Carbohydrate Chains of Human Thyrotropin Are Differentially Susceptible to Endoglycosidase Removal on Combined and Free Polypeptide Subunits

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ABSTRACT: The accessibility of the asparagine-linked carbohydrate chains of human thyrotropin (hTSH) and free α and β subunits was investigated by their susceptibility to endoglycosidases H and F as well as to peptide:*N*-glycosidase F. Iodinated hTSH or subunits were incubated with a commercial enzyme preparation containing both endoglycosidase F and *N*-glycosidase F activities and further analyzed by sodium dodecyl sulfate gel electrophoresis followed by quantitative autoradiography. We show that, working at the optimum of the *N*-glycosidase activity, the relative amount of endoglycosidase required for half-deglycosylation was 20-fold higher for native hTSH than for the reduced and dissociated subunits. Under nondenaturing conditions, the 18K β subunit of hTSH could be readily deglycosylated to a 14K species while the 22K α subunit was largely resistant. However, both subunits were converted to an apoprotein of similar apparent molecular weight of 14K following reduction of disulfide bonds. In contrast, the free α subunit of human choriogonadotropin appeared fully sensitive to carbohydrate removal under nonreducing conditions despite the presence of a partially deglycosylated 18K intermediate at low concentration of endoglycosidase. Similarly, both hTSH-α and hTSH-β could be completely deglycosylated after acid dissociation of the native hormone. While all three carbohydrate chains of hTSH are sensitive to pure peptide:*N*-glycosidase F, only one on α and the single oligosaccharide present on β in hTSH appeared to be cleaved by pure endoglycosidase F. Interestingly, one of the two carbohydrate chains present on α was also found to be susceptible to endoglycosidase H. These findings indicate that while the carbohydrate chain on β is not involved in $\alpha\beta$ association, the oligosaccharides on α are hindered when hTSH subunits are combined.

The glycoprotein hormones derived from the pituitary and placenta consist of two noncovalently associated subunits, designated α and β. Within a species, the primary structure of the α subunit is common to all these hormones while the β subunits are distinct and confer hormonal and immunological specificity to the dimeric complex. The conformation of glycoprotein hormones has been studied by using several probes such as chemical modifications and heterologous recombination, but no successful X-ray crystallography has yet been achieved [for a review, see Pierce and Parsons (1981)]. Several studies have shown conformational changes to occur during hormone dissociation that are reversed during assembly of subunits, but the exact location of these changes is still unknown. Since the biological activity of these hormones has been found to be lost upon chemical deglycosylation while the binding to their receptor is largely preserved (Manjurath & Sairam, 1982; Kalyan & Bahl, 1982; Sairam & Bargham, 1986; Amr et al., 1980), the possible involvement of the carbohydrate chains in these events is of particular interest. Indeed, several laboratories had previously shown that the carbohydrate (CHO)[1] chains of glycoprotein hormones are required for $\alpha\beta$ subunit assembly of mouse TSH (Weintraub et al., 1983) as well as for proper folding of bovine LH-β (Strickland & Pierce, 1985) and to a lesser extent for that of bovine LH-α (Strickland et al., 1985). TSH subunit combination was shown to occur very early during hormone biosynthesis together with the trimming of CHO precursor forms (Ronin et al., 1984) and is fully achieved as the CHO chains are further elongated to complex structures (Magner et al., 1984) that contain sulfate and/or sialic acid (Parsons & Pierce, 1980; Green et al., 1985a,b). This multistep maturation probably involves discrete conformational changes dependent on the dimeric hormonal complex since uncombined LH-α subunits undergo additional O-linked glycosylation which renders them unable to further combine with fully glycosylated LH-β (Strickland & Pierce, 1983).

Very recently, endoglycosidases of different specificities have been commercially available that are able to cleave complex as well as high-mannose asparagine-linked CHO chains and allow selective deglycosylation of native glycoproteins. While this work was in progress, two reports claimed that native equine pituitary and chorionic gonadotropins (Swedlow et al., 1985) as well as bovine TSH (Lee et al., 1986) were totally resistant to peptide:*N*-glycosidase F although free α-subunit could be partially deglycosylated by this enzyme. We now report on the successful enzymatic deglycosylation of human TSH by various endoglycosidase activities. Human TSH-α is composed of a 92 amino acid polypeptide reticulated with 5 disulfide bonds and 2 asparagine-linked CHO chains while hTSH-β is somewhat larger (112 amino acids) with a single glycosylated asparagine residue and 6 disulfide bonds (Pierce

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[1] Abbreviations: TSH, thyrotropin (thyroid-stimulating hormone); LH, lutropin (luteinizing hormone); h, human; b, bovine; m, mouse; hCG, human chorionic gonadotropin; Endo F, endo-β-*N*-acetylglucosaminidase F (EC 3.2.1.96); Endo H, endo-β-*N*-acetylglucosaminidase H (EC 3.2.1.96); PNGase F, peptide:*N*-glycosidase F (EC 3.2.2.18); CHO, carbohydrate(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ME, mercaptoethanol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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& Parsons, 1981). No CHO structure is presently available for TSH. In this regard, the examination of the susceptibility of CHO chains on combined and free subunits to endoglycosidase removal provides a new insight on their structural properties and more generally on the involvement of the glycosylation sites in the three-dimensional conformation of the $\alpha\beta$ hormone complex.

Materials and Methods

Supplies and Hormone Preparation. hTSH, hTSH-β, and hCG-α were from the National Institutes of Health (Bethesda, MD). Na^{125}I (13–17 mCi/μg) was from Amersham. Endo F types I and II as well as Endo H were purchased from Boehringer Mannheim (France). PNGase F was from Genzyme (Boston, MA). All the chemicals were of analytical grade.

Hormone Iodination. hTSH or hCG-α (5 μg) was iodinated with Iodogen (10 μg) and Na^{125}I (300 μCi) in 50 μL of 50 mM phosphate buffer, pH 7.5, containing 0.15 M NaCl for 1 min at room temperature. The reaction was stopped by dilution with 1 mL of 0.5% bovine serum albumin in the buffer, and isolation of the hormone was achieved by gel filtration on a Bio-Gel AcA column (1 × 50 cm) with a flow rate of 25 mL/h. The specific activity of ^{125}I-hTSH was 22 μCi/μg and was 62.4 μCi/μg for hCG-α. hTSH-β (5 μg) was labeled with chloramine T (10 μg) and Na^{125}I (500 μCi) in 20 μL of 50 mM phosphate buffer, pH 7.5, containing 0.15 M NaCl for 30 s at room temperature. The reaction was stopped by adding a mixture of 5 mM glycine and 5 mM tyrosine followed by 1 mL of 50 mM phosphate buffer containing 0.5% serum albumin and 0.01% NaN_3. ^{125}I-hTSH was purified on a Sephadex G-25 column (1.5 × 7 cm) with a specific activity of 37.4 μCi/μg.

All the iodinated hormone derivatives had been selected according to their reactivity toward specific polyclonal and monoclonal antibodies.

Endoglycosidase Digestion. Iodinated hTSH, hCG-α, or hTSH-β (25 000 cpm) was incubated for 24 h at 37 °C in 50 μL of 50 mM Tris-HCl buffer, pH 8.6, containing 25 mM EDTA, 0.05% serum albumin, and variable amounts of Endo F type II and Triton X-100 as mentioned. Under these conditions, the Endo F preparation exhibited the same deglycosylating activity toward ribonuclease B and fetuin, suggesting the prominent endoglycosidase acting at this pH to be of broad specificity like PNGase F. In contrast, fetuin failed to be deglycosylated at pH 4.0 while ribonuclease B was, indicating the presence of another endoglycosidase activity in the preparation, more likely to be that of Endo F itself as previously described (Plummer et al., 1984).

Treatment with Endo H or Endo F type I was carried out in 50 μL of sodium acetate buffer, pH 4.5 and 5.0, respectively, containing 0.15 M NaCl, 0.5% bovine serum albumin, 1% ME, and/or 1% Triton.

All the concentrations of endoglycosidases are given in units defined according to the commercial suppliers.

Polyacrylamide Gel Electrophoresis and Autoradiography. SDS slab gel electrophoresis was performed according to Laemmli on 15% polyacrylamide gels with a bis(acrylamide):acrylamide ratio of 1:37.5. The Bio-Rad protein standard kit was used for determination of molecular weight on the intact subunits and the deglycosylated products. After fixation, the gels were dried and exposed to Kodak X-OMAT film at −70 °C for variable periods of time. Autoradiograms were quantitated by using a Shimadzu gel scanner.

Acid Dissociation. Iodinated hTSH (200 000 cpm) was dissociated into labeled α and β subunits by incubation for 5 h at 37 °C in 0.1 M glycine buffer, pH 2.5. After neutralization, aliquots (25 000 cpm) of the incubation mixture were further treated with a supraoptimal concentration of Endo F under nondenaturing conditions as described above. The labeled products were finally analyzed by gel electrophoresis followed by autoradiography.

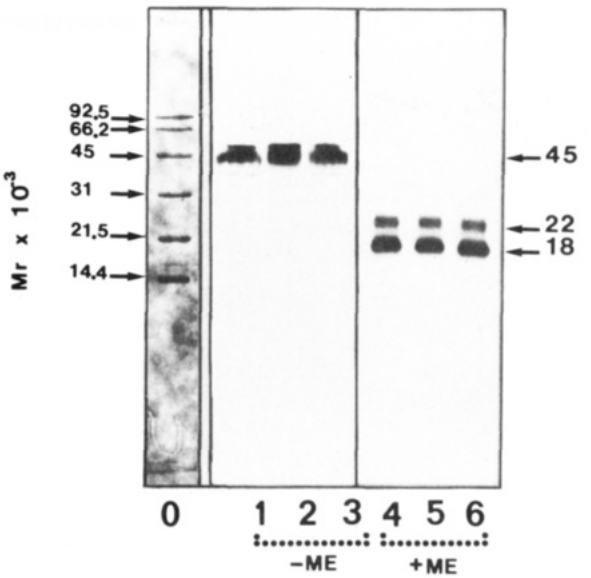

FIGURE 1: SDS gel electrophoresis of ^{125}I-hTSH. Lane 0 shows Bio-Rad standards: phosphorylase B (M_r 92 500), bovine serum albumin (M_r 66 200), ovalbumin (M_r 45 000), carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and lysozyme (M_r 14 400). Lane 1, ^{125}I-hTSH, lane 2, ^{125}I-hTSH preincubated with 1% Triton X-100; lane 3, ^{125}I-hTSH preincubated with 0.1% SDS; lane 4, ^{125}I-hTSH treated with 1% ME; lane 5, ^{125}I-hTSH treated with 1% ME and 1% Triton X-100; lane 6, ^{125}I-hTSH treated with 1% ME and 0.1% SDS.

Results

Iodinated human thyrotropin was treated with various endoglycosidases under nondissociating and dissociating conditions to compare the accessibility of the CHO chains in the combined and free subunits. As shown in Figure 1, hTSH remained as a dimeric $\alpha\beta$ complex of apparent M_r 45 000 in the presence of a nonionic detergent like Triton X-100 (lane 2) or Nonidet P-40 (data not shown) or even in the presence of a low amount of SDS (lane 3). In contrast, hTSH is fully dissociated under reducing conditions into an α subunit of apparent molecular size 22K and a β subunit of 18K (lanes 4–6).

Deglycosylation of hTSH under Reducing and Nonreducing Conditions. Since oligosaccharide-cleaving enzymes have generally been found poorly active on native glycoproteins (Chu, 1981), denaturation often appeared necessary to promote complete access to the glycosylation sites. We therefore investigated TSH deglycosylation by the presumed PNGase F activity present in the commercial Endo F preparation in the presence or absence of a disulfide bond reductive reagent. Under reducing conditions, both α and β subunits of hTSH appeared fully sensitive to endoglycosidase treatment (Figure 2). Increasing amounts of endoglycosidase led to the successive disappearance of the 22K and 18K bands (Figure 2A) together with the appearance of a new species comigrating with a protein marker of M_r 14 000. Quantitative estimation of the autoradiogram indicated that the half-maximal effect occurred for 0.2 milliunit of the enzyme preparation (Figure 2B).

In contrast, the 22K subunit was found largely resistant to Endo F treatment under nonreducing conditions while the 18K subunit disappeared as a function of the amount of endo-

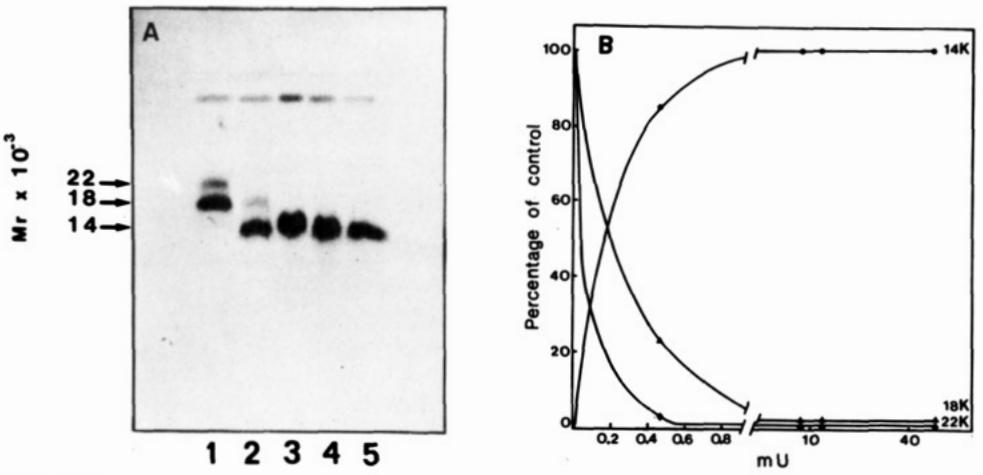

FIGURE 2: Endo F treatment of ^{125}I-hTSH under reducing conditions (1% Triton X-100, 0.1% SDS, and 1% ME). (Panel A) SDS–PAGE of the products: lane 1, no endoglycosidase; lane 2, 0.5 milliunit; lane 3, 2.5 milliunits; lane 4, 12 milliunits; lane 5, 48 milliunits of Endo F. (Panel B) Quantitative autoradiography of the data. The percentage of 22K and 18K deglycosylation was estimated from the ratio of each labeled species before (taken as 100%) and after treatment with Endo F. The percentage of the 14K component was deduced from the labeling of the species obtained under optimal Endo F treatment and taken as 100%.

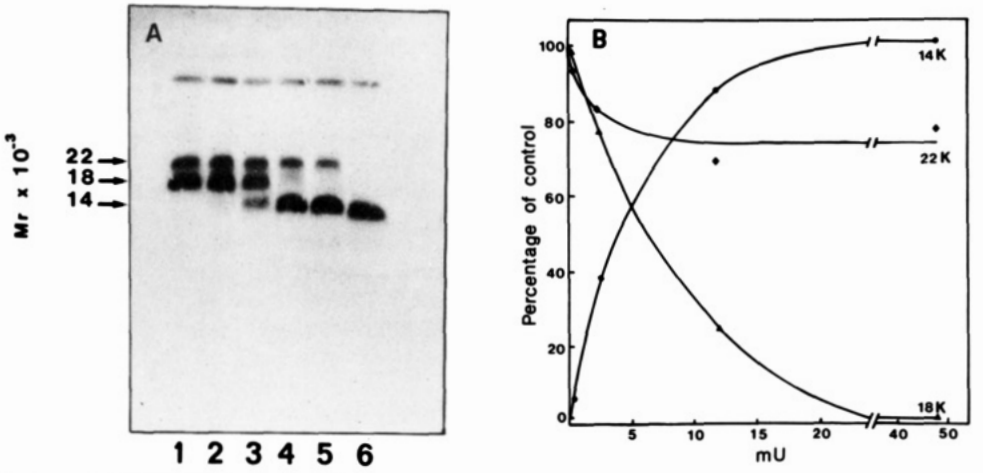

FIGURE 3: Endo F treatment of ^{125}I-hTSH under nondissociating nonreducing conditions (1% Triton X-100). (Panel A) SDS–PAGE of the products: lane 1, no endoglycosidase; lane 2, 0.5 milliunit; lane 3, 2.5 milliunits; lane 4, 12 milliunits; lane 5, 48 milliunits of Endo F; lane 6, 48 milliunits of Endo F + 1% ME. (Panel B) Quantitative autoradiography of the data.

glycosidase (Figure 3A, lanes 2–5). Addition of mercaptoethanol together with the highest concentration of Endo F resulted, however, in a successful deglycosylation of both subunits (lane 6). As can be deduced from the quantitative data (Figure 3B), the half-maximal dose of the enzyme preparation required under nonreducing conditions was increased up to 4 milliunits.

These findings indicate that CHO chains on native hTSH are far less accessible to enzymatic removal than those on free subunits, the three-dimensional structure of which had been destroyed by cleavage of the disulfide bonds.

Stability of the Deglycosylating Activity. Since it was recently reported that Endo F could be inactivated by SDS during prolonged incubation (Chu, 1986), we assessed whether partial deglycosylation of hTSH under nondissociating conditions could be due to some inactivation of the enzyme. Indeed, the presence of a supraoptimal concentration of endoglycosidase was able to deglycosylate the β subunit of hTSH in the absence of any detergent while little deglycosylation occurred in the presence of SDS (data not shown), indicating that the enzyme preparation was indeed largely inactivated by this detergent. Denaturing hTSH by mercaptoethanol did not overcome the loss of Endo F action while adding Triton to the incubation mixture fully restored the deglycosylating activity (data not shown).

To confirm further that the inactivation of Endo F occurred in the presence of SDS and was not dependent on the substrate itself, we also investigated the deglycosylation of free hTSH-β and hCG-α subunits under these conditions. Quantitation of the data are summarized in Table I. No significant loss of material was observed in all instances, suggesting that no degradation by protease activity was detectable in our incubations. Endo F was found to be inhibited by SDS to an extent of 80% on hTSH and 100% on free α subunit but only to 20% on β alone. Interestingly, the α subunit seemed to be deglycosylated to an 18K form while under reducing conditions a 14K species could be obtained (see also Figure 4), suggesting the occurrence of an intermediate step during CHO removal. Triton could satisfactorily overcome enzyme inactivation for all three substrates. Reducing the disulfide bridges in the presence of SDS did not increase the rate of deglycosylation on hTSH and hTSH-β but definitely enhanced the accumulation of an intermediate 18K form in the case of α subunit. These findings suggest that both the inactivating effect of SDS on the deglycosylating activity and the inaccessibility of CHO chains in the native protein hormone severely impaired the rate of deglycosylation. The stabilizing effect of Triton on the enzyme activity in conjunction with prior cleavage of the disulfide bonds of the substrate facilitated the complete deglycosylation of hTSH, α and β subunits.

Table I: Deglycosylation of hTSH and Free Subunits in the Presence of Detergents and Mercaptoethanol

Endo F treatment	%[a] of labeling in hTSH 22K	hTSH 18K	hTSH 14K	hTSH-β 18K	hTSH-β 14K	hCG-α 22K	hCG-α 18K	hCG-α 14K
without Endo F	40	60	0	100	0	100	0	0
with Endo F								
+0.1% SDS	40	36	24	21	79	89	11	0
+0.1% SDS + 1% Triton	28	6	66	0	100	0	0	100
+0.1% SDS + 1% ME	38	32	30	26	74	36	64	0
+1% Triton + 1% ME	0	11	89	0	100	0	11	89
+0.1% SDS + 1% Triton + 1% ME	0	4	96	0	100	0	0	100

[a] As deduced from quantitation of autoradiograms.

To assess further whether the length of endoglycosidase treatment was optimal in spite of enzyme instability, we carried out kinetic experiments in the presence and in the absence of Triton. Free hTSH-β subunit was selected as substrate since it contains a single CHO chain easily removed by Endo F under nondenaturing conditions. Using a supraoptimal dose of Endo F (12 milliunits), we observed the disappearance of the 18K band as a function of time with a concomitant accumulation of the 14K species (data not shown). The reaction was complete over a period of time of 15 h. An apparent molecular weight of 14 000 for deglycosylated β subunit is totally compatible with an apoprotein containing 113 amino acids (M_r 14 700) as deduced from the sequencing of the human β gene (Hayashizaki et al., 1985) and would therefore represent this subunit onto which the only CHO chain (M_r ~2500) has been cleaved.

Deglycosylation of Free α and β Subunits. Since the CHO chains present on α in hTSH appeared largely resistant to Endo F compared to that of β, we separately investigated the susceptibility of the uncombined α and β subunits. As within a species the α subunits are virtually identical among the glycoprotein hormone family, hCG-α was used as the source of free α subunit. Figure 4 shows the comparative deglycosylation of hTSH (panel A), hCG-α (panel B), and hTSH-β (panel C) under both reducing and nonreducing conditions. It can be seen that both hCG-α (lane 4) and hTSH-β (lane 8) were converted to a 14K species (lanes 6 and 9, respectively) following Endo F treatment under nonreducing conditions. Therefore, the 14K band observed for deglycosylated hTSH after cleavage of the disulfide bonds (lane 3) should represent a mixture of α- and β-apoproteins not resolved in our gel system. Interestingly, when hCG-α was treated with Endo F in the absence of detergent, an intermediate species of 18K could be detected (lane 5). Adding Triton to the incubation mixture allowed further deglycosylation of α to the apoprotein. As already suggested from biosynthetic studies with mouse thyrotropic tumors (Weintraub et al., 1983), this 18K form may represent an α subunit missing one CHO chain. The 14K form, however, is likely to correspond to the fully deglycosylated α subunit assuming that removal of one CHO chain results in a migration shift of approximately 4K. This is in good agreement with an apoprotein of M_r 14 400 as deduced from the sequencing of the human α gene (Fiddes & Goodman, 1981).

These findings further indicate that the CHO chains present on either free α or free β subunits are fully susceptible to enzymatic removal under nonreducing conditions whereas those of α were totally resistant on native hTSH. Moreover, these latter apparently display a different susceptibility to Endo F, suggesting that their CHO structures may not be identical.

Deglycosylation of hTSH following Subunit Dissociation. To elucidate whether the CHO chains on α could be inaccessible to Endo F due to polypeptide interactions in hTSH,

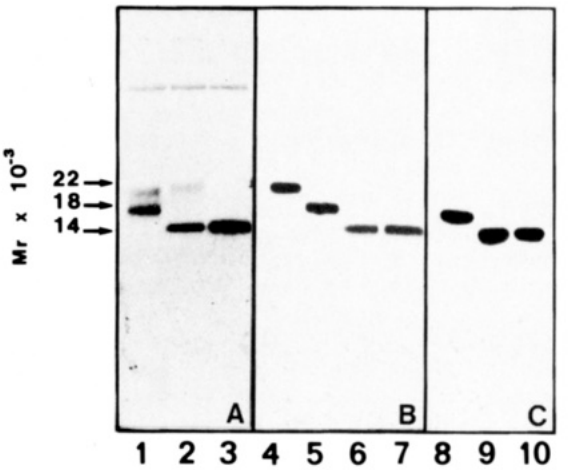

FIGURE 4: Comparison of Endo F treatment of ^{125}I-hTSH (panel A), free hCG-α (panel B), and free hTSH-β (panel C). The hormone and subunits were incubated in the absence (lanes 1, 4, and 8) or presence of 48 milliunits of endoglycosidase (lanes 5 and 9) with 1% Triton (lanes 2, 6, and 10) plus 1% ME (lanes 3 and 7).

we dissociated the hormone αβ complex at low pH and subsequently treated the uncombined hTSH-α and hTSH-β subunits with Endo F. Any CHO chain potentially masked by subunit interactions should be expected to be uncovered and susceptible to the deglycosylating enzyme under these conditions. Incubating hTSH at low pH and 37 °C over 4 h resulted in the dissociation of the hormone complex of 45K into a 25K component that appeared to be a mixture of α and β subunits after reduction of disulfide bonds (data not shown). As already found for hCG-α, the CHO chains of dissociated hTSH-α subunits proved to be highly susceptible to Endo F with a two-step deglycosylation process (data not shown).

These findings further support the idea that on the α subunit associated with β in hTSH, the two CHO chains are inacessible to Endo F because of some interactions resulting from subunit assembly. In contrast, the CHO chain present on β is apparently not involved in this process.

Deglycosylation of Unlabeled hTSH. To ascertain whether the iodination procedure could have artifactually influenced the susceptibility of hTSH to CHO removal, we scaled up our deglycosylation assay to microgram amounts of hormone. Silver staining of the polyacrylamide gels confirmed that treatment of 7 μg of hTSH with 1.5 units of Endo F under native conditions resulted in the disappearance of the 18K band with the concomitant presence of a 14K band (data not shown). Therefore, the bulky iodide atoms introduced in the amino acid side chains of the hormone did not have any steric hindrance on the accessibility of the CHO chains to Endo F.

Susceptibility of hTSH to Endo H, Pure Endo F, and PNGase F. To get a better understanding of the specificity of the deglycosylating activities present in the Endo F preparation used in this study, we separately analyzed the sus-

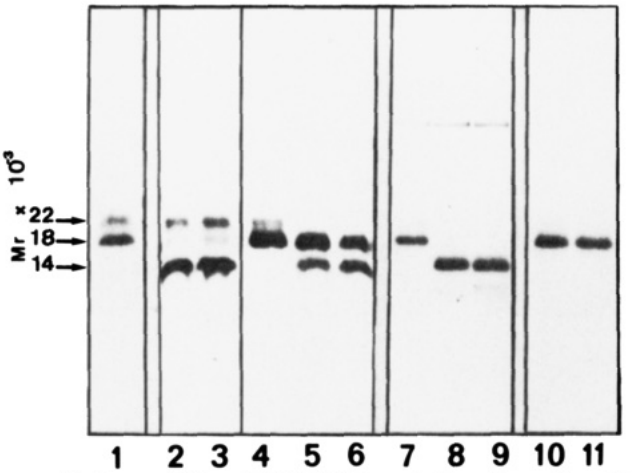

FIGURE 5: Susceptibility of ^{125}I-hTSH to endoglycosidases H and F and PNGase F. ^{125}I-hTSH was incubated either under nondenaturing conditions, in the absence (lane 1) or presence (lane 2, 50 milliunits; lane 3, 500 milliunits) of pure Endo F type I, or under denaturing conditions, in the presence of pure Endo F type I (lane 4, 5 milliunits; lane 5, 50 milliunits; lane 6, 500 milliunits), pure PNGase F (lane 7, 6.5 milliunits; lane 8, 50 milliunits; lane 9, 500 milliunits), or Endo H (lane 10, 10 microunits; lane 11, 100 microunits).

ceptibility of hTSH to pure Endo F and pure PNGase F provided by different commercial suppliers (Figure 5). No quantitative comparison could be made between all the enzyme preparations used in the present study since the endoglycosidases have not been thoroughly assayed on the same glycoprotein substrate. Incubation under native conditions (lanes 2–3) revealed that, in fact, the 18K β subunit is the polypeptide sensitive to pure Endo F in intact hTSH while the CHO chains present on α are inaccessible as previously shown in Figure 2. However, treatment of hTSH with pure Endo F at pH 5.0 under reducing conditions did not result in complete deglycosylation as found above with the so-called Endo F preparation incubated at pH 8.5. The 22K band completely disappeared as a function of pure Endo F concentration together with an increase in the 14K species, but the 18K band was always persistent (lanes 4–6). These findings show that only two out of the three CHO chains in hTSH are sensitive to pure Endo F. They suggest that β deglycosylation accounts for the 14K species observed under native conditions and that one CHO chain on α is responsible for a shift in the migration of this subunit from a 22K to an 18K species when Endo F treatment is performed under reducing conditions. In contrast, deglycosylation of the hormone with pure PNGase F at pH 8.5 (lanes 7–9) satisfactorily reproduced the data obtained with the so-called Endo F preparation used previously at pH 8.5. After cleavage of disulfide bonds, full deglycosylation was achieved since a single 14K species was observed as already seen in Figure 1. Interestingly, Endo H was found to have an effect on hTSH by probably removing one of the two CHO chains present on α under disulfide bond reduction. A single 18K band was observed, suggesting a comigration of the β subunit resistant to this enzyme with the α subunit missing one CHO chain (lanes 10–11).

Altogether, these findings indicate that the three CHO chains present on hTSH are probably of different structure. The oligosaccharide present on the β subunit is Endo H resistant but Endo F sensitive and thus may be of biantennary complex structure. One CHO chain on α is sensitive to Endo H and Endo F, suggesting that it contains at least the mannose-rich sequence required by Endo H (high-mannose or hybrid structure). The other α-linked CHO chain is probably more highly branched since it is resistant to both Endo H and Endo F but sensitive to PNGase F, known to cleave all complex as well as high-mannose asparagine-linked oligosaccharides.

Discussion

The current study presents a thorough investigation of enzymatic CHO removal on native hTSH compared to free α and β hormone subunits. We demonstrate that the two CHO chains present on α are of different structures but both resistant to endoglycosidase action in intact with hTSH and fully sensitive in the dissociated hormone. This suggests that at least their core portion should be interacting with peptide domains involved directly or indirectly in subunit assembly. In contrast, the β subunit appears readily deglycosylated in the native hormone as well as in its free form, indicating no special constraint arising from polypeptide association.

Glycoprotein hormones have already been deglycosylated by chemical means (Manjunath et al., 1982; Kalyan et al., 1982; Sairam et al., 1986; Amr et al., 1985). Loss of biological activity had been strikingly observed in all instances, although deglycosylation was often of poor yield probably owing to a denaturation and/or clips in the polypeptide chains. When possible, deglycosylation by endoglycosidases should be preferred to preserve the native form of the glycoprotein substrate but presents the disadvantage that the N-glycosidic linkage may be hindered by polypeptide folding structures. Such an accessibility factor has already been reported for other secretory nonendocrine glycoproteins (Chu et al., 1981) and might in part explain the failure of pure PNGase F to deglycosylate equine gonadotropins (Swedlow et al., 1986) or bTSH (Lee et al., 1986). In addition, since these very recent studies were carried out on microgram quantities of hormones, it would have been of prohibitive cost to thoroughly investigate the enzyme to substrate ratio as we have performed here on trace amounts of iodinated hormone. Indeed, the present study shows a 20 time difference in the amount of deglycosylating enzyme able to cleave the hTSH-β-linked CHO chain that is easily accessible in the native hormone as well as in the free subunit under nondenaturing conditions.

In addition to the accessibility, the structure of the CHO chains has also to be considered to optimize the deglycosylation procedure by endoglycosidase. Unfortunately, no information is available so far for TSH. Biantennary complex asparagine-linked CHO chains terminated with unusual sulfated GalNAc sequences had been recently described for bLH (Green et al., 1985a). In an in vitro sulfation assay, bTSH appeared to be a better acceptor than hTSH, suggesting that the underlying CHO structures from both species may be different but nevertheless contain the adequate residues to be sulfated (Green et al., 1985b). Whether sulfate-containing structures are resistant to Endo F or PNGase F remains also largely unknown. Although most of bLH carbohydrate chains are released by Endo F, up to 20% of them appeared resistant, however, when synthesized by bovine pituitary explants (Green et al., 1985), but no such endoglycosidase resistant CHO structure could be detected in our hTSH preparation. This further strengthens the idea that endoglycosidases may be of greater help than exoglycosidases in deglycosylating glycoprotein hormones containing CHO chains potentially capped with sulfate residues.

Regarding the nature of hTSH glycosylation, our present study points to three putative types of CHO structures. The oligosaccharide present on β is likely to be of the biantennary complex type (Tarentino et al., 1983). Surprisingly, one CHO chain on α in hTSH is fully susceptible to Endo H and Endo F, suggesting a high-mannose or hybrid structure. Indeed, such a hybrid mannose-rich structure designated as S_1/hybrid

was recently reported to represent as little as 8% of hTSH CHO chains susceptible to be labeled in vitro with sulfate (Green et al., 1985a). However, this might not be the same CHO structure in the human hormone since methylation analysis of hTSH-α revealed no terminal mannose residues (Nillson et al., 1986). More highly branched CHO structures must also be present on α since there appears also to be an oligosaccharide resistant to both Endo H and Endo F but susceptible to PNGase F. Therefore, these findings suggest for each glycosylation site of hTSH subunits a restricted pattern in CHO branching. While this paper was in preparation, it was proposed for two closely related glycoproteins, Mac-1 and LFA-1, present on the surface of lymphocytes that differential subunit association largely influences site-specific glycosylation (Dahms & Hart, 1986). This may very well also apply to pituitary glycoprotein hormones for which the combination of a specific β-polypeptide to the common α subunit might determine the final structure of each individual CHO chain.

The differential susceptibility of α-linked CHO chains to enzymatic removal appears related to whether or not α- and β-polypeptides are combined. Indeed, reversible conformational changes were shown to occur on each subunit of glycoprotein hormones upon dissociation (Strickland & Puett, 1982), and they may as well also unmask the core portions of α-linked CHO chains. Alternatively, since the glycosylation sites on α are located in the primary structure close to the sequence involved in subunit interaction (Pierce et al., 1981), the possibility exists that these CHO chains may be located between the α and β subunits. In immunoglobulins, they have been shown by X-ray crystallography to be in close contact between the two heavy chains in the hinge region (Sutton & Phillips, 1983). Any of these possibilities involves the CHO chains as possible regulatory candidates for the modulation of the overall conformation of the hormone dimer. Interestingly, distinct subpopulations of mTSH that probably differ in their CHO content have been reported to behave as agonists and antagonists of bTSH in the stimulation of adenylate cyclase (Joshi & Weintraub, 1983). As already demonstrated for LH, the CHO chains of the α subunit appear especially critical in this process, further supporting our observation that their spatial arrangement is essential for a functional $\alpha\beta$ dimer (Sairam & Bhargavi, 1986). If their structure and also the glycosylation of the β subunit are under endocrine control as recently described for mTSH (Gesundheit et al., 1986), there might be a subtle regulation of the biological activity of the hormone complex through the processing of its CHO chains.

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