Site-specific and complete enzymic deglycosylation of the native human chorionic gonadotropin α -subunit

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Numerous studies have shown that glycosylation of the α -subunit of human chorionic gonadotropin (α hCG) is essential for the biological activity of this hormone. To obtain detailed insight into the function of N-glycosylation, the availability of site-specifically and fully deglycosylated α -subunits obtained under non-denaturing conditions is a prerequisite. NMR spectroscopy in combination with FAB-mapping demonstrates that only Asn52 of the α -subunit is accessible to digestion by peptide-*N*-(*N*-acetyl- β -glucosaminyl)asparagine amidase F under native conditions. Treatment of native α hCG with endo- β -*N*-acetylglucosaminidase B results in full deglycosylation yielding α hCG with one GlcNAc residue at both Asn52 and Asn78.

Keywords. Glycoprotein; human chorionic gonadotropin; site-specific N-deglycosylation.

Human chorionic gonadotropin is a heterodimeric, placental glycoprotein hormone which binds to a G-protein-coupled receptor resulting in increased adenyl cyclase activity [1–3]. The subsequent increase in cAMP levels causes steroid synthesis and luteinization [4].

The α -subunit of human chorionic gonadotropin (α hCG) consists of 92 amino acids, contains five disulfide bridges and two N-linked glycans resulting in a molecular mass of about 14.6 kDa. The following structures have been reported to be attached to α hCG: Asn78 bears mono- or diantennary *N*-acetylglucosamine-type glycans while at Asn52 in addition to these glycans, hybrid-type glycans are also present [5, 6]. Glycosylation of this subunit, particularly at Asn52, has been shown to be essential for biological activity using a variety of approaches (reviewed in [7]).

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Abbreviations. α hCG, α -subunit of human chorionic gonadotropin; β hCG, β -subunit of human chorionic gonadotropin; PNGase F, peptide-*N*-(*N*-acetyl- β -glucosaminyl)asparagine amidase F; Endo B, H and F2, endo- β -*N*-acetylglucosaminidase B, H and F2; FAB-MS, fast-atom-bombardment mass spectrometry; fd α^{GlcNAc} , fully deglycosylated α hCG bearing only one GlcNAc residue at each glycosylation site; pd α^{GlcNAc} , site-specifically deglycosylated α hCG, one glycosylation site is deglycosylated by PNGase F (Endo H, GlcNAc referring to the residual GlcNAc); NOESY, two-dimensional nuclear Overhauser effect spectroscopy; TOCSY, total correlated spectroscopy; DQF-COSY, double-quantum-filtered correlated spectroscopy; MLEV, composite spin-lock pulse devised by M. Levitt; HSQC, ¹H-detected heteronuclear single quantum coherence spectroscopy; TPPI, time-proportional phase increment; CID, collisional induced dissociation; 1D and 2D, one- and two-dimensional.

Enzymes. Peptide-*N*-(*N*-acetyl- β -glucosaminyl)asparagine amidase F (EC 3.5.1.52); acylneuraminylhydrolase (EC 3.2.1.18); endo- β -*N*-acetylglucosaminidase F2, H and B (EC 3.2.1.96).

Detailed experiments on the nature of the functional importance of glycosylation of this subunit require site-specifically and completely deglycosylated forms. The harsh conditions of HF deglycosylation of human chorionic gonadotropin lead to partial cleavage of an acid-labile bond in the polypeptide backbone [8, 9] and to the removal of only 50% of the total carbohydrate [8]. Site-directed mutagenesis yields α hCG specifically non-glycosylated at either Asn52 or Asn78 or at both Asn residues [10, 11]. However, using this method the possibility of unnatural folding of the protein has to be considered, since there is evidence that protein-linked glycans are involved in achieving the correct folding of glycoproteins during biosynthesis [12, 13]. Enzymic deglycosylation is an obvious approach since it is performed after correct folding of the protein in the cell has been achieved. Deglycosylation using a combination of various exoglycosidases could be incomplete leading to a varied extent of residual glycosylation, and a residual molar ratio of Man/ α hCG of 1.5:1 has been reported [14].

Digestion of susceptible structures using peptide-*N*-(*N*-acetyl- β -glucosaminyl)asparagine amidase F (PNGase F) or endoglycosidases is generally complete if the conformation of the protein does not interfere. Therefore full deglycosylation is generally achieved when using PNGase F or endoglycosidases on denatured proteins [15, 16]. Obviously, denaturation produces a protein which is unsuitable for conformational or activity studies. Under non-denaturing conditions the integrity of the polypeptide is not disturbed with the exception of the conversion of Asn to Asp by PNGase F digestion. Treatment of native α hCG with PNGase F resulted in a reduction in molecular mass of only 3 kDa suggesting partial deglycosylation [17]. Using NMR spectroscopy and FAB-MS, we show that this deglycosylation by PNGase F is indeed partial and prove that it is site-specific for Asn52. Furthermore, we report full deglycosylation

of α hCG by an endoglycosidase using non-denaturing conditions.

EXPERIMENTAL PROCEDURES

Materials. An acetone precipitate of urine from pregnant women was kindly provided by Diosynth B.V. (Oss, The Netherlands). Recombinant PNGase F (200 U/ml), recombinant endo- β -N-acetylglucosaminidase H (Endo H; 1 U/ml) and acylneuraminylhydrolase (sialidase) from *Vibrio cholerae* (1 U/ml) were purchased from Boehringer Mannheim. Endo- β -N-acetylglucosaminidase F2 (Endo F2) from *Flavobacterium meningosepticum* (200 mU/ml) was purchased from Genzyme. Endo- β -N-acetylglucosaminidase B (Endo B) from the Basidiomycete *Sporotrichum dimorphosporum* (278 mU/ml) was a generous gift from Dr S. Bouquelet (Université des Sciences et Techniques de Lille Flandres-Artois, Villeneuve d'Ascq, France).

Purification of α hCG. Human chorionic gonadotropin was purified essentially as described [18]. In short, a portion of an acetone precipitate of urine from pregnant women, containing approximately 150 mg human chorionic gonadotropin, was dissolved in 80 ml 10 mM Tris/HCl pH 7.4, containing 2 M NH_4OAc (buffer A), and applied to an *n*-octyl-Sepharose CL-4B column (5 $\times$ 12.5 cm, Pharmacia), equilibrated in buffer A at a flow rate of 200 ml/h. After washing with 150 ml buffer A, proteins were eluted in 7.5 h at the same flow rate using a linear gradient of 5–40% (by vol.) ethanol in buffer A. Fractions were collected and assayed for human chorionic gonadotropin using SDS/PAGE [19]. In order to remove NH_4OAc , the pooled human chorionic gonadotropin-containing fractions were repeatedly diluted with 100 mM NH_4HCO_3 pH 7.2, with intermediate concentration by ultrafiltration over an Amicon YM10 76-mm filter, and finally lyophilized.

Purified human chorionic gonadotropin was dissociated and the subunits were separated by reverse-phase HPLC [18] (Vydac C₄ column, 1 $\times$ 25 cm, Separations Group, Hesperia CA) using a linear gradient of 18–36% (by vol.) acetonitrile (Rathburn, HPLC grade S) in 100 mM triethylamine/sodium phosphate pH 6.5. Fractions were collected and assayed for α hCG and β hCG using SDS/PAGE. Fractions containing α hCG or β hCG were pooled, and after evaporation of acetonitrile, salts were removed by gel chromatography (Bio-Gel P-2, fine, 2.5 $\times$ 60 cm, Bio-Rad) using 25 mM NH_4HCO_3 adjusted to pH 7.0 with HCl, as eluent. The void volume was collected and lyophilized. N-terminal sequencing of six amino acids identified α hCG and revealed a 5–7% contamination with β hCG. To check the capability of the isolated subunits to yield a functional protein, aliquots of each subunit preparation were used for recombination studies as described [20], and bio-activity proved to be restored (data not shown).

Reduction and S-carboxymethylation. α hCG was reduced and S-carboxymethylated using standard methods [21]. Briefly, 1 mg α hCG was dissolved in 500 μ l 1 M Tris/HCl pH 8.2, containing 6 M guanidinium/HCl, 1 mM EDTA and 34 mM dithiothreitol, and kept for 2 h at 37°C. After cooling to room temperature, iodoacetic acid (0.5 M in 0.5 M NaOH) was added to a final concentration of 68 mM and the mixture was incubated in the dark for 30 min. The reaction was quenched by adding excess 2-mercaptoethanol. Salt was removed using gel-filtration on Bio-Gel P-2.

Enzymic deglycosylation. For analysis on 12.5% SDS/PAGE, digestions were performed on an analytical scale on native or reduced and S-carboxymethylated α hCG. Samples (125 μ g α hCG), dissolved in 100 mM NaOAc pH 5.0, containing 10 mM EDTA, were digested with 0.17 U PNGase F, 5 mU

Endo H or 0.2 mU Endo F2 for 16 h at 37°C, in a final volume of 50 μ l. For NMR and MS studies, 30 mg native α hCG was digested with PNGase F using appropriately scaled-up conditions.

Digestion with Endo B was performed on a preparative scale. Prior to digestion terminal sialic acid was removed, because sialo-glycoproteins are not substrates for Endo B [22]. Native α hCG (30 mg), dissolved in 7.5 ml 50 mM NaOAc pH 5.0, containing 100 mM NaCl, 10 mM CaCl_2 , 0.5 mM phenylmethylsulfonyl fluoride, and leupeptin hydrogen sulfate, antipain hydrochloride, and pepstatin A at a concentration of 7 μ g/ml each, was incubated with sialidase (1.2 U) for 1 h at 37°C. Complete removal of sialic acid was shown using the glycan differentiation kit (Boehringer Mannheim). Following desialylation, NaN_3 , EDTA, *o*-phenanthroline and phenylmethylsulfonyl fluoride were added to the mixture to 1, 30, 10, and 0.5 mM, respectively. The reaction mixture (final volume 10 ml) was incubated with 135 mU Endo B for 16 h at 37°C.

Tryptic digestion. 200 μ g reduced and S-carboxymethylated α hCG was dissolved in 50 μ l 0.1 M NH_4HCO_3 adjusted to pH 7.3 with acetic acid, containing 1 mM NaN_3 and 0.1 mg/ml trypsin (treated with tosylphenylalanylchloromethane). The mixture was incubated for 18 h at 37°C and subsequently lyophilized.

Separation of site-specifically deglycosylated (pd α) and fully deglycosylated α hCG (fd α^{GlcNAc}) from their released glycans. The PNGase F digest, containing 30 mg α hCG (described above), was fractionated on a Sephadex G-50 SF column (2.5 $\times$ 70 cm, Pharmacia) equilibrated with 100 mM NH_4HCO_3 pH 7.0 at 25 ml/h. The Endo B digest (described above) was applied to a Sephadex G-50 SF column (1 $\times$ 40 cm) equilibrated with 50 mM NaCl, containing 0.1 mM each of EDTA, leupeptin, pepstatin, antipain and *o*-phenanthroline at 4.5 ml/h. Aliquots of collected fractions were assayed for glycans by high-pH anion-exchange chromatography using a CarboPac PA-1 column (25 cm $\times$ 4 mm, Dionex), with a linear gradient of 20–200 mM NaOAc in 100 mM NaOH in 30 min, at a flow rate of 1 ml/min [23, 24], and for α hCG using SDS/PAGE. Fractions containing released glycans, pd α or fd α^{GlcNAc} , were pooled and lyophilized.

NMR spectroscopy. Prior to NMR-spectroscopic analysis in $^2\text{H}_2\text{O}$, samples were exchanged twice in 99.9% $^2\text{H}_2\text{O}$ with intermediate lyophilization and finally dissolved in 450 μ l 99.96% $^2\text{H}_2\text{O}$ (Isotec Inc.), containing 0.1 M or 0.3 M NaCl. Alternatively, samples were dissolved in 450 μ l 0.1 M NaCl, 1 mM NaN_3 , 5–8% (by vol.) $^2\text{H}_2\text{O}$. The pH/ ^2H was adjusted to 5.3 by addition of dilute HCl or to 4.9 by addition of dilute ^2HCl (pH reading not adjusted for ^2H -isotope effect). Sample concentrations were estimated using absorbance at 280 nm and a molar absorption coefficient for α hCG of $6.4 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ [25]. One-dimensional (1D) ^1H -NMR spectra of protein samples were recorded with presaturation of the water resonance during the relaxation delay. These experiments were performed on a Varian Unity plus 750-MHz spectrometer (SON NMR Large Scale Facility, Utrecht University). All other NMR spectra were recorded on a Bruker AMX-500 or AMX-600 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University, The Netherlands or NSR Center, University of Nijmegen, The Netherlands). Two-dimensional (2D) NOE spectra [26] were recorded with a mixing time of 75 ms or 150 ms, and 2D TOCSY spectra were recorded using a 'clean' MLEV-17 spin-lock pulse sequence (15–156 ms) preceded by a 2.5-ms trim-pulse [27–29]. A DQF-COSY spectrum [30, 31] and natural abundance ^1H - ^{13}C HSQC and HSQC-TOCSY spectra were recorded as described [32]. Water-resonance suppression was achieved by presaturation during the relaxation delay and NOESY mixing time. In some experiments pulsed field gradients were applied for ad-

Fig. 1. SDS/PAGE analysis of enzymic digestions of reduced and *S*-carboxymethylated α hCG and native α hCG. The gel was stained with amido-black. (A) Enzymic digestions of reduced and *S*-carboxymethylated α hCG. Digestion with: lane 1, nothing; lane 2, PNGase F; lane 3, Endo F2; lane 4, Endo H. (B) Enzymic digestions of α hCG using non-denaturing conditions. Digestion with: lane 1, nothing; lane 2, sialidase VC; lane 3, sialidase VC and Endo B; lane 4, PNGase F; lane 5, Endo F2; lane 6, Endo H; lane 7, PNGase F followed by Endo F2; lane 8, Endo F2 followed by PNGase F; lane 9, Endo H followed by PNGase F; lane 10, buffer. The highest migrating band in lanes 1, 4, 6, 9 and 10 comigrates with β hCG and shows immunoreactivity with a specific antibody against β hCG. Furthermore, the presence of 5–7% contaminating β hCG was revealed by N-terminal sequencing. The upper two bands in lanes 5, 7 and 8 correspond to partially deglycosylated forms of β hCG.

ditional water-resonance and artefact suppression as follows: a sine-bell-shaped pulsed field gradient of 500 μ s was applied along the *z*-axis with a strength of 75 G/cm at the center of the sine bell, and was followed by a recovery delay of 500 μ s. To achieve phase-sensitive detection in the t_1 direction either the TPPI [33] or the States-TPPI method [34] was applied. 2D spectra were recorded with 400–600 t_1 experiments, and 80–120 free induction decays of 2048 (or 4096 for COSY) data points were collected per t_1 experiment. Data sets were processed using Bruker UXNMR software. In short, time-domain data were multiplied with a shifted squared sine bell function. After zero-filling and Fourier transformation, the resulting data sets of 1024 $\times$ 1024 (or 2048 $\times$ 2048) data points were baseline corrected in both frequency domains by fifth-order polynomial fits.

Fast-atom-bombardment mass spectrometry. Fast-atom-bombardment (FAB) mass spectra were acquired using a Jeol JMS SX/SX 102A four-sector mass spectrometer, operated at 10 kV accelerating voltage, equipped with a Jeol MS-FAB 10 D FAB gun operated at 10 mA emission current, producing a beam of 6 keV Xe atoms. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage. Data acquisition and processing were accomplished using Jeol Complement software. Collisional-induced dissociation (CID) mass spectra were acquired using a collision cell in the third-field-free region of the instrument and He as the collision gas. The pressure of the collision gas was adjusted to obtain a 50% reduction of the main beam. The lyophilized enzyme digests were redissolved in 10 μ l 5% (by vol.) acetic acid in water. Thioglycerol was used as the matrix. For each experiment approximately 1 μ l of the sample solution was loaded into the matrix. Formation of $[M+H]^+$ ions was promoted by adding 1 μ l trifluoroacetic acid to the matrix.

RESULTS

Deglycosylation of reduced and *S*-carboxymethylated α hCG. In order to probe the capability of various endo- β -*N*-acetylglucosaminidases to release glycans from α hCG, digestions were performed on reduced and *S*-carboxymethylated α hCG and evaluated using SDS/PAGE. Incubation of reduced and *S*-carboxymethylated α hCG with PNGase F preponderantly yields completely deglycosylated reduced and *S*-carboxymethylated α hCG (Fig. 1A, lane 2). Digestion of reduced and *S*-

carboxymethylated α hCG with Endo F2 (Fig. 1A, lane 3) yields a product comigrating on SDS/PAGE with completely deglycosylated reduced and *S*-carboxymethylated α hCG, indicating that Endo F2 removes both *N*-acetylglucosamine- and hybrid-type glycans from both glycosylation sites of α hCG under these conditions. The hybrid-type structures (Fig. 3, $I_{H/C}$) have not previously been reported as substrates for Endo F2 [15]. Following Endo H digestion, a portion of reduced and *S*-carboxymethylated α hCG remains unaffected while an additional band appears on the gel (Fig. 1A, lane 4), migrating between undigested reduced and *S*-carboxymethylated α hCG and completely deglycosylated reduced and *S*-carboxymethylated α hCG. Because Endo H only cleaves oligomannose- and certain hybrid-type glycans [35, 36], this observation suggests that the additional band corresponds to α hCG from which only the hybrid-type glycans of Asn52 have been removed (this form will be referred to as $pd\alpha^{GlcNAc}$).

Site-specific deglycosylation of native α hCG. When native α hCG is digested with PNGase F a major band is observed on SDS/PAGE (referred to as $pd\alpha$), which comigrates with $pd\alpha^{GlcNAc}$, indicating that this enzyme is unable to fully deglycosylate α hCG under these conditions (Fig. 1B, lane 4). An analogous experiment with Endo F2 indicates that this enzyme is also not capable of fully deglycosylating native α hCG (Fig. 1B, lane 5). Furthermore, subsequent action of these enzymes does not result in an additional shift on SDS/PAGE (Fig. 1B, lanes 7 and 8), showing no further deglycosylation. Digestion of native α hCG with Endo H results in the same bands on SDS/PAGE as those observed when reduced and *S*-carboxymethylated α hCG is digested with this enzyme (Fig. 1B, lane 6). Subsequent deglycosylation with PNGase F yields $pd\alpha$ (Fig. 1B, lane 9), suggesting that PNGase F, Endo F2 and Endo H all act on the same glycosylation site. This is probably Asn52, considering the substrate specificity of Endo H.

NMR spectroscopy was used to demonstrate that deglycosylation is indeed site-specific. Chemical shifts stemming from glycan protons were determined from the 1H - ^{13}C cross-peaks assigned in an HSQC spectrum of native α hCG, and used to identify the cross-peaks in homonuclear spectra [32]. A DQF-COSY spectrum and TOCSY spectra of native α hCG recorded with different mixing times were used to assign the cross-peaks of both Asn-linked GlcNAc residues based on the unique spin system (*N*-acetyl)NH-H2-H1-(Asn)N H [37]. In the TOCSY spectrum of native α hCG spin systems for Asn-linked GlcNAc residues are indicated on the H1 tracks at 5.01 and 5.30 ppm (Fig. 2B). In the TOCSY spectrum of $pd\alpha$ (Fig. 2A) the spin system on the H1 track at 5.30 ppm is still present, whereas the spin system of the other residue is not observed, proving that one of the sites has been specifically and completely deglycosylated. Inspection of the anomeric region of a 1D 1H -NMR spectrum of the pool of glycans released by PNGase F shows the presence of all glycans reported to be attached to α hCG [6], including the hybrid structures found only on Asn52 (see Fig. 4). This indicates that PNGase F specifically deglycosylates Asn52 under non-denaturing conditions. Therefore the track at 5.01 ppm is assigned to the GlcNAc residue attached to Asn52 accordingly.

Determination of the site of deglycosylation of α hCG by PNGase F using FAB-MS. The FAB-mapping method [38] was used to determine which of the two Asn residues of α hCG is deglycosylated by PNGase F. This method is based on the observation that, in a mixture of peptides and glycopeptides, pseudomolecular ions are only observed for peptides, while glycosylated peptides do not ionize. Positive-ion-mode FAB maps were obtained from tryptic digests of native α hCG and $pd\alpha$ after reduction and *S*-carboxymethylation (Fig. 3). $[M+H]^+$

Fig. 2. Expanded regions of 2D TOCSY spectra of $pd\alpha$ (A), αhCG (B) and $fd\alpha^{GlcNAc}$ (C) showing GlcNAc-1 H1 tracks. All samples contain 0.1 M NaCl. (A) $pd\alpha$ (5 mM) in H_2O , recorded at 328 K at 500 MHz with a mixing time of 55 ms. (B) Native αhCG (5 mM) in H_2O , recorded at 338 K at 600 MHz with a mixing time of 55 ms. (C) $fd\alpha^{GlcNAc}$ (2.5 mM) in H_2O , recorded at 328 K at 600 MHz with a mixing time of 60 ms. GlcNAc-1 H1 tracks are indicated by a dashed line for the GlcNAc residue attached to Asn52, and a solid line for the GlcNAc residue attached to Asn78. In A and C the cross-peak positions for native αhCG are indicated by dashed circles. For native αhCG , the spectrum recorded at 338 K instead of at 328 K is shown. This is because it is of better quality, probably due to the lower tumbling rate of αhCG compared to the deglycosylated forms and therefore αhCG displays a larger line width at the same temperature. The difference in temperature accounts for the slightly lower shift values of the amide protons in the spectrum of native αhCG . In Table 1 chemical shift values at 328 K are given.

pseudomolecular ions were observed at m/z 539, 719, 817, 838 and 847 in the spectra of both digests, corresponding to tryptic fragments T7, T5, T2, T8 and T4T5 (Fig. 3). Importantly, the spectrum obtained from the tryptic digest of $pd\alpha$ also contains an $[M+H]^+$ pseudomolecular ion at m/z 1358, which is absent from the spectrum obtained from the tryptic digest of native αhCG (Fig. 3). This ion corresponds to tryptic fragment T6 which contains an Asp residue derived from Asn52 upon deglycosylation (this m/z value could also theoretically correspond to an ion for a T7T8 incomplete digestion product). The sequence of the peptide having m/z 1358 was determined using CID MS/MS. Fragment ions were generated at m/z 133, 532, 639, 881, 956 and 1043 which correspond to the C_1' , B_5 , Y_5' , B_8 , Y_8' and

Fig. 3. Amino acid sequence of αhCG (A) and FAB maps obtained from tryptic digests of αhCG (B) and $pd\alpha$ (C) after reduction and S-carboxymethylation. Expected tryptic fragments with the m/z values for their $[M+H]^+$ are shown in the sequence (B refers to S-carboxymethyl cysteine). Asn is converted to Asp upon deglycosylation with PNGase F. The ion at m/z 1560 corresponds to residues 58–70 and results from nonspecific digestion.

Y_8' fragments of T6, respectively (nomenclature of fragment ions according to [39]). No fragment ions indicating the presence of T7T8 were observed. These data show that, following site-specific and complete deglycosylation of αhCG , tryptic digestion generates a peptide in which Asn52 had been deglycosylated and converted into Asp, whilst this deglycosylated T6 peptide is not observed in the tryptic digest of the native αhCG . It can thus be concluded that Asn52 of αhCG is deglycosylated by PNGase F using non-denaturing conditions. These results confirm that our assignment of the H1 track at 5.01 ppm in the TOCSY spectrum (Fig. 2) to the GlcNAc residue linked to Asn52 (see above) is indeed correct.

Full deglycosylation of native αhCG . Digestion of desialylated native αhCG with Endo B (Fig. 1B, lane 3) yields a product comigrating on SDS/PAGE with completely deglycosylated reduced and S-carboxymethylated αhCG , suggesting full deglycosylation by Endo B (referred to as $fd\alpha^{GlcNAc}$). The released glycans were separated from $fd\alpha^{GlcNAc}$ using gel-filtration and all expected carbohydrate chains [6] were identified in a 1D 1H -NMR spectrum (not shown). It should be noted that the mono-antennary N-acetylactosamine- and both hybrid-type structures (Fig. 4A, I_A – C) have not previously been reported to be substrates for Endo B [22, 40].

Fig. 4. 1D ^1H -NMR spectra from glycans released from native *ahCG* using PNGase F (B), native *ahCG* (C) and $\text{fd}\alpha^{\text{GlcNAc}}$ (D). (A) Structures of the N-linked glycans of *ahCG* [5, 6] are shown. Numbers of the residues are indicated in bold. $z = 0-1$; I_A : $a = 0$, $b = 0$; I_B : $a = 1$, $b = 0$; I_C : $a = 1$, $b = 1$. (B) 1D ^1H -NMR spectrum of glycans released from *ahCG* using PNGase F with an expansion of the region showing anomeric signals. The anomeric resonances for all constituent monosaccharides are labelled. (C) 1D ^1H -NMR spectrum of 5 mM native *ahCG*. (D) 1D ^1H -NMR spectrum of 5 mM $\text{fd}\alpha^{\text{GlcNAc}}$. Protein samples contain 95% (by vol.) $\text{H}_2\text{O}/\text{D}_2\text{O}$, 0.1 M NaCl and 1 mM NaN_3 . All spectra were recorded at a probe temperature of 328 K.

1D ^1H -NMR spectra of glycans display two characteristic regions with a high density of signals: the so-called 'bulk region' of 3.4–4.1 ppm and the *N*-acetyl region of 2.0–2.1 ppm (Fig. 4B). These regions also dominate the appearance of the spectrum of native *ahCG* (Fig. 4C), whereas no signals indicating the presence of glycans are observed in the spectrum of $\text{fd}\alpha^{\text{GlcNAc}}$ proving full deglycosylation by Endo B (Fig. 4D). Additional proof for the deglycosylation at both Asn52 and Asn78 is obtained from 2D ^1H -NMR spectra. The TOCSY and NOESY spectra of $\text{fd}\alpha^{\text{GlcNAc}}$ show only cross-peaks belonging to the two Asn-linked GlcNAc residues, whereas signals for all other monosaccharide constituents are absent. Comparison of the TOCSY spectrum of $\text{fd}\alpha^{\text{GlcNAc}}$ (Fig. 2C) with that of native *ahCG* (Fig. 2B) reveals that, after digestion with Endo B, the positions of the cross-peaks resulting from the Asn-linked GlcNAcs have changed completely. This indicates full deglycosylation and cleavage of GlcNAc-2→GlcNAc-1 linkages. The results clearly show that Endo B cleaves the glycans at both glycosylation sites of *ahCG* under non-denaturing conditions. To

Table 1. ^1H chemical shifts for the proximal GlcNAc residues linked to native *ahCG* or various glycopeptides. Compound 1, Asn78 of native *ahCG* (5 mM), determined at 328 K; 2, Asn52 of native *ahCG* (5 mM), recorded at 328 K; 3, *ahCG*-derived glycopeptide containing Asn78, recorded at 298 K [6]; 4, *ahCG*-derived glycopeptide containing Asn52, recorded at 298 K [6]; 5, hen-ovomucoid-derived glycopeptide recorded at 298 K [45]; 6, hen phosphitin recorded at 313 K [37]. n.d., not determined.

Compound	Chemical shift of						
	H1	H2	H3	H4	H5	NH(NAc)	Asn-N $^{\text{H}}$
	ppm						
1	5.30	3.93	3.62	3.68	3.18	8.09	8.53
2	5.01	3.79	3.83	3.65	3.54	7.99	8.39
3	5.02	3.84	3.74	3.66	n.d.	n.d.	n.d.
4	5.06	3.86	3.76	3.66	n.d.	n.d.	n.d.
5	5.03	3.85	3.76	3.66	3.56	8.28	8.68
6	5.02	3.81	3.74	n.d.	n.d.	8.09	8.56

our knowledge, this is the first report of full deglycosylation of *ahCG* under non-denaturing conditions using an endo- β -*N*-acetylglucosaminidase.

DISCUSSION

Glycans attached to Asn78 of native *ahCG* are not susceptible to cleavage by either PNGase F or Endo F2. Because this enzymic selectivity does not arise from the primary glycan structures, this suggests a higher degree of steric hindrance at Asn78 than at Asn52. Some support for this proposal can be derived from X-ray studies. In the crystal structure of HF-treated human chorionic gonadotropin [41, 42], electron densities are defined for the two proximal GlcNAc residues of both glycosylation sites of *ahCG*. The crystal structure shows that the GlcNAc residue attached to Asn52 is located at the surface of the protein, giving a possible explanation for the fact that Asn52 can be deglycosylated in solution. Although the orientation of the GlcNAc residue attached to Asn78 is less clearly indicated in the crystal structure, it is tempting to propose that PNGase F and Endo F2 are sterically hindered by the proximity of one or two polypeptide loops.

Differences in environment between the glycosylation sites can also be inferred from NMR spectra. Cross-peak intensities in TOCSY and NOESY spectra are influenced by mobility. In 2D TOCSY and NOESY spectra of native *ahCG* a difference in cross-peak intensities of the two GlcNAc-1 residues was observed, which may be explained by a lower mobility of the GlcNAc residue attached to Asn78 than to Asn52. Several studies have presented chemical shift data for Asn-linked GlcNAc residues. The chemical shifts reported for ring protons of GlcNAc-1 residues in several glycopeptides resemble each other very closely, indicating a similar environment, probably due to solvent exposure (Table 1). An analogous interpretation is accepted for amino acid side-chain NMR resonances in 'random coil' polypeptides [43, 44]. The chemical shifts of the ring protons of the GlcNAc residue attached to Asn52 are in the same range as for those attached to solvent-exposed glycopeptides (Table 1), suggesting that this GlcNAc residue is also solvent-exposed. However, the ^1H -chemical shifts of the GlcNAc residue attached to Asn78 deviate considerably from these values, particularly those of the H1, H3, and H5 atoms, which are all axially oriented facing the same side of the ring. Their deviating chemical shifts suggest that this face of the ring is influenced

by amino acid residues which are close by due to the local conformation of the protein. The orientation, mobility and environment of the proximal GlcNAc residues are now the subject of further studies.

The availability of completely deglycosylated and site-specifically deglycosylated native α hCG allows further studies of the influence of the glycans on the molecular aspects of hormonal activity (see also [46]). Comparison of pda with α hCG that is non-glycosylated at Asn52 using site-directed mutagenesis allows investigation of the effect of glycosylation at this site on the correct folding of the protein during biosynthesis. The homogeneity of fda^{GlcNAc} should probably yield better quality crystals diffracting to a higher resolution as compared with crystals of HF-treated protein in which deglycosylation is incomplete and heterogeneous. Currently, pda and fda^{GlcNAc} are being investigated using NMR spectroscopy to study the influence of glycosylation on the conformation of α hCG.

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