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^{1}H and ^{13}C NMR assignments for the glycans in glycoproteins by using ^{2}H/^{13}C-labeled glucose as a metabolic precursor

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Abstract

In order to understand the role of the glycans in glycoproteins in solution, structural information obtained by NMR spectroscopy is obviously required. However, the assignment of the NMR signals from the glycans in larger glycoproteins is still difficult, mainly due to the lack of appropriate methods for the assignment of the resonances originating from the glycans. By using [U-$^{13}C_6$,$^{2}H_7$]glucose as a metabolic precursor, we have successfully prepared a glycoprotein whose glycan is uniformly labeled with ^{13}C and partially with D at the sugar residues. The D to H exchange ratios at the C1-C6 positions of the sugar residues have been proven to provide useful information for the spectral assignments of the glycan in the glycoprotein. This is the first report on the residue-specific assignment of the anomeric resonances originating from a glycan attached to a glycoprotein by using the metabolic incorporation of hydrogen from the medium into a glycan labeled with [U-$^{13}C_6$,$^{2}H_7$]glucose.

The glycans in glycoproteins are often involved in important biological phenomena, such as cell–cell adhesion and protein traffic (Kobata, 1992; Lis and Sharon, 1993; Varki, 1993; Dwek, 1995). In order to understand the role of the glycans in glycoproteins in solution, structural information obtained by NMR spectroscopy is obviously required. Although several NMR studies on glycoproteins have been reported (de Beer et al., 1994, 1996; Wyss et al., 1995a,b; Weller et al., 1996), the assignment of the NMR signals from the glycans in glycoproteins with large molecular weight is still difficult. This is mainly due to the lack of appropriate methods for the assignment of the resonances originating from glycans in glycoproteins.

In a previous study, we have developed a systematic ^{13}C labeling method to assign the anomeric resonances of glycans attached to a glycoprotein by using the Fc fragment of immunoglobulin G (IgG), with a molecular weight of 50 K (Yamaguchi et al., 1998). This technique enabled us to successfully identify the anomeric resonances from the GlcNAc residues of the glycan attached to the Fc fragment in the ^{1}H-^{13}C HSQC spectrum. However, it was not possible to discriminate between the Man and Fuc resonances due to metabolic scrambling in the biosynthetic pathway. Therefore, it is necessary to develop a general method to assign the resonances originating from glycans in glycoproteins in a residue-specific manner.

Glucose, which is used as a precursor for all of the sugar residues within glycoproteins in cells, is metabolically converted to UDP-GlcNAc, GDP-Man, and GDP-Fuc by epimerization, oxidation, reduction, and hydrogen transfer (Topper, 1957; Rose and O'Connell, 1960, 1961; Rose, 1975; Midelfort and Rose, 1977; Chang et al., 1985, 1988; Golinelli-Pimpaneau et al., 1989; Oths et al., 1990; Varki, 1991, 1994; Seeholzer, 1993; Martin, 1998). It was also found that hydrogen from the medium is incorporated into the CH groups of the sugar residues during cell cultivation, and that the efficiency of the incorporation varies for the different positions of sugar rings and

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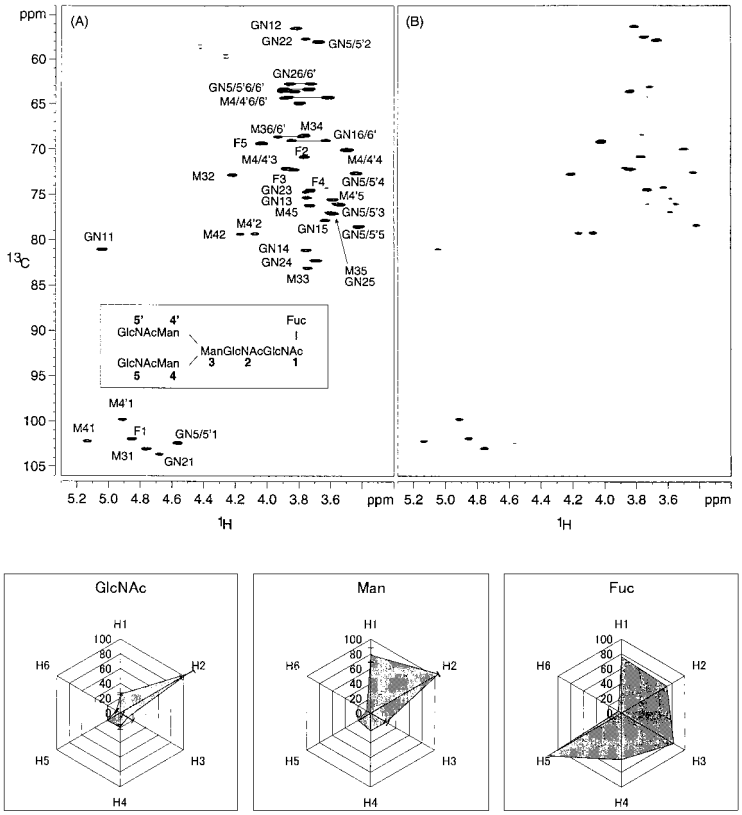

Figure 1. ^{1}H-^{13}C ct-HSQC spectra of glycopeptides metabolically labeled with (A) [U-$^{13}C_6$]glucose or (B) [U-$^{13}C_6$,$^{2}H_7$]glucose. The carbohydrate sequence is also shown in the left spectrum. The samples were dissolved in 5 mM sodium phosphate buffer (pH 7.3), containing 150 mM NaCl and 3 mM NaN_3 in 99.96% D_2O. (C) D to H exchange ratio of each oligosaccharide residue. The D to H exchange ratios were calculated by the intensities of the ^{1}H-^{13}C ct-HSQC spectra of the glycopeptides. The exchange ratios of the GlcNAc and Man residues are calculated as the averages of four GlcNAc and three Man residues, respectively. Standard deviations for GlcNAc and Man residues are also included. Standard deviations for GlcNAc H5, Man H4, H5, H6 were not calculated because of signal overlapping.

different types of sugar residues (Topper, 1957; Rose and O'Connell, 1960, 1961; Rose, 1975; Midelfort and Rose, 1977; Chang et al., 1985, 1988; Golinelli-Pimpaneau et al., 1989; Oths et al., 1990; Seeholzer, 1993). Accordingly, by using [U-$^{13}C_6$, $^{2}H_7$]glucose as a metabolic precursor, it is possible to prepare a glycoprotein whose glycan is uniformly labeled with ^{13}C and partially with D at the sugar residues. The degree of D to H exchange at the C1-C6 positions of the sugar residues is expected to provide useful information on the spectral assignments of the glycan in the glycoprotein. In the present study, we report a new method to assign the resonances originating from the glycan in the glycoprotein, by using [U-$^{13}C_6$, $^{2}H_7$]glucose as a metabolic precursor.

Firstly, we prepared IgG labeled with [U-$^{13}C_6$]glucose. The glycopeptide was isolated from the labeled IgG by enzymatic digestions according to the procedure previously described (Yamaguchi et al., 1998). Figure 1A shows the ^{1}H-^{13}C constant time (ct) HSQC spectrum of the glycopeptide. Complete assignments of the carbon and proton resonances of the glycan of the glycopeptide were successfully established by using ^{1}H-^{13}C ct-HSQC, 2D ct-HCCH-COSY (Constantine et al., 1993), and 2D ct-HCCH-TOCSY (Yu et al., 1993) measurements, and were in agreement with the previous report (Lu and Halbeek, 1996). The CH groups at the C1-C6 positions of the GlcNAc, Man, and Fuc residues of the glycan in the IgG were uniformly labeled with ^{13}C, with a high isotope enrichment of $>95\%$. This result indicates that the [U-$^{13}C_6$]glucose added to the medium is used as the main carbon source for the biosynthesis of the glycan of the glycoprotein.

In order to determine the D to H exchange ratios for the sugar residues, the glycopeptide labeled

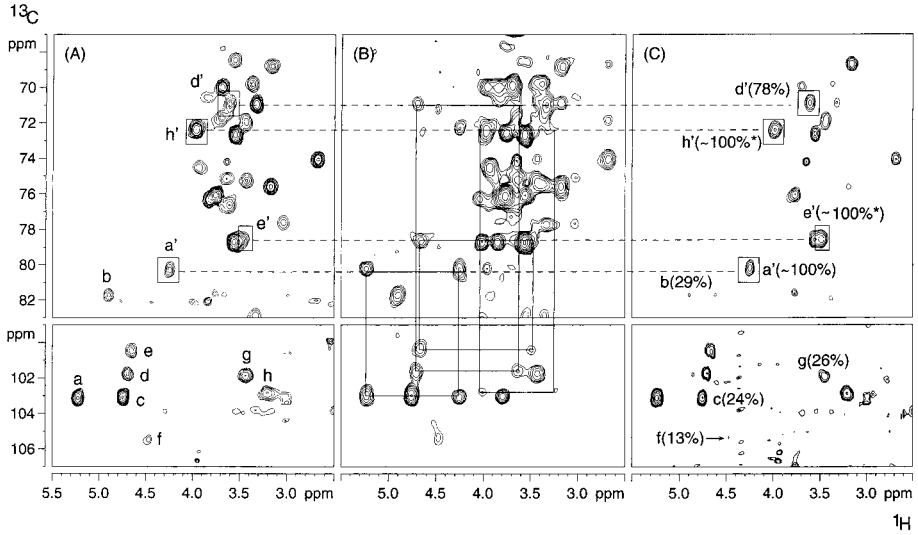

Figure 2. (A) ^{1}H-^{13}C HSQC and (B) ^{1}H-^{13}C 2D HCCH-COSY spectra of Fc metabolically labeled with [U-$^{13}C_6$]glucose. (C) ^{1}H-^{13}C HSQC spectrum of Fc metabolically labeled with [U-$^{13}C_6$,$^{2}H_7$]glucose. The D to H exchange ratios (%) for the Fc fragment are also shown in (C). The linewidths of the ^{1}H resonances were almost identical for both the (A) and (C) spectra, therefore, the effect of deuteration of the neighboring protons on the signal intensities was not taken into account. The exchange ratio for peak e′ with the asterisk was obtained from the spectrum with resolution enhancement by multiplying the data with a Gaussian function in the t2 dimension prior to Fourier transformation. The exchange ratio of peak h′ with the asterisk was calculated by subtraction of the intensity of the overlapping signal which was assigned to Man H3/C3 by a HCCH-COSY experiment along with the D to H exchange ratios. The exchange ratio of peak f includes errors due to accidental overlapping of the HDO signal. The Man/Fuc C2 signals (peaks a′, d′, e′, and h′) are indicated by squares.

with [U-$^{13}C_6$,$^{2}H_7$]glucose was prepared in a similar manner. Figure 1B shows the ^{1}H-^{13}C ct-HSQC spectrum of the glycopeptide thus obtained. Although [U-$^{13}C_6$,$^{2}H_7$]glucose was used for the stable-isotope labeling, the cross peaks originating from the sugar residues are obviously observed in the spectrum, due to the incorporation of the medium hydrogen during the metabolic conversion of [U-$^{13}C_6$,$^{2}H_7$]glucose to the GlcNAc, Man, and Fuc residues of the glycan. On the basis of a comparison of the peak intensities between both spectra (Figures 1A and 1B), the D to H exchange ratios at the C1-C6 positions for the GlcNAc, Man, Fuc residues were calculated. The results are summarized in Figure 1C.

As shown in Figure 1C, the D to H ratios for the GlcNAc and Fuc residues are significantly lower at the C1 and C2 positions, respectively. These findings indicate that the comparison of the D to H ratios at the C1 position between the glycopeptide and the glycoprotein can be used to identify the GlcNAc resonances, and that at the C2 position can be used for the Fuc resonance.

Figure 2A shows the ^{1}H-^{13}C HSQC spectrum observed for the Fc fragment prepared by using [U-$^{13}C_6$]glucose. Anomeric carbons of sugar residues in proteins resonate in distinct regions (∼82 ppm for a residue directly attached to Asn297, 100–106 ppm for the other residues in the sugar chain) from the C2-C6 signal region (Wyss et al., 1995). In addition to ^{13}C incorporation into the glycan (>95%), the peptide moiety was also labeled with ^{13}C to the extent of 11% for aliphatic carbons, less than 3% for aromatic carbons, and 6% for carbonyl carbons. As shown in Figure 2A, the peaks a, b, c, d, e, f, g, and h were therefore easily identified as anomeric signals. However, significant differences in chemical shifts were observed for most of the anomeric resonances between the Fc fragment (Figure 2A) and the isolated glycopeptide (Figure 1A). Therefore, it was not possible to assign most of the individual anomeric peaks of the Fc fragment in a residue-specific manner solely on the basis of a chemical shift comparison.

To determine the D to H exchange ratios for the Fc fragment, the ^{1}H-^{13}C HSQC spectrum of an Fc fragment prepared with using [U-$^{13}C_6$,$^{2}H_7$]glucose was recorded (Figure 2C). In this case, the peptide portion was labeled with ^{2}H, but the degree of enrichment was quite low (<5%). By comparing the intensities of the resonances between both spectra (Figures 2A and 2C), the D to H exchange ratios for the Fc fragment were calculated, and the ratios thus obtained are shown in Figure 2C.

The cross peaks b, c, f, and g exhibited significantly lower values of the D to H exchange ratio (Figure 2C). Therefore, by referring to the D to H exchange ratio, these cross peaks could be successfully assigned to the C1 position of the GlcNAc residues. The assignments established here are consistent with the previous report (Yamaguchi et al., 1998).

In order to identify the C2 resonances originating from the glycan attached to the Fc fragment, a 2D HCCH-COSY (Bax et al., 1990) experiment was carried out for the Fc fragment labeled with [U-$^{13}C_6$]glucose. Figure 2B shows the 2D HCCH-COSY spectrum observed for the labeled Fc fragment. On the basis of the analysis of the spectrum, the anomeric peaks a, d, e, and h could be connected to the C2 cross peaks a′, d′, e′, and h′, respectively. In Figure 2C, only C2 cross peak d′ indicates a ratio of 78%. Therefore, the cross peaks d and d′ could be assigned to the C1 and C2 positions of the Fuc residue, respectively, and the cross peaks a, e, and h, to the Man residues.

It should be noted that in the present measurement, the Fc fragment labeled with [U-$^{13}C_6$,$^{2}H_7$]glucose was prepared from the same IgG that was used for the preparation of the labeled glycopeptide described above. Consequently, the glycopeptide and the Fc fragment thus prepared have the same D to H exchange ratios. It is possible to assign the resonances for the glycan of the glycoprotein based on the D to H exchange ratios, even if the efficiency of the incorporation from the medium proton might vary with the conditions of the cell cultivation.

This strategy can be applied to other glycoproteins with some modifications. For glycoproteins with either a more complex glycan or more than one glycan, molecular biological techniques (e.g. site-directed mutagenesis, exoglycosidase digestion, and glycosyltransferase reaction) along with the strategy are required. In general, the NMR resonances for the C2-C6 positions of glycans in glycoproteins are observed with severe overlapping, and it is difficult to assign the resonances in a residue specific manner, even by HCCH-type NMR measurements. This method makes it possible not only to assign the anomeric resonances but also to extend the assignments to the C2-C6 resonances. The combination of the labeling method used in the present study and the NMR measurements would be especially useful for the spectral assignments of glycans in glycoproteins with larger molecular weight.

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