^1H NMR visibility of mammalian glycogen in solution

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ABSTRACT High-resolution ^1H NMR spectra of rabbit liver glycogen in $^2\text{H}_2\text{O}$ were obtained at 500 MHz, and several resonances were assigned by comparison with the chemical shifts of α -linked diglucose molecules. The NMR relaxation times T_1 and T_2 of glycogen in $^2\text{H}_2\text{O}$ were determined to be 1.1 and 0.029 s, respectively. The measured natural linewidth of the carbon-1 proton (12 ± 2 Hz) is in excellent agreement with that calculated from T_2 . The visibility measurements made by digesting glycogen and comparing glucose and glycogen signal intensities demonstrate that in spite of the very high molecular weight, all of the proton nuclei in glycogen contribute to the NMR spectrum. The result is not unexpected, since 100% NMR visibility was previously observed from the carbon nuclei of glycogen, due to the rapid intramolecular motions.

In mammals glucose is stored as glycogen in chains 11–14 residues long linked by α 1-4 glycosidic bonds and branched by α 1-6 glycosidic bonds. This forms a treelike molecule on a protein core, with a molecular weight of $\approx 10^7$, which is called a β particle (1, 2). Muscle electron micrographs show uniform β particles with a diameter of ≈ 200 Å, while liver glycogen appears to be composed of even larger aggregates of particles. Despite the large size of glycogen, its ^{13}C NMR resonances are 100% visible with well-resolved lines (relaxation time $T_2^* \approx 10$ ms) (3–5). It was shown in solution (3) that the relaxation times of the ^{13}C resonances could be fit approximately with a rigid rotator nearest-neighbor model with $\tau_c \approx 6$ ns, indicating extensive internal motion in the molecule. The sharp lines from the glycogen molecule have allowed measurements of glycogen concentration and ^{13}C enrichment in animals and humans (6–9). A recent, more detailed study of glycogen relaxation times as a function of magnetic field has shown that T_2 equals T_2^* and is roughly independent of field, while T_1 depends strongly upon field (unpublished results). The analysis has attributed the 100% visibility of glycogen ^{13}C NMR signals to the fast internal motions of the molecule.

These ubiquitous rapid intramolecular motions led us to expect that the ^1H NMR signals of glycogen might also be narrow and visible. In conducting solutions the signal/noise ratio of the H1-4 glycogen resonance should be 1600 times greater than the corresponding natural-abundance ^{13}C NMR C1-4 resonance, due to the 100-fold greater isotopic abundance and the 16-fold signal/noise enhancement per nucleus (10). Based on the current accuracy for measuring human muscle glycogen by natural-abundance ^{13}C NMR (11, 12), ± 5 mM in 15 min, performing the same measurements by ^1H NMR would improve the time resolution to seconds and thus allow detailed measurements of glycogen kinetics at high spatial resolution. Here we present results showing well-resolved proton spectra of glycogen in solution and demonstrate that the lines are 100% visible.

FIG. 1. The 500-MHz proton spectrum of glycogen in D_2O (25 mg/ml) at 310 K. HDO presaturation was applied for 10 s before the acquisition; the repetition time was 16 s; 90° pulse with four scans.

MATERIALS AND METHODS

Type III rabbit liver glycogen from Sigma was used without further purification. For spectral and relaxation measurements glycogen was dissolved directly in D_2O (99.9% D, where D is deuterium, ^2H) to final concentrations of ca. 25 mg/ml and 50 mg/ml. For the visibility study, a pD 4.17 buffer was prepared in D_2O with 40 mM potassium phthalate. A solution of glycogen and a deuterated internal standard, sodium 3-trimethylsilyl[2,2,3,3- D_4]propionate (TSP), in this buffer was divided into two portions. An excess of amyloglucosidase (powder) was added to one, and the solution was warmed to 45°C for 10 min to assist a complete enzymatic reaction. Equal volumes of both solutions were then transferred into two 5-mm NMR tubes. The tube containing enzyme was then kept at 37°C for 2 hr before the spectral measurement, to allow α and β glucose anomers to reach equilibrium. The tube without enzyme was used as the control. Three solutions with glycogen concentrations of 25, 50, and 70 mg/ml were prepared for the visibility study.

The proton spectrum of glycogen in D_2O was taken on a 500-MHz AM superconducting spectrometer system (Bruker). HDO suppression was done by a 10-s low-power presaturation pulse. The signal of the C1 proton (H1) of glycogen was fit by a single Lorentzian function with NMR1 curve-fitting software (New Methods Research, Syracuse, NY). The visibility and the relaxation measurements were performed on a 360-MHz AM superconducting spectrometer system (Bruker). T_1 and T_2 relaxation times were measured without HDO presaturation, by using inversion-recovery and spin-echo methods, respectively. Data were analyzed using a three-parameter fitting program from the spectrometer computer (Bruker) and NMR1

Table 1. ^1H NMR chemical shifts (relative to TSP) of rabbit liver glycogen, α -linked glucopyranose disaccharides, and α -D-glucose in D_2O

Proton	Chemical shift,* ppm				
	Glycogen [†]			α -Mal-	α -Isomal-
	α 1-4	α 1-6	Free ends	tose [‡] Glucose [‡]	tose [§] (α 1-6)
H1	5.39	4.99		5.23	5.41
H4			3.44	3.40	4.99

*Values for protons bound to glycosidic linked carbons are in italics.

[†]Measured in D_2O at 310 K at 500 MHz.

[‡]Measured in D_2O at 310 K at 360 MHz.

[§]Data from ref. 13.

software (New Methods Research). In the determination of the visibility, TSP was used as an internal standard. The percentage visibility of the protons in glycogen was obtained by comparing the integrated glycogen signals and the corresponding glucose peaks after hydrolysis. Both signals were normalized with respect to the TSP signal.

To assist the assignment of glycogen proton peaks, a 90.5-MHz natural-abundance ^{13}C NMR spectrum of glycogen (100 mg/ml) in D_2O was taken at 310 K using a 90° pulse and a relaxation delay of 1.9 s for 2000 scans. Proton decoupling was gated on only during acquisition. The ratio of the integrated signals of C1-4 and C4' (based on the assignment from ref. 3) was compared with that of the corresponding ^1H signals assigned in this work. The peak area analysis was made using Sigma-Scan software (Jandel Scientific, Corte Madera, CA).

RESULTS AND DISCUSSION

With HDO presaturation, rather sharp peaks are observed in the 500-mHz proton spectrum of glycogen at 310 K (Fig. 1). Tentative assignments of several peaks have been made by comparison with the chemical shifts previously reported for α -maltose, which is an α 1,4-linked glucose disaccharide (13), as well as with those measured for α -D-glucose at 360 MHz. These assignments are given in Table 1. As shown in Fig. 1, the H1-4, H1-6, and H4 peaks are well resolved. These correspond to protons bound to C1 in the primary α 1-4 glycosidic bond, C1 in the branching α 1-6 glycosidic bond,

FIG. 2. Computer fit of glycogen H1-4 signal with a single Lorentzian function. The fit converged after five iterations. Curve a, the experimental data; curve b, the fit; curve c, the difference spectrum, $a - b$.

Table 2. Percentage of rabbit liver glycogen that gives rise to high-resolution NMR signals

Signals integrated	Glycogen signals/glucose signals, %
H2 + H3 + H4 + H5 + 2H6	98 $\pm$ 3
H1-4 (+ H1-6)*	93 $\pm$ 4 (99 $\pm$ 4)

Results were averaged from five measurements on three samples with glycogen concentrations of 25, 50, and 70 mg/ml (in D_2O) at 310 K at 360 MHz.

*Calculated by taking α/β ratio = 41:59, a result from ^{13}C NMR measurement under the same experimental conditions. The number in parentheses was obtained by adding the peak at 4.99 ppm based on our assignment that it came from H1-6 protons.

and the free C4 nonreducing ends. The intensities of these resonances contain information about the branched structure of the molecule. The resonance from H1-4 is shifted 0.16 ppm downfield from its position in α -D-glucose due to the α 1-4 glycosidic linkage, while that from H1-6 is shifted 0.24 ppm upfield due to the α 1-6 glycosidic linkage. The ratio of the integrated peaks of H1-4, H4, and H1-6 is 1.0:0.11:0.064, which is similar to that measured from the ^{13}C spectrum, 1.0:0.099:0.09, which has been assigned previously (3). The area of C1-6 could not be determined as accurately due to its overlap with the C1-4 signal and lower signal/noise ratio. The comparison provides additional evidence for the assignment we have made. The other intense peaks in the region between 3.7 and 4.0 ppm are from the H6A, H6B, H5, H4-1, H3, and H2 protons, but definite assignments will require more specific identifications. The well-resolved H1-4 peak can be fitted with a single Lorentzian function (Fig. 2). The linewidth obtained from the fitting, after correction for the field inhomogeneity and line broadening, is 12 ± 2 Hz.

Relaxation time measurements were made for glycogen H1-4. Two independent measurements gave T_1 values of 1.12 s and 1.18 s. Standard deviations obtained from the curve fitting for all relaxation data are $<2\%$. A T_2 of 29 ms was calculated from the spin-echo measurement without HDO presaturation. This T_2 value gives a natural linewidth of 11 Hz, which is in excellent agreement with the measured linewidth of 12 ± 2 (shown in Fig. 2).

Visibility measurements were done without HDO presaturation in order to avoid leakage saturation of the glycogen and glucose peaks. The results, summarized in Table 2, indicate a 100% NMR visibility of the glycogen protons within the experimental error.

The 100% visibility and the narrow linewidth of the H1-4 resonance suggest that ^1H NMR can be used to measure glycogen concentration *in vivo* with greatly enhanced sensitivity over currently used ^{13}C NMR methods.

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