Derivation of the Bound-State Conformation of a Ligand in a Weakly Aligned Ligand−Protein Complex

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Most carbohydrate−protein interactions are characterized by weak affinities (K_d < 10^{-5} M). Under these circumstances, discrete resonances for the free and bound forms of the ligand are not observed in NMR spectra—the ligand is in fast exchange, giving rise to a resonance whose chemical shift is the weighted average of the shifts in each state. This phenomenon is advantageous since transferred nuclear Overhauser effect (TRNOE) connectivities that characterize the bound state can be observed between resonances of the free state by virtue of the exchange process, and these resonances possess favorable line widths and resonance intensities compared with the bound state.[1−12] In addition, it is commonplace to work with a very high ligand:protein ratio (typically 15:1), which gives rise to substantial TRNOEs without interference from protein resonances. Despite these advantages, the number of intraligand TRNOEs is often small,[11] and the bound-state conformation of the ligand can be determined with limited accuracy. Moreover, unless the locations of protons within the protein binding site are known, it becomes very difficult to utilize the full-relaxation matrix calculations that are required to account for spin-diffusion within the slowly tumbling complex. If this spin-diffusion process is not accounted for, erroneous conclusions regarding the bound-state conformation of the ligand can occur.[13,14] Here, we describe an alternative method for the derivation of the bound-state conformation of a ligand by measurement of residual dipolar couplings in the exchanging system.

Recently, Tjandra and Bax demonstrated that macromolecules dissolved in a dilute liquid crystalline (LC) medium composed of dihexanoylphosphatidylcholine (DHPC)/dimyristoylphosphatidylcholine (DMPC) bicelles exhibit a tunable degree of orientational alignment.[15] Under these circumstances, dipolar couplings between NMR active nuclei do not average to zero as in the isotropic case, and the measured dipolar coupling depends on the cylindrical coordinates describing the orientation of the relevant bond vector PQ in the principal axis system of the alignment tensor (**A**):[16]

$$D_{PQ}(\theta,\phi) = S\frac{\mu_o}{4\pi}\gamma_P\gamma_Q h\Big[A_a(3\cos^2\theta - 1) + \frac{3}{2}A_r\sin^2\theta\cos 2\phi\Big]/4\pi^2 r^3_{PQ}$$

where S is the generalized order parameter for internal motion of the bond vector PQ, μ_o is the magnetic permeability of vacuum, γ_P and γ_Q are the magnetogyric ratios of P and Q, h is Planck's constant, r_{PQ} is the distance between P and Q, A_a and A_r are the axial and rhombic components of **A**, and θ and ϕ are cylindrical coordinates describing the orientation of the vector PQ in the principal axis system of **A**. In the present study, we exploit the fact that the alignment of the ligand−protein complex will be substantially larger than that of the free ligand.[17] The measurable dipolar couplings in an LC solution of the ligand plus protein will thus be a weighted average of the couplings in the two states in the fast exchange regime, and since the values in each state will be very different, the values of the dipolar couplings in the bound state can readily be determined, provided a significant fraction of ligand is bound under the relevant experimental conditions. We illustrate this approach in the determination of the conformation of the trisaccharide ligand Galα1−4Galβ1−4Glc in ^{13}C-enriched form, when bound to the B-subunit homopentamer of the toxin derived from *Escherichia coli* O157.[18] It has recently been shown by TRNOE measurements that the trisaccharide binds principally to a single binding site per B-subunit monomer in solution.[11] The anomeric region of the ^{13}C−^{1}H HSQC spectrum of the trisaccharide (100 μM) in a 7.5% solution of DHPC:DMPC (1:3) at 35 °C is shown in Figure 1a. Shown in Figure 1b is a similar spectrum recorded after addition of B-subunit in 5-fold molar excess over the trisaccharide. In each case, the residual dipolar couplings can be measured for each ^{13}C−^{1}H bond vector in the glycan by comparison of the observed splittings in F_2 in Figure 1a and b (which correspond to $^1J_{C,H} + {}^1D_{C,H}$) with those observed for the free trisaccharide in DHPC:DMPC solution in the isotropic phase at 25 °C (which correspond to $^1J_{CH}$, data not shown). Knowledge of the affinity of the trisaccharide for the B-subunit ($K_d = 2 \times 10^{-3}$ M)[19] together with the concentrations of protein and ligand is sufficient to compute the fraction of ligand bound (19%), from which the

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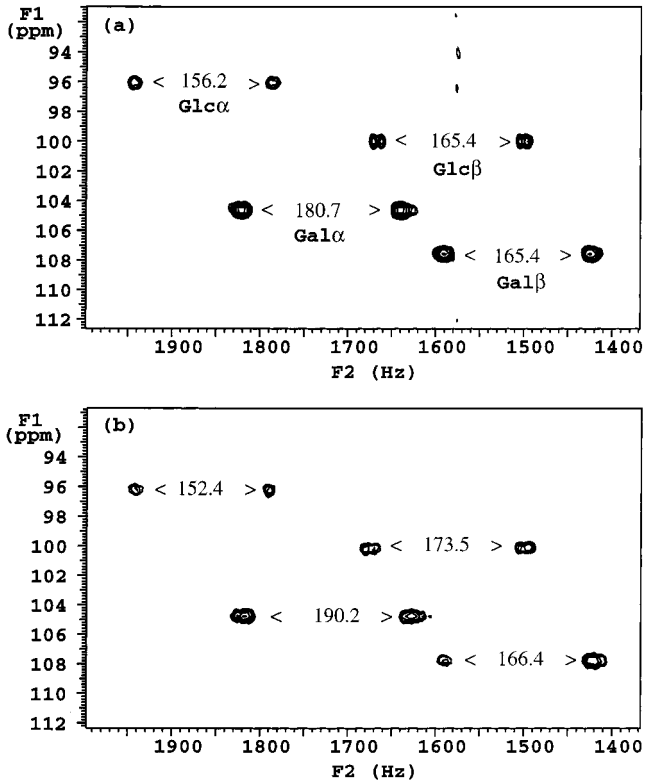

Figure 1. (a) Anomeric region of the $^{13}C-^{1}H$ HSQC spectrum of uniformly ^{13}C-enriched (~95%) Galα1−4Galβ1−4Glc (100 μM) in 7.5% (w/v) DHPC/DMPC (1:3) solution containing 50 mM NaCl, pH 7.0. No ^{13}C decoupling was used in the acquisition (F_2) dimension. (b) Similar spectrum recorded after the addition of B-subunit in 5-fold molar excess (based on binding sites).

residual dipolar couplings for the complex (100% bound ligand) can be derived (Table 1). These couplings can then be utilized in the derivation of the bound-state conformation of the ligand by use of conventional dynamical simulated annealing calculations incorporating dipolar coupling restraints.[20] The dipolar couplings listed in Table 1 were utilized in such calculations, using as input a series of 10 geometries of the trisaccharide with randomized glycosidic torsion angles. The magnitudes of the axial (A_a = −68.1) and rhombic (A_r = −6.8) components of **A** were determined by consideration of the extreme values of the measured dipolar couplings, followed by iterative fitting of these values to obtain the lowest restraint violations.[20] A single family of structures resulted from these simulations (Figure 2), whose rmsd for the heavy atoms of Galα and Galβ with respect to the average structure was better than 0.5 Å. The Glc residue was very disordered due to the limited number of measurable dipolar restraints for this residue. The conformation of the Galα1−4Gal moiety of this family of structures is similar to those observed in the crystal structure of the complex[21] and to the recently derived solution structure of the complex derived from full-relaxation matrix TRNOE calculations.[11] In the present case, the bound-state conformation of the ligand can be determined without interference from protein-mediated effects, and with substantially lower concentrations of materials than are required for TRNOE measurements.

We note that not only does this approach gives rise to angular information with which to define accurately the bound-state conformation of the ligand, but also it could be used to define the orientation of the ligand with respect to the protein, since all dipolar couplings in the complex are defined with respect to a single alignment tensor. For this purpose, it would be convenient to measure residual dipolar $^{15}N-^{1}H$ couplings in the ^{15}N-enriched protein[20] in the absence and presence of ^{13}C-enriched ligand. This approach offers the additional advantage that the axial and rhombic components of the alignment tensor for the complex can be more accurately defined from the powder pattern of observed couplings.[22]

Table 1. Residual Dipolar Couplings Measured for the Trisaccharide Galα1−4GalB1-4Glc in Free Solution and in Complex with the B-Subunit Homopentamer of the Toxin Derived from *E. coli* O157

	$^{1}D_{C,H}$ (Hz)[a]		
bond vector	free[b]	+B-subunit[c]	bound[d]
	Galα		
H-1−C-1	+8.7	+18.2	+57.9
H-2−C-2	−9.7	−12.6	−24.8
H-3−C-3	−11.3	−14.6	−28.4
H-4−C-4	+7.1	+14.7	+46.5
H-5−C-5	−9.6	−12.9	−26.9
	Galβ		
H-1−C-1	+0.6	+1.6	+5.8
H-2−C-2	nd[f]	nd	nd
H-3−C-3	+2.1	+2.1	+2.1
H-4−C-4	0.0	+8.8	+45.6
H-5−C-5	+1.7	+2.0	+3.1
	Glcβ[e]		
H-1−C-1	+3.6	+11.7	+45.6
H-4−C-4	+6.0	+13.4	+44.6

[a] Error in measurement = ±1 Hz. [b] Difference between C−H splittings measured for the trisaccharide (0.1 mM) in the isotropic (25 °C) and liquid crystalline phases (35 °C). [c] Difference between C−H splittings measured for the trisaccharide (0.1 mM) in the isotropic (25 °C) phase and B-subunit:trisaccharide (5:1 molar ratio based on binding sites) in the liquid cystalline phase (35 °C). [d] Calculated value of $^{1}D_{C,H}$ for 100% bound ligand. [e] Extreme resonance overlap with Glcα prevented determination of a complete set of endocyclic couplings for this residue with sufficient accuracy. [f] nd, not determined.

Figure 2. Family of 10 bound-state structure of Galα1−4Galβ1−4Glc obtained from dynamical simulated annealing calculation with dipolar restraints derived from the dipolar couplings listed in Table 1. Calculations were performed with 10 geometries of the trisaccharide with randomized glycosidic torsion angles as input, which were subjected to slow cooling from an intial temperature of 750 K. Force constants for dipolar couplings were slowly increased from 0.001 to 1.0 kcal mol^{-1} Hz^{-2} over the time course of the simulation. The force field utilized in these simulations is that described,[23] with all exo-anomeric torsion terms set to zero.

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