MOLECULAR DYNAMICS OF SUGARS BOUND TO WHEAT GERM AGGLUTININ, AS STUDIED BY DEUTERIUM NUCLEAR MAGNETIC RESONANCE

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ABSTRACT Deuterium nuclear magnetic resonance is used to delineate the molecular dynamics of sugars bound to a lectin. ^2H spin-spin relaxation times (from linewidth measurements) and reorientational correlation times are determined for *N*-acetylglucosamine specifically-labeled with ^2H in the *N*-acetyl group and at carbon-3 of the pyranoside ring, in the presence and absence of wheat germ agglutinin. The correlation time for the ^2H-label of *N*-acetylglucosamine-3-^2H in the bound state is the same as that of the protein (3×10^{-8}s), indicating that the six-membered ring has negligible motional freedom relative to the protein. The correlation time for the C^2H_3 group of *N*-acetyl-$^2H_3$-glucosamine (1.7×10^{-9}s) shows that the *N*-acetyl side chain is also immobilized in the binding site, the only motion available being rotation of the C^2H_3 group about its threefold axis.

INTRODUCTION

Lectins are widely used as probes for the investigation of cell surface carbohydrates involved in biological recognition between cells, and in phenomena such as the selective binding by cells of enzymes, hormones, and toxins; the immune response; the control of cell differentiation and growth; and in malignant transformation of cells (1). Recently, lectins have also been shown to be useful tools for the fractionation of lymphocytes into biologically distinct subpopulations (2). However, despite the rapidly increasing literature on the use of lectins in the study of cell surface carbohydrates, very little is as yet known in detail about the dimensions and specificities of the recognition sites of most of these proteins.

Nuclear magnetic resonance can provide detailed information on the interactions between carbohydrates and lectins. The relaxation times of ^1H and ^{13}C, for example, have been used to determine distances from the nucleus under observation in methyl glycosides to a paramagnetic site in Mn-concanavalin A (3–5). The ultimate goal of such studies is identification of the amino acid residues on the protein and the groupings on the sugar molecule involved in carbohydrate binding, and an understanding of the structure and dynamics of lectin-carbohydrate complexes. Groupings involved in sugar binding should be restricted in their rotational motion due to interactions, and thus probing the molecular dynamics in the presence and absence of protein or sugar should provide information on the involvement of the monitored groups in carbohydrate binding. We have therefore investigated the feasibility of

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substitution of ^1H by ^2H, and the use of ^2H NMR to delineate the molecular dynamics of sugars bound to a lectin.

^2H relaxation is dominated by the intramolecular quadrupole mechanism, which makes it ideal for the study of intramolecular rotational motion. Due to the sensitivity of linewidths to subtle changes in correlation times, ^2H NMR can give a detailed picture of rotational motion of specifically-^2H labeled groups of a molecule in the binding site of a protein (for a general review of the method, see reference 6). This technique has, for example, been used to study internal motions of ^2H-labeled EDTA binding to several proteins (7) and of methyl *N*-acetyl aminoglucosides binding to lysozyme (8). So far, however, it has not been applied to the study of molecular dynamics of carbohydrates bound to lectins.

As an example, we have determined ^2H relaxation times and rotational correlation times for *N*-acetylglucosamine[1] specifically labeled with ^2H in the *N*-acetyl group and at carbon-3 of the pyranoside ring, in the presence and absence of wheat germ agglutinin, a lectin specific for GlcNAc (9).

MATERIALS AND METHODS

Chemicals

D-Glucosamine hydrochloride was obtained from Pfanstiehl Inc. (Waukegan, Ill.). Acetic anhydride-$^2H_6$ was from Merck Sharp & Dohme Canada Ltd. (Montreal, P.Q.). ^2H-depleted water was from Aldrich Chemical Co. (Milwaukee, Wis.).

Preparation of ^2H-labeled Sugars

NAc-$^2H_3$-GlcNAc was synthesized by direct *N*-acetylation of glucosamine with acetic anhydride-$^2H_6$, according to the method of Horton (10). Purity was checked by paper chromatography in n-butanol/ethanol/water, vol/vol/vol 4:1:5, detection with (*a*) 2% $AgNO_3$ in acetone, and (*b*) NaOH in ethanol. GlcNAc-3-^2H, synthesized as described in reference 11, was a gift from Dr. D.R. Bundle of the Division of Biological Sciences, National Research Council, Ottawa.

Purification of WGA

Wheat germ was purchased locally. Crude WGA was obtained by acid extraction followed by precipitation with ammonium sulfate according to the procedure of Bassett (12). WGA was purified from this crude extract by affinity chromatography on ovomucoid-Sepharose in acetate buffer (0.1 M sodium acetate, 0.1 M NaCl, pH 5.0). The lectin was eluted from the column with 0.05 N HCl. The ovomucoid-Sepharose column was prepared by linking 500 mg of ovomucoid (trypsin inhibitor, Sigma Chemical Co., St. Louis, Mo.) to 10 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) according to the manufacturer's procedure. A 1 × 20 cm column bound ~200 mg of WGA. The yield of WGA was roughly 100 mg/lb of wheat germ. The lectin migrated as one band of 17,000 mol wt on a 15% SDS-polyacrylamide gel in the presence of mercaptoethanol.

NMR Samples

NMR samples were made up in aqueous buffer, containing 0.1 M sodium acetate, 0.1 M NaCl, at pH 5.0. For experiments with GlcNAc-3-^2H, samples were made up in ^2H-depleted water. The protein concentration was maintained at 0.1 mM in WGA monomers. The concentration of the deuterated sugar varied between 1 and 50 mM. All ^2H NMR spectra were recorded in 10-mm (2-ml sample

[1] *Abbreviations used in this paper:* GlcNAc, *N*-acetylglucosamine; NAc-$^2H_3$-GlcNAc, *N*-acetyl-$^2H_3$-glucosamine: GlcNAc-3-^2H, *N*-acetylglucosamine-3-^2H; WGA, wheat germ agglutinin.

volume) tubes on a Bruker CXP-300 spectrometer (Bruker Instruments Inc., Billerica, Mass.), at 46.1 MHz, using quadrature detection. The temperature of the probe was regulated by a Bruker BVT 1000 temperature unit and monitored with a thermocouple in the probe. In the experiments with NAc-$^2H_3$-GlcNAc, the observed line broadening effects were not large (up to ~8 Hz); they were therefore corrected for magnetic field inhomogeneity using the HDO half-bandwidth. Spectra were recorded with a recycle time of 1.8 s, with 8K data points. Linewidths at half height were measured from 16K zero-filled spectra, and are estimated to be accurate to ±0.5 Hz. In the experiments with GlcNAc-3-^2H, samples were made up in ^2H-depleted water, since the ^2H resonance of this sugar is very close to that of HDO. In view of the large broadenings observed for this derivative (up to 20 Hz), samples were not spun and spectra were recorded with 0.11 s recycle time and 1K data points. Line widths at half height were measured from 2K zero-filled spectra, and are estimated to be accurate to ±1 Hz.

THEORY

Broadening of the NMR resonance of a small molecule due to partial binding to a protein can be treated by the method of Swift and Connick (13). The observed spin-spin relaxation rate, $1/T_{2obs}$, for a nucleus undergoing chemical exchange between the free and protein-bound states, is given by:

$$\frac{1}{T_{2obs}} = \frac{1}{T_{2free}} + \frac{f}{T_{2M} + \tau_M}, \tag{1}$$

where $1/T_{2free}$ is the relaxation rate of the free sugar, T_{2M} is the spin-spin relaxation time in the bound environments, f is the fraction of sugar bound (ratio of bound to total sugar), and τ_m is the lifetime in the bound state ($1/k_{off}$). This equation assumes that the chemical shift difference between the free and bound state is negligible and that the free sugar is in large excess over the bound sugar ($f \ll 1$). In this case, only a single resonance is observed in the ^2H NMR spectrum, broadened due to exchange with a small fraction of bound sugar. For an excess of sugar, $[S] \gg n[P]$, the concentration of the sugar-protein complex is given by

$$[SP] = \frac{n[P] \cdot [S]}{K_d + [S]},$$

where $n[P]$ is the concentration of protein binding sites, $[S]$ is the total sugar concentration, and K_d represents the dissociation constant of the sugar-protein complex. With $f = [PS]/[S]$ and $1/\Delta\nu_{obs} = \pi(T_{2obs} - T_{2free})$ one obtains:

$$[S] = \frac{1}{\Delta\nu_{obs}} \frac{n[P]}{\pi(T_{2M} + \tau_m)} - K_d. \tag{2}$$

A plot of $[S]$ versus $1/\Delta\nu_{obs}$ yields the dissociation constant K_d from the y intercept, while the slope yields the bound linewidth, $\Delta\nu_{bound} = 1/\pi(T_{2M} + \tau_M)$. If the exchange rate $1/\tau_M$ is known, or can be neglected, the spin-spin relaxation time T_{2M} in the bound environment can be calculated. In the absence of internal motion, the spin-spin relaxation time for a ^2H nucleus in the bound state is given by (14):

$$\frac{1}{T_{2M}} = \frac{3}{8}\left(\frac{e^2qQ}{\hbar}\right)^2 f(\tau_c), \tag{3}$$

where

$$f(\tau_c) = \frac{1}{20}\left(6\tau_c + \frac{10\tau_c}{1 + \omega^2\tau_c^2} + \frac{4\tau_c}{1 + 4\omega^2\tau_c^2}\right), \tag{4}$$

and τ_c is the effective correlation time of the label in the bound environment, $e^2qQ/\hbar$ and ω are the quadrupole coupling constant and the resonance frequency (in radians per second), respectively. The quadrupole coupling constant of ^{2}H in a C—^{2}H bond is relatively insensitive to changes in molecular structure not affecting the C-^{2}H carbon itself, and therefore is not expected to alter on binding of the ^{2}H-labeled sugar to the protein (6). It may be taken equal to 174 kHz, as observed in many such compounds (6). From the T_{2M} value obtained from Eq. 2, the correlation time of the label can thus be calculated using Eq. 3. The requirement that relaxation is exponential outside the extreme narrowing region is satisfied for ^{2}H with a spin of $I = 1$ (14). Therefore, Eqs. 1 and 2 are valid even in the absence of extreme motional narrowing.

Effect of Internal Rotation

If internal rotation of the ^{2}H-label in the bound environment is present, the correlation time of the label depends on the rate of both internal rotation and overall rotation of the protein. Two correlations times have to be considered: τ_R for rotational diffusion of the protein (assumed to be isotropic), and τ_i for internal rotation. In the diffusion model for internal rotation, the effective correlation time for the label is (15):

$$\tau_c = \frac{1}{4}(3\cos^2\theta - 1)^2\tau_R + 3\sin^2 2\theta\left(\frac{1}{\tau_R} + \frac{1}{\tau_i}\right)^{-1} + \frac{3}{4}\sin^4\theta\left(\frac{1}{\tau_R} + \frac{1}{\tau_i}\right)^{-1}, \tag{5}$$

where θ is the angle between the axis of rotation and the label axis. If the rate of internal rotation is much faster than that of protein rotation, the last two terms in Eq. 5 can be neglected. In the case of a ^{2}H-labeled methyl group, the internal rotation could also be described by a jump model. In this model, instantaneous random jumps occur between three positions, 120° apart, at an average rate of $(3\tau_i)^{-1}$ (15). In this case the effective correlation time is given by:

$$\tau_c = \frac{1}{4}(3\cos^2\theta - 1)^2\tau_R + \frac{3}{4}(\sin^2 2\theta + \sin^4\theta)\left(\frac{1}{\tau_R} + \frac{1}{\tau_i}\right)^{-1}. \tag{6}$$

Again, if internal rotation is much faster than that of the protein, the last term in Eq. 6 can be neglected, and the same result is obtained as in the rotational diffusion description (Eq. 5). The effective correlation time of the label is thus simply a series of factors times the correlation time of the protein, which is assumed to undergo isotropic rotational diffusion. Each internal rotation, which is faster than protein rotation (by a factor of ten or more, as shown in reference 16), contributes a single factor $1/4(3\cos^2\theta - 1)^2$, where θ is the angle from the internal rotation axis of interest to the next internal rotation axis, or, for the last internal rotation, to the label axis of interest.

RESULTS AND DISCUSSION

WGA is specific for GlcNAc and has been shown to be a dimer of mol wt 34,000 at pH 5.0, with two carbohydrate binding sites per monomer (17). ^{1}H NMR studies of the binding of GlcNAc to WGA (18) have shown that WGA does not distinguish between the α- and the β-anomer of GlcNAc. Binding experiments were therefore carried out with the reducing sugars at pH 5.0. Figs. 1 *A* and *C* show the ^{2}H NMR spectra of NAc-$^2H_3$-GlcNAc and GLcNAc-3-^{2}H in buffer at pH 5.0. The ^{2}H signal of the C^2H_3 group of NAc-$^2H_3$-GlcNAc is narrow because of the short correlation time for reorientation of the methyl group (6×10^{-12}s, see Table I). Due to the much longer correlation time of 1.3×10^{-10}s for the ^{2}H label in the six-membered ring of GlcNAc-3-^{2}H, the ^{2}H signal for this compound is broad, with a half width of ~20 Hz. In the presence of WGA, the ^{2}H resonances of both sugars show considerable line broadening, as displayed in Figs. 1 *B* and *D*. For similar fractions bound as used in the present study (up to ~0.02), in ^{1}H NMR spectra, at 220 MHz and 36°C, the methyl protons of the *N*-acetyl group of GlcNAc experience an upfield shift of ~4 Hz upon binding to WGA (18). In the 46 MHz ^{2}H NMR spectra, however, the corresponding chemical shift change would be only ~0.8 Hz and is therefore negligible. Eqs. 1 and 2 are thus valid. Fig. 2 shows a plot, according to Eq. 2, of the total sugar concentration [*S*] versus the reciprocal line broadening, for the binding of the two specifically-deuterated sugars to WGA at 35°C. Dissociation constants (K_d), bound linewidths ($\Delta\nu_b$), and spin-spin relaxation times (T_{2M}) in the bound environment for the two sugars are summarized in Table I. The K_d values obtained in the present ^{2}H NMR study are in good agreement with those determined by ^{1}H NMR (18). The τ_M values used for the calculation of T_{2M} from the bound linewidths, obtained from ^{1}H NMR studies of the binding of GlcNAc to WGA (18), are included in Table I.

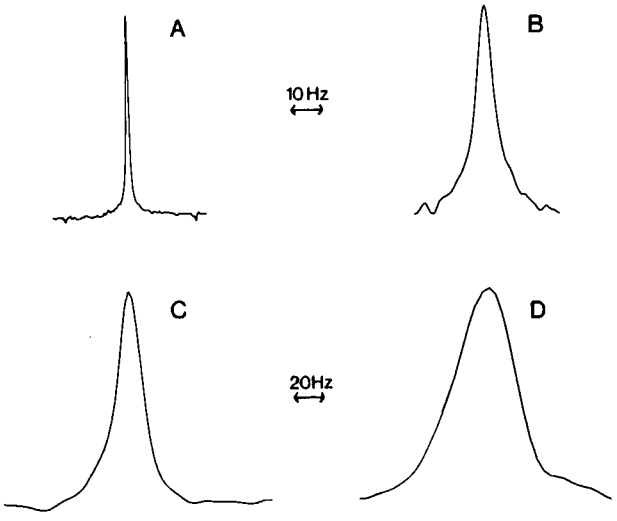

FIGURE 1 ^{2}H NMR spectra (46.1 MHz) of NAc-$^2H_3$-GlcNAc) and GlcNAc-3-^{2}H in the presence and absence of WGA. *A* and *C* are the spectra of the free sugars in 0.1 M acetate buffer, pH 5.0; *B* and *D* are the corresponding spectra of 1.2 mM NAc-$^2H_3$-GlcNAc and 3.7 mM Glc-NAc-3-^{2}H in the presence of 0.1 mM (monomer concentration) WGA. All spectra were recorded at 35°C.

TABLE I

LINEWIDTHS ($\Delta\nu$), SPIN-SPIN RELAXATION TIMES (T_2), AND ROTATIONAL CORRELATION TIMES (τ_c) FOR NAc-$^2H_3$-GlcNAc AND GlcNAc-3-^2H IN THE PRESENCE AND ABSENCE OF WGA

Sugar	T	$\Delta\nu_{free}$*	$\Delta\nu_{bound}$	T_{2free}‡	T_{2bound}	τ_{cfree}§	τ_{cbound}	τ_M‖	K_d
	(°K)	*(hertz)*	*(hertz)*	*(milli-seconds)*	*(milli-seconds)*	*(seconds)*	*(seconds)*	*(milli-seconds)*	*(milli-molar)*
NAc-$^2H_3$-GlcNAc	298	1	113.3	318	2.0	7×10^{-12}	1.3×10^{-9}	0.8	5.0
	308	0.82	198.8	388	1.6	6×10^{-12}	1.7×10^{-9}	0.1	9.3
GlcNAc-3-^2H	308	18.7	1017.4	17	0.21	1.3×10^{-10}	3×10^{-8}	0.1	8.0

*Calculated from the observed linewidth, using the HDO half-bandwidth as correction factor.
‡Calculated from the corrected linewidth, $T_2 = 1/\pi\Delta\nu$.
§Calculated from T_2, according to $1/T_2 = (3/8)\,(e^2qQ/\hbar)^2\,\tau_c$.
‖Determined by ^1H NMR (18).

Corrections for τ_M have to be made, since the observed ^2H nuclei are in the regime of intermediate exchange with the protein. In the case of GlcNAc-3-^2H, the experiment could not be carried out at 25°C, due to the short T_{2M} of 0.21 ms. At 25°C, the τ_M value (0.8 ms, Table I), would be longer than T_{2M}, and the ^2H nucleus would be in a slow exchange with the protein.

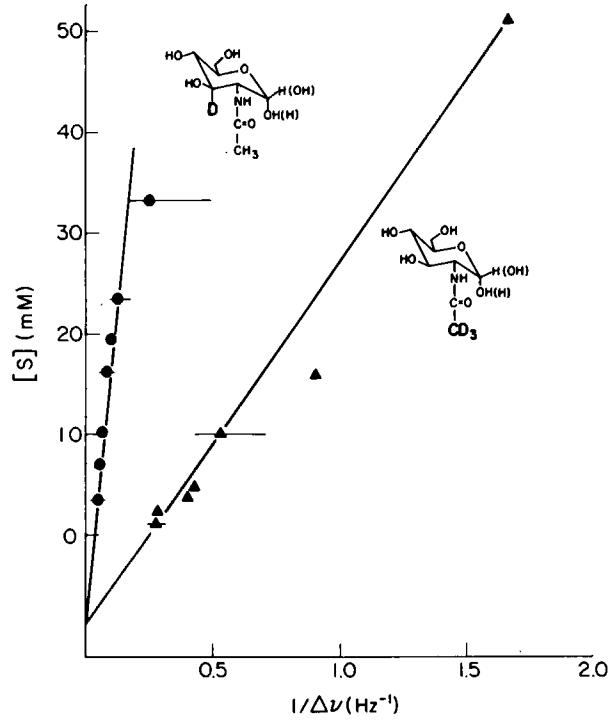

FIGURE 2 Plot of the total sugar concentration [S] versus the reciprocal line broadening $1/\Delta\nu$ for the binding of NAc-$^2H_3$-GlcNAc and GlcNAc-3-^2H to WGA at pH 5.0 and 35°C.

The correlation time for rotational diffusion of WGA was calculated using the Stokes-Einstein relationship, $\tau_R = 4\pi\eta r^3/3kT$, where r is the radius of the molecule and η is the viscosity of the solution. An effective radius of 25 Å was used in this calculation, as derived from the x-ray structure of WGA (19). The correlation time τ_R of WGA was calculated to be 1.6×10^{-8}s at 35°C. As seen from Table I, the correlation time for the ^{2}H-label in GlcNAc-3-^{2}H was found to be the same within a factor of two, i.e. 3×10^{-8}s, indicating that the six-membered ring does not have any significant degree of motion relative to the protein (any rapid motion within the binding site).

The correlation time for the ^{2}H-label in NAc-$^2H_3$-GlcNAc, 1.7×10^{-9}s (Table I), is much shorter than that of the protein, indicating some internal motion of the label in the bound site. As the label is in a methyl group, one could expect rapid rotation about the threefold axis. If rotation of the C^2H_3 group were at least ten times more rapid than that of the protein, both the rotational diffusion model (12) and the three jump model (12) for methyl-rotation would predict a correlation time of $\tau_{obs} = \tau_R\, 1/4\, (3\cos^2 109° - 1)^2 = 0.11\, \tau_R = 1.7 \times 10^{-9}$s, which is in excellent agreement with the τ_C calculated from the observed line broadening, 1.7×10^{-9}s. This suggests no other fast internal rotations are present in the *N*-acetyl side chain, as they would lead to a shorter predicted correlation time.

The linewidths, T_2 values, and correlation times for the ^{2}H labels of the two sugars free in solution, and in the binding site of WGA, are compared in Table I. They demonstrate a significant reduction of intramolecular motion of the sugar in the bound state. The pyranoside ring, and the *N*-acetyl side chain up to the methyl group, are immobilized on the protein; the only fast internal motion still present is rotation of the methyl group about its threefold axis. ^{2}H NMR spectra of solid samples of Nac-$^2H_3$-GlcNAc, and of methylglycosides ^{2}H-labeled in the methyl group, show quadrupole splittings of ~40 kHz, demonstrating rapid rotation of this group about its threefold axis even in the solid.[2] The ^{2}H NMR study shows clearly that the pyranoside ring binds as a rigid unit, and emphasizes the importance of the *N*-acetyl group for binding.

CONCLUSION

The present study shows that ^{2}H NMR can give a detailed picture of intramolecular motion of a sugar in the binding site of a lectin. Limitations of this technique, however, can arise due to the short relaxation times of ^{2}H and the large contribution of the exchange rate τ_M^{-1}. For motional information to be extracted, the line broadening effects must be dominated by T_{2M}, i.e., the observed ^{2}H nucleus must be in fast, or, if τ_M is known, intermediate exchange. Since ^{2}H relaxation times are very short for correlation times of $\sim 10^{-8}$s, the requirement for short τ_M values may be difficult to satisfy in some cases. However, as τ_M decreases with increasing temperature, intermediate or fast exchange conditions can be achieved by temperature elevation. ^{2}H NMR is an excellent, nonperturbing method for the study of internal rotations in a bound, small molecule. By using labeled groups of different shapes or sizes, it should be possible to see at what point internal rotations become restricted, thus revealing details of the

[2]Neurohr, K. J., and I. C. P. Smith. Unpublished results.

geometry of the binding site. Extension of the method by labeling of residues on the protein thought to be involved in carbohydrate binding is also possible.

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