¹³C NMR Visibility of Rabbit Muscle Glycogen *in Vivo*

ROLF GRUETTER, TOMAS A. PROLLA, AND ROBERT G. SHULMAN

Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, Connecticut 06510

Received March 1, 1991; revised April 25, 1991

The integrated ¹³C NMR intensity of the glycogen C1 resonance was measured in skeletal muscle (biceps femoris region) of nine rabbits under *in vivo* conditions. Concurrent chemical determinations of glycogen content showed that the *in vivo* signal was 1.02 ± 0.06 the intensity of analytical samples, where glycogen is known to be $\sim 100\%$ visible. © 1991 Academic Press, Inc.

INTRODUCTION

In vitro ¹³C NMR studies of glycogen in aqueous solution have shown that approximately 100% of the ¹³C carbon atoms contribute to the C1 resonance at 100.5 ppm (1) despite the large molecular weight of this macromolecule, which is of the order of 10⁷ Da. Glycogen is the main storage form of glucose in mammals and ¹³C NMR spectroscopy of glycogen is therefore of considerable interest in biomedical research (2-5).

¹³C NMR relaxation studies of hepatic glycogen *in vitro* at 295 K suggested that fast motions with a correlation time τ_c of the order of 10⁻⁹ s dominate the longitudinal relaxation time T_1 , whereas a slower motion which was attributed to molecular tumbling dominates the transverse relaxation time T_2 (6). It had also been previously suggested from indirect *in vivo* measurements that the relaxation times of the *visible* portion of the molecule do not vary in the liver under conditions of degradation or synthesis (7).

A study at 500 MHz showed that the ¹H NMR spectrum of glycogen was $\sim 100\%$ visible *in vitro* at 310 K, like the ¹³C resonance (8). It was possible to resolve the resonance of H1 in 1-4 glycosidic linkages, i.e., in glycogen chains, from those at branching points, i.e., in 1-6 linkages. However, recent proton NMR measurements showed that the area of the H1-6 protons doubled when the temperature was raised from 310 to 328 K (9). This indicated that the total NMR intensity of H1 *in vitro* is actually only $\sim 96\%$ visible at 310 K. A similar small reduction of intensity in the ¹³C NMR spectrum would be undetectable because of the lower signal-to-noise. However, the ¹H NMR experiments raised concern that parts of the molecule could be affected by interactions *in vivo* so as to reduce their visibility. In order to ascertain the limits of any such reduction *in vivo*, we have compared the ¹³C NMR signal of glycogen in solution to that observed in living rabbit muscle under normal physiological conditions.

MATERIALS AND METHODS

In vivo ¹³C NMR measurements of muscle glycogen were made in New Zealand white rabbits. At the beginning of the measurement α -blockers (phentolamine 5 mg/

rabbit, intraperitoneally) and β -blockers (propranolol 1 mg/kg, intravenous) were administered in order to suppress epinephrine-induced glycogenolysis. Anesthesia was achieved with an intraperitoneal injection of pentobarbital (40 mg/kg). This was followed by surgical removal of skin and adipose tissue above the biceps femoris muscle region. The rabbit was then tightly taped onto the Plexiglas separating the coil from the muscle.

The NMR experiments were performed in a 4.7-T Bruker Biospec spectrometer with a 30-cm-diameter bore. The homebuilt radiofrequency (rf) probe was a 20-mm-diameter ^{13}C coil and a concentric 40-mm-diameter ^1H surface coil. A sealed external reference sphere of 8 mm diameter containing a diluted aqueous solution of 99% enriched [^{13}C]formic acid (Aldrich Chemical Co., Milwaukee, WI) was attached in the center of the inner carbon coil. The outer proton coil was used for shimming, proton decoupling, and imaging. Only muscular tissue was present in the sensitive volume of the carbon coil, i.e., in a half-sphere of 20 mm radius, as judged from the subsequent freeze clamp of the tissue and from proton NMR images that were obtained in transaxial and sagittal planes using heavily T_1 -weighted gradient-echo imaging (10) with a repetition time of 60 ms and an echo time of 10 ms.

All *in vivo* and *in vitro* ^{13}C NMR spectra of glycogen were collected as the sum of 3000 scans obtained with a 50- μs rf pulse applied 500 Hz downfield from glycogen C1 at a repetition time of 176 ms. Radiofrequency power was adjusted to null the signal of the formate sphere with a 50- μs pulse applied on resonance. Ten watts of continuous wave (CW) proton decoupling was used only during the acquisition time, which was set to 26 ms.

The ^{13}C NMR spectra were obtained by Fourier transformation of the free induction decays (FID) that were multiplied by a 40-Hz line-broadening exponential function and zero-filled to 108 ms. The glycogen peak area was obtained by integrating from 96 to 104 ppm after correcting the baseline in the region from 80 to 120 ppm with a second-order polynomial. All spectral processing was performed with the spectrometer software DISNMR (Bruker, Karlsruhe, Germany). In order to eliminate effects of variable loading on the signal strength, integrated values of glycogen intensities were normalized to the area of the formate peak obtained with one 25- μs (90°) pulse after a 60-s waiting time. To reduce effects of spectrometer instability these reference spectra were obtained at the beginning and at the end of the ^{13}C NMR measurements of glycogen.

The *in vitro* NMR measurements were performed at 295 K on aqueous solutions of oyster glycogen (Sigma, St. Louis, MO). The glycogen concentration was varied by successively diluting a stock solution containing 98 mM glycogen, 50 mM KCl, and 2 mM NaN_3 with distilled water. This resulted in glycogen concentrations of 98, 70, 49, and 43 mM glucosyl units, respectively, which were determined as described below. The solutions were placed in an infusion bag to ensure that all the volume above the carbon coil was filled with solution, as verified with proton NMR imaging.

Enzymatic Determination of Tissue Glycogen Content

The muscle tissue above the carbon coil (~ 3 g of tissue) was freeze-clamped in liquid nitrogen immediately after the NMR measurements and stored at -20°C . No loss of glycogen was observed in the same muscle pieces analyzed several times. Muscle

glycogen content was determined in samples of 0.15–0.25 g according to an established enzymatic assay procedure (11), which can be briefly described as follows: After weighing, the tissue was immediately homogenized at high speed in five parts per weight of ice-cold perchloric acid (0.6 mol/liter) using an Ultra-Turrax homogenizer (Tekmar Co., Cincinnati, OH). KHCO_3 (0.1 ml; 1 mol/liter) was added to 0.2 ml homogenate in Eppendorf containers before 0.5 ml solution of glucoamylase (50 kU/liter) in acetate buffer (0.2 mol/liter, pH 4.8) was added. We verified that the analytical grade glucoamylase preparations from *Aspergillus niger* (Sigma Co., St. Louis, MO) were free from measurable contaminations of starch or glycogen by subjecting millipore water to the identical procedure as the glycogen solutions. The treatment of the glycogen solutions was identical to that of the muscle samples except that they were diluted in five parts per volume of perchloric acid, which resulted in the same dilution factors. Complete hydrolysis of glycogen was achieved by subsequent incubation at 40°C in a shaker bath for at least 2 h. Completeness of hydrolysis was verified by prolonging the incubation time to 3–4 h in a few glycogen samples. After centrifugation for 10–15 min, glucose concentration was determined enzymatically in the supernatant of both the homogenate and the digested solution by measuring the calibrated increase in absorbance at 339 nm in a spectrophotometer when 5 μl of hexokinase suspension (140 U/mg protein) was added to a solution of 100 μl of supernatant mixed with 1 ml of tetraethylammonium (TEA) buffer (0.3 mol/liter TEA, 4 mmol/liter MgSO_4 , pH 7.8) containing ATP (1 mmol/liter), β -NADP (0.9 mmol/liter), and glucose-6-phosphate dehydrogenase (330 U/mg protein). All chemicals were purchased from Sigma unless otherwise stated.

Muscle glycogen concentration was calculated from the glucose concentration of the digested solutions minus the concentration of the undigested tissue homogenates (background glucose, typically 1–2 mmol/kg wet wt) corrected for the respective dilutions.

RESULTS

^{13}C NMR spectra were recorded in nine rabbits and in four aqueous glycogen solutions. An expansion of a typical spectrum of rabbit skeletal muscle is shown in Fig. 1. The C1 resonance of glycogen (peak 2 in Fig. 1) is clearly visible at 100.5 ppm. When taking the 40-Hz line broadening into account, the linewidth of glycogen in Fig. 1 is 60 Hz corresponding to a T_2^* of 5 ms. The spectrum in Fig. 1 represents the sum of seven spectra that were acquired over a total time of ca. 70 min during which the signals remained stable during all *in vivo* and *in vitro* experiments.

In order to eliminate effects of variable loading on the signal strength we normalized the area under the C1 peak to that of the formate sphere measured in separate reference spectra. The normalized integral value was compared to the chemically determined glycogen content as shown in Fig. 2. The solid line goes through the origin and was obtained by linear regression from the measurements of four aqueous solutions (solid circles in Fig. 2). The nine *in vivo* measurements (open circles) lie on the calibration line within two standard errors of the measurements. The glycogen concentrations of the *in vivo* measurements were calculated from the normalized NMR intensity (y -axis in Fig. 2) and the calibration curve. Table 1 lists the *in vivo* concentrations determined from the intensity calibration and the concentrations obtained by the chemical assay. The ratio is 1.02 ± 0.06 (mean $\pm$ standard error) indicating equal signal intensity *in vivo* and *in vitro* at the same glycogen concentrations.

FIG. 1. Portion of an *in vivo* ^{13}C NMR spectrum of rabbit muscle. The spectrum represents the sum of 7×3000 scans obtained with proton decoupling and a 176-ms repetition time. Parameters were exactly as described under Materials and Methods except that the baseline was corrected here with a fourth-order polynomial between 145 and 45 ppm. Peak assignments are according to Refs. (5, 12): (1) fatty acyl chains, (2) glycogen C1, (3) glycogen C2–C5 and C2 of glycerol backbone, (4) glycogen C6 and C1, C3 of glycerol backbone, (5) trimethylamine groups (e.g., choline).

DISCUSSION

We have compared the ^{13}C NMR signal intensity of the C1 resonance of muscle glycogen *in vivo* to that in solution. At identical, enzymatically determined concentrations of glycogen, the *in vivo* signal equals the *in vitro* signal (Fig. 2, Table 1) when measured with identical NMR parameters and when adjusted for effects of variable

FIG. 2. Normalized glycogen signal vs enzymatic determination of glycogen content. Seven NMR measurements consisting of 3000 scans each were averaged for each point. The enzymatic determination was performed with seven muscle samples per rabbit (open circles). In the case of the aqueous solutions, three aliquots were assayed and averaged (solid circles). The solid line was obtained by fitting $y = ax$ to the four *in vitro* measurements since the NMR measurement as well as the chemical assay will yield true zero values in the absence of glycogen. Error bars indicate standard errors. Measurements were performed as described under Materials and Methods.

TABLE 1
Muscle Glycogen Concentration Determined by *in Vivo* NMR and Enzymatic Hydrolysis^a

Rabbit:	1	2	3	4	5	6	7	8	9	Mean
Enzymatic	61 ± 6 ^b	39 ± 2	48 ± 1	37 ± 1	37 ± 2	38 ± 3	41 ± 3	47 ± 2	42 ± 1	43 ± 3
NMR ^c	48 ± 5	52 ± 4	52 ± 5	40 ± 10 ^d	41 ± 2	32 ± 4	46 ± 4	41 ± 4	40 ± 3	44 ± 2
Ratio	0.79	1.33	1.08	1.08	1.11	0.84	1.12	0.87	0.95	1.02 ± 0.06

^a Seven measurements were performed per rabbit by NMR as well as by enzymatic hydrolysis as described under Materials and Methods. Concentration values are mean ± standard error in mmol glucosyl units/kg wet weight.

^b Only one muscle sample was available for assay and the error was set to the average standard deviation of the other rabbit measurements.

^c Concentrations were obtained by dividing the normalized intensity (*y*-axis in Fig. 2) by the slope of the solid line in Fig. 2.

^d Radiofrequency power had to be increased fourfold resulting in a halved signal-to-noise in the spectra of this rabbit.

loading. The signal intensity variations due to loading were small and corrected by normalizing the glycogen signal to that of the formate sphere attached at the carbon coil center. The flip-angle distribution was constant by adjusting rf power assuming constant penetration depths.

At the repetition time used, i.e., 176 ms, the signal is influenced by longitudinal relaxation since T_1 is approximately 150 ms at 4.7 T (6). The signal-to-noise of the spectra (Fig. 1), however, did not allow long repetition times. The effect of slightly different T_1 on the signal is difficult to estimate because of the spatially inhomogeneous B_1 of the surface coil. However, assuming a spatially uniform 90° excitation we calculate that a 10% change in T_1 influences the saturation factors only by 5% which is within the accuracy of the NMR measurements. We therefore assumed T_1 of glycogen in solution to be identical to T_1 in muscle *in vivo*. This is supported by the observation that T_1 of hepatic glycogen measured *in vivo* was close to T_1 measured at 295 K in solution (6). In addition, relaxation studies suggested that T_1 of glycogen is determined mostly by fast internal motions of the molecule at 295 K (6) and T_1 is therefore less likely to be affected by the overall motion or environment of the molecule.

The comparison of the *in vivo* signal with that from the reference glycogen solution depends strongly on identical sample volumes. Proton NMR imaging was used to verify that no major nonmuscular tissues, e.g., fat inclusions or bone, were in the sensitive volume of the carbon coil where superficial skin and adipose tissue were removed surgically.

We conclude that the C1 resonance of muscle glycogen is close to 100% visible *in vivo*, since glycogen has been shown to be approximately 100% visible in solution (1). An obvious implication of our study is that ^{13}C NMR can provide reliable muscle glycogen concentration measurements noninvasively in humans as well as in experimental animals.

ACKNOWLEDGMENTS

Financial support from the National Institutes of Health, Bethesda (Grant GN30287), is gratefully acknowledged. T. A. Prolla was supported by a National Science Foundation graduate fellowship. We also thank Drs. O. A. C. Petroff (drug dosages) and G. I. Shulman (chemical assay) for their support and advice.

REFERENCES

1. L. O. SILLERUD AND R. G. SHULMAN, *Biochemistry* **22**, 1087 (1983).
2. G. I. SHULMAN, D. L. ROTHMAN, T. JUE, P. STEIN, R. A. DEFONZO, AND R. G. SHULMAN, *N. Engl. J. Med.* **322**, 223 (1990).
3. V. REO, B. A. SIEGFRIED, AND J. J. H. ACKERMAN, *J. Biol. Chem.* **259**, 13,664 (1984).
4. P. JEHEHSON, D. DUBOC, G. BLOCH, AND A. SYROTA, "Proceedings, Society of Magnetic Resonance in Medicine, Ninth Annual Meeting, New York, 1990," p. 360.
5. N. BECKMANN, J. SEELIG, AND H. WICK, *Magn. Reson. Med.* **16**, 150 (1990).
6. L. ZANG, M. R. LAUGHLIN, D. L. ROTHMAN, AND R. G. SHULMAN, *Biochemistry* **29**, 6815 (1990).
7. R. A. SHALWITZ, N. V. REO, N. N. BECKER, AND J. J. H. ACKERMAN, *Magn. Reson. Med.* **5**, 462 (1987).
8. L. ZANG, D. L. ROTHMAN, AND R. G. SHULMAN, *Proc. Natl. Acad. Sci. USA* **87**, 1678 (1990).
9. L. ZANG, A. M. HOWSEMAN, AND R. G. SHULMAN, *Carbohydr. Res.*, in press, (1991).
10. A. HAASE, J. FRAHM, D. MATTHAEI, W. HÄNICKE, AND K.-D. MERBOLDT, *J. Magn. Reson.* **67**, 258 (1986).
11. BERGMAYER, H. U., BERGMAYER, J., GRASSL, M. (Eds.), "Methods of Enzymatic Analysis," Vol. VI, p. 1-18 Verlag Chemie, Weinheim, 1984.
12. P. CANIONI, J. R. ALGER, AND R. G. SHULMAN, *Biochemistry* **22**, 4974 (1983).