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# Glycosylation beyond the Asn78-Linked GlcNAc Residue Has a Significant Enhancing Effect on the Stability of the $\alpha$ Subunit of Human Chorionic Gonadotropin

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**The effects of glycosylation beyond the Asn-linked GlcNAc residues on the stability of the $\alpha$ subunit of human chorionic gonadotropin are investigated, using enzymatic deglycosylation and NMR spectroscopy. Comparison of thermal denaturation profiles of both the intact $\alpha$ subunit and the $\alpha$ subunit carrying only GlcNAc monomers at both Asn52 and Asn78 established a small but significant decrease in thermal stability of the deglycosylated form. Since there is no secondary structure around Asn52 in the free subunit these results demonstrate that glycosylation beyond the Asn78-linked GlcNAc residue enhances the thermal stability of the $\alpha$ subunit of hCG. This feature has implications for understanding the effect of glycosylation on protein stabilization in general.** 

Glycosylation of proteins has been shown to have many functions (reviewed by 1, 2). One of these functions, stabilization of the glycoprotein structure, has currently been reviewed (3). For example, NMR measurements of amide-proton/deuterium exchange rates indicate that N-glycosylation of bovine pancreatic ribonuclease (RNase B) leads to protection from solvent exchange for a large number of amino acid residues throughout the protein (4). In addition, circular dichroism analysis of RNase B and RNase A reveals a small stabilizing effect of glycosylation (B) on the protein structure (5). The N-linked glycan of a proteolytic fragment containing domain 1 of human CD2 plays an important role in maintaining the native structure (6), thereby credibly influencing the initial folding of CD2 (7). This stabilizing effect is mainly mediated by the Asn-linked GlcNAc residue, which displays many NOEs to the protein indicating an interaction between the protein and this residue (8).

Human chorionic gonadotropin is a glycoprotein hormone involved in the maintenance of the *corpus luteum* during early pregnancy, consisting of an $\alpha$ and a $\beta$ subunit. Studies performed by Matzuk *et al.* (9) using site-directed mutagenesis have shown that mutant $\alpha$ subunit of human chorionic gonadotropin ($\alpha$hCG), lacking the glycan at Asn78 is quickly degraded and poorly secreted. However, it is not known yet whether this is due to improper disulfide bridge formation, lacking of steric protection against proteolytic attack, absence of stabilization of protein conformation or a combination of these.

In the present study we have explored the effect of glycosylation of $\alpha$hCG beyond the Asn78-linked GlcNAc residue on the conformational stability. Enzymatically deglycosylated $\alpha$hCG, carrying only a GlcNAc monomer at each glycosylation site, was utilized for this purpose. Thermal denaturation profiles of both this modified $\alpha$hCG and the native subunit were determined.

## MATERIALS AND METHODS

*Sample preparation.* The purification and deglycosylation of the $\alpha$ subunit of urinary hCG was performed as described elsewhere (10). The protein concentration of samples was approximately 1 mM for the thermal stability measurements and 2.5 mM for the amide exchange experiments (concentrations were estimated using UV absorbance at 280 nm and a molar extinction coefficient for $\alpha$hCG of $6.4 \times 10^3$ $M^{-1}$ $cm^{-1}$; 11). Prior to NMR spectroscopic analysis in $^2H_2O$, samples were exchanged twice in 99.9% $^2H_2O$ with intermediate lyophilization, and finally dissolved in 500 $\mu$l 99.96% $^2H_2O$ (Isotec Inc.), containing 0.1 M NaCl. The p$^2$H was adjusted to 4.8 by addition of diluted $^2$HCl (pH-meter reading was not adjusted for the $^2$H-isotope effect).

*NMR spectroscopy.* $^1$H-NMR spectra were recorded on a Bruker AMX-600 or a Bruker AMX-500 spectrometer (NSR Center, University of Nijmegen, The Netherlands or Bijvoet Center, Utrecht Univer-

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Abbreviations used: ($\alpha$)hCG, ($\alpha$ subunit of) human chorionic gonadotropin; fd$\alpha$, fully deglycosylated $\alpha$hCG carrying a GlcNAc monomer at both glycosylation sites; TOCSY, total correlated spectroscopy; MLEV, composite spin-lock pulse devised by M. Levitt; PNGase F, peptide-$N^4$-(*N*-acetyl-$\beta$-glucosaminyl)asparagine amidase F.



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sity, The Netherlands). Externally, probe temperatures were exactly determined using the chemical shift difference between 2,2-dimethylpropionic acid and $^1HO^2H$. The water resonance was suppressed by low-power presaturation during the relaxation delay. In addition, suppression of the $^1HO^2H$ signal in 1D spectra was performed by a modified water eliminated Fourier transform (WEFT) sequence (12). In $^1H$ total correlated spectroscopy (TOCSY) experiments presaturation was followed by a 90° ($\phi_1$=x,−x)−Δ(=1 μs)−90°($\phi_2$=2(x),2(−x)) pulse scheme and a homo-spoil pulse of 10 ms with a consecutive recovery delay of 15 ms. 2D $^1H$ TOCSY spectra (13) were recorded with a mixing time of 25 ms using an MLEV-17 spin-lock pulse sequence (14, 15) preceded by a 2.5 ms trim-pulse. The 90° pulse length and the duration of the "clean delay" in the MLEV-17 sequence were adjusted to 25-30 μs. Chemical shifts are expressed by reference to internal acetone ($\delta_H$ 2.225). Data sets were processed using Bruker UXNMR software. Resolution enhancement was achieved by multiplication of data with a phase shifted sine-squared bell window function, prior to Fourier transformation. Amide NH exchange experiments were carried out at 300K and 323K. Hydrogen exchange was initiated by dissolution of freeze-dried αhCG (previously incubated at 323K in $^1H_2O$ containing 100 mM NaCl and 1 mM $NaN_3$) in 99.96 % $^2H_2O$ (Isotec Inc.) to give a protein concentration of 2.5 mM. 1D spectra and 2D TOCSY spectra were recorded sequentially starting 30 min after the initiation of hydrogen exchange.

## RESULTS

Following enzymatic treatment with endoglycosidase B fully deglycosylated αhCG (fdα) was obtained, carrying at Asn52 and Asn78 only a GlcNAc monomer (10). An almost complete resonance assignment of both αhCG and fdα has been achieved (16). It is our experience that prolonged storage in solution of fdα leads to a faster deterioration and aggregation than for the intact subunit. Both effects are not observed for the partially deglycosylated protein lacking the complete glycan at Asn52, only (prepared by peptide-$N^4$-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) digestion; 10). When tested with bovine lactotransferrin as a substrate no protease activity could be detected in the endoglycosidase B preparation (S. Bouquelet, personal communication). Thus, the possibility that any degradation after deglycosylation is due to the introduction of proteases during the enzymatic treatment is unlikely.

In order to determine whether glycosylation influences the dynamic fluctuations in the structure of αhCG, amide NH exchange rates were studied. αhCG was fully protonated by incubation in $^1H_2O$ containing 100 mM NaCl and 1 mM $NaN_3$ at 323K for 18 h. After lyophilization, hydrogen exchange was induced by dissolution in $^2H_2O$. Then, immediately, the pH was adjusted to 4.9 and 2D TOCSY and 1D spectra were recorded at 300K. Already in the first 1D spectrum virtually all NH signals had disappeared (Figure 1). Because many signals are severely broadened at this temperature, the experiment was repeated as before but the NMR measurements were performed at 323K. As a result some residual NH peaks became observable in the 1D spectrum. Inspection of the consecutively recorded

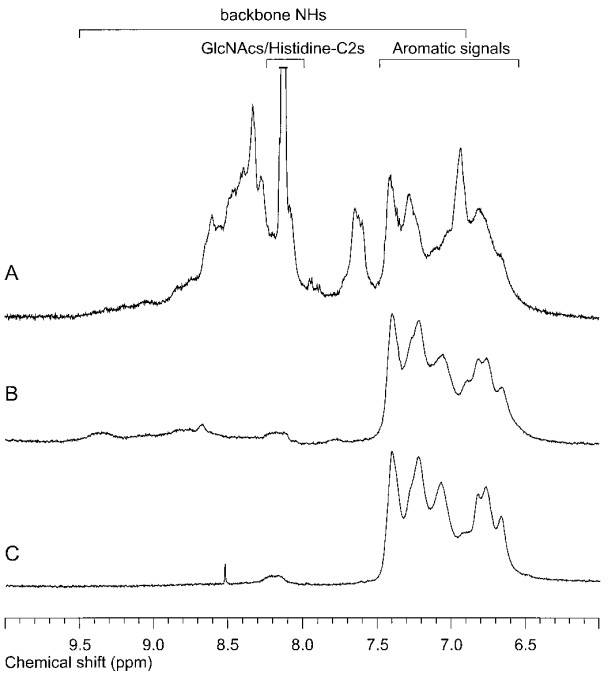


**FIG. 1.** 1D $^1H$-NMR spectra from native αhCG. (A) Fully protonated sample; (B) directly after dissolution in $^2H_2O$; (C) fully deuterated sample. All spectra were recorded at a probe temperature of 300K.

2D TOCSY revealed that these peaks were not attributable to slow exchange. This conclusion is supported by the observation that prolonged incubation at 323K did not lead to a decrease in signal intensities. The resonance at δ 8.05 originates from the C-2 proton of His79 which is reported not to exchange in the free α subunit in $^2H_2O$ at pH 5 (16, 17). The other visible peaks could be assigned to the NHs of the Neu5Ac and GlcNAc residues attached to the trimannosyl-*N*,*N*′-diacetylchitobiose core structure. These residues have a higher mobility than the protein resulting in smaller linewidths. In combination with the presence of some residual $^1H_2O$ this may explain why these signals are still present after dissolution in $^2H_2O$. No other cross-correlations to any NH protons were detected.

In order to probe the influence of glycosylation on the thermal stability of αhCG, denaturation profiles were determined both of αhCG and of its deglycosylated counterpart. Val76 was chosen as a reporter because it is located in one of the two β-sheets determined for αhCG in solution and since Val76 γH′ displays an NOE to the methyl group of the NAc-function of the Asn78-linked GlcNAc residue (16). The chemical shifts of these protons clearly deviate from random coil values and can easily be observed in a 1D spectrum as non-overlapped signals. Therefore, the chemical shifts of Val76 γH and γH′ (at 0.29 and 0.41 ppm) were monitored as a function of the temperature at 313, 318.5,

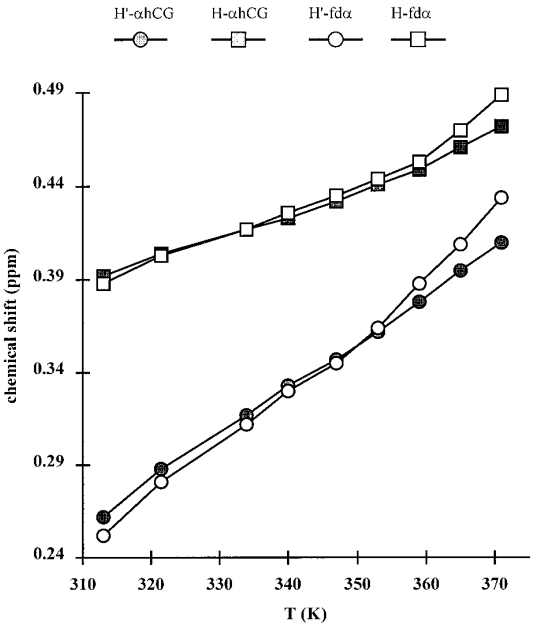


**FIG. 2.** Thermal denaturation profiles of native $\alpha$hCG (filled symbols) and fd$\alpha$ (open symbols). Val76$\gamma$H′ (circles) and Val76$\gamma$H (squares) chemical shifts were determined at increasing temperatures.

321.5, 328, 334, 340, 347, 353, 359, 365, and 371K, respectively (Figure 2).

At high temperatures the protein is expected to attain random coil conformation and the chemical shift values of $\gamma$H and $\gamma$H′ are expected to go to random coil values (0.94 and 0.97 ppm, respectively). Even at the highest temperature measured (371K), however, the chemical shifts of $\gamma$H/$\gamma$H′ are still far away from the random coil values. Nevertheless, for both proteins there is a definite tendency of the chemical shifts to move towards random coil values. Interestingly, when comparing the thermal denaturation profiles of $\alpha$hCG and fd$\alpha$ a significant difference arises, starting at 353K. From that temperature on, the deglycosylated form denatures faster than the fully glycosylated form.

## DISCUSSION

Glycosylation at Asn78 prevents aggregation of free $\alpha$hCG and protects the protein from degradation, as can be concluded from differences in physical behavior after prolonged storage in solution between fd$\alpha$ and $\alpha$hCG deglycosylated at Asn52. Because disulfide-bridges are not affected during the enzymatic treatment used for deglycosylation, this effect is not due to a change in protein folding but can be directly attributed to the glycan itself. A previous study has described an increased chymotrypsin susceptibility of hCG upon deglycosylation but there it remained unclear whether one of the glycans contributes specifically to this effect (18). Determination of amide-proton/deuterium exchange rates to study the dynamic fluctuations of $\alpha$hCG was not possible for this molecule. At low temperature no NH signals are observable in NMR spectra because they are either severely broadened or already exchanged. In addition, when spectra are recorded at temperatures high enough to give suitable line widths still no signals can be detected, indicating that the solvent exchange rates of the amide protons are too high to be observed. This feature is probably caused by the fact that $\alpha$hCG is not a globular protein and that NH-bridges in $\beta$-sheets are not very strong.

From the thermal stability measurements it can be concluded that the thermal stability of $\alpha$hCG is very high, which may be the consequence of the presence of the cystine knot in the center of the protein (19-21). When the stabilities of the native protein and the fully deglycosylated $\alpha$ subunit are compared, it should be noted that the fully deglycosylated $\alpha$hCG still carries a GlcNAc monomer both at Asn52 and Asn78. Since there is no secondary structure around Asn52 in free $\alpha$hCG, the effect reported here can specifically be attributed to the glycan attached to Asn78. Thus we show that the part of the N-glycan outside the Asn78 linked GlcNAc residue gives a small but significant increase in thermal stability.

For the proteinase inhibitor PMP-C it has been reported that the presence of a single fucose-residue at Thr9 is responsible for an increase in thermal stability (22). Especially since the Asn78 linked GlcNAc residue has many NOEs to the protein (16), it is interesting to study if $\alpha$hCG lacking this residue can be obtained and its thermal denaturation profile determined.

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