Localization of the Binding Site for the Oligosaccharide Moiety of Gb_3 on Verotoxin 1 Using NMR Residual Dipolar Coupling Measurements†

Gary S. Thompson,‡ Hiroki Shimizu,‡ Steve W. Homans,*,‡ and Art Donohue-Rolfe§

School of Biochemistry and Molecular Biology, University of Leeds, LS2 9JT, U.K, and Department of Comparative Medicine, Tufts University School of Veterinary Medicine, 200 Westboro Road, North Grafton, Massachusetts 01536

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ABSTRACT: By use of NMR residual dipolar coupling measurements in a dilute liquid-crystalline solvent, the solution structure has been determined of the complex between the oligosaccharide moiety of globotriaosylceramide (Gb_3-OS) and the B-subunit homopentamer of verotoxin 1 (VTB). The dipolar coupling data indicate that Gb_3-OS binds in a single binding site per monomer, which is identical to one of three sites inferred from the X-ray structure of the same complex. We find no evidence within experimental error for occupancy at either of the two additional binding sites observed per monomer in the crystal structure.

The *Escherichia coli* verotoxins are responsible for microvascular disorders such as haemolytic uremic syndrome (*1*), which is the principal cause of acute pediatric renal failure. The toxins comprise an enzymatic A subunit in association with a B subunit homopentamer that binds to a specific cell-surface carbohydrate (*2*−*4*) which is globotriaosylceramide (Gb_3;[1] Galα1−4Galβ1−4Glc-Cer) in the case of verotoxin-1. The inhibition of this interaction is the basis of a new proposed therapy (*5*). The crystal and NMR structures of verotoxin-1 B subunit (VTB) have been solved in the absence (*6*, *7*) and presence of the oligosaccharide moiety of Gb_3 (Gb_3-OS) (*8*, *9*). The crystal structure of the complex exhibits three binding sites for the glycan. The first, termed site 1, is similar to the site predicted by Nyholm et al. (*10*, *11*) and is characterized by a stacking interaction of Galβ with the side chain of Phe 30. The second site (site 2) is topologically equivalent to the binding sites found in the other OB fold proteins, and is similar to a second site predicted by Nyholm et al. The third site (site 3) involves a stacking interaction of Galβ on the side chain of Trp 34. In our recent study on the solution structure of the complex (*9*), we found that only site 2 is predominantly occupied in solution. This is supported by a recent study using fluorescence resonance energy transfer (*12*) which suggests that a coumarin Gb_3 analogue binds in a site analogous to site 2, but with a different orientation to that observed in the crystal structure. The transferred nuclear Overhauser effect (TR-NOE) techniques that were used in our NMR study are of insufficient accuracy to permit a distinction between the two proposed orientations. Here, we make use of NMR residual dipolar coupling measurements on the complex in the weakly aligned state which provides convincing evidence that site 2 is occupied in solution, with an orientation of the glycan that is indistinguishable, within experimental error, from that observed in the crystal.

MATERIALS AND METHODS

Protein and Ligand Preparation. VTB was expressed and purified as described (*13*, *14*). Uniformly ^{13}C-enriched Gb_3-OS was prepared from uniformly ^{13}C-enriched glucose by chemical synthesis using a route previously developed in our laboratory (*15*). Gb_3-OS was prepared for NMR studies by dissolution of 0.21 mg. of lyophilised glycan in 603 μL of 7.5% (w/v) dimyristoylphosphatidylcholine/dihexanoylphosphatidylcholine (DMPC/DHPC) (3:1 w/w) in 99.96% D_2O doped with 1 mM tetradecyltrimethylammonium bromide (TTAB). Prior to dissolution in the liquid-crystalline medium, the amount of glycan was verified by ^1H NMR following dissolution in 99.96% D_2O in the presence of 1 mM methanol as standard. For titration studies, the Gb_3 in DMPC/DHPC solution was added directly to aliquots of lyophilised VTB (1.16 mg dry weight). The latter were prepared by lyophilisation of a standard solution of VTB whose concentration was determined by optical density (ϵ_{280} 1 mg/mL = 1.073). Following each addition of VTB the degree of magnetic alignment of DMPC/DHPC bicelles was assessed by measurement of the residual quadrupolar splitting of the solvent deuterium resonance (*16*).

Measurement of Residual Dipolar Couplings. One-bond ^{13}C-^1H residual dipolar couplings for uniformly ^{13}C-enriched Gb_3-OS were measured by use of conventional HSQC experiments in the absence of ^{13}C decoupling in the F2 dimension. Spectral widths were 1200 Hz in the F_2 (^1H) and 10 kHz in the F_1 (^{13}C) dimension, with 4096 and 256 complex datapoints, respectively. Prior to Fourier transformation, data were apodized with cosine-bell weighting functions and zero-filled once, giving a final dataset of 4096 × 256 real datapoints. Residual dipolar couplings for the

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* To whom correspondence should be addressed Phone: 0113 233 3125. Fax: 0113 233 3167. E-mail: s.w.homans@leeds.ac.uk.

‡ University of Leeds.

§ Tufts University School of Veterinary Medicine.

[1] Abbreviations: DHPC, dihexanoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; Gb_3, globotriaosylceramide; Gb_3-OS, globotriaosylceramide trisaccharide; VTB, verotoxin-1 B subunit.

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free ligand were determined from the difference in splittings of the ligand in DMPC/DHPC solution at 25 and 35 °C, corresponding to the isotropic and liquid crystalline phases, respectively. The change in residual dipolar couplings following titration of the ligand with VTB was similarly followed at 35 °C.

Data Analysis and Determination of Order Tensor Components. Values of one-bond ^{13}C-^{1}H residual dipolar couplings for the ligand in the bound-state were determined from a plot of fraction of ligand bound versus measured residual dipolar coupling, corrected for the residual dipolar coupling of the free ligand. The former was determined from the known concentrations of VTB and Gb_3-OS, together with the K_d for the association (2×10^{-3} M) as reported (*17*). The residual dipolar couplings for the bound ligand were determined by extrapolation to 100% bound ligand. While in principle there are three binding sites per VTB monomer for Gb_3-OS (*8*), in previous work (*9*) we showed that the K_ds for Gb_3-OS at two of these sites are >6 mM. At the ligand:protein ratios used in the present study, the fraction of ligand bound at these lower affinity sites is $<\sim$3.5%, and hence the use of a single K_d value is a good approximation. The axial and rhombic components of the alignment tensor were determined from the measured residual dipolar couplings by use of singular value decomposition as described (*18*). The principal axis direction of the orientation tensor was taken as the symmetry axis of VTB (*19*). All structural computations were undertaken using the program XPLOR (*20*) modified for structural refinement using residual dipolar couplings with a fixed external axis (*21*). Energy minimization was achieved using the standard conjugate gradient minimizer within XPLOR until the norm of the gradient of the total energy was less than 0.001 kcal/mol.

RESULTS AND DISCUSSION

The approach that we have chosen to adopt in order to determine the mode of binding of Gb_3 to VTB involves the measurement of residual ^{13}C-^{1}H dipolar couplings for the ^{13}C-enriched ligand in the complex. As has been adequately demonstrated, residual dipolar couplings can readily be measured for solutes in lyotropic liquid-crystalline media such as DMPC/DHPC (*16*). In the system under study here, the ligand is in fast exchange on the NMR time scale between the free and bound states, and the measured residual dipolar coupling represents a weighted average over these states. To determine the residual dipolar coupling values for the bound-state of the ligand, the latter was thus titrated with VTB. The measured residual dipolar coupling, corrected for the contribution from the ligand in the unbound state, was then plotted versus fraction of ligand bound. The residual dipolar couplings for the bound state were then determined by extrapolation to 100% bound ligand (Figure 1), and are given in Table 1. It is notable, particularly in the case of Galβ C-2, that the measured residual dipolar coupling differs from those derived from other bond vectors (i.e., C1−H1, C3−H3, C5−H5) which one would presume to be collinear. This phenomenon has been observed in other studies (*19*) and in the present case can be rationalized by noting that small changes in this particular orientation of these vectors ($\sim$5°) can reduce the measured dipolar coupling by 50%. To extract structural information from these dipolar couplings, the orientation of the principal frame and order parameters of the alignment tensor for the complex must be known. As demonstrated recently by Prestegard and co-workers (*18*), these can be obtained by use of singular value decomposition. For this purpose, the five residual dipolar coupling values measured for the Galα residue of the trisaccharide were used as input to the program ORDERTEN_SVD (*18*), giving rise to the principal component of the orientation tensor Szz = -1.1×10^{-2} and a rhombic component $R = 0.23 \pm 0.2$. Given that VTB has 5-fold axial symmetry, the principal axis of the alignment tensor lies by definition along the symmetry axis of the molecule (*19*). Thus, these parameters are sufficient to enable calculation of theoretical residual dipolar couplings for the trisaccharide in each of the three

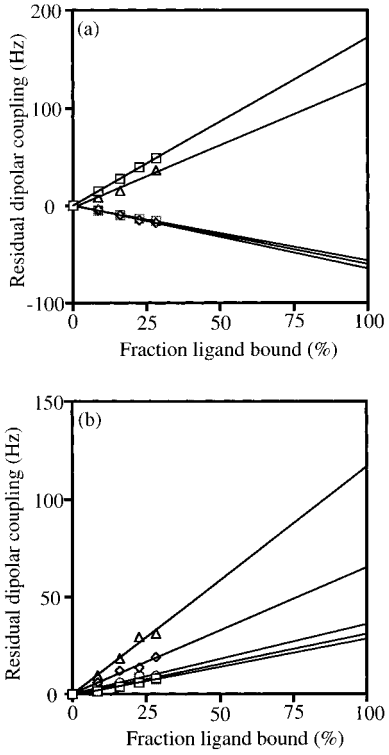

FIGURE 1: Plots of measured residual dipolar coupling versus fraction ligand bound in a titration of VTB with Gb_3-OS in a dilute (7.5%) liquid-crystalline medium comprising DHPC/DMPC (1:3) doped with 1 mM TTAB. (a) Residual dipolar couplings for Galα: (□) C1−H1; (◇) C2 −H2; (○) C3−H3; (△) C4−H4; (⊞) C5−H5. (b) Residual dipolar couplings for Galβ: (□) C1−H1; (◇) C2−H2; (○) C3−H3; (△) C4−H4; (⊞) C5−H5.

Table 1: Experimental Residual Dipolar Couplings Measured for Gb_3 in Complex with VTB versus Theoretical Values Derived from the Crystal Structure of the Complex

	residual dipolar coupling (Hz)			
atoms	crystal site 1	site 2	site 3	exptl[a]
Galα H1−C1	−96.1	161.1	−46.0	172.4
Galα H2−C2	44.6	−67.2	32.1	−65.3
Galα H3−C3	48.3	−54.9	31.4	−60.0
Galα H4−C4	−104.2	148.0	−60.5	127.5
Galα H5−C5	18.2	−71.0	28.0	−56.0
Galβ H1−C1	53.2	66.6	−98.5	29.0
Galβ H2−C2	−4.1	69.2	−113.0	64.8
Galβ H3−C3	−37.0	68.7	−105.4	35.8
Galβ H4−C4	−41.6	103.9	247.3	110.6
Galβ H5−C5	6.9	69.3	−107.3	38.2

[a] Estimated error in these measurements is ±10%.

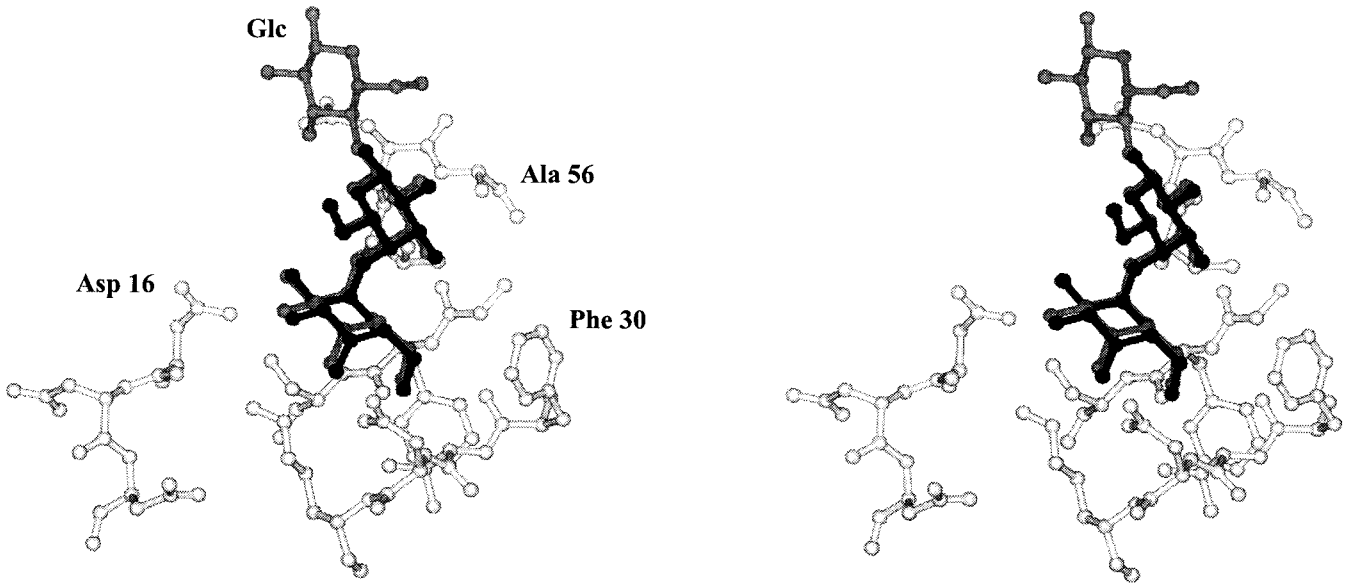

FIGURE 2: Stereodiagram illustrating the structure of Galα1−4Galβ1−4Glc in site 2 of the crystal structure of the Gb_3-OS−VTB complex (gray) compared with the structure of the Galα1−4Gal fragment (black) derived from the present study.

binding-sites observed in the crystal structure (Table 1). Residual dipolar coupling values for the Glc residue of the trisaccharide were not included in these analyses due to overlap between resonances of each anomer. It can readily be seen that only one of the binding sites in the crystal structure is comparable with experimentally determined residual dipolar couplings in both magnitude and sign, namely site 2. The solution structure of the complex was thus determined by minimizing the site 2 glycan orientation with respect to the symmetry axis of the protein using the experimental residual dipolar couplings as restraints and with the crystal structure as the starting conformation. As can be seen in Figure 2, the solution data give rise to an orientation of the glycan in site 2 that is essentially identical to the crystal structure. Since no restraint violations resulted from minimization, it is clear from Table 1 that the residual dipolar couplings are very sensitive to the small structural differences between the crystal and solution structure at site 2. It is therefore extremely unlikely that an alternative conformation in this site would be consistent with the measured residual dipolar couplings. Moreover, these data support our earlier observations based on TRNOE measurements (*9*) that site 2 is the only site that is substantially occupied in solution. In contrast to TRNOE measurements, residual dipolar couplings are not influenced by the phenomenon of spin diffusion, and hence the structure of the complex can be delineated with confidence without knowledge of the disposition of nuclear spins within the protein binding site. This is clearly of paramount importance when studying complexes where the location of the binding site is unknown.

Our NMR observations appear at first sight to be at variance with the crystallographic study. However, the cocrystal was obtained from a solution containing 50 mM ligand (*8*), and hence it would be anticipated that sites with very high K_ds (> 1 mM) would be occupied in the crystal. In contrast, a much lower concentration of ligand (0.70 mM) was utilized in our NMR study. With regard to physiological relevance of the binding sites observed it must be borne in mind that the association observed in the present study is monovalent in character, whereas recognition of Gb_3 at the cell surface is likely to be a polyvalent association. It is well-known that polyvalent associations give rise to greater affinities than the equivalent monovalent association and are generally additive in terms of free energy minus a contribution that Jencks has referred to as the "connection Gibbs energy" (*22*). Since the association in turn is a logarithmic function of the standard free energy of binding, in practical terms the weaker binding affinities exhibited at sites I and III may contribute to binding under physiological conditions. From the point of view of design of ligand analogues, however, a suitable strategy might be to focus on the high-affinity site. In this regard, it is noteworthy that the STARFISH inhibitor of the Gb_3-VTB interaction developed by Bundle and co-workers to bridge sites 1 and 2, in fact binds exclusively to site 2 in two adjacent VTB molecule in the crystal structure (*23*).

The approach described here represents a general method for the delineation of the structures of ligand−protein complexes. In this particular application, the use of ^{13}C-enriched ligand is mandatory, since the protein concentration far exceeds the ligand concentration in the later stages of the titration of Gb_3-OS with VTB. However, if lower accuracy in the measured residual dipolar couplings for 100% bound-ligand can be tolerated, the latter can be determined from a single-point measurement at lower protein:ligand ratios. Measurement of heteronuclear residual dipolar couplings then becomes feasible at natural abundance, especially with the advent of cryo-probe technology.

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