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Assignment of resonances for 'acute-phase' glycoproteins in high resolution proton NMR spectra of human blood plasma

J.D. Bell, J.C.C. Brown, J.K. Nicholson and P.J. Sadler

Department of Chemistry, Birkbeck College, Univerity of London, Malet Street, London WC1E 7HX, England

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Broad resonances at 2.04 and 2.08 ppm in 500 MHz Hahn spin-echo ^{1}H NMR spectra of human blood plasma are assigned to the *N*-acetyl groups of mobile carbohydrate side-chains (largely *N*-acetylglucosamine and *N*-acetylneuraminic acid) of glycoproteins such as α_1-acid glycoprotein. Their intensities in spin-echo spectra correlate with clinical conditions in which an elevation of the level of 'acute-phase' glycoproteins is expected, and so may be of value in the study of certain diseases.

^{1}H-NMR; Plasma; Glycoprotein; *N*-acetylated sugar; Acute-phase protein

1. INTRODUCTION

High resolution ^{1}H NMR spectra of human blood plasma can provide useful biochemical and clinical information [1–3]. By suppression of broad resonances from high molecular mass plasma constituents (such as albumin), using spin-echo Fourier transform (SEFT) methods, it is possible to obtain a profile of mobile protons, mostly low molecular mass non-protein-bound metabolites, present in human plasma at concentrations >0.1 mM [1,2].

We have previously assigned most of the major resonances in ^{1}H NMR spectra of human and animal plasmas [1]. However, comparatively broad peaks centred at 2.04 ppm and at 2.08 ppm have not been assigned to any common low molecular mass component, and have aroused particular interest by the constancy of both their relative intensities and their chemical shifts in the many plasma samples we have so far studied.

Correspondence address: P.J. Sadler, Department of Chemistry, Birkbeck College, University of London, Malet Street, London WC1E 7HX, England

We report here the assignment of these peaks to the *N*-acetyl protons of highly mobile (in NMR terms) *N*-acetylated carbohydrate side-chains associated with 'acute-phase' plasma glycoproteins. The intensities of the resonances from these protons appear to be perturbed in spin-echo spectra of plasma taken from subjects with certain abnormal clinical conditions. Further studies of these signals may shed new light on the biochemistry of acute-phase glycoproteins and give insight into associated diseases or pathological processes.

2. MATERIALS AND METHODS

2.1. *Proton NMR spectroscopy*

All spectra were acquired using a Bruker AM500 spectrometer operating at 500 MHz with quadrature detection, probe temperature 298 K.

Broad protein resonances from plasma were suppressed using the Hahn spin-echo pulse sequence (SEFT), (90°-*t*-180°-*t*-collect), with a *t* value of 60 ms [1,4,5]. Spectra were typically the result of 32–48 repetitions of this sequence with a 5-s delay between cycles. The large water signal was suppressed, where present, by the application

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of continuous secondary irradiation at water frequency with the decoupler coils. An exponential function corresponding to a line-broadening of 1 Hz was applied to the FID prior to Fourier transformation.

2.2. *Sample collection and preparation*

Human plasma glycoproteins (α_1-acid glycoprotein, α_1-antitrypsin, transferrin, haptoglobin and immunoglobulins) were purchased from Sigma. Each glycoprotein was dissolved in deuterium oxide (D_2O) and the pH of the solution adjusted to pH 7.2 (meter reading) with 10 M NaOD. Both single pulse and SEFT spectra were obtained from these samples using either endogenous alanine (δ = 1.48 ppm) or added sodium 3-(trimethylsilyl)-2,3-tetradeutero-propionate, TSP (δ = 0.00 ppm), as a chemical shift reference. Spectra were similarly acquired from samples of *N*-acetylneuraminic acid and *N*-acetylglucosamine (Sigma).

Blood was obtained from a number of consenting healthy volunteers (> 10 adults, both males and females) and placed in sterile plastic vials containing lithium heparin. The blood was centrifuged at 4°C immediately and the plasma separated and stored at −20°C. Samples were thawed at room temperature prior to NMR measurement. Samples were also collected from subjects undergoing diagnostic tests for various clinical disorders including six patients with monoclonal gammopathy, i.e. raised immunoglobulin (IgG, IgA, IgM and IgE) levels, one subject with melanoma, and five subjects with rheumatoid arthritis. Paired plasma samples obtained at birth from both mother and cord were also collected (> 10 pairs).

To estimate the concentration of (NMR-observable) acute-phase glycoproteins in blood plasma by NMR, standard additions were made of a mixture of α_1-acid glycoprotein, α_1-antitrypsin, haptoglobin and transferrin respectively (The percentages of *N*-acetylglucosamine and sialic acid in these proteins (w/w) are 25.0, 3.4, 10.0 and 3.6, respectively [6]. Therefore α_1-acid glycoprotein is likely to be the main source of *N*-acetyl sugars.) with a composition close to the average reported for human plasma [7] (0.8:3.0:1.0:3.0 by wt). Peak intensities were compared by weighing paper traces.

Plasma and standard α_1-acid glycoprotein samples were treated with the enzyme neuraminidase (*Vibrio cholerae*, Boehringer Mannheim; 300 units per ml of sample) for 4 h, followed by treatment with β-galactosidase (bovine liver, Sigma; 10 units per ml) and β-*N*-acetylglucosaminidase (Jack beans, Sigma; 5 units per ml) for 12 h, at 37°C. The resulting solutions were ultrafiltered with Amicon centrifree micropartition devices (< 10 kDa cut-off). ^{1}H NMR spectra were acquired for these reaction mixtures, as well as their ultrafiltrates.

3. RESULTS

The 'aliphatic' region (0–4.2 ppm) of the SEFT 500 MHz ^{1}H NMR spectrum of human α_1-acid glycoprotein is shown in fig.1a. Broad resonances from the protein component of the glycoprotein are suppressed in the SEFT spectrum, only signals from protons with long T_2 values, which can be associated with very mobile protons, are observed.

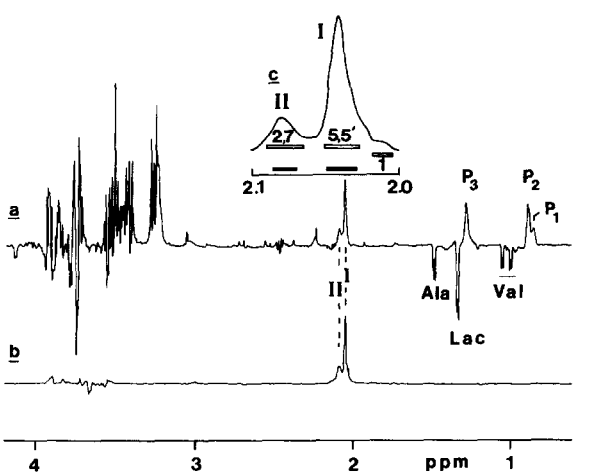

Fig.1. 500 MHz spin-echo (t = 60 ms) ^{1}H NMR spectra of the aliphatic region of (a) normal human blood plasma and (b) α_1-acid glycoprotein in D_2O. In (a) most of the assignments of the peaks for plasma have been reported previously [1,2]. Peaks P_1 and P_2, and P_3 arise from the CH_3 and CH_2 groups, respectively, of lipoprotein components. The inset (c) shows that the reported chemical shift ranges [10,11,13] for the *N*-acetyl groups of *N*-acetylglucosamine (▭) and *N*-acetylneuraminic acid (▬) of the isolated carbohydrates of human plasma glycoproteins cover the range from 2.0–2.1 ppm and can be correlated with the position of the sugar in the branching chains (1,2,5,5',7).

The most intense resonance in the spectrum is comparatively broad and centred at 2.04 ppm with a weaker shoulder centred at 2.08 ppm. Weak signals are also observed in the 3.0–4.0 ppm region of the SEFT spectrum, where signals from sugar CH protons usually occur. Similar resonances were observed in the spectra of the other plasma glycoproteins studied (see section 2), except for immunoglobulins for which none were seen under these conditions.

The 'aliphatic' region of the SEFT 500 MHz ^{1}H NMR spectrum of normal human plasma is shown in fig.1b. The majority of the signals in this spectrum have been identified previously [1,2]. The distinctive broad resonances centred at 2.04 ppm (resonance I: $\Delta\nu_{1/2}$ = 8.0 Hz) and 2.08 ppm (resonance II: $\Delta\nu_{1/2}$ = 11.9 Hz) are almost identical to those seen for α_1-acid glycoprotein (fig.1a). The plasma components which give rise to these signals did not pass through an ultrafiltration membrane with a cut-off of 10 kDa. By 'spiking' a plasma sample with α_1-acid glycoprotein, a clear increase in intensity of these signals was observed (not shown). Similar results were obtained by spiking with other 'acute-phase reactive' plasma glycoproteins, including α_1-antitrypsin, haptoglobin and transferrin. These proteins all have molecular masses in the range 40–100 kDa [7].

Addition of known quantities of a plasma glycoprotein mixture (see section 2) suggested that the concentration of glycoproteins responsible for the signals I and II was ~13 mg/ml in the plasma of normal subjects.

The resonances of the sugar ring protons of the glycoproteins were not clearly discernable in any of the plasma spectra studied, because of extensive overlap with the more intense signals from glucose.

In fig.2a and b, SEFT spectra of plasma before and after treatment with a mixture of neuraminidase, galactosidase and *N*-acetylglucosaminidase are compared. A number of new resonances are clearly observed in the latter spectrum, including a marked increase in intensity of the signal at 2.06 ppm. Similar results were obtained with enzymatically treated glycoprotein solutions. ^{1}H NMR spectra of the ultrafiltrate (<10 kDa) obtained after these enzymatic reactions, suggested that the new resonances could be assigned to 'free' *N*-acetylneuraminic acid and *N*-acetylglucosamine. The signal intensity of the resonance at 2.04 ppm (I) was reduced to less than 30% of its original intensity after the enzymatic treatment of plasma and glycoprotein samples.

Fig.3a and b compare the SEFT ^{1}H NMR spectra of mother and paired cord plasma obtained at birth. The intensities of peaks I and II in the spectrum of maternal plasma exceed those from the

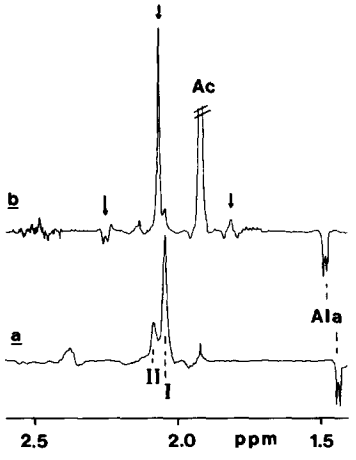

Fig.2. 500 MHz ^{1}H NMR spin-echo spectra of human blood plasma (a) before and (b) after treatment (at pH 5) with neuraminidase followed by galactosidase and glucosaminidase (see section 2). The plasma had been freeze-dried to remove H_2O which caused a rise in pH (to 8.6) and the small shift of the Ala CH_3 resonance [1]. The appearance of resonances for free sugars after enzyme treatment is indicated by arrows in b.

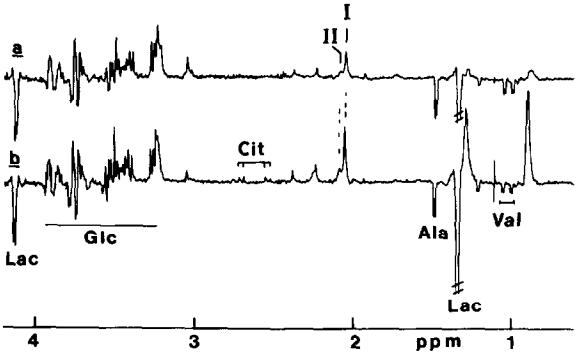

Fig.3. 500 MHz ^{1}H NMR spin-echo spectra of a matched pair of (a) cord and (b) maternal blood plasma taken at birth. Note the large increase in intensity of the *N*-acetyl signals in b. The spectra have been plotted with the Ala CH_3 resonance as the intensity standard. The other differences in these spectra will be discussed elsewhere (Brown, J.C.C. et al., to be published).

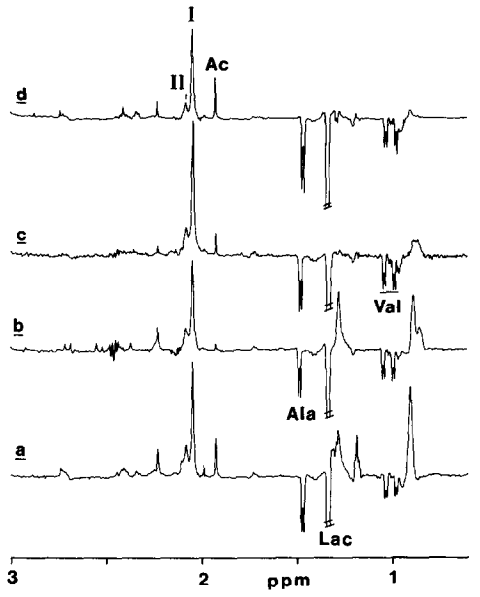

Fig.4. 500 MHz ^{1}H NMR spin-echo spectra of human blood plasma from the following subjects: (a) melanoma, (b) normal, (c) rheumatoid arthritic and (d) monoclonal gammopathy (elevated IgG levels). Changes in the relative intensities of the *N*-acetyl resonances I and II are discussed in the text. The significance of changes in the intensities of other resonances will be discussed elsewhere.

cord plasma by a factor of 2.2 ± 0.5 in the 10 pairs studied (Brown, J.C.C. et al., unpublished). The intensities of these peaks in spectra of maternal plasma also appear to exceed those in spectra from non-pregnant women.

Fig.4 shows a similar comparison of spectra for plasmas obtained from a healthy subject, and subjects with melanoma, rheumatoid arthritis and a monoclonal gammopathy. The intensities of peaks I and II in the spectra of plasma from subjects with melanoma and rheumatoid arthritis exceed those of the normal plasma. These peaks appeared normal for subjects with monoclonal gammopathy (IgG).

4. DISCUSSION

The enzymes neuraminidase, galactosidase and *N*-acetylglucosaminidase cleave the terminal *N*-acetylneuraminic acid, galactose and *N*-acetylglucosamine residues, respectively, of polysaccharides [8]. The addition of these enzymes to plasma samples and glycoprotein solutions gave rise to several new resonances in the spectrum, which were assigned to *N*-acetylneuraminic acid and *N*-acetylglucosamine, both of which are components of most plasma glycoproteins [9–11]. Thus, the plasma signals centred at 2.04 (I) and 2.08 (II) ppm appear to arise from the *N*-acetyl groups of *N*-acetylglucosamine and *N*-acetylneuraminic acid and give rise to the sharp *N*-acetyl peak at 2.06 ppm after enzymatic treatment.

Previous studies on the extracted carbohydrate moieties of plasma glycoproteins (including α_1-acid glycoprotein) have shown that these can give rise to peaks for *N*-acetyl groups with shifts in the chemical shift range 2.00–2.10 ppm depending on their position in the branched polysaccharide chain (see inset to fig.1). This accounts for the apparent broadness (and yet long T_2 values, ~450 ms) of peaks I and II [9–13].

The carbohydrate side-chains of these glycoproteins appear to be highly mobile compared to the protein. Indeed, they are not seen in electron density maps of crystalline lactoferrin, due to their high temperature factors (B. Anderson, personal communication).

Human plasma contains many *N*-acetylated glycoproteins including α_1-antitrypsin, transferrin, haptoglobin, α_1-acid glycoprotein (acute-phase glycoproteins), immunoglobulins and fibrinogen [8,12,13]. Our studies suggest that the resonances at 2.04–2.08 ppm (I and II) in human plasma spectra originate from *N*-acetyl groups of *N*-acetylated carbohydrate side-chains present in the acute-phase reactive plasma glycoproteins. The concentration of some of these plasma glycoproteins are known to change in a number of clinical disorders, e.g. inflammation, cancer, rheumatoid arthritis, some liver diseases, trauma, and also during pregnancy and at birth [14–19].

Providing appropriate checks are made for possible distortions due to differential T_2 relaxation effects, comparison of the areas of glycoprotein *N*-acetyl signals with those of added standards (e.g. alanine) can give an indication of differences in glycoprotein levels in plasma samples.

Thus, the ^{1}H NMR measurements suggested that raised levels of acute-phase glycoproteins are present in maternal plasma compared to cord plasma. This agrees with literature reports that maternal plasma glycoprotein concentrations increase at

birth while cord levels are one-third those of normal adults. However, the clinical significance of these changes is unclear [14].

The association between human cancer and increased levels of circulating blood glycoproteins, in particular *N*-acetylneuraminic acid levels, is well established [17–19]. The ^{1}H NMR spectrum of plasma from a subject with melanoma showed the expected increase in the relative intensities of peaks I and II.

Similar increases in intensity of the glycoprotein signals were observed in the spectra of plasmas from all the subjects with rheumatoid arthritis. Such changes in plasma glycoprotein concentrations in arthritis subjects have also been detected by classical methods [14,15].

The measurement of the acute-phase reactive plasma glycoproteins is of considerable value in the detection, prognosis and therapeutic monitoring of patients with tissue damage. Resonances assignable to *N*-acetyl groups of glycoproteins have also been observed in the ^{1}H NMR spectra of rat urine (Bell, J.D. et al., unpublished), synovial fluid (Bell, J.D. et al., unpublished) and plasma from a number of mammals [1].

Further ^{1}H NMR studies of human plasma are likely to be useful in understanding the changes in plasma levels of glycoproteins with *N*-acetylated side-chains and associated clinical disorders.

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