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Rapid and simple approach for the NMR resonance assignment of the carbohydrate chains of an intact glycoprotein

Application of gradient-enhanced natural abundance ^{1}H-^{13}C HSQC and HSQC-TOCSY to the α-subunit of human chorionic gonadotropin

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Abstract

The structure assessment of an intact glycoprotein in solution requires an extensive assignment of the carbohydrate NMR resonances. However, assignment of homonuclear spectra is very complicated because of the severe overlap of protein and carbohydrate signals. Application of pulsed field gradients allowed high quality natural abundance ^{1}H-^{13}C HSQC and HSQC-TOCSY spectra to be recorded of the α-subunit of human chorionic gonadotropin. Most carbohydrate ^{1}H-^{13}C correlations appear in a distinct region between the aromatic region and the protein C^{α}-H^{α} region. The enormous reduction in overlap led to fast and unambiguous assignment of the anomeric ^{1}H-^{13}C correlations. Subsequently, correlations of the monosaccharide skeleton atoms were readily assigned in the HSQC-TOCSY spectrum.

Key words: Glycoprotein; α-subunit; Human chorionic gonadotropin; Pulsed field gradients; Nuclear magnetic resonance; ^{1}H-^{13}C correlation spectroscopy

1. Introduction

The functional significance of oligosaccharide chains of numerous glycoproteins in higher organisms is well established and has been comprehensively reviewed [1,2]. Despite the recent explosive development of multidimensional NMR techniques, not much is known about the structure of glycoproteins in solution. Determination of the structure of a glycoprotein requires an extensive assignment of the NMR resonances of the carbohydrate chains, in addition to those of the protein. The 'structural reporter group' concept has proven to be a very useful approach in the ^{1}H NMR resonance assignment of oligosaccharide structures [3,4]. In homonuclear two dimensional (2D) spectra these structural reporter groups are used as starting points for the assignment of the resonances of the corresponding skeleton protons [5–8]. However, in homonuclear 2D spectra of intact glycoproteins, straightforward assignment of the oligosaccharide resonances is complicated because the anomeric proton resonances coincide with the region containing most protein backbone C^{α}-proton resonances [9–11]. Inspection of a database of NMR spectral data of proteins reveals that most α-carbons are found within the ^{13}C chemical shift range of 44–64 ppm [12,13], while most carbons of the carbohydrates resonate within the range of 61–105 ppm [14]. Taking advantage of this chemical shift difference, several groups have reported the partial ^{13}C chemical shift assignment of carbohydrates linked to a glycoprotein by application of natural abundance one dimensional (1D) ^{13}C NMR [15]. Recently, Medvedeva et al. [16] showed that the use of pulsed field gradients greatly improves the quality of heteronuclear spectra of proteins at the ^{13}C natural abundance level. Here, we demonstrate that gradient-enhanced natural abundance ^{1}H-^{13}C HSQC and HSQC-TOCSY spectra of an intact glycoprotein greatly facilitate the NMR resonance assignment of the oligosaccharide chains. In the framework of our study of the structure of the α-subunit of human chorionic gonadotropin (α-hCG), we demonstrate the approach for this glycoprotein. hCG is a member of the family of heterodimeric glycoprotein hormones, and is involved in maintaining the corpus luteum in the early weeks of pregnancy [17,18]. The α-subunit of 92 amino acids is N-glycosylated at Asn-52 and Asn-78, and contains either *N*-acetyllactosamine or hybrid type oligosaccharide chains [1,19–22].

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Abbreviations: NMR, nuclear magnetic resonance; HSQC, ^{1}H-detected heteronuclear single quantum coherence spectroscopy; TOCSY, total correlation spectroscopy; INEPT, insensitive nuclei enhanced by polarization transfer; MLEV, M. Levitt; GARP, globally optimized alternating-phase rectangular pulses; hCG, human chorionic gonadotropin; Gal, D-galactose; Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; NeuAc, *N*-acetyl-D-neuraminic acid.

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2. Materials and methods

hCG was kindly provided by Diosynth BV (Oss, The Netherlands), and was further purified as described by Hiyama et al. [23] with minor modifications. In short, the crude hCG precipitate was dissolved in 2 M NH_4Ac, 10 mM Tris-HCl, pH 7.4, and applied to an *n*-octyl–Sepharose column (12.5 × 5 cm), which was equilibrated in the same buffer. Proteins were eluted with a linear gradient of 5–40% (v/v) ethanol in this buffer. Fractions containing hCG were combined, desalted and concentrated by ultrafiltration before lyophilization. Subsequently, the hormone was separated into its subunits essentially as described [21]. α-hCG concentrations were estimated by UV absorbance using a molar extinction coefficient of $\varepsilon_{280\ nm} = 6.4 \times 10^{-3}\ M^{-1} \cdot cm^{-1}$ [17].

The pulse sequence for the 2D ^1H-^{13}C HSQC experiment was essentially as described by Bodenhausen and Ruben [24], 90(^1H)-Δ_1-180(^1H),180(^{13}C)-Δ_1-90(^1H)-90(^{13}C)-t_1/2-180(^1H)-t_1/2-90(^{13}C)-90(^1H)-Δ_2-180(^1H),180(^{13}C)-Δ_2-acquisition. The value of the delays Δ_1 and Δ_2 (1/4 J_{CH}) in the INEPT sequence [25] was set to 1.7 ms. Pulsed field gradients were included in each delay Δ_1 (G1), in each delay Δ_2 (G4) and between 90(^1H) and 90(^{13}C) pulses flanking the t_1 evolution period (G2 and G3, respectively), to suppress artifacts and to reduce the need for phase cycling [26]. Sine-bell-shaped pulsed field gradients were applied along the *z*-axis with a shielded gradient coil. All gradients had a duration of 500 μs, followed by a recovery delay of 500 μs. The gradient strength at the center of the sine bell were as follows: G1 = 37.5 G/cm, G2 = −G3 = −52.5 G/cm, G4 = 25 G/cm. Furthermore, during the relaxation delay of 0.6 s, low power H_2O irradiation was applied, and a spin lock pulse with a duration of 500 μs was implemented just before acquisition [27]. Decoupling during acquisition was achieved by the GARP sequence [28].

The pulse sequence of the 2D gradient-enhanced HSQC-TOCSY consisted of a gradient-enhanced HSQC building block ($\Delta_1 = \Delta_2 = 1.55$ ms) plus a clean-MLEV TOCSY transfer step [29–31]. The mixing time was 60 ms. Phases of the pulses in the MLEV sequence were adjusted to compensate for the difference in power levels. The 180° pulse in the MLEV sequence had a value of 41.2 μs, and every low power 90° pulse was followed by a delay time of 20.6 μs. During the recovery delay, low power H_2O irradiation was applied for 750 ms.

3. Results and discussion

In Fig. 1 the 750 MHz 1D ^1H NMR spectrum of a 5 mM solution of α-hCG is shown. The presence of carbohydrates is recognized by the intense signals in the characteristic bulk region at 3.4–4.3 ppm, and by the methyl singlets of *N*-acetylated monosaccharide constituents around 2 ppm. In the region 4.4–5.2 ppm severe overlap of the carbohydrate anomeric signals with those stemming from C^α-protons of the protein backbone is observed. In fact, only the anomeric signals of some α-Man residues can be distinguished from the protein signals by their narrow line width, as indicated by the arrows in Fig. 1.

The gradient-enhanced natural abundance HSQC spectrum of α-hCG, depicted in Fig. 2A, was recorded over 16 h using a 5 mM solution of α-hCG. The high quality of this spectrum was achieved by the combination of spoiler gradients and phase cycling to suppress pulse artifacts [16,26,32]. In Fig. 2A it can be observed that most cross-peaks stemming from the carbohydrates appear in a window between the aromatic side chain region and the region containing $^1H^\alpha$-$^{13}C^\alpha$ correlations of the protein backbone. This was already predicted by Bush [6], but this should be the first report showing results of the application of gradient-enhanced natural abundance heteronuclear experiments to a glycoprotein. It should be noted that the cross-peaks originating from most carbohydrate residues are more intense than those from the protein backbone, probably reflecting the difference in mobility. Thus, the severe overlap of carbohydrate and protein signals observed in homonuclear spectra is effectively reduced in the HSQC spectrum. A specific region (δ_H = 4.4–5.2, δ_c = 97–105) is observed in the spectrum containing all but the Asn-linked, anomeric ^1H-^{13}C correlations (Fig. 2B). The anomeric ^1H-^{13}C correlation of one of the Asn-linked GlcNAc residues is observed at 5.01 ppm/79.6 ppm (indicated in Fig. 2A by an arrow). The intensity of the cross-peak of the other Asn-linked GlcNAc residue was too low to be observed. Inspection of the anomeric region reveals several ^1H-^{13}C correlations (Fig. 2B) which were readily assigned based on comparison with ^1H and ^{13}C spectral data of reference compounds [15,19,21,33,34] and with knowledge of the oligosaccharide structures reported for α-hCG (Fig. 2C) [1,19–22].

In comparing the resonance assignment of a free oligosaccharide, some important differences are observed in analyzing homonuclear spectra of oligosaccharides linked to a glycoprotein, in addition to the severe overlap of protein and carbohydrate cross-peaks as mentioned above. NMR analysis of an oligosaccharide is normally done after release from the protein, and subsequent extensive fractionation (c.f. [35]), whereas NMR experiments on the glycoprotein are performed on the whole population of glycoforms. Because α-hCG contains both *N*-acetyllactosamine and hybrid type structures [1,19–22], several different types of Man residues are present. In homonuclear spectra of α-hCG the anomeric resonances of several α-Man residues appear within 0.04 ppm of each other. Substitution of Man residues at C-2 results in an upfield ^{13}C chemical shift of the anomeric carbon with respect to the other Man residues, facilitating the assignment (Fig. 2B) [15]. Another feature affecting the appearance of the spectra is the line width, directly related to the size of the molecule. Whereas the anomeric resonances of GlcNAc-**2** and GlcNAc-**5/5′** residues are well-resolved in homonuclear spectra of a free oligosaccharide, they could not be distinguished from each other in spectra of α-hCG. In contrast, the anomeric ^1H-^{13}C correlations of these GlcNAc residues are clearly separated in the HSQC spectrum (Fig. 2B). Thus, in addition to the effective separation of protein and carbohydrate NMR cross-peaks, the ^{13}C chemical shift dispersion reduces overlap among carbohydrate correlations as well. Obviously, the ^{13}C chemical shift values themselves also contribute to the NMR resonance assignment.

Most other carbohydrate NMR cross-peaks are observed in the region from 3.4 to 4.3 ppm (^1H) and from 51 to 83 ppm (^{13}C), indicated by the dotted box in

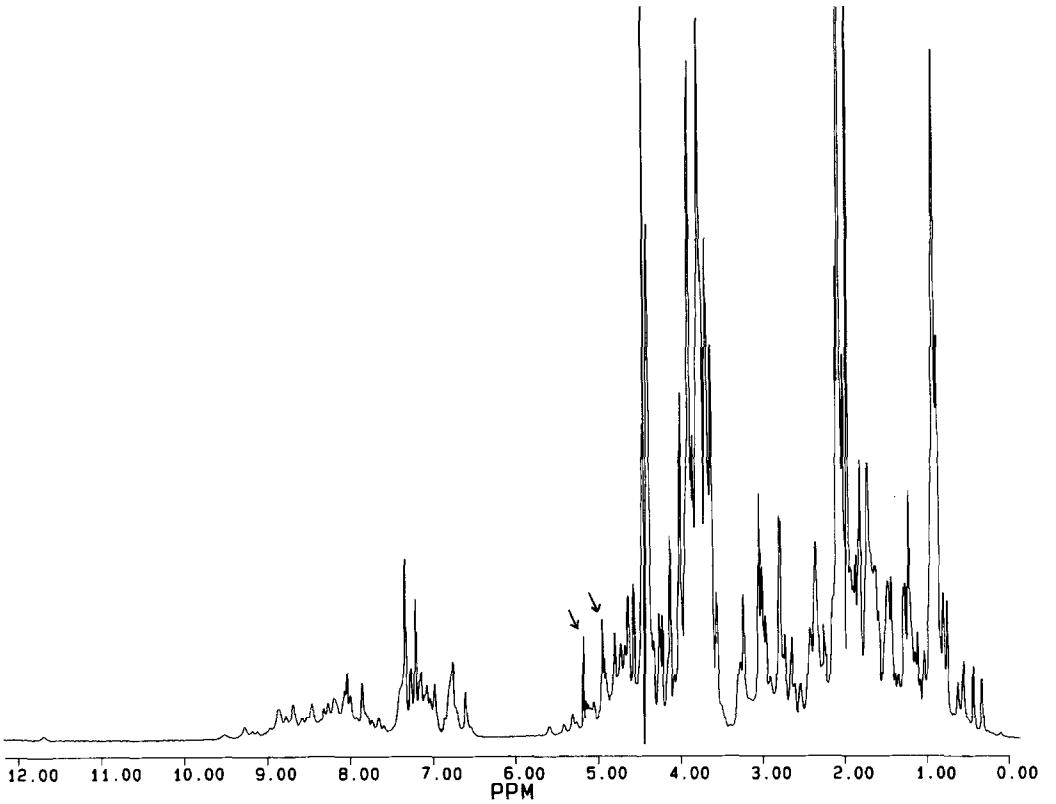

Fig. 1. 1D ^{1}H NMR spectrum of 5 mM α-hCG in 95% (v/v) $H_2O/^2H_2O$, 0.3 M NaCl, 1 mM NaN_3. The pH was adjusted to 5.1 by addition of dilute HCl. The experiment was performed on a Varian Unity plus 750 MHz spectrometer at a probe temperature of 338 K. The spectrum was recorded with a 1D NOE sequence with presaturation of the H_2O resonance during the relaxation delay. The ^{1}H carrier frequency was set at 750.368 MHz. The spectral width was 12,000 Hz. In total 64 FIDs of 2,048 complex data points were acquired. The data were processed using the TRITON NMR software package (Bijvoet Center, Department of NMR Spectroscopy). In short, the time domain data were multiplied by a phase shifted squared sine bell window, and baseline corrected after Fourier transformation and zero filling to 4,096 data points with a fifth order polynomal fit. The Man-**4** and the Man-**4′** anomeric resonances are indicated by an arrow.

Fig. 2A. To correlate the anomeric atoms with their respective skeleton atoms, a gradient-enhanced natural abundance 2D ^{1}H-^{13}C HSQC-TOCSY experiment was performed on the 5 mM α-hCG sample. A high quality HSQC-TOCSY spectrum with sufficient resolution in both directions was obtained within a reasonable acquisition time (60 h). The part of the spectrum containing most carbohydrate NMR cross-peaks, and a corresponding part of the HSQC spectrum are shown in Fig. 3. To illustrate the assignment procedure, the assignment of a Gal residue, which is sialylated at C3, is described. On the H1 track through the anomeric ^{1}H-^{13}C correlation at δ_H/δ_C = 4.52/104.0 (cross-peak a in Fig. 3) three relayed cross-peaks are observed, with ^{13}C chemical shifts of 76.8 ppm (b), 70.5 ppm (c) and 68.8 ppm (d), respectively. On each of the ^{13}C tracks through the cross-peaks b–d, a connectivity pattern of four protons is observed (H1–H4). Inspection of the HSQC spectrum then identifies the ^{1}H-^{13}C correlations of this Gal residue (e,f,g). The C3 (e) atom is readily assigned because of the downfield shift to 76.8 ppm, which is typical of a 3-substitution. The homonuclear $^3J_{HH}$ coupling patterns observed for the relayed cross-peaks on the ^{13}C tracks, and spectral data of (α2-3)sialyllactose were used to discriminate between the H2/C2 (f) cross-peak and the H4/C4 (g) cross-peak [34].

Assignment of the NMR cross-peaks stemming from Man residues of an intact glycoprotein can be troublesome, because of the weak TOCSY transfer due to the small homonuclear coupling constants $^3J_{12}$ and $^3J_{23}$ [10,11]. To show the quality of the HSQC-TOCSY spectrum the assignment pathway of Man-**4** is outlined. A weak cross-peak (i) on the H1 track through the anomeric correlation (h at δ_H/δ_C = 5.13/100.7) and a cross-peak (j) on the ^{13}C1 track leads to assignment of the ^{1}H-^{13}C correlation of the H2/C2 pair (k at δ_H/δ_C = 4.17/78.0). On the H2 track through this cross-peak three relayed cross-peaks (l–n) are observed. Inspection of the HSQC spectrum identifies the corresponding ^{1}H-^{13}C correlations on the respective ^{13}C tracks (o–q), and comparison with chemical shift data of the Man-**4** residue in a diantennary decasaccharide [33] makes the assignment possible (H3/C3,p;H4/C4,q;H5/C5,o). The ^{1}H-^{13}C correlations of oligosaccharide hydroxymethyl groups appear

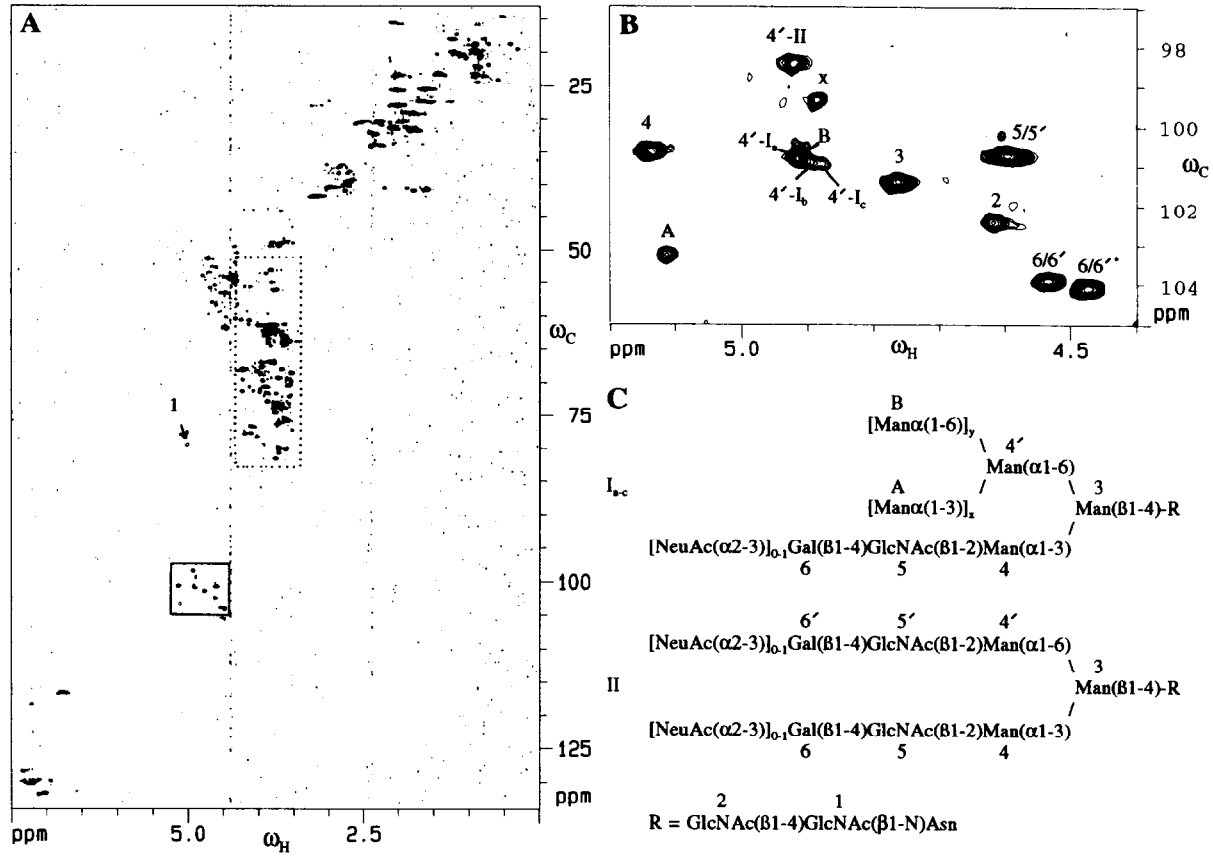

Fig. 2. (A) ^{1}H-^{13}C 2D HSQC spectrum of 5 mM α-hCG in 99.95% ^2H_2O, 0.1 M NaCl, 1 mM NaN_3. The pH was adjusted to 4.7 by adding dilute ^{2}HCl (no correction was made to the pH meter reading for the deuterium isotope effect). The experiment was performed on a Bruker AMX-600 spectrometer equipped with a 5 mm shielded-gradient triple resonance probe at a probe temperature of 328 K. The ^{13}C carrier frequency was placed at 150.91625 MHz. The HSQC experiment was recorded with 1,024 experiments in the t_1 direction. Per experiment 64 free induction decays of 2048 data points were recorded. The ^{1}H carrier frequency was placed at 600.140784 MHz. Quadrature detection in the t_1 dimension was achieved by the States-TPPI method [36]. The spectral width was 7,042 Hz in the t_2 direction, and 21,741 Hz in the t_1 direction. The data set was processed with the Bruker UXNMR software package. In short, time domain data were multiplied with a phase shifted squared sine bell function. The data set of 2k × 2k data points, resulting after Fourier transformation and zero filling, was baseline corrected in both directions with a fifth order polynomal fit. Chemical shifts are given by reference to acetone (δ_H = 2.225) and sodium 3-(trimethylsilyl)-propionate (δ_C = 0). The arrow indicates the anomeric ^{1}H-^{13}C correlation of one of the Asn-linked GlcNAc-1 residues. The area within the dotted box contains most carbohydrate ^{1}H-^{13}C correlations. The anomeric region lies within the solid box, and is enlarged in B. (B) Anomeric region of the ^{1}H-^{13}C HSQC spectrum (the notation is depicted in Fig. 2C, and taken from Vliegenthart et al. [3]). **6/6'*** indicates the ^{1}H-^{13}C correlation of asialo Gal-**6/6'** residues. The Man ^{1}H-^{13}C correlation indicated by × has an upfield ^{13}C chemical shift relative to Man-**4'** of structures I_{a-c} (C), but could not be assigned as yet based on spectral data [15,19,21,33,34] of the free oligosaccharide structures reported for α-hCG [1,19–22]. (C) Oligosaccharide chains reported for α-hCG [1,19–22]. I_a, x = 0, y = 0; I_b, x = 1, y = 0; I_c, x = 1, y = 1.

in a region also containing some protein H^{α}-C^{α} correlations, and cross-peaks stemming from some amino acid side chains (c.f. Ser, Thr). The typical pattern of two hydrogens attached to a single carbon in the HSQC spectrum, the relayed coherence from the hydroxymethyl protons to carbohydrate ring protons, and the similar connectivity pattern on the ^{13}C5 track in the HSQC-TOCSY spectrum makes assignment of the hydroxymethyl carbons unambiguously. For example, the Man-**4** H6 and H6′ resonances were assigned based on the relayed correlations (r and s) observed on the ^{13}C5 track at 74.8 ppm. The corresponding C6 atom was identified by the connectivity patterns in the HSQC and the HSQC-TOCSY spectrum, observed at 62.9 ppm.

Apart from the hydroxymethyl ^{1}H-^{13}C correlations, the H2/C2 cross-peaks of GlcNAc residues also appear in a region crowded with cross-peaks stemming from the protein. In this case the relayed connectivity to their corresponding anomeric ^{1}H-^{13}C correlations is used for assignment, as indicated in Fig. 3 for the GlcNAc-**5/5′** residues (v, at δ_H/δ_C = 4.59/56.2). Despite the small differences in ^{1}H chemical shift of the GlcNAc-**5,5′** H2, H3 and H4 atoms, as can be seen on the ^{13}C1 track (δ_C = 100.8, indicated by an arrow), assignment can be carried out accurately because of the large ^{13}C chemical shift dispersion as is observed on the H1 track at 4.59 ppm (v(H2),u(H3),t(H4)).

In conclusion, to determine the structure of a

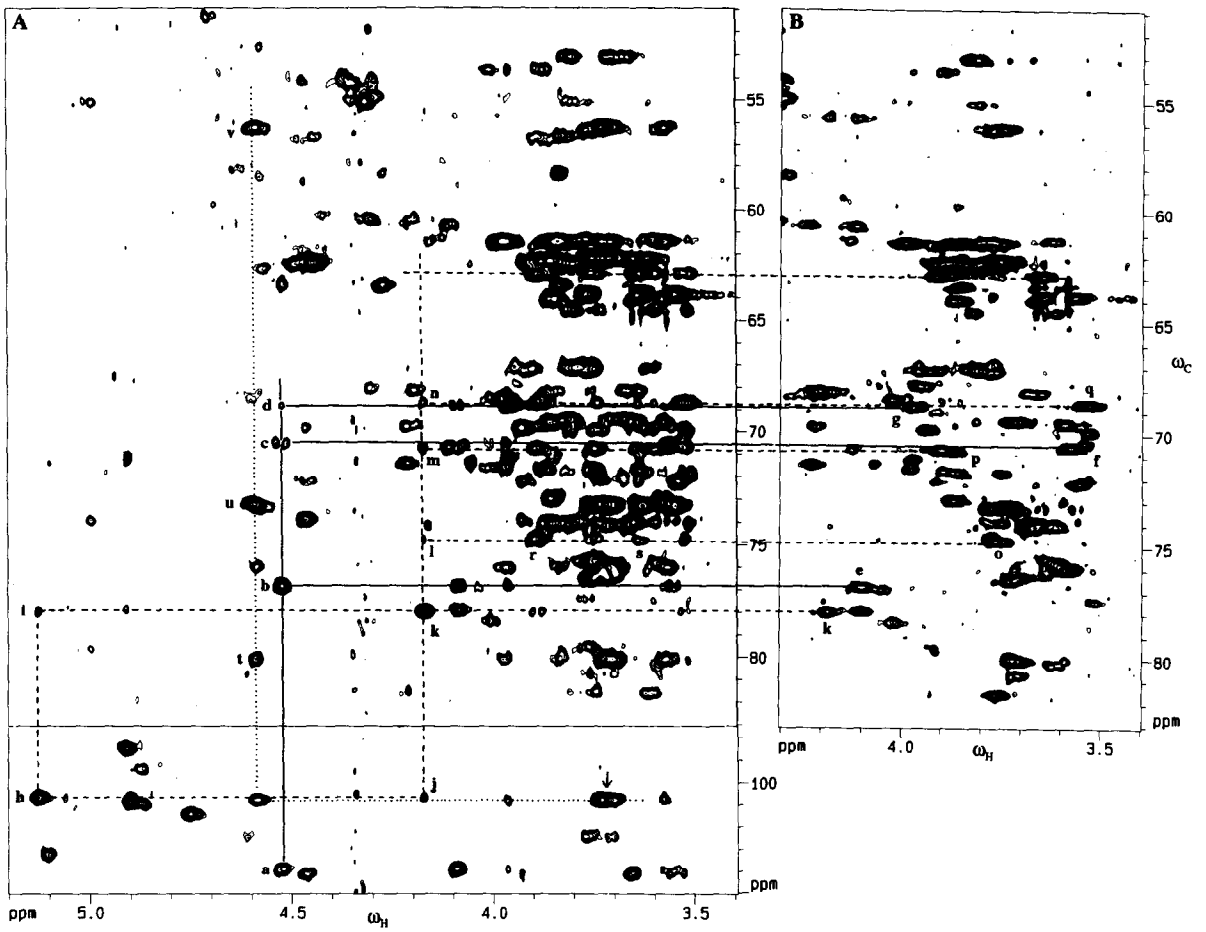

Fig. 3. (A) Part of the ^1H-^{13}C 2D HSQC-TOCSY spectrum of α-hCG and (B) a corresponding part of the HSQC spectrum. Experimental conditions were as described in the legend of Fig. 2 with the following differences and additions. In the t_1 direction 679 experiments were recorded, and per experiment 338 free induction decays of 2,048 data points were collected. The ^1H carrier frequency was placed at 600.140774 MHz. The spectral width was 6,024 Hz in the t_2 direction, and 21,741 Hz in the t_1 direction. The data set was processed as described for the HSQC spectrum. Correlation pathways are shown for Gal-**6/6′** (solid line) and Man-**4** (dashed line). The GlcNAc-**5/5′** C1 and H1 track are also depicted (dotted lines). The arrow indicates relayed connectivities of GlcNAc-**5/5′** H1 to H2, H3 and H4.

glycoprotein in solution, or to study the conformation of oligosaccharide chains attached to a glycoprotein, an extensive assignment of the carbohydrate NMR resonances is required. The severe overlap of carbohydrate and protein signals observed in homonuclear spectra makes assignment of these spectra a tedious, and often impossible, task. The application of gradient-enhanced natural abundance heteronuclear experiments greatly simplifies this assignment. Even at a moderate glycoprotein concentration (5 mM) and with moderate acquisition times, high quality spectra are obtained. Most cross-peaks stemming from carbohydrates attached to an intact glycoprotein appear in a region (δ_H = 3.4–5.2, δ_C = 61–105) in the spectrum essentially devoid of cross-peaks of the protein backbone. Anomeric ^1H-^{13}C correlations are readily assigned, also taking advantage of the reduced overlap among themselves. Furthermore, the heteronuclear relayed experiment powerfully connects these anomeric correlations to their respective skeleton atoms. This approach is an important step in the implementation of gradient-enhanced heteronuclear experiments, recorded at natural abundance, in the NMR resonance assignment of a glycoprotein.

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