Structural and Conformational Analysis of Glycan Moieties *in Situ* on Isotopically $^{13}C,^{15}N$-Enriched Recombinant Human Chorionic Gonadotropin†

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ABSTRACT: The conformational properties in solution of the glycans on the α subunit of recombinant human chorionic gonadotropin are described, using high-resolution multinuclear NMR studies on uniformly $^{13}C,^{15}N$-enriched recombinant glycoprotein expressed in CHO cells. The glycan important for full biological activity of hCG, namely, that at Asn 52, appears to extend into solution both in the isolated α subunit and in complex with the *β* subunit. The disposition of this glycan with respect to the protein backbone suggests that glycosylation maintains full biological activity of hCG either by interacting with a lectin-like region of the hCG receptor or by reducing the affinity of the hormone for the hCG receptor and preventing its down-regulation.

The glycan moieties of many glycoproteins appear to be essential for full biological activity of the parent protein. The essential role of glycohormone glycans is well-known [reviewed by Sairam (1989)] and was investigated in detail in human chorionic gonadatropin (hCG) by Calvo and Ryan (1985). These workers observed that glycopeptides from hCG inhibited adenylate cyclase activation in corporeal luteum slices in a competitive manner, thus suggesting a bipartite structure for the functional receptor consisting of separate subsites which bind protein and carbohydrate and which must be cross-linked for effective signal transduction. More recent work has demonstrated that one of the two N-linked glycans on the α subunit, namely, that at Asn 52, is of primary importance for biological activity (Matzuk et al., 1989).

Recently, the crystal structure of hCG has been published, which suggests a putative receptor-binding region on the protein (Lapthorn et al., 1994; Wu et al., 1994). Unfortunately, however, structural details of the N-linked glycan at Asn 52 could not be determined in view of the requirement for HF deglycosylation of the protein prior to crystallization. We therefore have attempted to define the conformational behavior of the glycan on the intact glycoprotein by high-resolution NMR methods. Such methods have been proven to be of value in the study of a variety of glycoproteins, and indeed preliminary data have been published for the glycan *in situ* on the isolated α subunit of native hCG (de Beer et al., 1994). In the latter study, the carbohydrate resonances were detected by use of heteronuclear ($^{13}C-^{1}H$) NMR studies at natural abundance, exploiting the fact that carbohydrate carbon resonances appear in a region of the spectrum devoid of protein resonances. However, due to the low inherent sensitivity of natural abundance methods, the majority of resonance assignments for the glycan, a prerequisite for structural studies, could not be obtained. In the present work we analyze recombinant hCG expressed in mammalian cells which has been uniformly enriched with ^{13}C and ^{15}N. The high level of enrichment with ^{13}C enables essentially complete proton and carbon resonance assignments to be obtained for the N-linked glycan at Asn 52, using a novel approach based upon a conventional heteronuclear NMR technique known as HCCH-TOCSY (Bax et al., 1990). These assignments enable the primary sequence of the glycan to be delineated *in situ* both on the α subunit and in the intact glycoprotein, and a comparison of these resonance shifts with those in the free glycan together with line-width considerations suggests a rather dynamic behavior of the glycan in each case. The facile nature of the assignment protocol suggests that this approach may be of general value in the analysis of glycans *in situ* on intermediate-sized recombinant glycoproteins. To our knowledge this is the first NMR study of a uniformly isotopically $^{13}C,^{15}N$-enriched glycoprotein.

METHODS

Sample Preparation. Uniformly isotopically labeled hCG and subunits were obtained by expression of hCG by transfected CHO cells (Lustbader et al., 1995) grown with a newly developed medium made from algal hydrolysates containing isotope-labeled amino acids. The expressed, labeled hCG was purified and separated into subunits by reverse-phase HPLC as described (Lustbader et al., 1995). Details concerning expression of uniformly labeled hCG as well as its characteristics are described in a separate report (Lustbader et al., 1996).

A sample (5.7 mg) of the uniformly $^{13}C,^{15}N$-enriched α subunit of hCG was utilized for studies on the α subunit alone. A partially enriched sample of hCG was obtained by incubation of this sample with 7 mg of native *β* subunit in 0.2 M ammonium bicarbonate at 37 °C for 4 h, followed by lyophilization. All samples were dissolved in 0.7 mL of

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99.96% ^2H_2O, pH 5.5, containing 0.3 M NaCl for all NMR studies (de Beer et al., 1994) except HNCA spectroscopy, where 95% (v/v) $H_2O/^2H_2O$ replaced ^2H_2O.

Enzymatic resialylation of the asialo-α subunit of hCG in the holoprotein containing isotopically enriched α subunit was achieved by incubation of the holoprotein at 37 °C for 24 h with 100 milliunits of α2−6 sialyltransferase in 100 mM phosphate buffer (pH 7.4) containing 10 mM CMP-NeuNAc.

NMR Measurements. Two-dimensional HCCH-TOCSY spectra were recorded at a nominal probe temperature of 45 °C using the three-dimensional pulse scheme of Bax et al. (1990), by maintaining the t_1 evolution period constant. The delays τ, $\delta 1$, and $\delta 2$ in this sequence were 1.5, 1.1, and 1.1 ms, respectively. A total of 512 complex points and 256 complex points were acquired in the t_2 and t_1 dimensions, respectively, with spectral widths of 1200 and 15000 Hz. The ^1H carrier was located at 4.55 ppm, and the ^{13}C carrier was located at 82 ppm, i.e., between the anomeric (C-1) carbon resonance positions (∼100 ppm) and the endocyclic carbon resonance positions (∼70 ppm) of the glycans, rather than in the conventional location (∼43 ppm) for the assignment of protein side chain resonances. A modest (6 KHz) DIPSI spin-lock field of duration 24 ms resulted in efficient suppression of protein resonances together with virtually complete coherence transfer through the carbon skeleton of each monosaccharide residue. A constant-time version of this experiment has been devised and applied to ^{13}C-enriched glucans (Yu et al., 1993). However, in the present application constant time evolution is best avoided in view of the relatively short spin−spin relaxation times of ^{13}C resonances of protein-bound glycan residues.

Two-dimensional constant-time HNCA spectra were recorded at a nominal probe temperature of 65 °C using the pulse scheme described by Grzesiek and Bax (1992), keeping the t_2 (^{15}N) evolution time constant. This elevated temperature was chosen following the work of de Beer et al. (1994) in order to minimize resonance line widths which in turn improves considerably the sensitivity of the HNCA experiment. Spectral widths of 3600 and 10 000 Hz were utilized with 1024 and 128 complex datapoints in the F_3 and F_1 dimensions, respectively. A total of 512 transients were recorded for each t_1 increment, with a relaxation delay of 1 s giving a total acquisition time of ∼24 h. The carrier was located at 8.5 ppm in F_3 and 55 ppm in F_1. Prior to two-dimensional Fourier transformation, data were apodized with Gaussian broadening functions (or with a $\pi/8$ phase-shifted sine-bell function in t_1 in the case of the resolution-enhanced spectrum), and the t_1 dimension was zero-filled to 256 complex points.

Three-dimensional ^{13}C-edited NOESY spectra were acquired at a nominal probe temperature of 45 °C as a compromise between usable resonance line widths and maintenance of physiological conditions, using conventional NOESY-HSQC with a mixing time of 140 ms and with spectral widths of 7900, 2000, and 2000 Hz in F_1 (^1H), F_2 (^{15}N), and F_3 (^1H) and 64, 16, and 1024 complex points in t_1, t_2, and t_3, respectively. The carrier was located at 4.4 ppm in F_1, 78 ppm in F_2, and 4.4 ppm in F_3. Prior to Fourier transformation, data were apodized with cosine-bell functions, and the F_1 and F_2 dimensions were zero-filled to 128 and 32 complex points, respectively.

Molecular Modeling. The X-ray crystal structure of hCG represents only the partial glycosylation at Asn 52. Thus it was necessary to model build the glycan onto the crystal structure of the protein. The coordinates of the glycan were model built using InsightII (Biosym Technologies), using values for the glycosidic dihedral angles reported previously (Rutherford & Homans, 1994). The resulting glycan fragment was then attached to the side chain of Asn 52, making a bond between Nδ2 (Asn 52) and C1 of the reducing terminal GlcNAc by a simple transformation. Rotations were performed around the bonds involving the Asn 52 atoms, viz, Cα−Cβ, Cβ−Cγ, and Nδ2−C1(GlcNAc) in steps of 30° while maintaining the glycosidic dihedral angles fixed, resulting in creation of an ensemble of 12^3 structures of the glycan attached to Asn 52. Each of these conformers was examined for steric contacts between the atoms of the glycan and the protein, based on a procedure by Ramachandran et al. (1968), described as follows: A limiting distance of approach for a pair of nonbonded atoms, less than the sum of their van der Waals radii, was developed by these workers using a "steep-well" potential. Any conformation in which the interacting atoms fell within this distance was considered disallowed with proper allowance for the pairs having potential of making hydrogen bonds. This resulted in 30 sets of "allowed" dihedral values for the Asn−GlcNAc linkage. Torsion angles about the carbohydrate−protein linkage are defined as χ_1 = N, Cα, Cβ, Cγ, χ_2 = Cα, Cβ, Cγ, Nδ$_2$, ϕ' = H1, C1, Nδ$_2$, Cγ, where H1 and C1 represent the anomeric proton and carbon atoms of the reducing terminal GlcNAc residue.

Molecular Dynamics Simulations. The conformer (60°, 180°, 30°) was arbitrarily chosen from the set of 30 allowed dihedral values as input for a restrained MD simulation. All the calculations were performed using Discover 3.0 (Biosym Technologies). The force-field parameters used were those of Ha et al. (1988) with the incorporation by Homans (1990) for oligosaccharides but with all torsional terms across the glycosidic linkage set to zero. The parameters for the bond between Nδ2 (Asn52) and C1 (GlcNAc) were treated analogously to those of the glycosidic linkages in nucleic acid residues in AMBER. A solvent sphere of 25 Å was constructed around the C1 atom of the GlcNAc residue attached to Asn 52 incorporating 1726 water molecules. For the purpose of energy minimization and MD simulation, only those amino acid residues falling within 12 Å from the backbone (N) of Asn 52, the carbohydrate, and the solvent molecules (amounting to 6046 atoms) were allowed to move. The system was energy minimized with the NOE restraints observed experimentally, for 200 cycles using the "steepest descent" algorithm, and then with the "conjugate gradient" method for an additional 9000 cycles. The nonbonded interactions were calculated with a cutoff of 14 Å. The system was equilibrated at 300 K for 10 ps with a time step of 0.5 fs. In order to prevent solvent "evaporation", the water atoms in the shell between 15 and 25 Å were restrained with a force of 500 kcal/Å. The production run was then carried out until 125 ps, storing the history every 0.5 fs. The final 100 ps of the history was taken up for the analysis.

RESULTS

The one-dimensional ^1H NMR spectrum of the ^{13}C- and ^{15}N-enriched α subunit acquired with broad-band ^{13}C decoupling is shown in Figure 1A. While there are clearly a

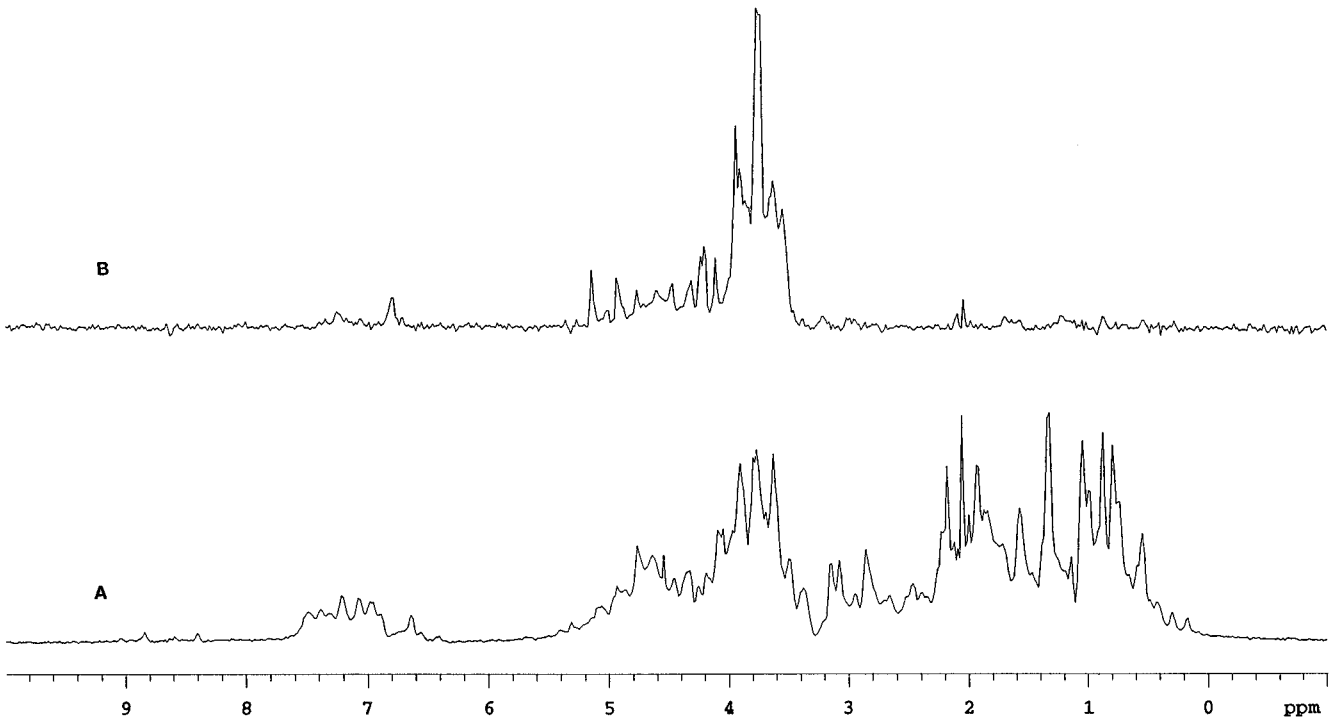


FIGURE 1: (A) 500 MHz ^{1}H NMR spectrum of the uniformly ^{13}C,^{15}N-enriched α subunit of hCG obtained with broad-band ^{13}C decoupling. (B) One-dimensional HCCH-TOCSY spectrum, obtained under conditions designed for selective observation of carbohydrate resonances. See text for details.

significant number of resonances in the spectral window which characterizes the majority of the resonances for free glycans, it is not possible to distinguish these from protein resonances in the proton spectrum. However, the ^{1}H and ^{13}C resonances for the glycan moieties can be observed selectively with efficient suppression of protein resonances by exploitation of the fact that ^{13}C resonances for carbohydrate moieties do not appear in the same region of the spectrum as those derived from the protein. Such "carbohydrate filtration" can be achieved using a technique termed HCCH-TOCSY originally developed for the assignment of side chain resonances in isotopically enriched proteins (see Methods). The resulting spectrum is essentially devoid of protein resonances, as shown in Figure 1B. While in principle the structural features of the glycans can be delineated by inspection of the anomeric proton resonance positions in the 1D spectrum using conventional "fingerprinting" (Vliegenthart et al., 1983), in practice it is much more efficient to acquire a two-dimensional spectrum comprising ^{13}C resonances in the F_1 dimension and ^{1}H resonances in the F_2 dimension, whereby both the proton and carbon assignments can be utilized for structural analysis. As shown in Figure 2A, the resulting two-dimensional spectrum displays carbon resonances for a given monosaccharide residue in the glycans in the F_1 dimension at a given proton shift in F_2. Provided the spin-lock time is sufficiently long, coherence transfer between all coupled carbons takes place, giving rise to a complete carbon spectrum for each monosaccharide in a suitable trace parallel to F_1. Similarly, all of the proton resonances for a given monosaccharide residue appear in a suitable trace parallel to F_2.

By consideration of proton resonance positions it is clear that the major glycan in the recombinant α subunit is of the asialobiantennary complex type, as evidenced by the identity (within experimental error) of the characteristic anomeric proton fingerprint resonances in comparison with those of the free glycan (Vliegenthart et al., 1983). Additional supporting evidence for this structural assignment is provided by the carbon resonance assignments for each monosaccharide, since mannose, galactose, and *N*-acetylglucosamine exhibit characteristic carbon resonance positions as monosaccharides (Jansson et al., 1989). In relative terms, these are much less sensitive than the proton resonances to glycan sequence and hence can be used to confirm monosaccharide type. An important exception is the resonance position of the glycosylated carbon, which invariably is shifted substantially to lower field in comparison with the shift in the monosaccharide. This provides important confirmatory evidence for the proposed structure. For example, the carbon resonances for Man-4 obtained from the trace parallel to F_1 at $F_2 = 5.13$ ppm are close to the shifts observed in free mannose. However, a resonance at the characteristic shift position of C-2 is absent, and instead a resonance is apparent at $F_1 = 77.8$ ppm, thus confirming that this mannose is glycosylated at the 2 position. Similarly, the carbon resonances for Man-3 obtained from the trace parallel to F_1 at $F_2 = 4.76$ ppm compare with the shifts observed in free mannose, with the exception of C-3 and C-6 which are both shifted to lower field, the latter overlapping C-4. This confirms the glycosylation of Man-3 at C-3 and C-6. Of particular relevance are the carbon shifts of the C-2 resonances of each of the GlcNAc resonances, which resonate at characteristically high field. *N*-Acetylhexoses can obviously never be glycosylated at C-2, and hence this characteristic shift is a valuable reporter of this type of residue.

As anticipated, the resonance shifts of GlcNAc-1 and to a lesser extent GlcNAc-2 differ from those of the free glycan, as a consequence of covalent association with the α subunit protein. These resonances are also significantly broader than those corresponding to monosaccharide residues in the antennae, which is a characteristic of the proximity of the

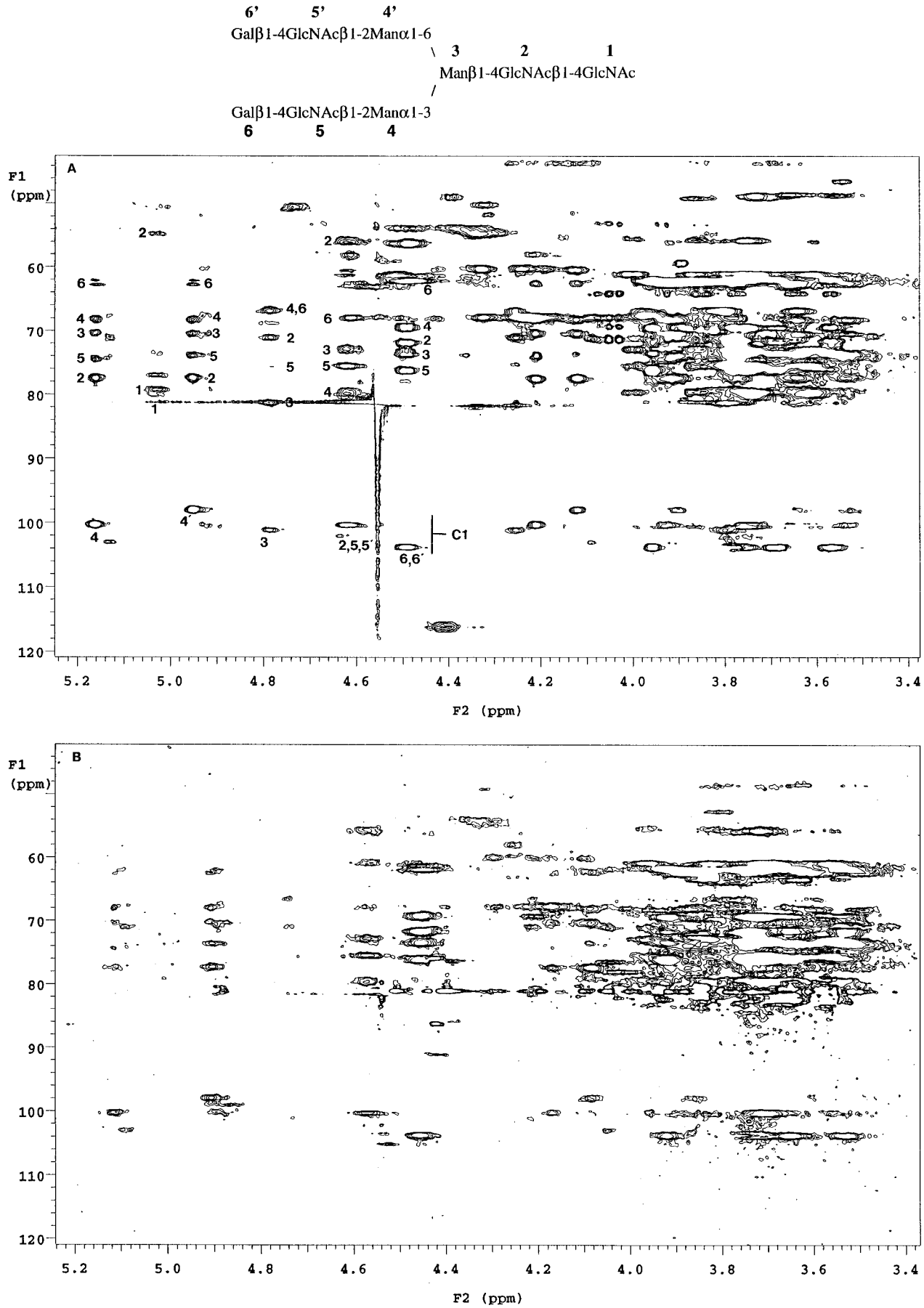


FIGURE 2: (A) Two-dimensional ($F_1 = {}^{13}C$, $F_2 = {}^{1}H$) HCCH-TOCSY spectrum of the hCG α subunit, obtained under conditions designed for selective observation of carbohydrate resonances. The deduced major glycan sequence at the two glycosylation sites is shown above the figure. Resonances from both glycans overlap. The figure below the C-1/H-1 cross peaks at ~100 ppm refers to the analogous residue in the glycan sequence above the figure, and the cross peaks are labeled in the F_1 dimension with the relevant carbon atom. (B) Spectrum acquired under conditions identical to those in (A) but following reassociation of the α subunit with the unlabeled β subunit.

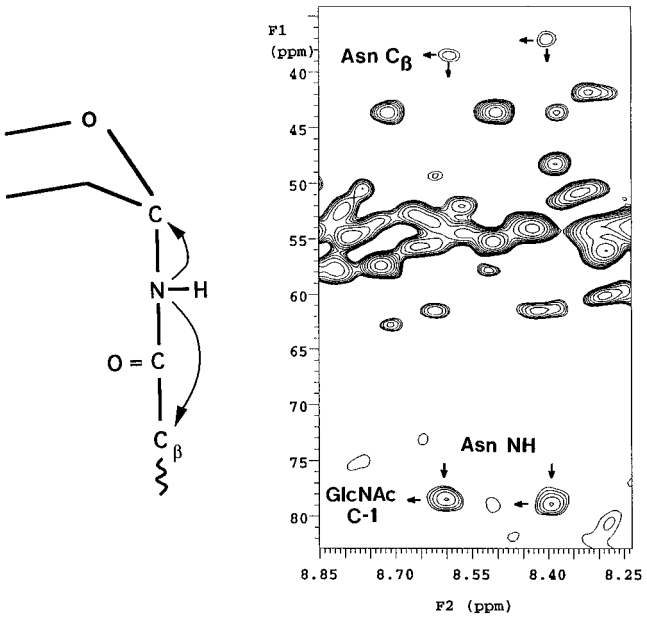


FIGURE 3: Region of the HNCA spectrum of the uniformly ^{13}C,^{15}N-enriched α subunit of hCG showing the correlations between GlcNAc-1 C-1 (see Figure 2 for nomenclature) and NH and Cβ of each glycosylated Asn residue. A diagrammatic illustration of the correlation pathway is shown at the left.

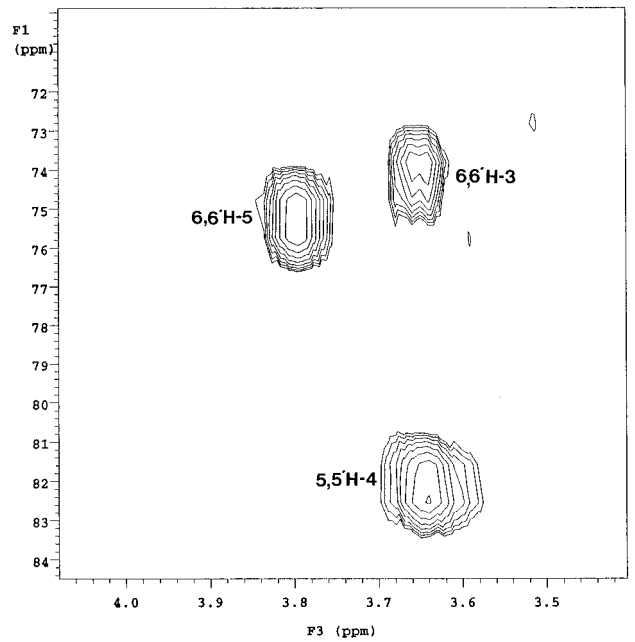


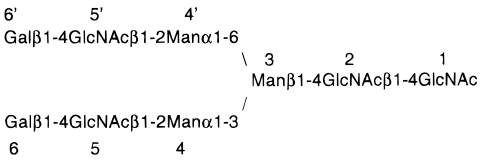
FIGURE 4: Slice from the $^{13}C-^{1}H$ NOESY-HSQC spectrum of the resialylated hCG α subunit at $F_2 = 4.44$ ppm (H-1 of Gal-6,6′; see Figure 2 for nomenclature), showing intraresidue NOEs to Gal H-5 and H-3 and a well-resolved interresidue NOE to GlcNAc-5,5′ H-4.

former to the slowly tumbling protein. As a corollary, these data suggest that there is significant internal motion within the glycan chain, in a manner analogous to that observed previously for the oligomannose glycan of bovine pancreatic ribonuclease B (Rutherford et al., 1993).

In addition to resonances derived from the major biantennary glycan, a series of weaker resonances can also be observed which are particularly prominent in the region of the proton and carbon resonance positions of Man-4 and Man-4′ and clearly correspond with these residues in a different glycan. A clue to the identity of the glycan giving rise to these resonances is given by the relevant carbon shifts. Of note is the absence of low-field carbon resonances assignable to C-4 for both Man-4 and Man-4′. In addition, the absence of weaker "ghost" resonances for either GlcNAc-5,5′ or Gal-6,6′ suggests that the glycan might be composed solely of the Manα1−6(Manα1−3)Manβ1−4GlcNAcβ1−4GlcNAc core sequence. In support of this, those proton resonances which can be detected for this glycan correspond essentially identically in shift with those for the authentic free glycan (S. W. Homans, unpublished data). Alternatively, there may be two discrete glycans with approximately equal populations, each of which terminates with Manα in only one of the antennae.

Assignment of Glycan Sequences. Since the α subunit of native hCG contains two N-linked glycans, the question arises whether the truncated glycan represents the full repertoire at either glycosylation site 52 or 78, in which case that site is not fully glycosylated by virtue of the much weaker intensities of the resonances from the truncated glycan, or whether the major glycan at each glycosylation site is of the biantennary type, whose resonances overlap. In order to resolve this issue, it is necessary to establish a covalent connectivity between the C-1 or H-1 of the proximal GlcNAc residues of each glycan with the respective asparagine side chain in the protein backbone. Since the glycoprotein under study is uniformly enriched with ^{13}C and ^{15}N, this connectivity can conveniently be defined by use of an HNCA experiment, originally devised for the correlation of backbone NH, N, and Cα resonances in proteins (Grzesiek & Bax, 1992). In application to the GlcNAc−Asn linkage, this technique in two-dimensional form gives correlations between the C-1 resonance of GlcNAc and the NH of the Asn side chain, as illustrated in Figure 3. It is seen that two discrete C-1−NH correlations are observed with approximately equal intensity. Since coherence transfer in the HNCA experiment relies upon one-bond correlations, cross peak intensity is to first order independent of local conformation and hence equal cross peak intensities provide unequivocal proof that the major resonances in the HCCH spectrum derive from the overlap of resonances from two biantennary glycans, one at each glycosylation site. Shift degeneracy is observed as far as the C-1 resonances of each GlcNAc, which are also almost degenerate and cannot be distinguished in the F_1 dimension of the HCCH spectrum due to insufficient digital resolution. It is of interest to note also that weak two-bond $^{15}N-^{13}C$ correlations can be detected in the HNCA

Table 1: NOE Restraints Used in the Restrained MD Simulation of hCG



NOE	size[a]	NOE	size[a]
2-H1−1-H4	S	5-H1−4-H1	S
3-H1−2-H4	S	5-H1−4-H2	S
3-H1−2-H6	W	4′-H1−3-H6	S
4-H1−3-H3	S	4′-H1−3-H6′	W
4-H1−3-H2	W	4′-H1−3-H5	W
3-H2−4-H5	M	5′-H1−4′-H2	S
		5′-H1− 4′-H1	S

[a] S = strong, 1.8 Å < r < 2.7 Å. M = medium, 2.7 Å < r < 3.3 Å. W = weak, 3.3 Å < r < 5.0 Å.

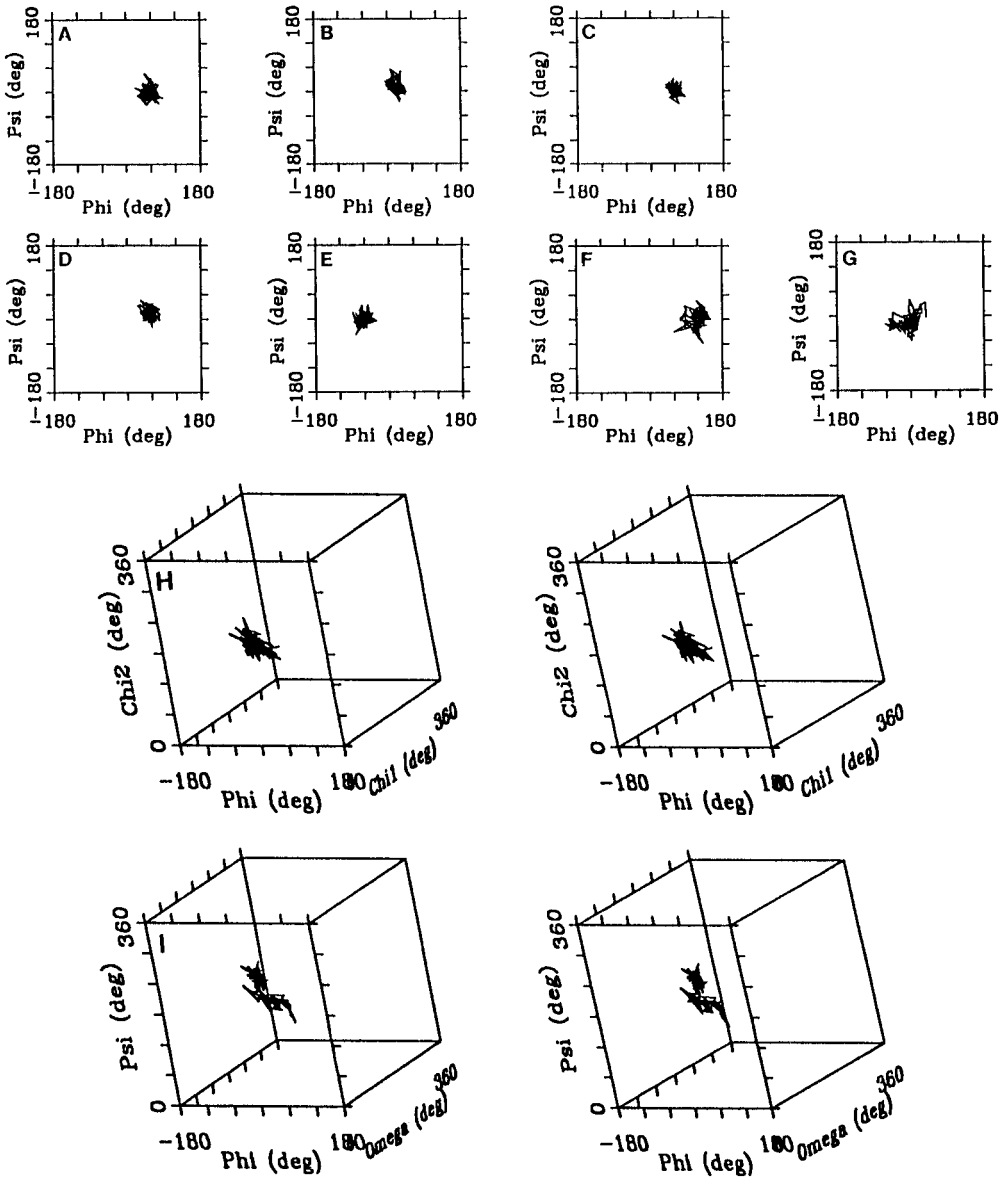


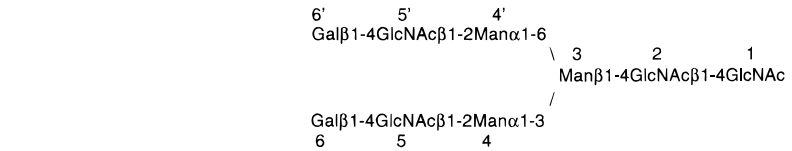
FIGURE 5: Plots of ϕ vs ψ for each glycosidic linkage in the biantennary glycan at Asn 52 derived from the last 100 ps of a 125 ps restrained molecular dynamics simulation of the hCG holoprotein. Also shown are plots of χ_1, χ_2, and ϕ' for the Asn 52 side chain and the N-glycosidic linkage. Panels refer to ϕ vs ψ for (A) Galβ1−4GlcNAc (α1−3 antenna), (B) GlcNAcβ1−2Man (α1−3 antenna), (C) Galβ1−4GlcNAc (α1−6 antenna), (D) GlcNAcβ1−2Man (α1−6 antenna), (E) Manα1−3Man, (F) Manβ1−4GlcNAc, and (G) GlcNAcβ1−4GlcNAc. (H) Stereoview of χ_1 vs χ_2 vs. ϕ' for Asn 52. (I) Stereoview of ϕ vs ψ vs ω for Manα1−6Man.

spectrum, which directly provide discrete assignments for Cβ of each Asn residue. This information could clearly be used to obtain site-specific assignments for each glycan once the protein resonances are assigned (work in progress). However, since the major glycan at each site is identical, this information is redundant in the present study.

Glycan Conformation in Intact hCG. Having established that the major glycan at each glycosylation site of the α subunit is of biantennary complex type, we sought to determine whether the conformational behavior of either glycan was modified in intact hCG. Since the β subunit of hCG is heavily glycosylated, we chose to recombine the uniformly enriched ^{13}C,^{15}N α subunit with the natural abundance ^{13}C,^{15}N β subunit. In this manner it is possible to observe selectively the carbohydrate resonances of the α subunit in the holoprotein using the HCCH-TOCSY technique described above, as shown in Figure 2b. It is immediately apparent that a significant number of resonances are substantially broadened as a result of the much larger rotational tumbling time of the holoprotein. In particular, the resonances of GlcNAc-1 virtually disappear, due to the proximity of GlcNAc-1 to the slowly tumbling protein. The resonances of the residues in both the α1−6 and α1−3 can, however, be readily observed, and by comparison with Figure 2A it is seen that there are no significant resonance shifts indicative of either modified conformational behavior of the glycans or interaction with the β subunit protein.

It is notable that the resonances of Man-4 are substantially broader than those of Man-4′. We interpret this as a natural consequence of the overall influence of the slowly tumbling protein, and the lesser line broadening of the resonances of Man-4′ can be attributed to the greater conformational mobility of the α1−6 antenna. In principle, it would be possible to quantify the extent of torsional fluctuations of the glycan in terms of the order parameter for individual $^{13}C-^{1}H$ vectors within each monosaccharide residue (Rutherford et al., 1993). However, in the present study the sensitivity of the techniques required for the measurement

Table 2: Initial and Time-Averaged Values of Torsion Angles of the Glycan at Asn 52 of hCG over the Time Course of 100 ps MD Simulation



	ϕ (deg)		ψ (deg)		ω (deg)	
linkage	initial	average	initial	average	initial	average
Gal-6−GlcNAc-5	60	58 (12)[a]	0	−2 (13)		
GlcNAc-5−Man-4	50	23 (11)	0	11 (13)		
Man-4−Man-3	−50	−62 (11)	−20	−1 (11)		
Gal-6′−GlcNAc-5′	60	60 (9)	0	2 (10)		
GlcNAc-5′−Man-4′	50	55 (11)	0	10 (12)		
Man-4′−Man-3	−60	−72 (11)[b]	180	−173 (13)	180	169 (12)
		31 (19)				63 (16)
Man-3−GlcNAc-2	60	120 (16)	0	2 (18)		
GlcNAc-2−GlcNAc-1	60	−5 (19)	0	−16 (17)		
χ_1	60	73 (12)				
χ_2	180	172 (14)				
ϕ'	30	−24 (17)				

[a] RMS deviations are given in parentheses. [b] Torsion angles ϕ and ω about this linkage adopt two values with an occupancy of 68% and 32%, respectively.

of the relevant ^{13}C relaxation parameters in the complex was insufficient for reliable quantitation.

Taken together, the above data suggest that the glycans at Asn 52 and Asn 78 in hCG do not interact with the protein and extend into solution with conformations similar to those observed in free solution. However, both glycans on the recombinant hCG studied here differ from those which are found in urinary hCG, namely, with regard to the absence of terminal Neu5Ac residues. The absence of sialic acid in the recombinant protein is at least partially a result of cleavage of these residues during the acid conditions used to prepare the α subunit. Since it has been reported that full biological activity is only apparent in hCG containing either the Neu5Acα2−6 or Neu5Acα2−3 group (Amano & Kobata, 1993), the possibility therefore remains that a specific carbohydrate conformation or carbohydrate−protein interaction which is necessary for full biological activity is manifest only in the fully sialylated glycoprotein. In order to address this point, Neu5Ac residues were enzymatically reintroduced into the holoprotein as Neu5Acα2−6Gal groupings. That the resialylation was complete was evidenced from the absence of resonances of the C-6's of Gal−6,6′ in the HCCH-TOCSY spectrum of the resialylated holoprotein (not shown). These resonances, which are found at ∼61.8 ppm in the asialoglycoprotein, are shifted substantially downfield as a consequence of glycosylation. However, no other substantial shift changes or line broadening were observed, indicating that the presence of terminating Neu5Ac residues does not alter the conformational behavior of the glycans.

In order to obtain more quantitative information on the conformational behavior of the glycan relevant to biological function at Asn 52, NOE connectivities were examined in both the asialo and the resialylated holoprotein by use of the ^{13}C NOESY-HSQC technique. Qualitatively, the observed NOE connectivities were in each case identical to those observed in the free glycan (Rutherford and Homans 1994). As anticipated, the relevant connectivities could more readily be extracted from the three-dimensional spectrum than from earlier studies on the free glycan (Rutherford & Homans, 1994), where severe resonance overlap was apparent in the ^{1}H−^{1}H NOESY spectrum despite the much narrower line widths. The increased resolution afforded by the ^{13}C dimension is readily apparent, for example, in Figure 4. This illustrates an F_1/F_3 slice from the ^{13}C NOESY-HSQC spectrum of the sialylated holoprotein at the F_2 resonance frequency of GlcNAc-6,6′ (4.44 ppm). Two intraresidue NOEs can readily be observed between Gal-6,6′ H-1 to H-3 and H-5. In addition, an interresidue NOE is observed between Gal-6,6′ H-1 to GlcNAc-5,5′ H-4. This information would not be apparent in the two-dimensional ^{1}H−^{1}H NOESY spectrum of the glycoprotein since the resonances of Gal-6,6′ H-3 and GlcNAc-5,5′ H-4 would clearly not be distinguishable due to a combination of line broadening and near identity of proton shifts. Despite the additional resolution afforded by the ^{13}C dimension, no NOE connectivities were observable between the glycan and the protein. This provides further evidence that the glycan exists in an extended conformation and probably enjoys considerable conformational freedom. Since the above NMR data were acquired at 45 °C in order to obtain usable line widths, then strictly it might be argued that a weak carbohydrate−protein interaction exists at physiological temperature which is disrupted under the experimental conditions employed. However, since the homonuclear and heteronuclear chemical shift pattern of the glycan in either the α subunit or the dimer was essentially unaffected by temperature over the range 30−65 °C (except for the expected exchange-related upfield shifts of amide proton resonances), we consider this possibility highly unlikely.

In order to probe the conformational space available to the glycan at Asn 52, the glycoprotein was subjected to a restrained molecular dynamics simulation using the NOE restraints obtained from the NOESY-HSQC spectrum (Table 1), with explicit inclusion of solvent water. As is apparent from plots of ϕ vs ψ for each glycosidic linkage over the time course of the simulation (Figure 5 and Table 2), the average conformation of the oligosaccharide is similar to that observed for the glycan in free solution (Rutherford & Homans, 1994) and further supports the experimental

observation that there is little constraint imposed by the protein. The torsion angles at the carbohydrate−protein linkage adopt average values of $\chi1 \sim +73°$, $\chi2 \sim +172°$, and $\phi' \sim -24°$, compared with those in the crystal structure (Wu et al., 1994) of +63°, +132°, and 78°, respectively (Table 2). Notably, the value of ϕ' is not consistent with the exoanomeric effect [reviewed by Bock (1983)] for a β-glycoside in either the crystal or solution structures, which suggests a local influence of the protein backbone on this torsion angle. The value of ϕ' predicted from the MD simulation is in good agreement with that determined from measurement of $^3J_{N\delta2,H\delta2}$ (6.0 ± 0.8 Hz) for each glycan, derived from the F_2 dimension of the resolution-enhanced HNCA spectrum, which translates to an experimental value of $\phi' \sim -40°$ using the appropriate Karplus equation (Wüthrich, 1986). In contrast, the measured value of $^3J_{N\delta2,H\delta2}$ is not consistent with the conformation about ϕ' in the crystal structure, which predicts a value of ~2.4 Hz. The values of χ_1 and χ_2 could not be validated experimentally, and it is probable that averaging occurs about these torsion angles, given that a variety of values of χ_1 and χ_2 are "allowed" on the basis of grid-search calculations (see Methods) and that conformational space is certainly not adequately sampled in the MD simulation which was of restricted length due to constraints of computational time. A representative conformation of the glycan at Asn 52 in covalent association with protein is shown in Figure 6A, and the extent of torsional fluctuation of this glycan over the time course of the molecular dynamics simulation is shown in Figure 6B.

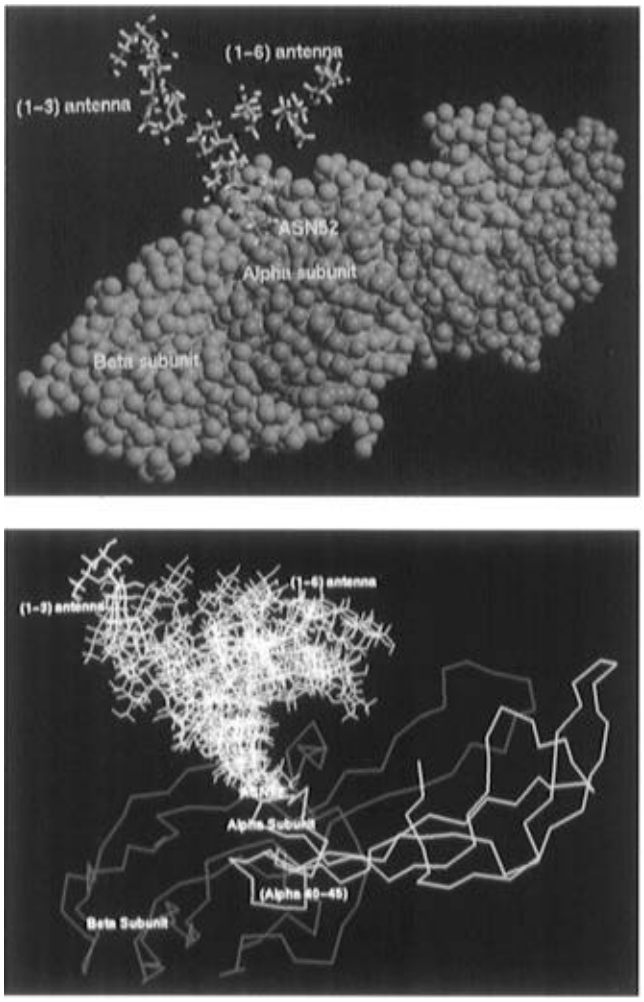


FIGURE 6: (A, top) Space-filling model of the conformation at 90 ps through the molecular dynamics simulation of the biantennary glycan at Asn 52 on the hCG holoprotein. Solvent water molecules have been omitted for clarity. (B, bottom) Representative molecular dynamics conformers of the biantennary glycan at Asn 52 on the hCG holoprotein to illustrate the extent of torsional fluctuation of the glycan over the time course of the simulation. The glycan is shown in yellow, the β subunit is in red, the α subunit is in blue, and the recognition determinant (Mengeling et al., 1995) recognized by the GalNAc-4-sulfotransferase (residues 40−45) on the α subunit is in green. The figures were generated with Raster3D version 2.0 (Merritt & Murphy, 1994).

DISCUSSION

The conformations of the glycans on the hCG α subunit, both for this subunit alone and in association with the β subunit, do not appear to differ substantially from the solution behavior of the free glycans, determined from measurement of ^{1}H−^{1}H NOESY connectivities within the glycans of the glycoprotein and from near identities of chemical shifts. No evidence could be obtained for any noncovalent interactions between the glycan at Asn 52 and the protein—no carbohydrate−protein NOE connectivities could be observed. The ^{13}C line widths of monosaccharide residues in each antenna of the glycan were substantially smaller than those in the core, which together with the results of restrained MD simulations on the glycoprotein suggest that each glycan enjoys considerable motional flexibility similar to that observed in the free solution state.

In the context of the earlier work of Calvo and Ryan (1985), the above observations would be consistent with a model of the receptor which recognizes the sterically unhindered oligosaccharide in a binding site different from that which binds the protein. However, given that an α2−3 or α2−6 terminated biantennary glycan is equally effective at maintaining maximal biological activity, it is likely that the negative charge on the carboxylate, rather than a specific conformational feature of the carbohydrate, is important for activity. It might be postulated that this dual carbohydrate/protein recognition is necessary to generate a conformational change in the receptor in order to transduce a signal across the membrane. In thermodynamic terms, this would be consistent with the observation that deglycosylation of hCG increases the affinity for the receptor. A conformational change in the hormone following deglycosylation is considered unlikely given the disposition of the carbohydrate at Asn 52.

Baenziger (personal communication) has proposed an alternative model whereby the deglycosylation and resulting increased affinity of hCG for the receptor results in its down-regulation. In this model the function of carbohydrate at Asn 52 is to reduce the affinity for the receptor, which would not require a specific binding site for carbohydrate. This model would also explain the observation that antibody binding to the β subunit of deglycosylated hCG can convert the antagonist to an agonist, since small structural changes or steric constraints induced by the antibody might readily reduce the affinity of deglycosylated hCG. The structural data presented in the current work are consistent with either of these models. A detailed understanding of the molecular basis of hormone action may require structural analysis of the complex between the hormone and the extracellular domain of its receptor.

Recently, it has been demonstrated (Mengeling et al., 1995) that the GalNAc-4-sulfotransferase which introduces terminal SO_4-GalNAc residues into the nonreducing termini of biantennary glycans on the α subunit of LH and FSH (but not hCG) recognizes, and has an absolute requirement for, a sequence of six residues on the α subunit (residues 40−45). These residues (highlighted in Figure 6) are not proximal to the nonreducing termini of the glycans at Asn 52 or Asn 78 on the α subunit of hCG. It is feasible that the orientations of the glycans on the α subunits of LH and FSH are different from hCG, resulting in a more proximal association of the nonreducing termini with the critical protein sequence. However, since the α subunits are identical in all three proteins, and the β subunits show substantial homology, it is more likely that the dispositions of the α-subunit glycans are similar in LH and FSH to those in hCG. In this event the above data would suggest that the recognition of residues 40−45 is at a site on the transferase which is distal from the active site responsible for transferase activity, as discussed in detail by Manzella et al. (1996).

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