NATURAL ABUNDANCE ^{13}C NMR SPECTRA OF SOME GLYCOSYLATED AMINO ACIDS

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1. Introduction

We have shown that natural-abundance ^{13}C NMR spectroscopy can be used to study the structure and dynamic behavior of the carbohydrate residues of aqueous glycoproteins [1]. Furthermore, ^{13}C NMR can be used to study conformational differences, if any, between a glycoprotein and the corresponding unglycosylated protein [2]. There are no reports in the literature of chemical shifts and assignments of the ^{13}C resonances of small glycopeptides. This type of information will be very valuable in the interpretation of ^{13}C NMR spectra of glycoproteins. Here we present chemical shifts and assignments of ^{13}C resonances for two derivatives of GlcNAc, i.e., β-GlcNAc–Asn and β-GlcNAc–Ser. We chose β-GlcNAc–Asn for this initial study of ^{13}C chemical shifts of model compounds because it is a common subunit in many glycoproteins [3,4]. Even though β-GlcNAc–Ser is not normally found in glycoproteins [3,4], we chose to initiate our studies of ^{13}C NMR spectra of model compounds with the use of a single type of carbohydrate residue. Our conclusions are readily extrapolated to the commonly occurring α-GalNAc–Ser unit [3,4].

2. Materials and methods

GlcNAc, β-GlcNAc–Asn, and *N*-carbobenzoxy–L-serine were purchased from Sigma Chem. Co., St Louis, MO and used without further purification. β-GlcNAc–Ser was synthesized by the method in [5], starting with GlcNAc and *N*-carbobenzoxy–L-serine. After purification by thin-layer chromatography on silica gel, our material had a melting (decomposition) point of 220°C, lower than the reported value of 234–236°C. The ^{13}C NMR spectrum (fig. 1C) inidicated the presence of an impurity; we have reason to believe that the impurity is GlcNAc(β1→6)β–GlcNAc–Ser.

Proton-decoupled natural-abundance ^{13}C NMR spectra were recorded at 67.9 MHz (63.4 kG) with the use of a 15 mm probe, essentially as in [1]. A recycle time of 5 s was used for all spectra. Chemical shifts are reported in ppm downfield from the ^{13}C resonance of Me_4Si, and have an estimated precision of ± 0.03 ppm. They were measured digitally with respect to a trace of internal dioxane, added only when recording spectra for chemical shift measurements. The chemical shift of dioxane was taken as 67.86 ppm.

Abbreviations: GlcNAc, *N*-acetylglucosamine; β-GlcNAc–Asn, $N^{\delta 2}$-(2-acetamido-1,2-dideoxy-β-D-glucopyranosyl)–L-asparagine; β-GlcNAc–Ser, O^{γ}-(2-acetamido-2-deoxy-β-D-glucopyranosyl)–L-serine, α-GalNAc–Ser, O^{γ}-(2-acetamido-2-deoxy-α-D-galactopyranosyl)–L-serine; Me_4Si, tetramethylsilane

3. Results and discussion

Figures 1B,C show the region of aliphatic carbons in the natural-abundance proton-decoupled ^{13}C NMR spectra at 67.9 MHz (63.4 kG) of β-GlcNAc–Asn and β-GlcNAc–Ser, respectively, at pH 6.5 (chemical shifts of carbonyl carbons are given in the legend of fig. 1). For comparison purposes, fig. 1A shows the corresponding chemical shifts of L-asparagine [6], L-serine [6], and the β-pyranose anomer of GlcNAc [7–9]. The assignments of fig. 1B follow by inspection from fig. 1A. We observe the expected [10] large

upfield shift of the anomeric carbon resonance of β-GlcNAc (fig. 1A) upon formation of the *N*-glycosidic linkage in β-GlcNAc–Asn (fig. 1B). As a result, we do not have one-to-one assignments for carbons 1 and 5 of the carbohydrate moiety of β-GlcNAc–Asn (see fig. 1B).

The resonances of C^{α} and C^{β} of the serine moiety of β-GlcNAc–Ser could not be assigned unambiguously by inspection, because of their proximity to resonances of the sugar moiety (fig. 1C). However, the effect of high pH yielded specific assignments for C^{α} and C^{β} of the amino acid moiety: When raised from pH 6.5–8.5 (fig. 1C), the resonances originally at 56.25 and 69.20 ppm moved downfield by about 0.4 and 1.9 ppm, respectively, while the other resonances of nonanomeric carbons did not move appre-

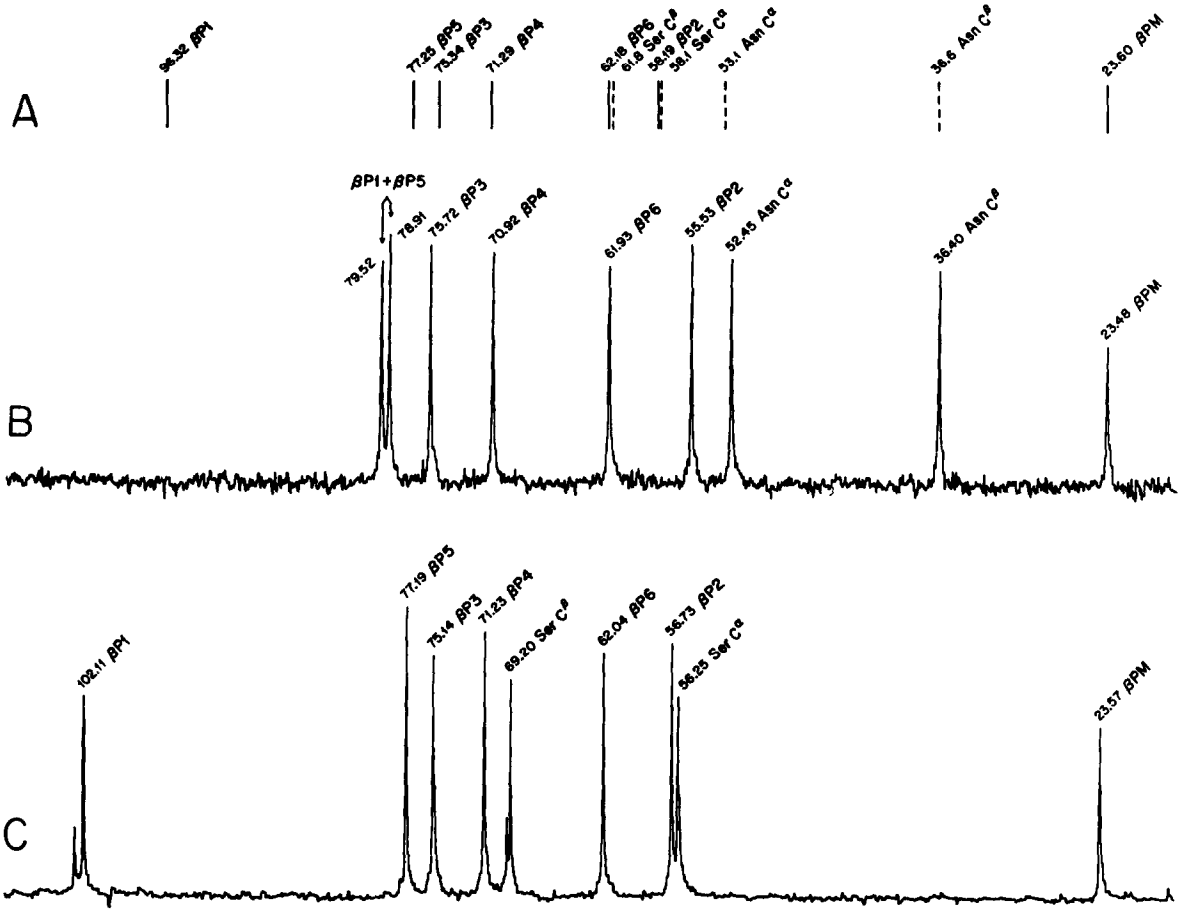

Fig.1. (A) Chemical shifts (in ppm downfield from the ^{13}C resonance of Me_4Si) of the β-pyranose anomer of GlcNAc (solid lines) and the amino acids L-asparagine and L-serine (dashed lines). Carbon 1 of β-GlcNAc is designated by βP1, (with analogous designations for carbons 2–6); the methyl carbon is designated by βPM. The chemical shifts of GlcNAc were measured by us (0.76 M compound in H_2O (pH 6.6), 29°C, 512 scans). The assignments for GlcNAc are taken from [7–9]. The chemical shifts for the amino acids are taken from [6], converted to the Me_4Si scale. Carbonyl resonances are at 176.03 (GlcNAc), 175.2 (C^{O} of L-asparagine), 176.2 (C^{γ} of L-asparagine) and 173.6 ppm (L-serine). (B) Region of aliphatic carbons in natural-abundance proton-decoupled ^{13}C NMR spectrum (at 67.9 MHz) of 19 mM β-GlcNAc–Asn in H_2O (pH 6.5), 29°C, after 4096 scans. Carbonyl resonances are at 173.92, 174.28, and 176.23 ppm. (C) Spectrum of 36 mM β-GlcNAc–Ser at 31°C with all other conditions as in spectrum B. Carbonyl resonances are at 176.25 and 172.69 ppm. The small peaks in spectrum C arise from an impurity (see section 2).

ciably*. It is known that the resonances of C^{α} and C^{β} of L-serine move downfield by 1.0 and 4.5 ppm, respectively, when going from the zwitterionic to the anionic state of the amino acid [11]. If we take into account that the pertinent pK is ~9.2 [12], it follows that the effect of pH on the resonances at 56.25 and 69.20 ppm (in fig. 1C) is fully consistent with assignments to C^{α} and C^{β}, respectively, of the serine moiety of β-GlcNAc–Ser. The other assignments in fig. 1C follow by inspection from a comparison with fig. 1A.

It is not necessarily safe to assume that the chemical shifts of the sugar moieties of β-GlcNAc–Asn and β-GlcNAc–Ser are similar to the corresponding chemical shifts in glycoproteins.

(1) It is necessary to take into account the effects of glycosidic bonds to other carbohydrate residues in oligosaccharide side chains, especially for the carbons involved in the glycosidic linkages [1,13].

(2) It is possible, in principle, that peptide bond formation affects the chemical shifts of the carbohydrate moiety. However, we expect this effect to be small, on the basis of the pH dependence of the ^{13}C chemical shifts of the GlcNAc moiety of β-GlcNAc–Asn: These chemical shifts are essentially the same (within ± 0.2 ppm) for the cationic, zwitterionic, and anionic states of the glycosylated amino acid, which is a good indication that peptide bond formation will also have a very small effect on the chemical shifts of the sugar moiety.

(3) It may be necessary to take into account the effect of protein folding: We have preliminary indications that the ^{13}C chemical shifts of carbohydrate residues attached to (or located near) the polypeptide backbone may be strongly influenced by protein folding [14]. However, solvent-exposed sugar residues are not significantly affected by protein folding [1].

Figure 1 has several important implications for studies of glycoproteins by ^{13}C NMR:

(i) The resonances of anomeric carbons which participate in N-glycosidic linkages will not show up in the so-called 'anomeric region' of the spectrum (~90–110 ppm). Therefore, the integrated intensity of the 'anomeric region' will only yield the total number of carbohydrate residues whose anomeric carbons participate in O-glycosidic linkages [1].

(ii) The resonances of anomeric carbons which are involved in N-glycosidic linkages will be relatively difficult to identify, because of their proximity to nonanomeric carbon resonances.

(iii) The C^{β} resonance of a serine residue is expected to move ~7 ppm downfield upon formation of the O-glycosidic linkage. In contrast, the chemical shifts of all carbons (even C^{γ}) of an asparagine residue should change relatively little ($\lesssim$ 2 ppm) upon formation of the N-glycosidic linkage. Therefore, it should be possible, in principle, to use ^{13}C NMR to identity the number of serine and threonine residues (but probably not asparagine residues) which are points of attachment for carbohydrate side chains. In practice, the resonances of C^{β} of glycosylated serine residues may be difficult to identify because of their proximity to various resonances of nonanomeric carbons of various carbohydrate residues (see fig. 1C) [1].

(iv) When the anomeric carbon of a carbohydrate residue participates in an O-glycosidic or N-glycosidic linkage to an amino acid residue, the chemical shifts of carbons 3, 4 and 6 should be relatively unaffected ($\lesssim$ 0.4 ppm change); the chemical shifts of carbons 2 and 5 may change appreciably ($\gtrsim$ 1 ppm) in some cases but not always (see carbon 5 of the sugar moiety of β-GlcNAc–Ser in fig.1 C).

* The resonance of the anomeric carbon moved ~0.2 ppm downfield

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