2$ resonance has a linewidth of ~50 Hz, while the α1-3Man $^{13}C_2$ resonance is 13Hz.

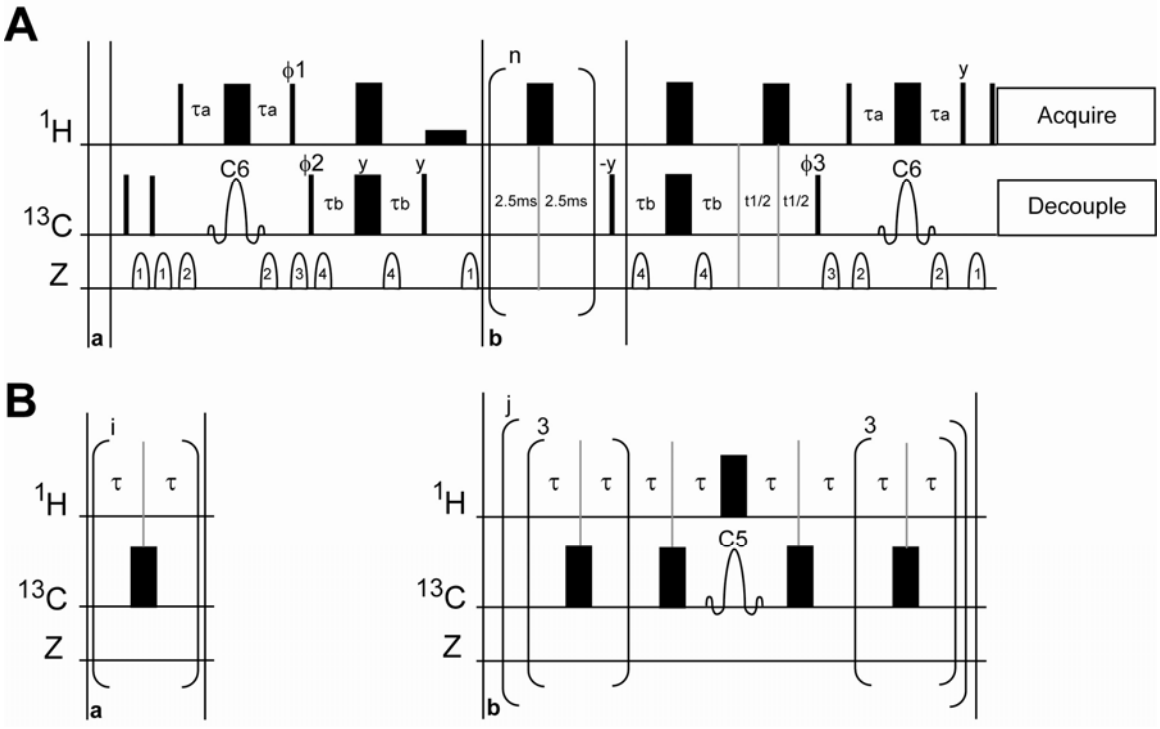
Supplementary Table **1**. Static field proton linewidth comparisons

^{1}H Linewidth (14.0T) / ^{1}H Linewidth (21.1T)

	α1-3 Gal	α1-6 Gal
H2	85%	62%
H3	78%	n.d.*
H4	79%	64%
H5	79%	66%
H6	62%	69%

*- α1-6 Gal H3 linewidths could not be accurately measured due to overlap

Supplementary Methods



Supplementary Figure **3**. Pulse sequences utilized for the Gal $^{13}C_6$ spin relaxation measurements. Narrow (wide) bars represent 90° (180°) radio frequency (RF) pulses. (**A**) R_1 measurements were obtained using a variation of the Varian Biopack ^{13}C-HSQC sequence where the ^{13}C refocusing pulses on the first and final INEPT periods (marked with "C6") were replaced with 180° Gaussian-shaped refocusing pulses centered at 64 ppm with a 6 ppm bandwidth to specifically refocus $^{13}C_6$ nuclei. Heteronuclear decoupling during ^{1}H acquisition was achieved with a Wurst 80ppm sequence. (**B**) R_2 measurements were achieved using a variation of the sequence shown in (**A**) by replacement of the portion in sections "**a**" and "**b**." Section "**a**" is used to ensure constant RF sample heating by applying the same number of ^{13}C refocusing pulses following the recycling delay. Gal C_5-C_6 ^{1}J coupling is removed when $^{13}C_6$ magnetization is transverse during the "**b**" section by a 180° square-shaped refocusing pulse centered at 78 ppm with an 11 ppm bandwidth and marked as "C5". Delays τa and τb are set to $1/(4^1J)$ and $1/(8^1J)$, respectively, with ^{1}J = 155ppm; in **B** τ = 0.3125ms. Z-gradient pulses were (1) 1.75 G/cm for 0.5ms, (2) 3.5 G/cm for 0.5ms, (3) 20 G/cm for 1ms, and (4) 10 G/cm for 0.5ms. Phase cycling used was ϕ1 = {4y , 4-y}, ϕ2 = {x , -x}, ϕ3 = { x , x , -x, -x }, ϕrec = { x , -x , -x , x , -x , x , x , -x }. $R_{1\rho}$ measurements were made using the sequence shown in **A** with the default $T_{1\rho}$ settings for section **b** specified in the Varian Biopack sequence.

Preparation of UDP-^{13}C-galactose

UDP-$^{13}C_U$-galactose and UDP-$^{13}C_2$-galactose were prepared with 10 mM of either $^{13}C_U$-D-galactose or $^{13}C_2$-D-galactose in a reaction containing 100 mM Tris, 10 mM magnesium chloride, 10 mM ATP, 10 mM UDP-glucose, 20 mM NAD+, 10% (v/v) galactokinase[1] (Prof. Maor Bar-Peled, University of Georgia), 0.24 mg/ml galactose-1-phosphate uridylyltransferase, 1.6 U/μL phosphoglucomutase, 1 U/μL glucose-6-phosphate dehydrogenase, pH 8.0. The reaction mixture was incubated at 37°C for four hours and progress was monitored by 1D ^{1}H NMR spectroscopy. Following complete conversion of galactose to UDP-galactose, the reaction mixture was passed through a 10 kDa filter (Millipore, Billerica, MA) to remove enzymes, and lyophilized.

Calculation of the low-energy glycan conformation

Biantennary Fc glycan and galactose-*N*-acetylglucosamine-mannose trisaccharide structures were calculated and energy minimized using the tools on the GLYCAM website (glycam.ccrc.uga.edu)[2]. Glycosidic torsion angles for the trisaccharide and the same trisaccharide units of the glycan were nearly identical.

Calculation of Fc correlation time

It will be useful to compare experimental measurements of spin relaxation times and derived correlations times to those expected based on simple theoretical models. The expected correlation time of the Fc fragment was estimated using an experimental value for the radius of hydration (r_H) = 31.9Å^3 and an isotropically-tumbling, spherical model[4]:

$$\tau_c = \frac{4\pi\eta_w r_H^{\ 3}}{3k_B T} \qquad \text{(S1)}$$

where η_w is the density of D_2O at 50°C (6.53 x10^{-4} N s m^{-2}) [5], k_B is the Boltzman constant and T is the temperature in degrees Kelvin.

Calculation of theoretical R_1 and R_2 for Gal $^{13}C_6$ and $^{13}C_2$

Values for the expected R_1 rates of C_6 in uniformly ^{13}C-labeled Gal were calculated by neglecting cross-correlation effects and summing dipole-dipole interaction contributions to longitudinal relaxation:

$$R_1(C_6) = 2(DD_{H6C6}) + DD_{C6C5} \qquad \text{(S2)}$$

where DD_{XY} is the dipolar coupling between nuclei X and Y. Contribution to R_1 from CSA[6] and DD_{HC} interactions from protons attached to neighboring carbons are expected to be negligible; the DD terms were calculated from[4]:

$$DD_{IS} = \frac{d_{00}}{4}\left[J(\omega_I - \omega_S) + 3J(\omega_I) + 6J(\omega_I + \omega_S)\right] \qquad \text{(S3)}$$

$$J(\omega_I) = \left(\frac{2}{5}\right)\frac{\tau_c}{1+\omega_I^2\tau_c^2} \quad \text{(S4)}$$

$$d_{00} = \left(\frac{\mu_0}{4\pi}\right)^2 \hbar^2\gamma_I^2\gamma_S^2 r_{IS}^{-6} \quad \text{(S5)}$$

ω_I is the frequency of spin I in rad s^{-1}, τ_c is the correlation time of the spin, μ_o is the permeability of free space, $\hbar$ is Plank's constant divided by 2π, γ is the gyromagnetic ratio and r_{IS} is the distance from spin I to S.

R_1 values for the $^{13}C_2$ in an otherwise ^{12}C-labeled Gal residue were calculated using:

$$R_1(C_2) = DD_{C2H2} \quad \text{(S6)}$$

For R_2 rates cross-correlation effects and remote interactions are again neglected. In the experiments performed R_2 rates pertain to averages over oscillation between in-phase and anti-phase transverse magnetization. A calculation which neglects differences in relaxation is adequate for comparison purposes and these effects were also neglected. Rates for $^{13}C_6$ relaxation rates in uniformly ^{13}C-labeled Gal and were calculated from:

$$R_2(C_6) = 2(DD_{H6C6}) + DD_{C6C5} \quad \text{(S7)}$$

Here,

$$DD_{IS} = \frac{d_{00}}{8}\left[4J(0) + J(\omega_I - \omega_S) + 3J(\omega_I) + 6J(\omega_s) + 6J(\omega_I + \omega_S)\right] \quad \text{(S8)}$$

d_{00} and $J(\omega)$ terms are described by equations (S5) and (S4), respectively. Contribution from R_{EX} was ignored for this analysis; DD_{HC} interactions from protons on neighboring carbons, CSA and R_2 inhomogeneity are again expected to be negligible[4,6].

R_2 rates for $^{13}C_2$ in an otherwise ^{12}C-labeled Gal residue were calculated using:

$$R_2(C_2) = DD_{H2C2} \quad \text{(S9)}$$

<u>Chemical shift extraction from temperature dependences</u>

An equation to describe the chemical shift of a resonance in fast exchange at a given temperature in terms of enthalpy and entropy was derived by assuming two-state exchange between states A and B and temperature independent entropy and enthalpy values. Starting with equations relating the free energy (G) of a system to entropy(S), enthalpy (H) and K_{eq}:

$$\Delta G = -RT\ln K_{eq} = \Delta H - T\Delta S \quad \text{(S10)}$$

Rearrange to

$$K_{eq} = e^{\frac{-\Delta G}{RT}} = e^{-\left(\frac{1}{R}\right)\left(\frac{\Delta H}{T}-\Delta S\right)} \qquad \text{(S11)}$$

In terms of p_B, the population of state B K_{eq} can also be written:

$$K_{eq} = \frac{p_B}{1-p_B} \qquad \text{which can be rearranged to } \; p_B = \frac{1}{\frac{1}{K_{eq}}+1} \qquad \text{(S12)}$$

Substituting the result of Eq (S11) in Eq (S12):

$$p_B = \frac{1}{\frac{1}{e^{-\left(\frac{1}{R}\right)\left(\frac{\Delta H}{T}-\Delta S\right)}}+1} \qquad \text{(S13)}$$

The relationship of the observed chemical shift at a given temperate (δ_X) to the chemical shift of state A (δ_A) is then:

$$\delta_X = \delta_A - p_B(\delta_A - \delta_B) \qquad \text{(S14)}$$

Substituting equation (S13) into equation (S14):

$$\delta_X = \delta_A - \frac{\delta_A - \delta_B}{\frac{1}{e^{-\left(\frac{1}{R}\right)\left(\frac{\Delta H}{T}-\Delta S\right)}}+1} \qquad \text{(3)}$$

<u>Calculation of p_A using CPMG and chemical shift data</u>

If the chemical shift of either state A or B is known, the φ_{EX} value (derived from fitting Eq (1) to the relaxation dispersion data) may be deconvoluted to calculate values for p_A, p_B and $\Delta\omega$:

Rearrange Eq 2 to solve for $\Delta\omega$, where $p_B = 1\text{-}p_A$

$$\Delta\omega = \sqrt{\frac{\varphi_{EX}}{p_A(1-p_A)}} \qquad \text{(S15)}$$

Also, recast Eq (S14) in terms of p_A, $\Delta\omega$ and ω_o, then rearrange to solve for $\Delta\omega$:

$$\delta_X = \delta_A - (1-p_A)\frac{\Delta\omega}{\omega_O}; \qquad \Delta\omega = -\omega_O\frac{(\delta_X - \delta_A)}{(1-p_A)} \qquad \text{(S16)}$$

The results of Eqs (S15) and (S16) are set equal to one another and solved for p_A:

$$p_A = \frac{1}{\frac{\omega_0^2 (\delta_X - \delta_A)^2}{\varphi_{EX}} + 1} \quad (4)$$

A value for p_A may be determined with Eq (4) using the chemical shift of state A (δ_A), and the observed φ_{EX} and δ_X values measured at a given temperature; the Larmor resonance frequency (ω_0) is a field-dependent constant in units of MHz. The chemical shift of state B (δ_B) may then be determined using the relationship

$$\delta_B = \delta_A + \Delta\omega/(2\pi\omega_0) \quad (S17)$$

Supplement References

1 Yang, T. *et al.*, Identification of galacturonic acid-1-phosphate kinase, a new member of the GHMP kinase superfamily in plants, and comparison with galactose-1-phosphate kinase. *J Biol Chem* **284**, 21526-21535 (2009).

2 Kirschner, K.N. *et al.*, GLYCAM06: A generalizable Biomolecular force field. Carbohydrates. *Journal of Computational Chemistry* **29**, 622-655 (2008).

3 Armstrong, J.K., Wenby, R.B., Meiselman, H.J., & Fisher, T.C., The hydrodynamic radii of macromolecules and their effect on red blood cell aggregation. *Biophys J* **87**, 4259-4270 (2004).

4 Cavanagh, J., *Protein NMR spectroscopy : principles and practice*, 2nd ed. (Academic Press, Amsterdam ; Boston, 2007).

5 Hardy, R.C. & Cottington, R.L., Viscosity of Deuterium Oxide and Water from 5-Degrees to 125-Degrees-C. *J Chem Phys* **17**, 509-510 (1949).

6 Shao, L., Yates, J.R., & Titman, J.J., Carbon-13 Chemical Shift Tensors of Disaccharides: Measurement, Computation and Assignment. *J Phys Chem A* **111**, 13126-13132 (2007).