Studies on the relevance of the glycan at Asn-52 of the α-subunit of human chorionic gonadotropin in the αβ dimer

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Glycosylation of Asn-52 of the α-subunit (αAsn-52) is required for bioactivity of the αβ-dimeric human chorionic gonadotropin (hCG), although at a molecular level the effect of the glycan at αAsn-52 is not yet understood. To study the role of this glycan for heterodimer stability, the β-subunit was recombined in solution with either the α-subunit or the α-subunit enzymically deglycosylated at αAsn-52. Enzymic deglycosylation avoids modification of the glycans at αAsn-78 and disturbing the protein folding. The efficiency of recombination after 16 h is 80%, independent of whether αAsn-52 is glycosylated or not. The dissociation constant of the hCG complex, with or without the glycan at αAsn-52, is less than 1×10^{-5} s^{-1}, indicating that the glycan at αAsn-52 does not contribute significantly to the stability of the dimer. CD and NMR spectra indicate a local conformational difference between both αβ-dimeric hCG variants, most probably involving amino acids of the hCG β-subunit close to the glycan at αAsn-52. These data explain the native-like receptor-binding abilities of hCG lacking the glycan at αAsn-52. It is proposed that for bioactivity the glycan at αAsn-52 is necessary for inducing and stabilizing a conformational change in hCG upon binding to the receptor, resulting in activation of the signal-transduction pathway.

Key words: glycoprotein structure, glycosylation, hCG, surface plasmon resonance.

INTRODUCTION

Human chorionic gonadotropin (hCG) is a placental glycoprotein hormone that acts through binding to a G-protein-coupled receptor, leading to increased adenylate cyclase activity [1–3]. The increase in cAMP level stimulates the corpus luteum to produce progesterone until the placenta itself acquires the ability to produce this pregnancy-sustaining steroid [4]. hCG is a heterodimer consisting of two non-covalently associated subunits, α and β, each encoded by a different gene, located on chromosomes 19 and 7, respectively. Biological activity depends on the association of these subunits. The final and rate-determining step in the folding and assembly of hCG is the formation

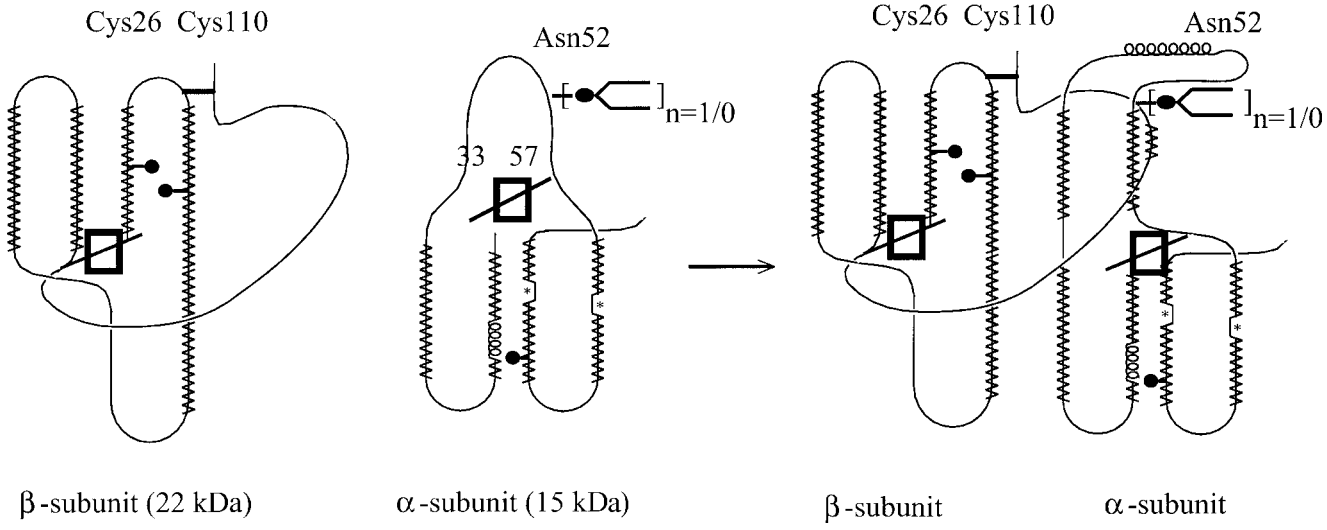

Figure 1 Schematic representation of recombination of the α- and β-subunits of hCG

The rate-determining and final step of the heterodimer formation involves oxidation of the disulphide bond βCys-26–βCys-110 to lock the α-subunit. The cystine knots are located in the centre of the structures (boxes). The black circles indicate the attachment sites of the Asn-linked carbohydrates. Only the glycan at αAsn-52 is shown; $n = 1$, αhCG[glycan^{52,78}]; $n = 0$, αhCG[glycan^{78}].

Abbreviations used: 2D, two-dimensional; hCG, human chorionic gonadotropin; αhCG[glycan^{52,78}], native-hCG-derived α-subunit containing N-glycans at Asn-52 and Asn-78; αhCG[glycan^{78}], partially deglycosylated αhCG containing a glycan at Asn-78; αAsn, an Asn residue of the α-subunit; βCys, a Cys residue of the β-subunit; βhCG, native-hCG-derived β-subunit containing N-glycans at Asn-13 and Asn-30; rehCG, recombined hCG; rehCG[αglycan^{78}], recombined hCG without the glycan at αAsn-52; PNGase F, peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F; RU, response units; SPR, surface plasmon resonance.

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of the cystine bond Cys-26–Cys-110 of the β-subunit (Figure 1). After dissociation of hCG into its subunits, the fully bioactive hormone can be regained by recombination of the subunits [5]. Striking structural elements of both subunits are the cystine knot motifs located in the centre of each subunit [6–8]. Native hCG has four N-linked carbohydrate chains, two in the α-subunit at Asn-52 and Asn-78 (αhCG[glycan^{52,78}]) and two in the β-subunit at Asn-13 and Asn-30 (βhCG). The mono- and di-antennary glycan structures are mainly sialylated, representing 10 % of the total weight of hCG [9].

Removal of the terminal sialic acids diminished the biological potency of hCG *in vivo*, due to a drastic reduction in the circulating half-life [10]. HF-treated hCG, wherein the asparagine-linked carbohydrate chains are predominantly truncated to $(GlcNAc)_2$-Asn, binds to the receptor, but does not trigger hormonal activity [11–13]. Site-directed mutagenesis experiments comprising the replacement of Asn-52 of the α-subunit (αAsn-52) by Gln-52 revealed that the glycan at αAsn-52 is essential for activity [14]. In this respect it is interesting that the crystal structure of HF-treated hCG shows only the carbohydrate chain at αAsn-52 to have interactions at the subunit interface. This glycan has contacts with the β-subunit residues Tyr-59, Val-62, Phe-64, Ala-83 and Thr-97 of the determinant loop [6,7].

Although it has been concluded that the glycan at αAsn-52 is involved in the bioactivity of hCG, its effect at a molecular level is not yet understood, and so far three models have been suggested. Based on the above-mentioned mutagenesis studies, Matzuk et al. [14] suggested that removal of the glycan at αAsn-52 results in a conformational change in hCG that abolishes its bioactivity. In an alternative explanation, a lectin-like membrane component was proposed to be involved in signal transduction in addition to the hCG receptor [15,16]. This model would explain the observation that removal of sialic acid reduces cAMP production to 50 %, even though sialic acid is not involved in receptor recognition [17]. However, Heikoop et al. [18] suggested that the glycan at αAsn-52 may only be involved in (thermo) stability of the heterodimer. Their mutagenesis studies with a single gene mutant of hCG, stabilized by intersubunit disulphide bonds, showed the bioactivity to be independent of the presence of the glycan at αAsn-52.

In the present study, we evaluated the role of the glycan at αAsn-52 at the molecular level in heterodimer formation, stability and conformation. In contrast to previous studies, in our approach the glycan at Asn-52 of the α-subunit was removed specifically by an enzymic method under native conditions, avoiding modification of the glycans at αAsn-78 and disturbing protein folding [19]. The α-subunit, with or without the glycan at αAsn-52, was recombined with the β-subunit in solution. The recombined subunits were isolated by size-exclusion chromatography and the heterodimer conformation studied by NMR spectroscopy, CD and enhanced proteolysis to gain insight into the effect of the glycan at αAsn-52 on the hormone structure. Furthermore, surface plasmon resonance (SPR) was applied to study heterodimer formation and stability by determining the association and dissociation kinetics, respectively, in both the presence and absence of the glycan at αAsn-52. In this context, we have developed a method, using SPR, in which the reduction and oxidation of the β-subunit cystine bond Cys-26–Cys-110 is enabled during the measurement to mimic conditions *in vivo*.

MATERIALS AND METHODS

Sample preparation

The purification of hCG from human urine, separation into its subunits and specific deglycosylation of the α-subunit by peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase F) has been described earlier [19]. αhCG[glycan^{52,78}] or partially deglycosylated αhCG containing a glycan at Asn-78 (αhCG[glycan^{78}]) were mixed with βhCG at concentrations of 1.5 mg/ml α-subunit and 1 mg/ml β-subunit in 10 mM phosphate-buffer solution (pH 7.4), containing 2.7 mM KCl and 137 mM NaCl (Fluka; referred to henceforth as PBS), and recombined at 37 °C for 16 h [20]. The recombined hCG molecules were separated from the remaining α-subunits by gel-permeation chromatography on a column (16 mm × 600 mm) of Superdex G-75, equilibrated and eluted with 150 mM ammonium bicarbonate at a flow rate of 0.2 ml/min with UV detection at 278 nm. The fractions containing recombined hCG were pooled and lyophilized. Similar recombination experiments were performed in PBS including 6.37 mM cysteamine and 3.63 mM cystamine as a redox couple, using a recombination time of 20 min.

SPR

A BIAcore 2000 instrument, BIAevaluation 3.0 software, CM5 sensorchips and an amino coupling kit were obtained from Pharmacia Biosensor AB (Uppsala, Sweden) [21]. The experiments were performed in PBS. The sensor surfaces were prepared as follows. After equilibration with PBS, the sensor surface was activated with a 7 min pulse of a 1:1 mixture of freshly prepared 0.05 M *N*-hydroxysuccinimide and 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl)-carbodi-imide at a flow rate of 5 μl/min. αhCG[glycan^{52,78}], αhCG[glycan^{78}], βhCG and hCG were bound to the activated surface during two 1 min pulses of sample (100 μg/ml) in 10 mM sodium acetate buffer, pH 6.0. Blocking of the remaining *N*-hydroxysuccinimide esters was performed by the addition of 1.0 M ethanolamine hydrochloride, pH 8.5, for 14 min. The blank channel was prepared in an analogous manner.

To measure the binding of βhCG to the αhCG variants, the following experiments were performed at 37 °C.

Recombination in PBS

Flow cell 1 of the sensorchip was a control surface (no protein), flow cell 2 contained ≈ 500 response units (RU) of αhCG[glycan^{52,78}], flow cell 3 contained ≈ 450 RU of αhCG[glycan^{78}] and flow cell 4 contained ≈ 500 RU of βhCG as a reference. A 20 μM βhCG solution in PBS was flowed across the surface at a rate of 1 μl/min. βhCG was injected five times for 250 min in a cyclic procedure, and the dissociation time between these injections was 10 min, resulting in an effective association time of almost 21 h. Regeneration was performed following this procedure using a 4 min pulse of 6 M guanidinium chloride followed by a 2 min pulse of 1 M NaCl. The running buffer was PBS.

Recombination in PBS in the presence of a redox couple

This procedure was similar to the method described above except that βhCG was dissolved in PBS including 6.37 mM cysteamine and 3.63 mM cystamine. The flow rate was increased to 5 μl/min and the injection time of βhCG was 40 min [5].

Kinetics of recombination of αhCG[glycan^{52,78}] or αhCG[glycan^{78}] with the β-subunit

Flow cell 1 of the sensorchip contained ≈ 800 RU of αhCG[glycan^{52,78}] or αhCG[glycan^{78}], flow cell 2 contained ≈ 400 RU of αhCG[glycan^{52,78}] or αhCG[glycan^{78}], flow cell 3 contained ≈ 100 RU of αhCG[glycan^{52,78}] or αhCG[glycan^{78}] and flow cell 4 was a control surface (no protein). To calculate the rate constants, the β-subunit was diluted appropriately (1–20 μM) in PBS including 6.37 mM cysteamine and 3.63 mM cystamine.

The flow rate was 5 μl/min and the injection time of βhCG was 40 min. Regeneration was performed using a 7 min pulse of 6 M guanidinium chloride followed by a 4 min pulse of 2 M NaCl.

Antibody recognition of correctly recombined hormone

Polyclonal antibodies from goat and rabbit that were specific for native hCG (cross-reactivity of less than 5% for βhCG) were used. Each antibody was injected at a flow rate of 5 μl/min for 5 min before and after recombination. Regeneration was performed using a 4 min pulse of 6 M guanidinium chloride followed by a 2 min pulse of 1 M NaCl. Finally, native hCG was coupled to a chip (250 RU) and the antibody reaction was monitored again.

Association and dissociation rate constants were calculated by non-linear fitting of the primary sensorgram data using the BIAevaluation 3.0 software. The closeness of fit for each estimated parameter is described by the statistical value χ^2.

Conformational analysis

Protein samples for enhanced proteolysis were dissolved in PBS (1.5 mg/ml). To an aliquot of 50 μl either 1 μl of trypsin (0.3 mg/ml) or 1 μl of chymotrypsin (2 mg/ml) was added. After incubation at 37 °C, after increasing time intervals aliquots of 10 μl of protein digest were denatured by adding 1 μl of 2-mercaptoethanol followed by boiling for 5 min. Digests were separated by gel electrophoresis utilizing an SDS/PAGE gel containing 15% acrylamide.

CD samples of recombined hCG were prepared by adding 250 μl of 5 mM ammonium bicarbonate to 0.5 mg of lyophilized protein. CD spectra were recorded without any further sample processing. For all samples, CD measurements were carried out on a Jasco J-600 spectropolarimeter, using a 0.2 mm pathlength cell, 1 nm bandwith, 0.2 nm resolution, 1 s response time and a scan speed of 20 nm/min. The spectra were recorded at room temperature.

Protein samples for NMR spectroscopy were dissolved in H_2O or ^2H_2O containing 0.1 M NaCl; the pH was adjusted to 6.0 (H_2O) or 5.6 (^2H_2O; the pH meter reading was not corrected for the ^2H isotope effect). Sample concentration was ≈ 1 mM (15 mg of protein). One-dimensional NMR spectra were recorded at 298, 308, 318 or 328 K with a Bruker DRX-500 or DRX-600 spectrometer (Bijvoet Center, Utrecht University, Utrecht, The Netherlands, and NSR Center, University of Nijmegen, Nijmegen, The Netherlands, respectively). Two-dimensional (2D) NMR spectra were recorded at 318 K. 2D ^1H TOCSY spectra [22] were recorded with a mixing time of 15 ms, and 2D ^1H NOESY spectra [23] were recorded with mixing times of 40 or 60 ms. Chemical shifts were expressed by reference to internal acetone (δ_H 2.225). NMR spectra were processed using software developed in-house (J. A. van Kuik, Bijvoet Center, Utrecht University).

RESULTS

Recombination of hCG subunits in PBS

To investigate the involvement of the glycan at αAsn-52 in the formation and stabilization of the heterodimer, native αhCG [glycan^{52,78}] or αhCG[glycan^{78}] and βhCG subunits were recombined in a PBS solution with a 2:1 molar excess of α-subunit. The efficiency of subunit recombination was estimated by gel filtration on Superdex G-75 from the amount of recombined hCG formed from known quantities of the two subunits during incubation for 16 h (Figures 2a and 2b). Native αhCG [glycan^{52,78}] and αhCG[glycan^{78}] recombined with the β-subunit with identical efficiency (≈ 80%), indicating the glycan at αAsn-52 to be non-essential for heterodimer formation. Remarkably, recombination of αhCG[glycan^{78}] with βhCG was faster than recombination of native αhCG[glycan^{52,78}] with βhCG; a small percentage recombined immediately upon incubation (see also [24]). Furthermore, the heterodimer formed was stable during the course of the experiment, independently of the presence of glycan at αAsn-52.

Recombination of hCG subunits in PBS monitored by SPR

The recombination of αhCG variants and βhCG was followed in real time in an SPR study. The αhCG[glycan^{52,78}] and αhCG[glycan^{78}] subunits were covalently bound to the carboxymethylated dextran layer of two different flow channels. The β-subunit was attached to a third flow channel as a reference surface and a fourth channel was treated identically but without attachment of protein, as a control channel. A 20 μM β-subunit solution in PBS was flowed across the four surfaces to enable recombination (for details, see the Materials and methods section).

The SPR experiments performed within a time frame of 1 h indicated only weak interactions between the subunits and negligible recombination (results not shown). This is in agreement with the observation that recombination of αhCG[glycan^{52,78}] and βhCG *in vitro* takes up to 24 h [5]. To approach this long recombination time, an SPR experiment was carried out at a low flow rate and in a cyclic procedure (for details, see the Materials and methods section) allowing the subunits to interact for ≈ 21 h. The sensorgram in Figure 3 reveals some interesting characteristics of the recombination process *in vitro*. (i) Recombination was very slow, since after a fast initial interaction of the subunits the binding response increased slowly with a k_a value of ≈ 10 $M^{-1} \cdot s^{-1}$.

(ii) Recombination of αhCG[glycan^{78}] with βhCG occurred faster than that of native αhCG[glycan^{52,78}], since the sensorgram of αhCG[glycan^{78}] shows that the greater part of recombination occurred within the first 4 h, while in the case of αhCG[glycan^{52,78}] it took up to 12 h. A similar feature for αhCG[glycan^{78}] has been observed in recombination studies in solution (Figure 2b). Structural properties [6,7] can be held responsible for these observations. The recombination of native αhCG[glycan^{52,78}] with βhCG is severely hindered, since the long peptide loop of α-subunit residues 33–57 and the extended glycan chain at αAsn-52 have to fit through the seat-belt-like arm of β-subunit residues 93–110 (Figure 1). In αhCG[glycan^{78}] the bulky glycan chain has been removed and thereby recombination can proceed more quickly.

(iii) The overall efficiency of recombination of αhCG[glycan^{78}] or αhCG[glycan^{52,78}] with βhCG is similar but low, since < 5% of the theoretically maximal response of 800 RU of βhCG bound to the αhCG surfaces. Likely explanations for the low efficiency of recombination, in comparison with experiments in solution, are steric hindrance by the surface and unfavourable orientation of the α-subunit at the surface of the chip. The complex formed was rather stable, as it was resistant to short regeneration with 6 M guanidinium chloride and 1 M NaCl. Using polyclonal antibodies specific for intact hCG it was possible to confirm that the complex formed was indeed correctly folded hCG (Figure 3). The antibody-binding response at the flow cells containing either αhCG[glycan^{52,78}] or αhCG[glycan^{78}] increased by ≈ 15 RU after 21 h of contact with the β-subunit. This response was similar to that of a flow cell containing the same amount (250 RU) of freshly prepared hCG (results not shown). At the surface

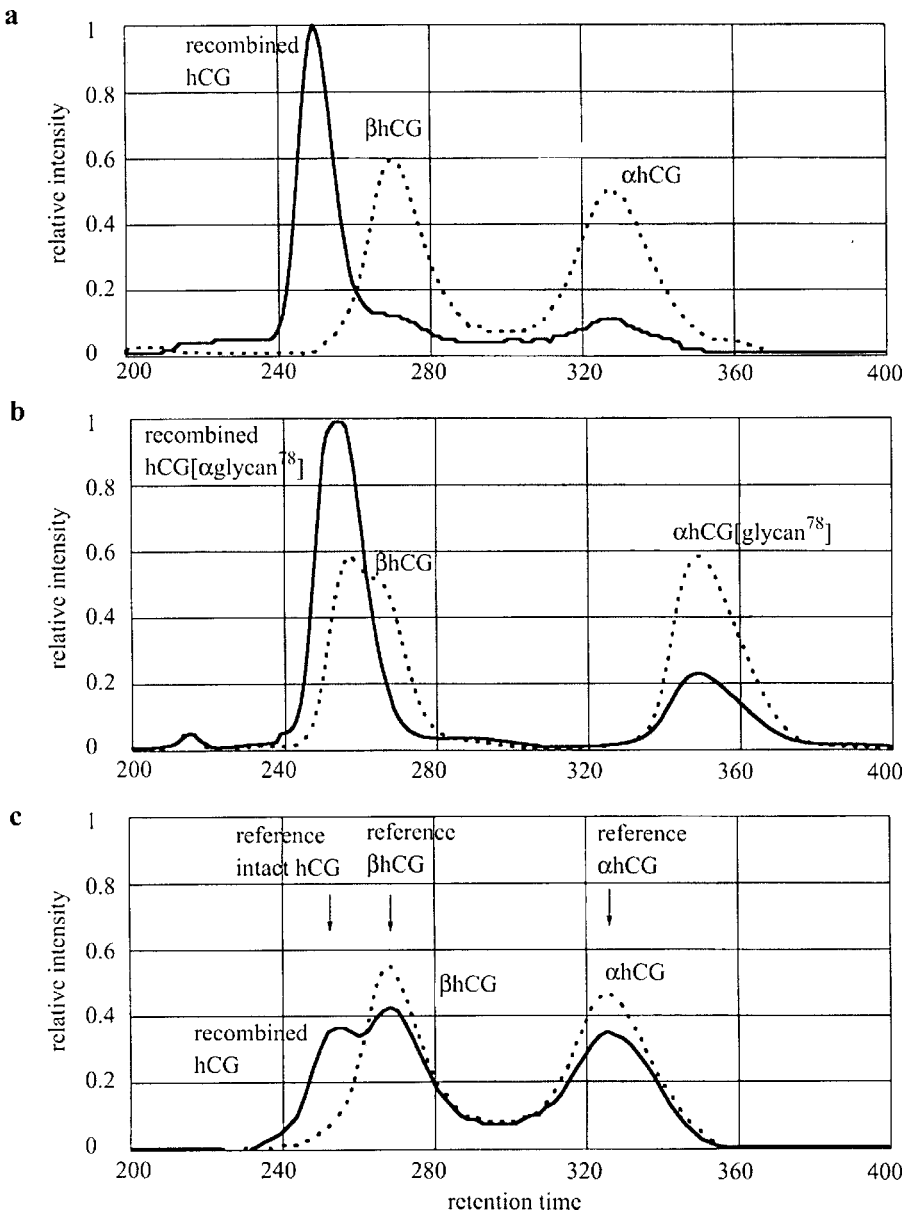

Figure 2 FPLC profile on Superdex G-75 following recombination of the hCG subunits in solution

(**a**) Recombination of native αhCG[glycan^{52,78}] and βhCG in PBS; (**b**) recombination of αhCG[glycan^{78}] and βhCG in PBS; (**c**) recombination of native αhCG[glycan^{52,78}] and βhCG in PBS including a redox couple. Profiles immediately after incubation are shown with a dotted line. Profiles shown by solid lines in (**a**) and (**b**) are after 16 h of incubation, and in (**c**) after 20 min of incubation. For experimental details, see the Materials and methods section.

containing immobilized βhCG no increase in response was observed after 21 h of contact with the β-subunit.

Recombination of hCG subunits in PBS containing a redox couple

Studies *in vivo* have shown that formation of the heterodimer occurs during protein folding [5]. The rate-determining and final step involves formation of the disulphide bond βCys-26–βCys-110 (where βCys is a Cys residue of the β-subunit) to lock the α-subunit (Figure 1). An appropriate *in vitro* model for assembly of hCG is the use of a buffer system containing the redox couple cysteamine/cystamine ($t_{1/2} = 12$ min), which catalyses the assembly rate to a value similar to that observed in intact cells ($t_{1/2} = 8$ min) [5]. The FPLC profile on Superdex G-75 obtained after 20 min of incubation of the native subunits (α/β molar ratio, 2:1) in redox buffer is shown in Figure 2(c). The efficiency of recombination was $\approx 50\,\%$. The CD spectrum of the recombined subunits was in agreement with that of freshly isolated hCG (for details, see Figure 4a).

Recombination of hCG subunits in PBS containing a redox couple monitored by SPR

A SPR method has been developed to monitor the association and dissociation kinetics of the hCG subunits under conditions that mimic protein folding *in vivo*. These conditions were achieved by allowing reduction and oxidation of the βCys-26–βCys-110 cystine bond by dissolving the β-subunit in a buffer containing a

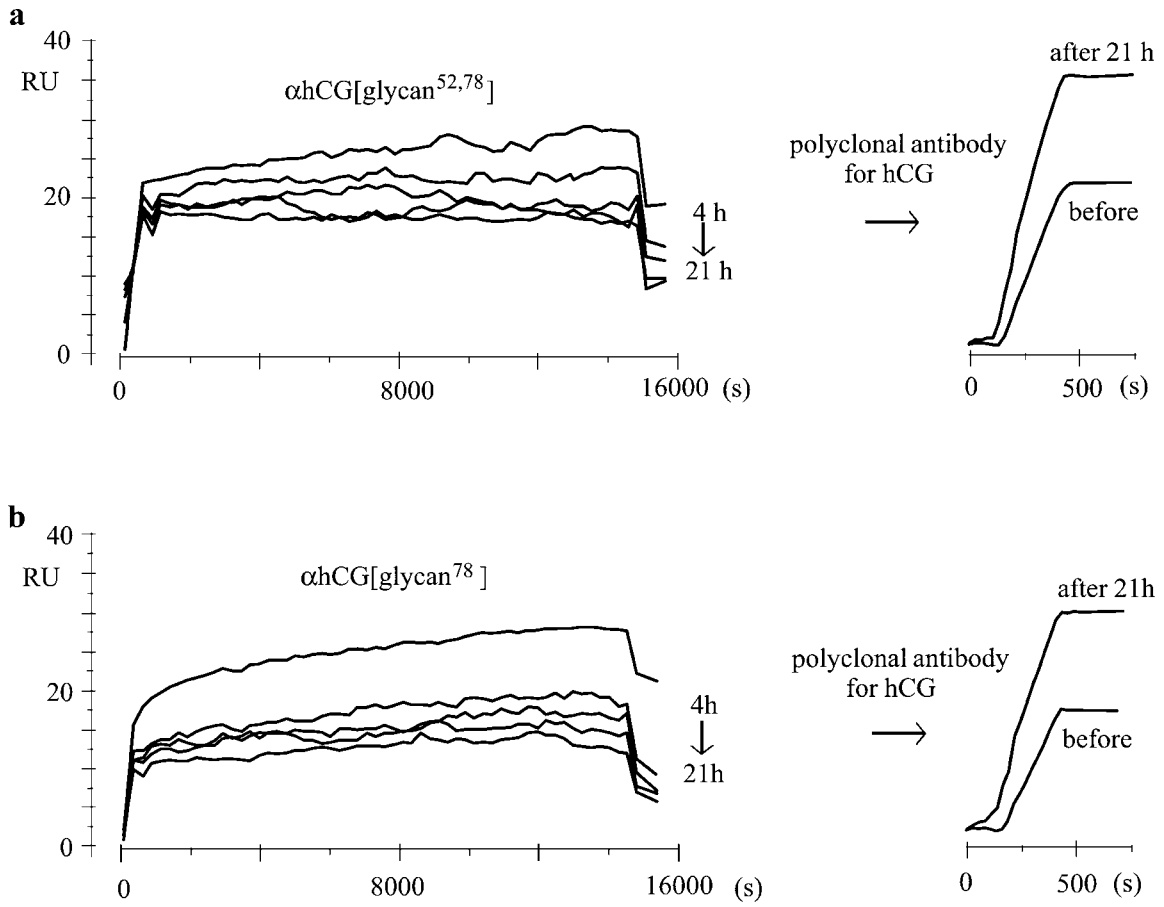

Figure 3 SPR sensorgrams in the absence of a redox couple

Binding of βhCG to αhCG[glycan^{52,78}] (**a**) and to αhCG[glycan^{78}] (**b**) in PBS. The upper lines in the sensorgrams represent the recombination kinetics during the first 4 h, the lower lines represent recombination during 4–8 h, 8–12 h, 12–16 h and 16–≈ 21 h, respectively. After a short regeneration with 6 M guanidinium chloride and 1 M NaCl, goat polyclonal antibodies for hCG were flowed across the surface (right-hand graphs, upper lines); the lower lines on the right show the background binding of this antibody to αhCG[glycan^{52,78}] and αhCG[glycan^{78}], respectively, before recombination. For experimental details, see the Materials and methods section.

redox couple. Otherwise the conditions were the same as those described above. It should be noted that the α-subunit is only exposed to the redox couple during injection of βhCG (for details, see the Materials and methods section). The sensorgrams are shown in Figure 5(a), and some interesting characteristics were noted. (i) Recombination occurs much more quickly than that in PBS without a redox couple. (ii) The association rates of αhCG[glycan^{52,78}] or αhCG[glycan^{78}] with βhCG were similar, as indicated by the slopes of the sensorgrams. (iii) The absence of dissociation is indicative of stable heterodimer formation.

The phenomena (i) and (ii) can be explained by reduced steric hindrance of the interaction between loop 33–57 of the α-subunit and the β-subunit seat-belt residues 93–110. This is due to a dynamic equilibrium between opening and closing of the disulphide bond βCys-26–βCys-110 by the redox couple (Figure 1).

Polyclonal antibodies were used to confirm the presence of correctly folded hCG. After 40 min of recombination with the β-subunit the level of antibody binding increased by 25–35 RU. The antibody response at the reference surface containing immobilized β-subunit remained unaltered.

In principle, the presence of the redox couple could introduce non-native disulphide bonds. However, this possibility is unlikely because the X-ray structures of hCG [6,7] and NMR data of the α-subunit [25] show that the disulphide bonds are hidden inside the protein; only the disulphide bond βCys-26–βCys-110 is exposed to the buffer. Furthermore, taking into account that (i) homodimers of βhCG were not observed when βhCG was flowed across a surface containing immobilized βhCG, (ii) high loading of a sensorchip hindered and eventually stopped recombination, (iii) frequently used sensorchips showed reduced recombination ability, (iv) thorough regeneration of the sensorchip with 6 M guanidinium chloride almost completely disrupted the αβ dimer [26] and (v) recombination of αhCG[glycan^{52,78}] and βhCG was not observed when αhCG[glycan^{52,78}] was flowed across a surface containing immobilized βhCG (results not shown, but see Figure 6 for details), it can be concluded that the correct αβ dimer has been formed. This study shows that redox conditions similar to those *in vivo* can be developed for use in SPR experiments.

Kinetics of recombination of hCG subunits in PBS containing a redox couple

To gain insight into the kinetics of recombination of the subunits and the stability of the αβ dimer, SPR experiments were performed at different β-subunit concentrations (Figure 5b). An

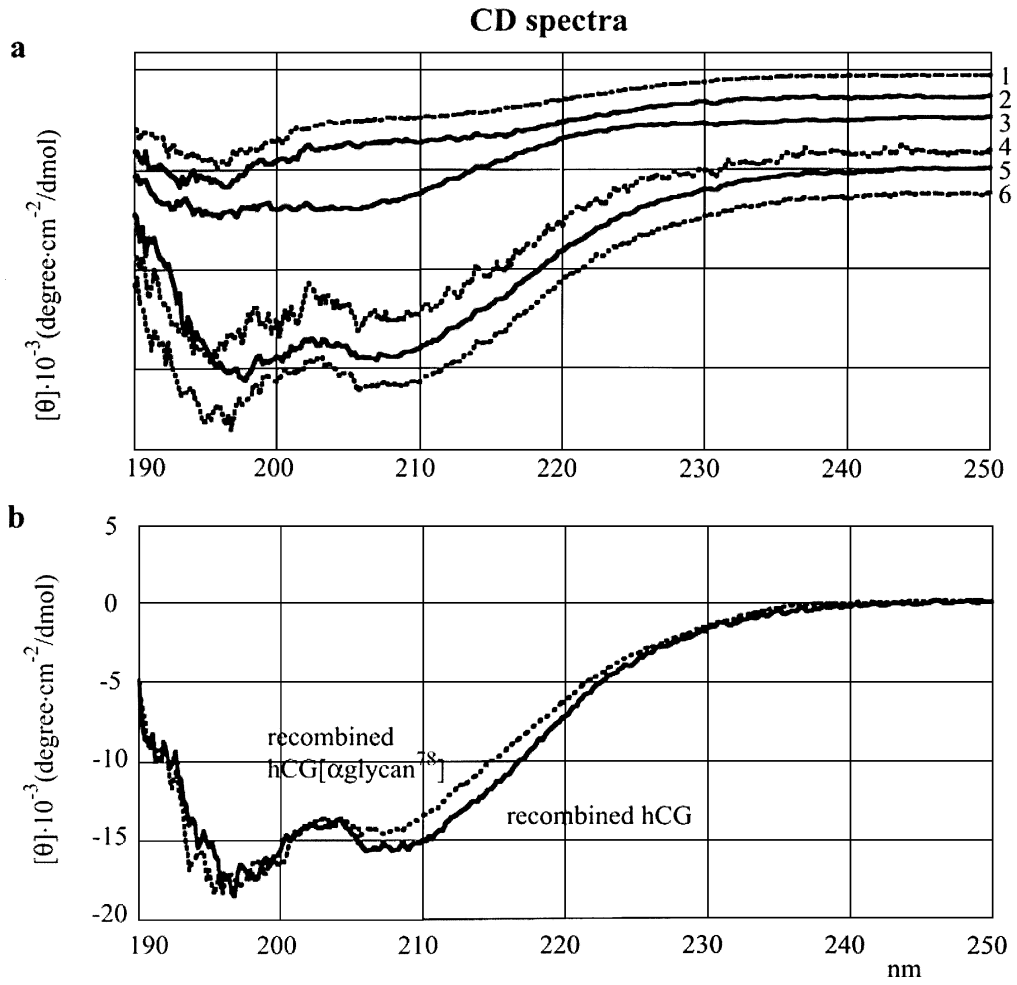

Figure 4 CD spectra of different hCG samples

(**a**) Curve 1, αhCG[glycan^{78}]; curve 2, αhCG[glycan^{52,78}]; curve 3, βhCG; curve 4, rehCG prepared in PBS with a redox couple; curve 5, freshly isolated hCG; curve 6, rehCG prepared in PBS without a redox couple. (**b**) rehCG (solid line) and rehCG[αglycan^{78}] (dotted line), both prepared in PBS without a redox couple. CD spectra below 200 nm are very sensitive to the presence of salts and other contaminants. The differences in the CD spectra of freshly isolated hCG (**a**, curve 5) and rehCG (**a**, curves 4 and 6) can probably be explained by the fact that freshly isolated native hCG was purified only on *n*-octyl-Sepharose CL-4B [19]. For rehCG the free α- and β-subunits prior to recombination were prepared from native hCG by dissociation followed by separation on reversed-phase HPLC (Vydac C_4) [19]; after recombination, rehCG was purified on Superdex G-75. Therefore, improved purity of rehCG and slightly different buffer conditions cause the difference in the CD spectra below 200 nm.

important observation is that both complexes, recombined hCG (rehCG) and recombined hCG without the glycan at αAsn-52 (rehCG[αglycan^{78}]), were very stable, resulting in dissociation rates near zero ($0 < k_d < 1 \times 10^{-5}$ s^{-1}). The association rates were rather similar for both αhCG variants ($100\ M^{-1} \cdot s^{-1} < k_a < 1 \times 10^3\ M^{-1} \cdot s^{-1}$). The recombination can be fitted assuming a 1:1 binding profile; however, particularly for native αhCG[glycan^{52,78}], a second binding phenomenon with a faster association but with a lower affinity appears to be present. One might speculate that this is related to heterodimer recombination in which the disulphide bond βCys-26–βCys-110 has not yet been formed. The SPR data concerning recombination of the α- and β-subunits of hCG are summarized in a model shown in Figure 6.

The outcome of the SPR experiments indicates that large structural rearrangements are involved in the recombination process. Further evidence for this conclusion was obtained from SPR experiments performed at 4 °C, 25 °C (results not shown) and 37 °C, since an increased efficiency of recombination leading to a more stable complex was observed at higher temperatures. Concerning the role of the glycan at αAsn-52, we can conclude that this glycan is not essential for heterodimer formation and stability.

Conformational analysis of recombined hCG in the presence or absence of the glycan at αAsn-52

Previous studies have shown that the α-subunit undergoes large conformational changes upon dissociation of the αβ dimer. The loop containing residues 33–57, including the glycosylated Asn-52 of the hCG-derived α-subunit, becomes flexible and disordered, as determined by NMR [25,27]. In contrast, X-ray studies of chemically deglycosylated hCG have shown that this part of the α-subunit around the glycan at αAsn-52 is well-defined in the αβ dimer (Figure 1). To investigate whether refolding of the α-subunit in the presence of the β-subunit occurs only when αAsn-52 is glycosylated, enhanced proteolysis was applied as a tool to probe the presence of large random-coil

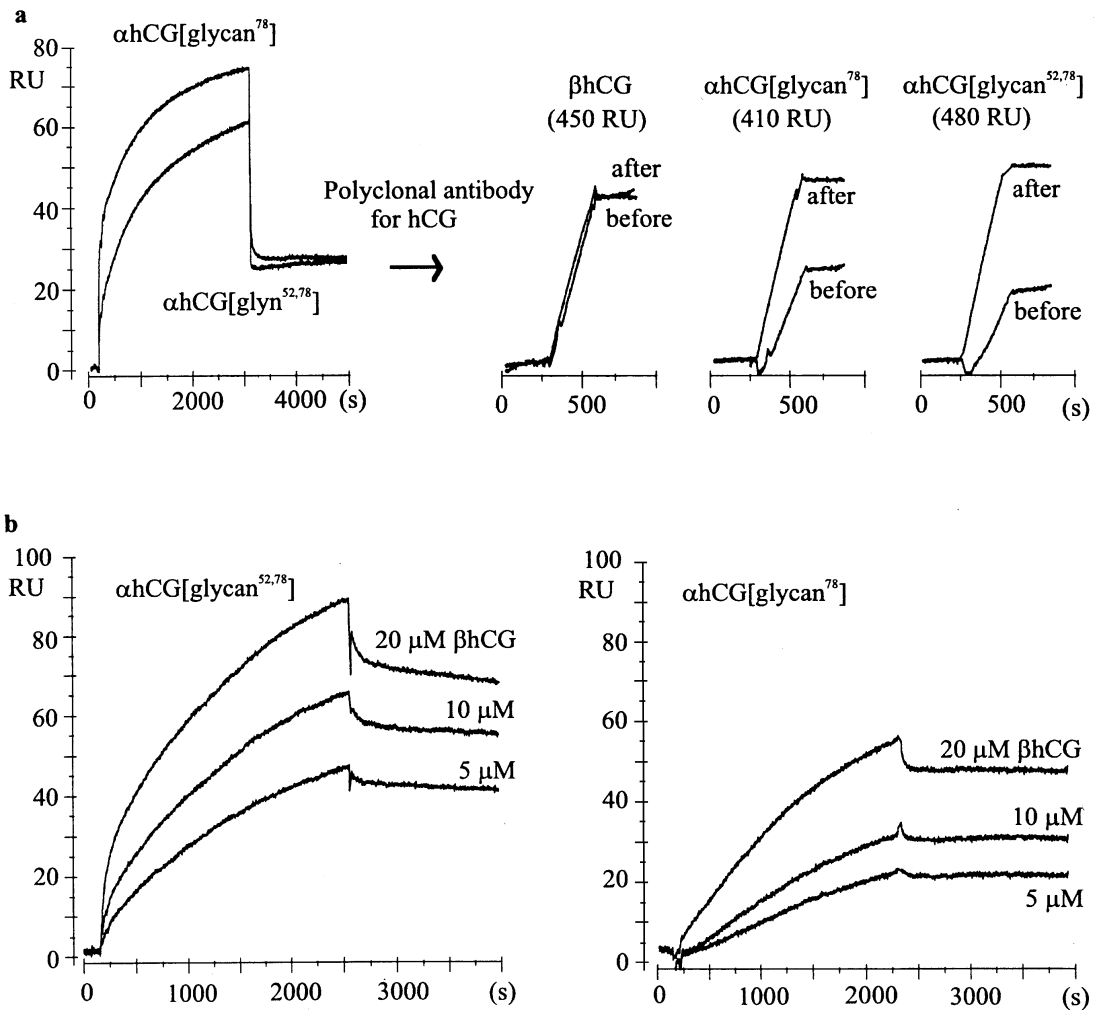

Figure 5 SPR sensorgrams in the presence of a redox couple

(**a**) Binding of βhCG to αhCG[glycan^{52,78}] and αhCG[glycan^{78}] in PBS with a redox couple. The subsequent sensorgrams show the recognition by goat polyclonal antibodies for hCG before and after recombination with βhCG for the sensorchip channels coated with βhCG, αhCG[glycan^{78}] or αhCG[glycan^{52,78}], respectively. (**b**) Binding of βhCG to αhCG[glycan^{52,78}] (left-hand panel) and αhCG[glycan^{78}] (right-hand panel) at three different concentrations in PBS with redox couple. For experimental details (≈ 800 RU of αhCG[glycan^{52,78}] or αhCG[glycan^{78}]), see the Materials and methods section.

structures. Enhanced proteolysis experiments of the free α-subunit showed rapid cleavage of the disordered α-subunit loop 33–57, independent of glycosylation at αAsn-52. rehCG and rehCG[αglycan^{78}] were incubated with chymotrypsin or trypsin and analysed by SDS/PAGE. Both hCG variants proved to be well protected against proteases, since even after 1 h of incubation the intact subunits were still present, except for the unstructured C-terminus of the β-subunit (results not shown). A loss of structural integrity of rehCG upon removal of the glycan at αAsn-52 was therefore not found by these experiments.

Various CD studies have been performed to determine the overall conformation of the hCG-derived subunits and the heterodimer of hCG [28,29]. The effect of glycosylation of hCG on the total CD is somewhat unclear. It has been described that the *N*-acetyl groups of *N*-acetylglucosamine and *N*-acetylneuraminic acid residues of a free glycan chain contribute only between 1 and 5% of the total CD of hCG [28,30]. However, it has been suggested that covalent binding of the glycan leads to enhanced optical activity between 207 and 208.5 nm, as a result of restriction of rotational freedom and enhancement of chromophore interactions [29]. In this study CD spectra were recorded to compare the conformations of rehCG[αglycan^{78}] and rehCG. The CD spectra of the hCG-derived α-subunits (Figure 4a) with or without the glycan at αAsn-52 were very similar, indicating that enzymic removal of this glycan does not affect the conformation of the protein. This observation is in agreement with the NMR structure of the hCG-derived α-subunit, since the glycan at αAsn-52 is located in a disordered loop [25,27]. The contribution of this glycan itself to the total CD of αhCG [glycan^{52,78}] is expected to be small, since this glycan is very flexible and is not in close contact with the disordered protein core. The CD spectra of rehCG were not influenced by the recombination protocol followed (with or without redox couple; Figure 4). Both sets of spectra show that upon recombination of the subunits the aperiodic structure of αhCG[glycan^{52,78}] decreases. This is in agreement with previous CD studies on isolated hCG [28,29] and the X-ray structures [6,7]. The negative circular dichroic extreme at 207.5 nm is composed of two bands, one at 215 nm and the other one between 207 and 208.5 nm. The 215 nm band is assigned to the peptide in the β-sheet con-

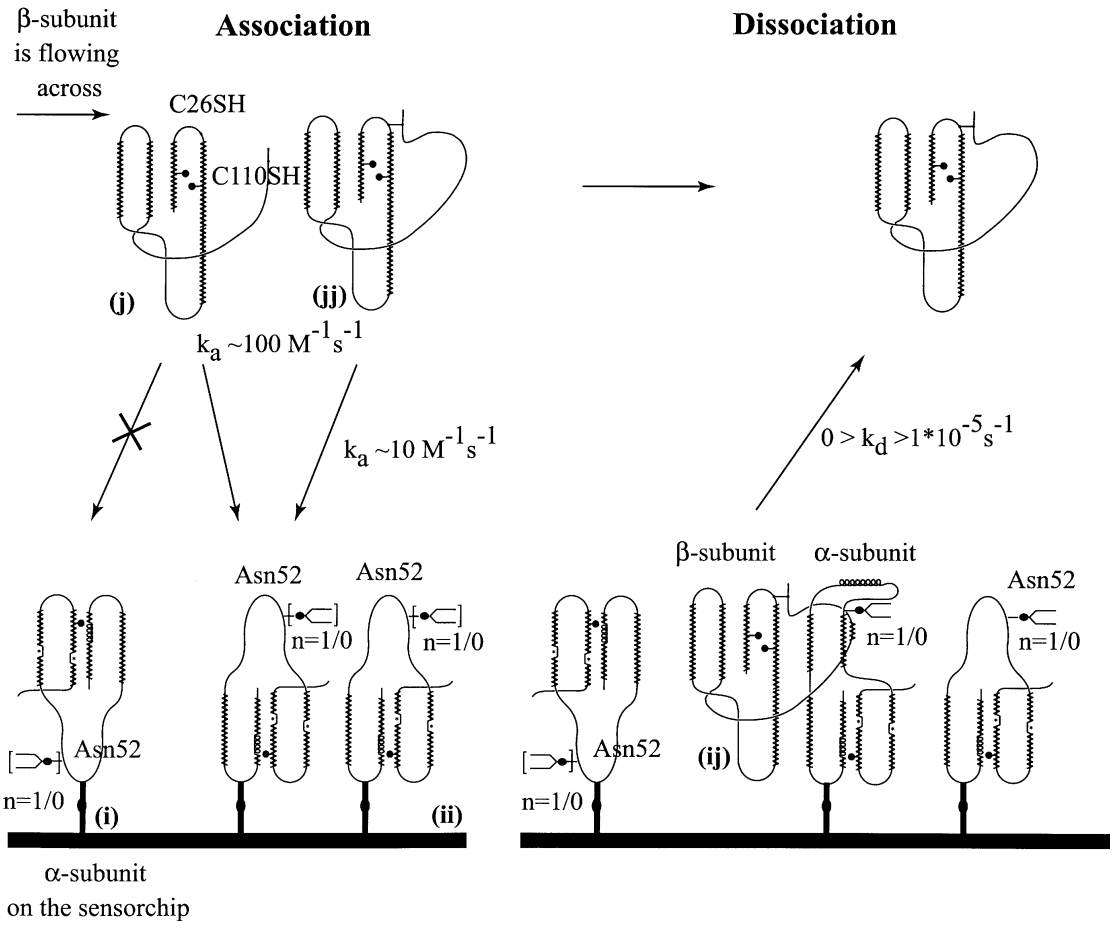

Figure 6 Model for recombination of the α- and β-subunits as studied by SPR

The α-subunit with or without the glycan at Asn-52 is attached to the sensorchip via lysine residues. Recombination with the β-subunit can only occur when the loop around the glycosylation site Asn-52 is not sterically hindered by either the sensorchip (i) or a second α-subunit (ii). This is in agreement with the observation that at higher loading of the sensorchip ($>$ 1000 RU) the efficiency of recombination decreases. It has been observed that a chip containing immobilized βhCG does not recombine with αhCG[glycan^{52,78}]. Also in this case steric hindrance is an obvious explanation since a large part of βhCG, including the seat-belt residues, are required to recombine with αhCG[glycan^{52,78}]. When a βhCG solution is flowed across an αhCG[glycan^{52,78}]-coated sensorchip in the presence of the redox couple the recombination with the α-subunit increases by more than a factor of 10 in comparison with recombination in a buffer without a redox couple. This observation can be explained by reduced steric hindrance of the interaction between the loop 33–57 of the α-subunit and the β-subunit seat-belt residues 93–110. This is due to a dynamic equilibrium between opening (j) and closing (jj) of the disulphide bond βCys-26–βCys-110 by the redox couple. During dissociation the PBS running buffer is flowed across the surface, washing away the unbound or incorrectly bound β-subunits. Dissociation of the recombined hCG (ij) is very slow due to a combination of hydrophobic and physical interactions, including the seat-belt structure of the residues of the β-subunit 93–110 after closing the βCys-26–βCys-110 disulphide bond.

formation. The lower-wavelength band has a contribution from the *N*-acetyl groups of the glycan part, as described above [29]. Based on the X-ray structures of hCG [6,7] at least the first two *N*-acetylglucosamine residues at αAsn-52 possess restricted rotational freedom and are in direct contact with the protein-core. Therefore, their optical activity might be enhanced in comparison with the situation for the hCG-derived α-subunit. The CD spectrum of rehCG[αglycan^{78}] is slightly different to that of the recombined native subunits (Figure 4b) due to either a reduced β-sheet content or to the absence of the *N*-acetyl groups of the glycan at αAsn-52.

The above-described biochemical and biophysical studies have not indicated large, overall, conformational differences between recombined hCG in the presence or absence of the glycan at αAsn-52. To gain more insight into local conformational changes of rehCG[αglycan^{78}] in comparison with rehCG, NMR experiments were performed. Although hCG is a large protein (37 kDa) in terms of NMR spectroscopy, resulting in line broadening of the resonances, interesting data could be obtained from the side-chain interactions between aromatic and aliphatic amino acids especially (Figure 7). The one-dimensional NMR spectra of rehCG and rehCG[αglycan^{78}] are similar. In combination with the fast proton exchange in ^2H_2O (less then 30 min) in both samples, these data point to similar overall structures for rehCG and rehCG[αglycan^{78}]. However, differences have been observed for a resonance around 0.55 p.p.m. and for resonances in the area of the aromatic side chain. In the 2D NOESY spectra of rehCG[αglycan^{78}] an extra set of cross peaks are observed between 6.9 and 7.2 p.p.m. and at 0.55 p.p.m., indicating strong interactions between aromatic and aliphatic amino acids (Figure 7). Based on the X-ray structure, five residues with aromatic or aliphatic side chains have direct contacts with the glycan at αAsn-52, namely β-subunit Tyr-59, Val-62, Phe-64, Ala-83 and Thr-97 (Figure 8). The differences between the NMR spectra of rehCG and rehCG[αglycan^{78}] are ascribed to the removal of the glycan chain.

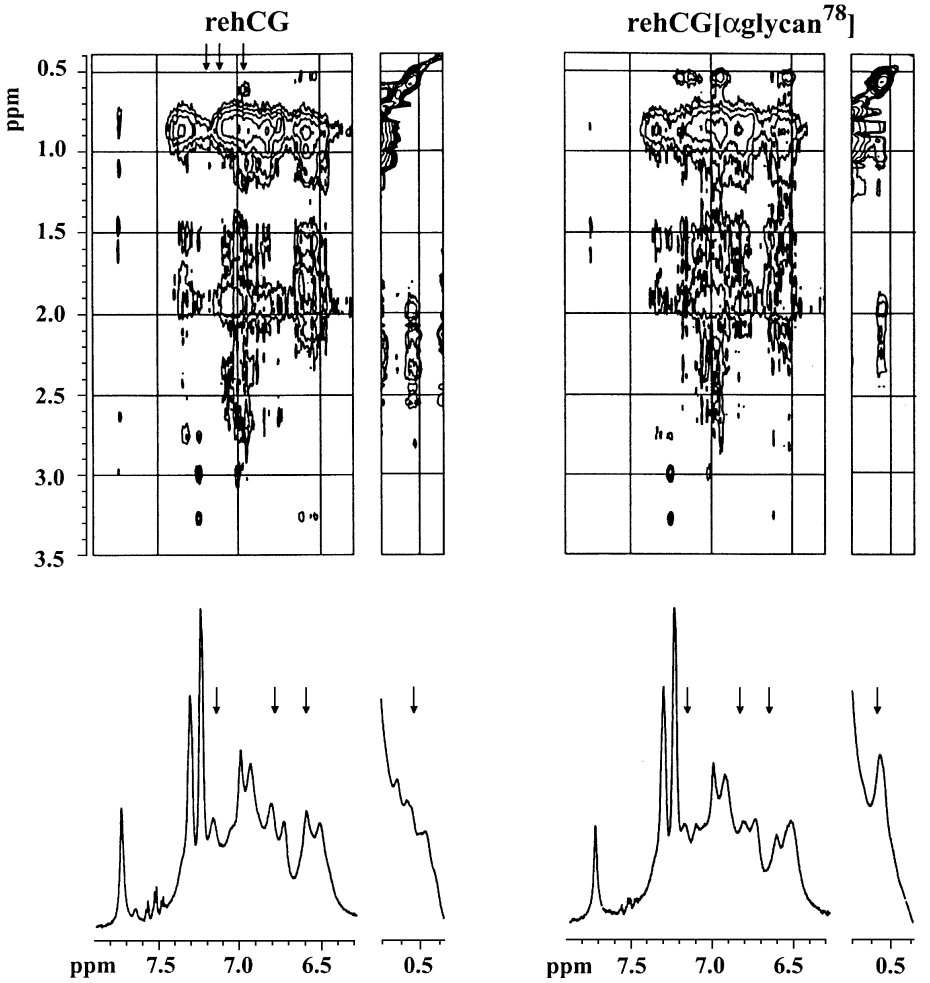

Figure 7 Comparison of a part of the one-dimensional ^1H and 2D ^1H NOE spectra of rehCG (left-hand panel) and rehCG[αglycan^{78}] (right-hand panel)

The spectra were recorded at 318 K with a mixing time of 60 ms (NOESY) in ^2H_2O with a Bruker DRX-600 spectrometer. Differences in chemical shift and signal intensity are indicated by arrows.

In summary, the CD and the NMR spectra of rehCG and rehCG[αglycan^{78}] indicate local and small conformational differences between both hCG variants, probably involving residues with aromatic and aliphatic side chains close to the glycan at αAsn-52.

DISCUSSION

Different hypotheses have been put forward to explain the role of glycosylation on the bioactivity of hCG. A complicating factor in evaluating the results of different research groups is the method by which the deglycosylated material is prepared. Chemical methods have generally been used to deglycosylate hCG, but these are non-selective, and could also damage the protein moiety [24,31]. More recently, site-directed mutagenesis has been performed to remove selectively the glycan at αAsn-52 [14,18,30]. For the present study the glycan at αAsn-52 has been removed specifically by using an enzymic method involving PNGase F. Here, disturbance of protein folding and undesired modifications of the glycan chains at αAsn-78 were avoided; only a conversion of αAsn-52 into αAsp-52 occurred.

Our findings contradict the hypothesis that the glycan at αAsn-52 is relevant for the stability of the heterodimer [18]. We showed that both αhCG[glycan^{52,78}] and αhCG[glycan^{78}] could recombine perfectly with βhCG in PBS buffer. This is corroborated by the observation that during the course of the NMR experiments (≈ 5 days) at a temperature of 45 °C and pH 6 dissociation was less than 20 % and similar for both hCG variants. Furthermore, the SPR results confirm that the glycan at αAsn-52 is not essential for heterodimer stability, since the dissociation rate was less than 1×10^{-5} s^{-1} and shown to be independent of glycosylation. This is in contrast to previous studies [24,30], wherein the dissociation rates of differently glycosylated forms of hCG were determined. These dissociation rates ranged from 1.5×10^{-5} s^{-1} for the native form to 9.4×10^{-5} s^{-1} for hCG lacking the glycan at αAsn-52. However, these measurements were carried out at pH 3 by a fluorescence method using 1-anilino-8-naphthalene sulphonate [20]. At lower pH values (< 3) the dissociation rates increase further [24]. Structural features of hCG can explain these observations [6,7], since upon partial unfolding at low pH (< 3) [32] the glycosylated αAsn-52 loop has to go through the seat-belt of the β-subunit to dissociate (Figure 1). The bulky and hydrated glycan chain will hamper the dissociation, but upon desialylation, partial deglycosylation or complete removal of the glycan at αAsn-52 the dissociation rate gradually increases [24]. Therefore, these data do not support the hypothesis that the function of this glycan is

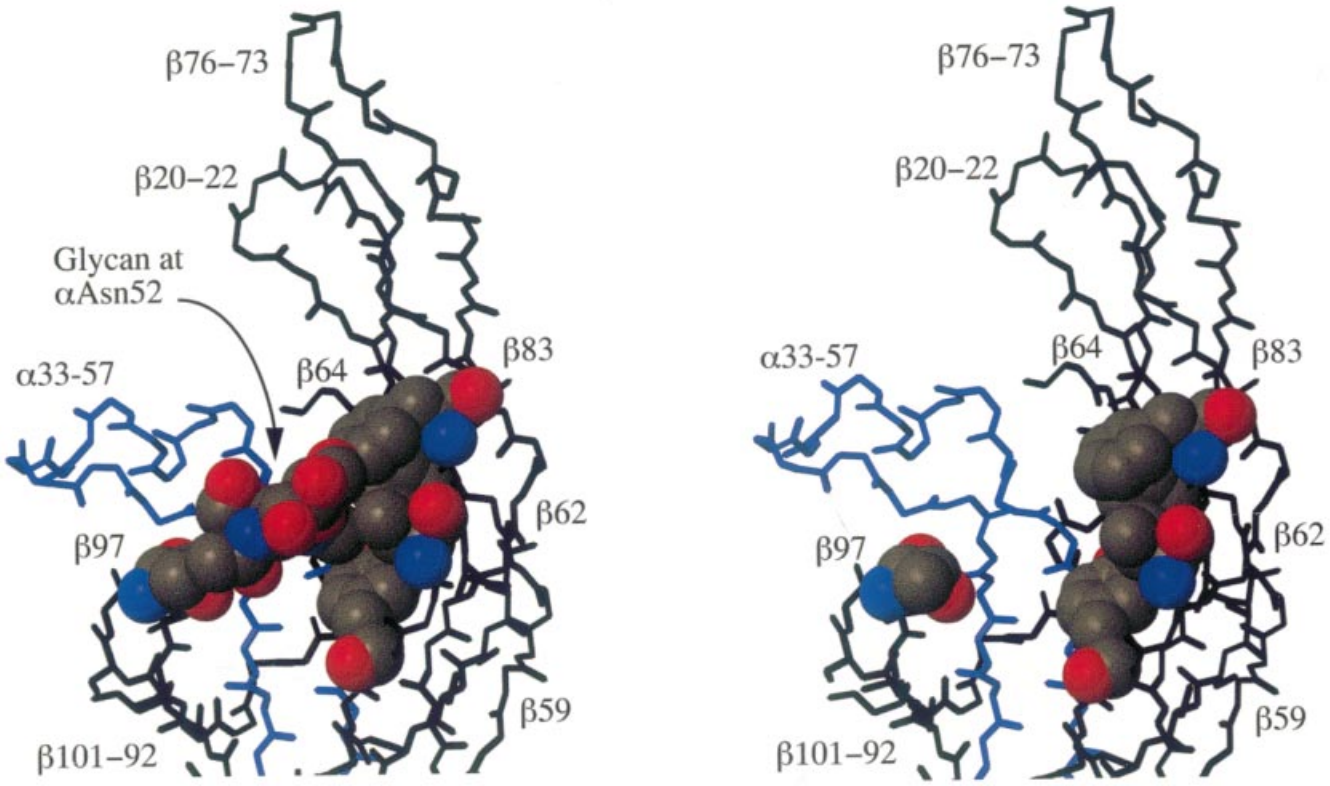

Figure 8 Enlarged X-ray structure of hCG around the glycosylation site at αAsn-52

The backbones of the α- (blue) and β-subunits (black) are shown in the presence (left-hand panel) and absence (right-hand panel) of αAsn-52-bound GlcNAc and (β1-4)-linked GlcNAc in space-filling form. The amino acids that have direct contact with the glycan are also displayed in space-filling form.

to stabilize the heterodimer, especially not under naturally occurring conditions.

An alternative explanation for the loss of bioactivity of hCG upon removal of the glycan at αAsn-52 involves a conformational change [14]. In the present study, a series of experiments have been performed on enzymically deglycosylated hCG to test this hypothesis.

Large conformational changes can be ruled out, since enhanced proteolysis experiments showed that hCG enzymically deglycosylated at Asn-52 is protected against the proteases trypsin and chymotrypsin as well as native hCG. In the past, small differences in proteolytic degradation have been observed upon chemical deglycosylation [24], but these differences can be attributed to a random deglycosylation upon chemical treatment. However, the CD spectrum of rehCG[αglycan[78]] and the CD spectrum of chemically deglycosylated hCG [31] might indicate a small conformational change upon deglycosylation. More evidence that enzymic deglycosylation of αAsn-52 has a local effect on the conformation of hCG was obtained from NMR spectroscopy. Taking into account the X-ray structure of hCG, different orientations of the β-subunit residues Tyr-59, Val-62, Phe-64 and Ala-83 are most probably responsible for the changes in the NMR spectra upon deglycosylation (Figure 8). Remarkably, the structure of hCG specifically deglycosylated at αAsn-52 by mutating αAsn-52 to αGln-52 showed a very different CD spectrum [30]. The CD spectrum of this mutant indicated a strong decrease in β-sheet structure, suggesting an important structural role for the glycan at αAsn-52. Possibly, this can be attributed to *in vivo* folding problems of hCG in the absence of the glycan at αAsn-52, thereby causing large differences in the hCG structure.

From our study and previous data we conclude that only minor conformational changes occur upon removal of the glycan at αAsn-52. Furthermore, it has been reported that chemically deglycosylated hCG [having at least $(GlcNAc)_2$-αAsn-52] binds to the receptor, but that signal transduction is abolished [6,7]. Studies on protein–carbohydrate interactions in glycoproteins have shown that mainly the inner carbohydrate residues have intramolecular contacts with the protein and therefore only these carbohydrate residues may account for a stabilizing or structural role [6,7,25,33,34]. These data support our conclusion that the loss of bioactivity of hCG upon removal of the glycan at αAsn-52 is not due to a conformational change or reduced stability of the heterodimer.

It has been shown by peptide mapping, site-directed mutagenesis studies and epitope mapping of the hCG–receptor complex that residues at the αβ interface around glycosylation site αAsn-52 and the seat-belt residues are important for strong receptor binding [35–38]. The residues at the C-terminus of the α-subunit, comprising the residues 88–91, and αPhe-18 on the opposite face of the hCG molecule, are involved in cAMP production [39,40]. Furthermore, there is experimental evidence that upon binding to the receptor the conformation of hCG changes and that consequently the receptor is activated [39,41–43]. We postulate that the bulky and extended glycan at αAsn-52 could have a function in inducing and stabilizing a conformational change in hCG upon binding to the receptor. This model would imply that the introduction of disulphide

bonds between α5–β8, α35–β35 and α37–β33 as performed by Heikoop et al. [18] could stabilize the active conformation of hCG, thereby creating a molecule that is bioactive even in the absence of the glycan at αAsn-52. In this respect elucidation of the three-dimensional structure of these hCG variants in solution could provide insight into the conformation of native hCG bound to the receptor.

This research has been supported financially by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (NWO-CW). We gratefully acknowledge Professor G. W. K. van Dedem for providing crude preparations of hCG. We thank Dr M. de Planque for his assistance in the CD measurements.

REFERENCES

1 Pierce, J. G. and Parsons, T. F. (1981) Glycoprotein hormones: structure and function. Annu. Rev. Biochem. **50**, 465–495
2 McFarland, K. C., Sprengel, R., Philips, H. S., Kohler, M., Rosemblit, N., Nikolics, K., Segaloff, D. L. and Seeburg, P. H. (1989) Lutropin–choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science **245**, 494–499
3 Taylor, C. W. (1990) The role of glycoproteins in transmembrane signalling. Biochem. J. **272**, 1–13
4 Talwar, G. P. (1979) Human chorionic gonadotropin and ovarian and placental steroidogenesis. J. Steroid Biochem. **11**, 27–34
5 Huth, J. R., Feng, W. and Ruddon, R. W. (1994) Redox conditions for stimulation of *in vitro* folding and assembly of the glycoprotein hormone chorionic gonadotropin. Biotechnol. Bioeng. **44**, 66–72
6 Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J. and Isaacs, N. W. (1994) Crystal structure of human chorionic gonadotropin. Nature (London) **369**, 455–461
7 Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E. and Hendrickson, W. A. (1994) Structure of human chorionic gonadotropin at 2.6 Å resolution from MAD analysis of the selenomethionyl protein. Structure **2**, 545–558
8 Erbel, P. J. A., Karimi-Nejad, Y., De Beer, T., Boelens, R., Kamerling, J. P. and Vliegenthart, J. F. G. (1999) Solution structure of the α-subunit of human chorionic gonadotropin. Eur. J. Biochem. **260**, 490–498
9 Weisshaar, G., Hiyama, J. and Renwick, A. G. C. (1991) Site-specific N-glycosylation of human chorionic gonadotrophin – structural analysis of glycopeptides by one- and two-dimensional ^{1}H NMR spectroscopy. Glycobiology **1**, 393–404
10 Tsuruhara, T., Dufau, M. L., Hickman, J. and Catt, K. J. (1972) Biological properties of hCG after removal of terminal sialic acid and galactose residues. Endocrinology **91**, 296–301
11 Moyle, W. R. (1975) Role of the carbohydrate of human chorionic gonadotropin in the mechanism of hormone action. J. Biol. Chem. **250**, 9163–9169
12 Channing, C. P., Sakai, C. N. and Bahl, O. P. (1978) Role of the carbohydrate residues of human chorionic gonadotropin in binding and stimulation of adenosine 3′,5′-monophospate accumulation by porcine granulosa cells. Endocrinology **103**, 341–348
13 Sairam, M. R. and Bhargavi, G. N. (1985) A role for glycosylation of the α subunit in transduction of biological signal in glycoprotein hormones. Science **229**, 65–67
14 Matzuk, M. M., Keene, J. L. and Boime, I. (1989) Site specificity of the chorionic gonadotropin N-linked oligosaccharides in signal transduction. J. Biol. Chem. **264**, 2409–2414
15 Calvo, F. O. and Ryan, R. J. (1985) Inhibition of adenylyl cyclase activity in rat corpora luteal tissue by glycopeptides of human chorionic gonadotropin. Biochemistry **24**, 1953–1959
16 Kobata, A. and Takeuchi, M. (1999) Structure, pathology and function of the N-linked sugar chains of human chorionic gonadotropin. Biochim. Biophys. Acta **1455**, 315–326
17 Amano, J., Sato, S., Nishimura, R., Mochizuki, M. and Kobata, A. (1989) Sialic acids, but not their linkage to galactose residues, are required for full expression of the biological activity of human chorionic gonadotropin. J. Biochem. (Tokyo) **105**, 339–340
18 Heikoop, J. C., Van den Boogaart, P., De Leeuw, R., Rose, U. M., Mulders, J. W. M. and Grootenhuis, P. D. J. (1998) Partially deglycosylated human choriogonadotropin, stabilized by intersubunit disulfide bonds, shows full bioactivity. Eur. J. Biochem. **253**, 354–356
19 Van Zuylen, C. W. E. M., De Beer, T., Rademaker, G. J., Haverkamp, J., Thomas-Oates, J. E., Hård, K., Kamerling, J. P. and Vliegenthart, J. F. G. (1995) Site-specific and complete enzymic deglycosylation of the native human chorionic gonadotropin α-subunit. Eur. J. Biochem. **231**, 754–760
20 Ingham, K. C., Weintraub, B. D. and Edelhoch, H. (1976) Kinetics of recombination of the subunits of human chorionic gonadotropin. Effect of subunit concentration. Biochemistry **15**, 1720–1726
21 Jönsson, U. and Malmqvist, M. (1992) Real time biospecific interaction analysis. The integration of surface plasmon resonance. Detection, general biospecific interface chemistry and microfluidics into one analytical system. Adv. Biosensors **2**, 291–336
22 Braunschweiler, L. and Ernst, R. R. (1983) Coherence transfer by isotropic mixing: application to protein correlation spectroscopy. J. Magn. Reson. **53**, 521–528
23 Jeener, J., Meier, B. H., Bachman, P. and Ernst, R. R. (1979) Investigation of exchange processes by two-dimensional NMR spectroscopy. J. Chem. Phys. **71**, 4546–4553
24 Merz, W. E. (1988) Evidence for impaired subunit interaction in chemically deglycosylated human chorionic gonadotropin. Biochem. Biophys. Res. Commun. **156**, 1271–1278
25 Erbel, P. J. A., Karimi-Nejad, Y., van Kuik, J. A., Boelens, R., Kamerling, J. P. and Vliegenthart, J. F. G. (2000) Effects of the N-linked glycans on the 3D structure of the free α-subunit of human chorionic gonadotropin. Biochemistry **39**, 6012–6021
26 Parsons, T. F., Strickland, T. W. and Pierce, J. G. (1984) Rapid and easy separation of the subunits of bovine and human glycoprotein hormones by use of high performance liquid chromatography. Endocrinology **114**, 2223–2227
27 De Beer, T., Van Zuylen, C. W. E. M., Leeflang, B. R., Hård, K., Boelens, R., Kaptein, R., Kamerling, J. P. and Vliegenthart, J. F. G. (1996) NMR studies of the free α subunit of human chorionic gonadotropin; structural influences of N-glycosylation and the β subunit on the conformation of the α subunit. Eur. J. Biochem. **241**, 229–242
28 Garnier, J., Salesse, R. and Pernollet, J. C. (1974) Reversible folding of human chorionic gonadotropin at acid pH or upon recombination of the α and β subunits. FEBS Lett. **45**, 166–171
29 Holladay, L. A. and Puett, D. (1975) Gonadotropin and subunit conformation. Arch. Biochem. Biophys. **171**, 708–720
30 Purohit, S., Shao, K., Balasubramanian, S. V. and Bahl, O. P. (1997) Mutants of human chorionic gonadotropin lacking N-glycosyl chains in the α-subunit. 1. Mechanism for the differential action of the N-linked carbohydrates. Biochemistry **36**, 12355–12363
31 Keutmann, H. T. (1983) Chemically deglycosylated human chorionic gonadotropin subunits: characterization and biological properties. Biochemistry **22**, 3067–3072
32 Parsons, T. F. and Pierce, J. G. (1983) Studies of histidine residues of human and bovine glycoprotein hormones by nuclear magnetic resonance. Int. J. Pept. Prot. Res. **21**, 522–535
33 Wyss, D. F., Choi, J. S., Li, J., Knoppers, M. H., Willis, K. J., Arulanadam, A. R. N., Smolyar, A., Reinherz, E. L. and Wagner, G. (1995) Conformation and function of the N-linked glycan in the adhesion domain of human CD2. Science **269**, 1273–1278
34 Goverman, J. M., Parsons, T. F. and Pierce, J. G. (1982) Enzymatic deglycosylation of the subunits of chorionic gonadotropin – effects on formation of tertiary structure and biological activity. J. Biol. Chem. **257**, 15059–15064
35 Charlesworth, M. C., McCormick, D. J., Madden, B. and Ryan, R. J. (1987) Inhibition of human choriotropin binding to receptor by human choriotropin α peptides. J. Biol. Chem. **262**, 13409–13416
36 Salesse, R., Bidart, J., Troalen, F., Bellet, D. and Garniet, J. (1990) Peptide mapping of intersubunit and receptor interactions of human choriogonadotropin. Mol. Cell. Endocrinol. **68**, 113–119
37 Remy, J., Couture, L., Pantel, J., Haertle, T., Rabesona, H., Bozon, V., Pajot-Augy, E., Robert, P., Salesse, R. and Bidart, J. (1996) Mapping of hCG-receptor complexes. Mol. Cell. Endocrinol. **125**, 79–91
38 Myers, R. V., Wang, Y. and Moyle, W. R. (2000) The surface of α-subunit loop 1 distant from the subunit interface is exposed in the hCG lutropin receptor complex. Biochim. Biophys. Acta **1475**, 390–394
39 Ryu, K. S., Chang, I. J. and Ji, T. H. (1996) Molecular mechanism of LH/CG receptor activation. Mol. Cell. Endocrinol. **125**, 93–100
40 Shao, K. and Bahl, O. P. (1997) Effect of modification of the β-hairpin and long loops simultaneously in both α- and β-subunits on the function of human choriogonadotropin: part II. Mol. Cell. Endocrinol. **127**, 179–187
41 Kikuchi, T., Koyama, M., Miyai, K., Kimura, T., Nishikiori, N., Kimura, T., Azuma, C., Kusunoki, M., Saji, F. and Tanizawa, O. (1994) Loss of biological activity of human chorionic gonadotropin (hCG) by the amino acid substitution on the ''GMGCC'' region of the α-subunit. Mol. Cell. Endocrinol. **102**, 1–7
42 Bhowmick, N., Huang, J., Puett, D., Isaacs, N. W. and Lapthorn, A. J. (1996) Determination of residues important in hormone binding to the extracellular domain of the luteinizing hormone/chorionic gonadotropin receptor by site-directed mutagenesis and modeling. Mol. Endocrinol. **10**, 1147–1159
43 Hong, S., Ji, I. and Ji, T. H. (1999) The β-subunit of human choriogonadotropin interacts with the exodomain of the luteinizing hormone/choriogonadotropin receptor and changes its interaction with the α-subunit. Mol. Endocrinol. **13**, 1285–1294

Received 12 October 2001/4 February 2002; accepted 13 March 2002