

Solution conformation of the biantennary N-linked oligosaccharide of human serotransferrin using ^1H NMR nuclear Overhauser effect measurements

S.W. Homans, R.A. Dwek, D.L. Fernandes and T.W. Rademacher

Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, England

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The conformation in solution of the biantennary complex type oligosaccharide unit derived from human serotransferrin has been investigated using ^1H - ^1H Nuclear Overhauser Effect (NOE) measurements at 300 MHz. From quantitation of the NOE, the $\alpha(1-3)$ antenna is shown to exist in a preferred solution conformation with respect to the mannosyl-chitobiose core. The flexibility of the $\alpha(1-6)$ arm, together with the absence of NOE data between this arm and the core, indicates that, in contrast to the $\alpha(1-3)$ arm the $\alpha(1-6)$ arm has no preferred conformation with respect to the core.

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| <i>Oligosaccharide</i> | <i>Oligosaccharide conformation</i> | <i>Nuclear Overhauser effect</i> |
| <i>Nuclear magnetic resonance</i> | | <i>Human serotransferrin</i> |

1. INTRODUCTION

Interest in this laboratory is centred upon the functional significance of complex N-glycosidically-linked oligosaccharide moieties of serum glycoproteins. It is now clear that in certain biological systems oligosaccharides of this type perform a key role as either structural or recognition units [1,2]. Any postulated role must however involve either carbohydrate-carbohydrate or carbohydrate-protein interactions between units of defined conformation.

Recently the X-ray crystallographic analysis of a number of glycoproteins has been completed. Interestingly, the conformation of the biantennary complex sugars of these glycoproteins – rabbit IgG Fc [3], human IgG Fc [4] and haemagglutinin [5] – are all different. Of particular interest is the lack of intra-oligosaccharide hydrogen bonds between the

$\alpha(1-3)$ and $\alpha(1-6)$ arms and the mannosyl-chitobiosyl core. All of these complex oligosaccharides make extensive interactions with the protein or, in the case of rabbit Fc, protein and oligosaccharide [3]. In contrast, the high-mannose oligosaccharide on haemagglutinin does not interact with the protein and assumes a conformation in which hydrogen-bonding between the Man 3 and Man 4 residues may play a stabilizing role [5]. This has led us to speculate that although the conformation of an oligosaccharide is restricted by the primary sequence, carbohydrate-protein or carbohydrate-carbohydrate interactions may play a role in shaping the oligosaccharide into either a structural or recognition unit. Indeed, model building has predicted a double character of rigidity and flexibility for this class of oligosaccharide but no experimental data supporting this prediction have been forthcoming [6].

The purpose of this communication is to show how the conformation in solution of peptide-free complex oligosaccharides can be investigated using proton nuclear Overhauser effect (NOE) measurements and that some degree of rigid conformation

Abbreviations: NOE, nuclear Overhauser effect; NMR, nuclear magnetic resonance; HST, human serotransferrin; Man, D-mannopyranose; Gal, D-galactopyranose; GlcNAc, 2-acetamido,2-deoxy D-glucopyranose

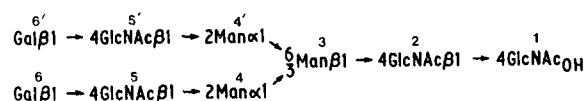


Fig. 1. Structure of the reduced biantennary asialo-oligosaccharide unit of human serotransferrin used for NMR studies as prepared by hydrazinolysis. The tri-antennary oligosaccharides (7% of total carbohydrate recovered) from HST and hydrazine-modified oligosaccharides (3%) were removed using P-4 (-400 mesh 1.5 cm \times 2 m) gel permeation chromatography and the asialo-oligosaccharide was produced by incubation with *Arthrobacter ureafaciens* neuraminidase (Nakari Chemicals, Kyoto). The reduced carbohydrate was produced using NaBH₄ and NaB³H₄ (New England Nuclear) to act as a tracer during purification.

can be demonstrated. This is illustrated for the oligosaccharide from human serotransferrin (HST) in fig. 1. This represents a simple complete form of the complex type structures found in nature [7], and therefore acts as a basic structure to which NOE measurements on different oligosaccharides may be compared.

2. THEORY

The theoretical treatment of the NOE in a two-spin system has been adequately treated in [8]. This may be generalised by considering a multi-spin system as a sum of pairwise interactions, where the relaxation effects of each pair of spins are simply added. For a spin i , coupled to a group of spins j , it can be shown that [8]:

$$d\langle I_{zi} \rangle / dt = -R_i(\langle I_{zi} \rangle - I_{oi}) - \sum_{j \neq i} \sigma_{ij}(\langle I_{zj} \rangle - I_{oj}) \quad (1)$$

where:

$\langle I_{zi} \rangle \propto$ intensity of resonance i with concomitant saturation of spins j ;

I_{oi} = equilibrium value of this magnetisation;

R_i = total direct relaxation rate of spin i , and is roughly equal to T_{1i}^{-1} ;

σ_{ij} = cross relaxation rate between spin i and spin j .

We use here the conventions of [8] in defining an expression for the NOE in multi-spin systems; d labels the observed spin; s labels the saturated spins, and n defines the spins not saturated not including d . The fractional enhancement of spin d on saturation of spins s is defined as:

$$f_d(s) = [\langle I_{zd} \rangle - I_{od}] / I_{od} \quad (2)$$

Equation (1) may be solved for $f_d(s)$, by setting $\langle I_{zs} \rangle = 0$, and using the steady state assumption for spin d ; $d\langle I_{zd} \rangle / dt = 0$. This gives:

$$f_d(s) = \sum_s (\sigma_{ds} I_{os} / R_d I_{od}) - R_d^{-1} \sum_n (\sigma_{dn} I_{on} f_n(s) / I_{od}) \quad (3)$$

Here, the effects of cross-correlation are neglected.

3. MATERIALS AND METHODS

The intact asialo-oligosaccharide from HST (Sigma) (fig. 1) was obtained by preparative scale hydrazinolysis [9] and purified by paper chromatography, paper electrophoresis and P-4 (-400 mesh) gel permeation chromatography as in [10]. Samples for NMR studies were prepared by passing through columns of Dowex AG50 \times 12, AG3.4A and Chelex 100 (BioRad), followed by filtration through a 0.5 μ m Teflon filter (Millipore Ltd). The oligosaccharide (20 mg) was deuterated by repetitive dissolution in 99.96% D₂O (low in paramagnetic ions) (Aldrich) with intermediate flash evaporation. Samples were finally dissolved in 400 μ l 99.996% D₂O (Aldrich) and deoxygenated by repeated evacuation and exchange with dry oxygen-free argon gas prepared by catalytic O₂ removal on BTS catalyst (Fluka). All samples for NMR were sealed anaerobically under dry argon within 5 mm bore precision-glass NMR tubes (Wilmad). Fourier transform NMR spectra were recorded at 300 MHz on a Bruker WH-300 spectrometer operating in the NOE difference mode and at 470 MHz using the Oxford Enzyme Group spectrometer at probe temperatures of 20°C. In NOE measurements, care was taken to ensure total saturation of the irradiated resonance and fractional enhancements were measured directly from difference spectra, where the intensity of the saturated resonance was assumed to be unity. Longitudinal relaxation times were measured using the inversion recovery method and the ratio of the values of these at two frequencies (470 and 300 MHz) was used to obtain an approximate value for τ_c .

4. RESULTS

Using the magnitudes of the NOE's between relevant protons (fig. 2a) in eq. (3), allows the

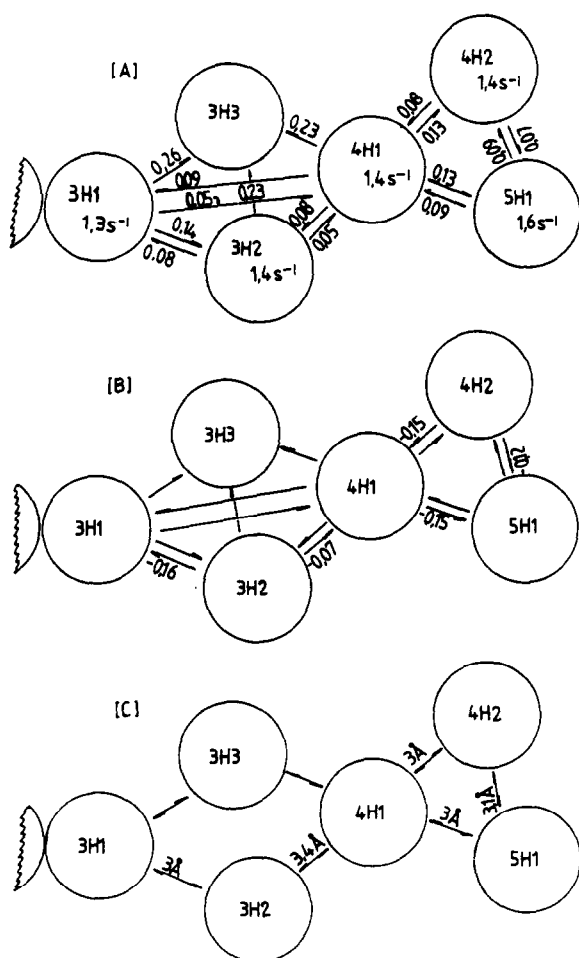


Fig. 2. The relative orientation of the protons of the $\alpha(1-3)$ arm of HST with respect to those of the core. The H3 resonance of mannose 3 was assigned on the basis of 2-dimensional NMR studies [14]: (A) values of the negative NOE between respective protons; (B) corresponding values of σ_{ij} ; (C) interproton distances calculated from (B).

values of σ_{ij} shown in fig. 2b to be calculated and hence the distances between protons may be found (fig. 2c). For this purpose R_d was approximated as $T_1 d^{-1}$. The Man 4 H1–Man 4 H2 interproton distance was determined from the crystal structure of methyl α -D-mannoside [11]. This provides a calibration distance for use in subsequent calculations. The value of $\sigma_{(4H1-4H2)}$, using this distance, was calculated as -0.15 s^{-1} . This corresponds to a calculated value for τ_c of $2 \times 10^{-9} \text{ s}$ which is in good agreement with the measured value of $1 \times 10^{-9} \text{ s}$.

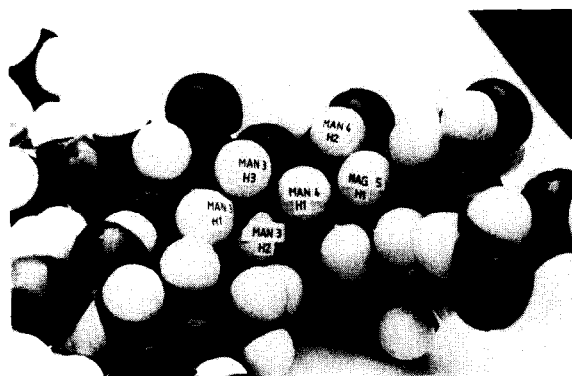


Fig. 3. The solution conformation of the $\alpha(1-3)$ arm relative to the mannosyl-chitobiosyl core based upon NOE measurements.

The distance information of fig. 2c is sufficient to define the spatial orientation of the $\alpha(1-3)$ arm with respect to the Man 3 residue. The orientations of protons Man 4' H1, Man 4' H2 and GlcNAc 5' H1 of the $\alpha(1-6)$ arm were also determined using calculations similar to those above. From these results (fig. 2) we emphasize that the position of Man 4 H1 must lie equidistant to Man 3 H2 and Man 3 H3. In addition, spatial positioning of the $\alpha(1-3)$ arm in a manner consistent with the magnitudes of the NOE's, suggests that there could be a hydrogen bond between the Man 3 C2-OH and the Man 4 C6 hydroxyl. The $\alpha(1-3)$ arm is thus fixed in the orientation shown in fig. 3 and is defined by a spin system consisting of the 6 protons: Man 3 H1, Man 3 H3, Man 3 H2, Man 4 H1, Man 4 H2 and GlcNAc 5 H1. Because of the magnitudes of the NOE's and the r^{-6} dependence of this effect the conformation described above represents the preferred spatial orientation of this oligosaccharide in solution.

5. DISCUSSION

These results, in combination with model building studies, indicate that the $\alpha(1-3)$ arm of the oligosaccharide exists in a well-defined orientation with respect to the Man 3 residue in the core. However, the present studies do not indicate the presence of any detectable through-space interactions between the $\alpha(1-6)$ arm and the mannosyl-chitobiosyl core. It is tempting, therefore, to speculate that in free solution the $\alpha(1-6)$ arm is not rigidly fixed

with respect to the rest of the molecule – this is not unreasonable considering the flexibility of $\alpha(1-6)$ linkages. Any NOE would be averaged away if the $\alpha(1-6)$ arm exhibited freedom of motion about the Man 4'–Man 3 linkage. The $\alpha(1-6)$ arm could thus be positioned to allow extensive interactions with the protein – a concept supported by the recent X-ray data [3–5]. In contrast, the rigid structure of the core and the $\alpha(1-3)$ arm probably represent a minimal energy conformer which may be stabilized by both hydrogen bonds and van der Waals' interactions. Interestingly, none of the conformations of the $\alpha(1-3)$ arms from the X-ray studies mentioned above or from the crystallographic data on α -D-Man(1–3)– β -D-Man(1–4)–D-GlcNAc [12] are consistent with the magnitudes of the NOE's observed here. However, the crystal conformation of the $\alpha(1-3)$ arm of the high-mannose oligosaccharide on haemagglutinin which does not interact with the protein is almost identical to the solution structure described here [5].

We have here assumed that the mannosyl-chitobiosyl core is a rigid structure, which is consistent with the results of our model building studies and also those in [6]. This rigidity is conferred by hydrogen bonds linking GlcNAc 1 C3-OH to the GlcNAc 2 ring oxygen and/or GlcNAc 2 C6-OH, and GlcNAc 2 C3-OH to the Man 3 ring oxygen [5,6]. On similar grounds, together with the limited crystal structure data available on complex type oligosaccharides of similar structure [3–5], hydrogen bonds may be defined linking GlcNAc 5 C3-OH–Gal 6 C2 oxygen, and GlcNAc 5' C3-OH–Gal 6' C2 oxygen within the $\alpha(1-3)$ and $\alpha(1-6)$ arms.

In investigating the possible structural and functional significance of these conclusions, our studies are continuing with attempts to define any conformational differences between glycoprotein-derived oligosaccharides of different structure. Preliminary results utilizing the above techniques as well as 2-dimensional NMR techniques have unexpectedly indicated that in free solution the spatial orientation of the $\alpha(1-3)$ arm may be invariant [13].

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