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Note

A ^{13}C-detection NMR approach for large glycoproteins

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ABSTRACT

NMR spectroscopy has great potential to provide us with information on structure and dynamics at atomic resolution of glycoproteins in solution. In larger glycoproteins, however, the detrimental fast ^{1}H transverse relaxation renders the conventional ^{1}H-detected NMR experiments difficult. ^{13}C direct detection potentially offers a valuable alternative to ^{1}H detection to overcome the fast T_2 relaxation. Here, we applied ^{13}C-detected NMR methods to observe the NMR signals of ^{13}C-labeled glycans attached to the Fc fragment of immunoglobulin G with a molecular mass of 56 kDa. Spectral analysis revealed that a ^{13}C-detected ^{13}C–^{13}C NOESY experiment is highly useful for spectral assignments of the glycans of large glycoproteins. This approach would be, in part, complementary to ^{13}C–^{13}C TOCSY and ^{1}H-detection experiments.

Recent advances in structural glycomics have enabled us to collect information on glycoforms, that is, sequences, linkage, positions, and profiling of glycans, of glycoproteins in a systematic manner.[1–5] One of the central issues in the next stage of structural glycomics is to provide a structural basis of the biological functions of the individual glycoforms of glycoproteins. NMR spectroscopy has great potential to provide us with information on structure and dynamics at atomic resolution of glycoproteins in solution. For detailed NMR analyses of glycoproteins, as in other biomacromolecules, application of stable isotope labeling techniques is essential. To date, ^{1}H-detected NMR methods, combined with stable isotope labeling techniques, have been successfully applied to spectral assignments of small glycoproteins or glycopeptides.[6,7] In larger glycoproteins, however, the detrimental fast ^{1}H transverse relaxation renders NMR experiments difficult. ^{13}C direct detection potentially offers a valuable alternative to ^{1}H detection to overcome the fast T_2 relaxation problem by virtue of the small magnetic moment of the ^{13}C nucleus. The development of cryogenic probes optimized for ^{13}C observation has made applications of ^{13}C-detected methods to paramagnetic proteins[8,9] and large biomacromolecules[10,11] possible. On the other hand, we have been developing stable isotope labeling methods of glycoproteins.[12,13] Here, we report an application of ^{13}C-detected NMR experiments to spectroscopic analyses of large glycoproteins using the Fc portion of immunoglobulin G (IgG) with a molecular mass of 56 kDa as model glycoprotein.

^{13}C-detected experiments are expected to have several advantages for the NMR spectral assignments of the carbohydrate peaks of glycoproteins. In general, the ^{1}H signals of glycans, except for those originating from the anomeric position, are severely overlapped in a narrow spectral region (3–4 ppm), while most ^{13}C signals are dispersed over a wider chemical shift range (50–110 ppm).[14–18] Observation of two-dimensional ^{13}C–^{13}C spectra is, therefore, anticipated to facilitate the assignments of glycan NMR peaks. Secondly, the ^{13}C–^{13}C shift correlations obtained from the ^{13}C-detected experiments can be helpful in classifying the carbohydrate signals simply by comparison with the reported ^{13}C chemical shift values.[14–18] This is due to the fact that ^{13}C chemical shift values of a sugar residue primarily depend on the covalent structures, but are not largely affected by the glycosidic linkage conformations and any other environmental factors. This is in contrast to ^{1}H chemical shifts, which are influenced, for example, by the interactions with the polypeptide chains.[14,19,20]

Figure 1 shows examples of the spectral patterns of intra-residue ^{13}C–^{13}C connectivities expected from the previously reported ^{13}C chemical shift values.[16,18,19] Hence, by inspection of the intra-residue ^{13}C–^{13}C connectivity patterns, the peaks can be easily classified into monosaccharide types with specific linkage configurations. In the case of the bi-antennary complex-type glycans

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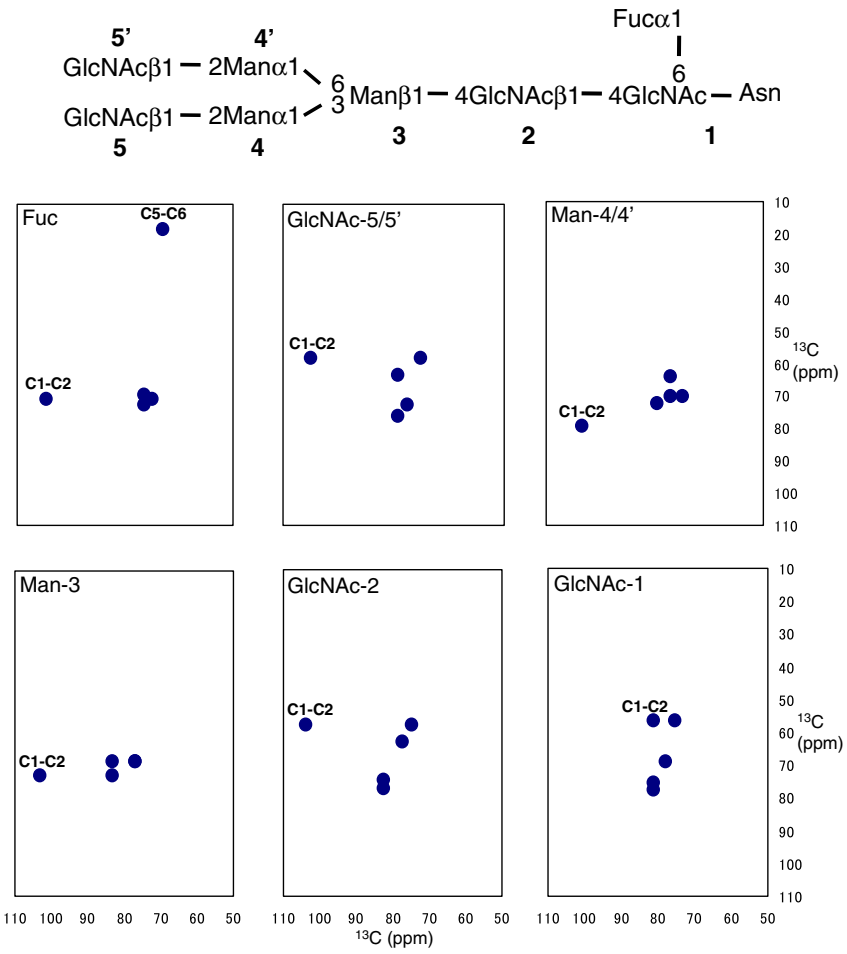

Figure 1. Two-dimensional ^{13}C–^{13}C NOESY patterns expected for the sugar residues, Fuc, GlcNAc-5/5′, Man-4/4′, Man-3, GlcNAc-2, and GlcNAc-1, in a bianntenary complex type oligosaccharide (top) based on the ^{13}C chemical shift values of the sugar residues in glycopeptides derived from ^{13}C-labeled IgG-Fc.[18] The oligosaccharide structure and nomenclature of the sugar residues of IgG-Fc used in the present study are also shown.

expressed on IgG-Fc, the peaks originating from the Fuc residue can be identified based on the unique spectral positions of the C5/C6 connectivity (ca. 70 ppm/18 ppm). The C1–C2 connectivities can be used for the identification of other sugar residues (GlcNAc and Man). C1–C2 correlations (ca. 103 ppm/58 ppm) are hallmark of GlcNAc residues (GlcNAc-2, -5 and -5′), and the GlcNAc residue attached to asparagine residue (GlcNAc-1) can be identified by its unique C1/C2 connectivity (ca. 81 ppm/57 ppm). Man-4/4′ and Man-3/Fuc are distinguished by their C1–C2 connectivities (ca. 100 ppm/80 ppm and 100 ppm/70 ppm, respectively). In cases where the spectral patterns deviate significantly from these expected ones, ^{13}C assignments have to be made with great care because it might reflect some exceptional conformational dependence on ^{13}C shifts. In such circumstances, peak classification should be verified by another method, for example, residue-selective ^{13}C-labeling of the glycans.[12,13,20]

Figure 2 shows the 2D ^{13}C–^{13}C NOESY spectrum of the ^{13}C-labeled Fc fragment of mouse IgG2b, which was prepared by cultivating antibody-producing cells in a synthetic medium containing [u-$^{13}C_6$]glucose.[12,13] After optimization, a mixing time of 600 ms was used for detecting one bond correlations in the ^{13}C–^{13}C NOESY spectrum, because the spectra obtained at longer mixing times gave significantly fewer peaks, none of which reflected more than two bond connectivities (Supplementary data, Fig. S1). In addition to the carbohydrate residues, the amino acid residues metabolically derived from glucose, that is, Ala, Ser, Glx, and Asx, gave ^{13}C–^{13}C NOESY peaks in the spectrum. As anticipated from Figure 1, the cross peak at 68 ppm/16 ppm was assigned to the C5/C6 connectivity of the Fuc, while the peaks from the GlcNAc residues were identified based on the C2 chemical shift value (ca. 55 ppm). The peaks of GlcNAc-2 were discriminated from those of GlcNAc-5/5′ on the basis of their C4 chemical shifts, which are expected to be shifted downfield by 10 ppm with respect to that of GlcNAc-5/5′ when Man-3 is attached thereto. The peak from GlcNAc-1, which is linked to the asparagine residue, was easily assigned because of its characteristic C1 chemical shift value, that is, ca. 80 ppm. The carbonyl/methyl carbon correlations (174/23 ppm) of the GlcNAc side chains were also observed in the spectra (Fig. 2A). The Man-3 and Man-4/4′ peaks were distinguished by inspection of the C2 chemical shift, which is expected to be shifted 10 ppm downfield by the attachment of the GlcNAc residues (GlcNAc-5/5′). Thus, the ^{13}C–^{13}C NOESY data allowed us to apply the expected spectral pattern (Fig. 1) to the peak classification and thereby offered assignments of all sugar residues except for the discriminations of Man-4 from Man-4′ and GlcNAc-5 from GlcNAc-5′.

In previous studies, we have completed assignments of the ^{1}H–^{13}C HSQC peaks originating from the anomeric positions of the Fc glycans by combined use of the ^{1}H-detected ^{1}H–^{13}C HMQC-NOESY and HCCH-TOCSY/COSY experiments and residue-selective ^{13}C-labeling.[12,13] These data allow us to assign the ^{13}C–^{13}C peaks of Man-4, Man-4′, GlcNAc-5, and GlcNAc-5′ unambiguously.

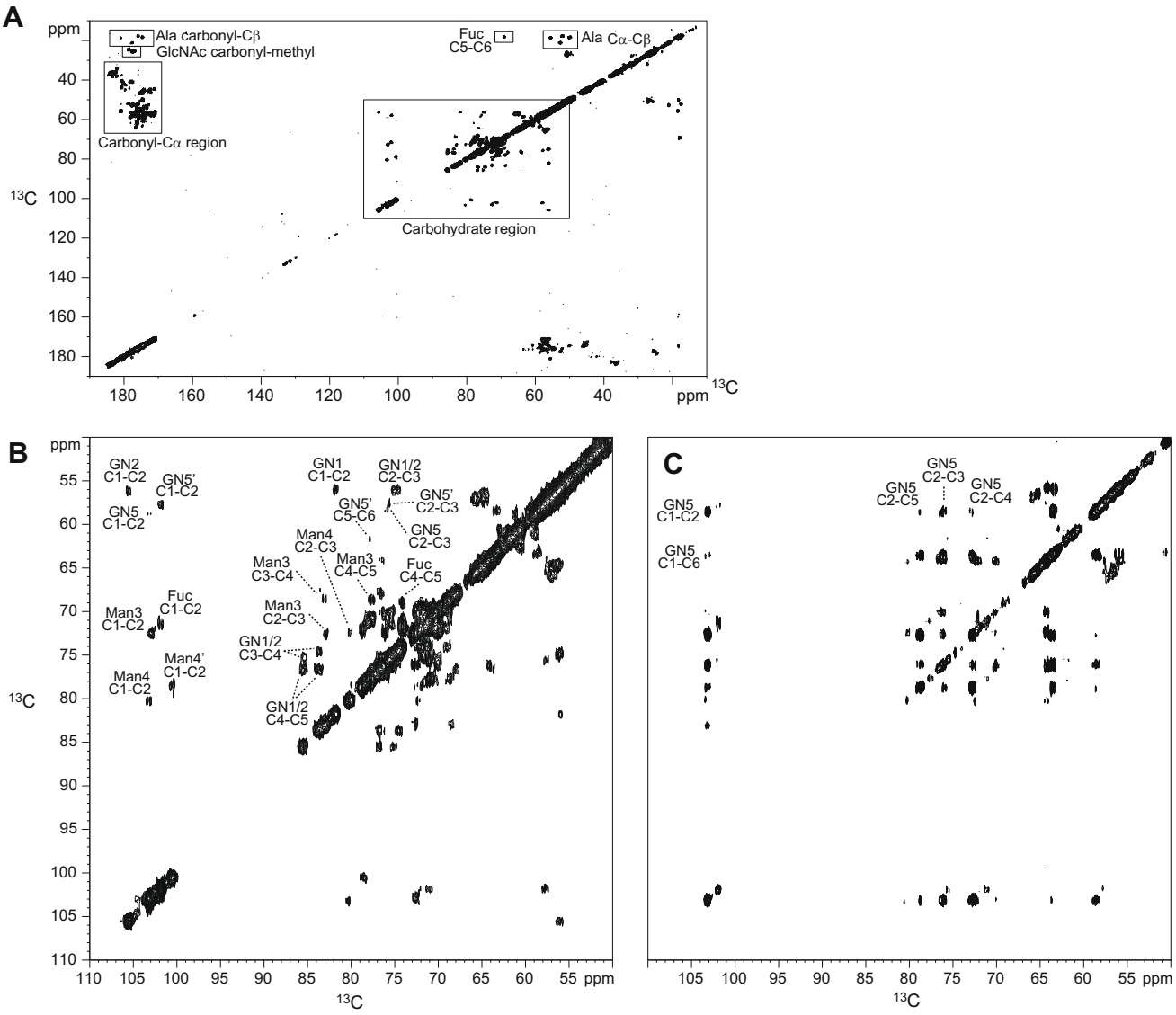

Figure 2. The entire (A) and oligosaccharide region (B) of the two-dimensional ^{13}C–^{13}C NOESY spectrum of ^{13}C-labeled IgG-Fc. The data were acquired at 47 °C on a CH dual cryogenic probe at the ^{13}C observation frequency of 125 MHz. The mixing time was 600 ms, and the total experimental time was 48 h. (C) ^{13}C–^{13}C TOCSY spectrum of ^{13}C-labeled IgG-Fc obtained in 19 h. The magnetization transfer was performed with the FLOPSY pulse sequence with a mixing time of 1.2 s. Key: GN, *N*-acetyl-D-glucosamine; Man, D-mannose; Fuc, L-fucose.

In the present ^{13}C-detected NOESY experiment, ^{13}C–^{13}C magnetization transfer is efficiently achieved through dipolar–dipolar interaction in a large glycoprotein due to slower molecular tumbling. Hence, relative intensities of the C1/C2 NOE peaks likely reflect the local mobilities of the sugar residues. For example, the peak intensities are significantly different between GlcNAc-5 (weak) and GlcNAc-5′ (strong), indicating that GlcNAc-5 exhibits a higher degree of freedom in internal motion than GlcNAc-5′. This is consistent with the previous reports demonstrating the difference in mobility of the two branches of the Fc glycan probed by using the ^{1}H–^{13}C and ^{1}H–^{15}N HSQC peaks as spectroscopic probes.[12,13,21]

We also conducted ^{13}C–^{13}C TOCSY experiments to identify the intra-residue correlations (Fig. 1C). It is of note that the ^{13}C–^{13}C TOCSY spectrum exhibited extensive intra-residue connectivities starting from C1 of GlcNAc-5, but barely gave the corresponding peaks for GlcNAc-5′. This indicates that ^{13}C–^{13}C TOCSY experiments provide us with information only on the flexible portions of the glycans of larger glycoproteins in contrast to ^{13}C–^{13}C NOESY.

In summary, as demonstrated here, simple ^{13}C-detected ^{13}C–^{13}C NOESY experiments are highly useful for spectral assignments of the glycans of large glycoproteins. This approach is complementary to ^{13}C–^{13}C TOCSY and ^{1}H-detection experiments.

1. Experimental

1.1. Materials

D-[$^{13}C_6$]Glucose was purchased from Shoko Co., Ltd Galactosidase (*Streptococcus* 6646K) was from Seikagaku Corp. Cell line 7D7, which produces mouse monoclonal anti-progesterone IgG2b(κ), was kindly provided by Drs. Tadao Terao and Jun-ichi Sawada.[22]

1.2. Preparation of ^{13}C-labeled IgG-Fc

^{13}C-Labeled IgG was prepared by cultivating hybridoma cells producing the mouse monoclonal IgG2b in a serum-free medium containing [u-$^{13}C_6$]glucose as described previously.[12,13] Briefly, 7D7 cells were cultured with a modified Nissui NYSF 404 serum-free media including 2 g/L of [u-$^{13}C_6$]glucose at 37 °C in a humidified atmosphere of 5% CO_2–95% air. After cell growth, the

supernatant was concentrated and applied to an Affi-gel protein A column (Bio-Rad). A typical yield was 70 mg of purified IgG2b per liter of cell culture. The CH groups at the C1–C6 positions of the GlcNAc, Man, and Fuc residues of the glycan in the IgG2b were uniformly labeled with ^{13}C with a high isotope enrichment (>95%). The Fc fragment was cleaved from IgG2b by V8 protease digestion and was purified with an Affi-gel protein A column.[23]

1.3. NMR measurements

^{13}C-Labeled Fc glycoprotein was dissolved in 0.5 mL of 5 mM sodium phosphate buffer pH 7.3 containing 200 mM NaCl, and in $^{2}H_2O$ (99%). The final protein concentration was 0.74 mM. NMR experiments were carried out on a Bruker Avance 500 equipped with a CH dual cryogenic probe. The probe temperature was set to 47 °C. ^{1}H and ^{13}C chemical shifts were given in ppm from external 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.12.003.

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