

The asparagine-linked oligosaccharides at individual glycosylation sites in human thyrotrophin

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The asparagine-linked carbohydrate structures at each of the three glycosylation sites of human thyrotrophin were investigated by 400 MHz ¹H-NMR spectroscopy. Highly purified, biologically active human thyrotrophin (hTSH) was dissociated into its subunits hTSH α (glycosylated at Asn 52 and Asn 78) and hTSH β (glycosylated at Asn 23). The α -subunit was further treated with trypsin which gave two glycopeptides that were subsequently separated by reverse-phase HPLC and identified by amino acid sequence analysis. The oligosaccharides were liberated from hTSH α glycopeptides and from intact hTSH β by hydrazinolysis, and were fractionated as alditols by anion-exchange and ion-suppression amine-adsorption HPLC preparatory to structural analysis. The *N*-glycans present on hTSH were mainly diantennary complex-type structures with a common Man α 1-3 branch that terminated with 4-*O*-sulphated GalNAc. The Man α 1-6 branch displayed structural heterogeneity in the terminal sequence, with chiefly α 2-3-sialylated Gal and/or 4-*O*-sulphated GalNAc. The relative amounts of the two major complete diantennary oligosaccharides and their core fucosylation differed according to glycosylation site; the sulphated/sialylated diantennary oligosaccharide was most abundant at the two sites on the α -subunit, whereas the disulphated, core-fucosylated oligosaccharide was more plentiful on the β -subunit. Some interesting structural features, not previously reported for the *N*-glycans of hTSH, included 3-*O*-sulphated galactose (SO₄-3Gal) and peripheral fucose (Fuc α 1-3GlcNAc) in the Man α 1-6 branch of some diantennary structures; the former suggests the presence of a hitherto uncharacterized galactose-3-*O*-sulphotransferase in thyrotroph cells of the human anterior pituitary gland.

Key words: human thyrotrophin/*N*-glycans/¹H-NMR spectroscopy/site-specific glycosylation

Introduction

Thyrotrophin, a glycoprotein hormone produced by thyrotroph cells of the anterior pituitary gland, is a vital component of the regulatory mechanism that maintains the structure and function of the thyroid gland and thereby influences metabolism.

In common with the structurally related hormones follicle-stimulating hormone, luteinizing hormone and chorionic gonadotrophin, the biologically active form of thyrotrophin is a heterodimer that consists of two non-covalently linked subunits, designated α and β , both of which are highly cross-linked by disulphide bonds. While the

peptide component of the α -subunit is virtually identical in all four hormones within a given species, and is highly conserved within that species, the β -subunit confers hormonal specificity.

The α -subunit of human thyrotrophin (hTSH α) contains two glycosylation sites at Asn 52 and Asn 78, respectively, whereas the site of attachment of carbohydrate in the β -subunit (hTSH β) is Asn 23 (Pierce and Parsons, 1981).

Advances in methods of analysis in carbohydrate chemistry and biochemistry, especially within the last 20 years (e.g. Vliegthart *et al.*, 1983; Kobata, 1984; Dell, 1987), have provided a very large corpus of information on the oligosaccharide components of the glycoprotein hormones which are essential for biological activity [see reviews by Baenziger and Green (1988), Ryan *et al.*, (1988), Hartree and Renwick (1992)].

The carbohydrate structures of human thyrotrophin attracted the early interest of Kim *et al.* (1967), Pierce *et al.* (1971), Cornell and Pierce (1973) and Hara *et al.* (1978), but results varied between laboratories, probably because of differences in methods and preparations used for analysis, the variable occurrence of charged species and microheterogeneity in the oligosaccharide chains, factors that were unknown or poorly understood at the time. Further information from sugar and methylation analysis was reported by Nilsson *et al.* (1986), and the first detailed structures of the native human hormone were reported by Green and Baenziger (1988a, b). However, full appreciation of the mode of action of any hormone demands complete knowledge of its structure and, to this end, we now report our studies on the *N*-glycans at individual glycosylation sites in human thyrotrophin.

Results

Isolation of hTSH subunits

Figure 1a shows a typical HPLC profile for hTSH obtained after treatment with 6 M guanidine HCl. Under the conditions employed, the dissociated hormone was resolved into three peaks. The two major peaks with retention times of ~21.5 and 28 min were identified as hTSH α and hTSH β , respectively, from their amino acid compositions. N-Terminal sequence analyses gave only one sequence for each subunit (hTSH α , Val-Gln-Asp-; hTSH β , Phe-(Cys)-Ile-Pro), which were in agreement with the reported N-terminal sequences for hTSH subunits (Sairam, 1983). The small peak that eluted at 18 min gave the identical N-terminal sequence as that of the α -subunit, which indicated a form of hTSH α . Reverse-phase HPLC analysis, which effected complete separation of the α - and β -subunits, showed only a single peak for each subunit preparation, when 25 μ g of each sample were analysed at 206 nm (Figure 1b). At this low wave length, 0.25 μ g of each subunit was detectable as a distinct peak using the absorbance range 0.1 absorbance unit full scale (AUFS), but no subunit preparation showed > ~1% cross-contamination. However, because this method did not permit complete separation of intact hTSH and

its β -subunit, sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was used under reducing conditions to examine possible contamination of the β -subunit with any remaining intact hormone. Each subunit (5–10 μ g) stained with Coomassie Blue showed essentially single bands at distinct positions (apparent M_r , 21.5 kDa for α 1 and 2, 18.0 kDa for β) which indicated that dissociation and isolation of subunits under the conditions used were complete. No structural difference was discerned during the above analyses between the minor and major forms of hTSH α (α 1 and α 2, respectively) and since they may have represented heterogeneous forms with different carbohydrate structures, both were combined in subsequent studies without further characterization. The yields of subunits starting from 145 mg of purified hTSH were 8 mg hTSH α 1, 42 mg hTSH α 2 and 40 mg hTSH β .

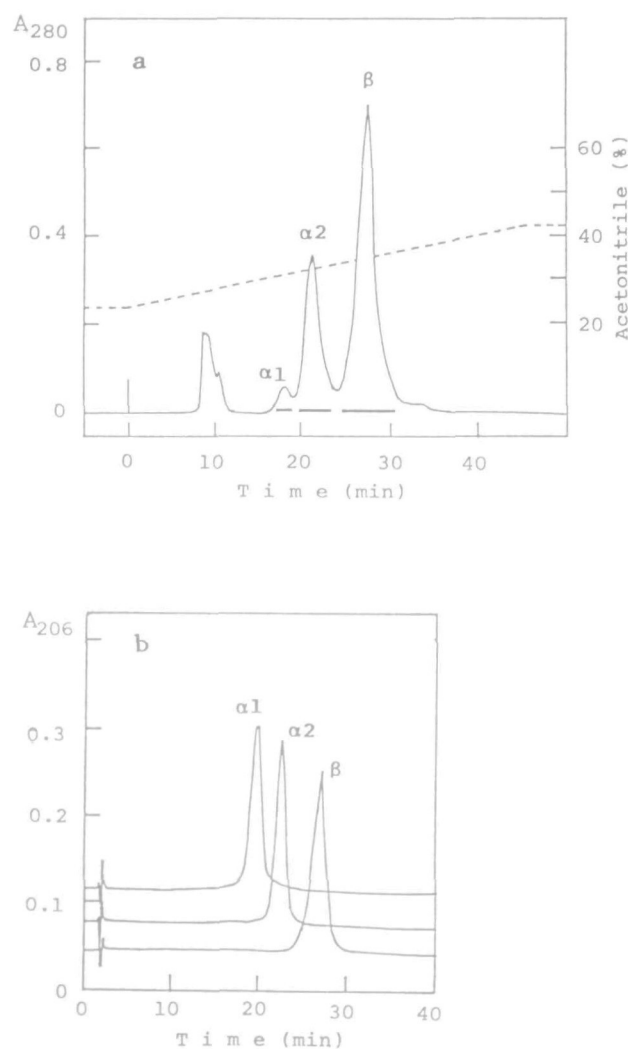


Fig. 1. Separation of hTSH α and hTSH β by reverse-phase HPLC. (a) Following dissociation with guanidine HCl, hTSH was applied in aliquots (10 mg) to a Vydac Protein C₄ column (1 $\times$ 25 cm) and eluted with a 45 min linear gradient of acetonitrile (24–42%) in 0.1 M triethylamine phosphate buffer (pH 6.5) at a flow rate of 1.6 ml/min. Subunit peaks, α 1, α 2 and β , were collected from each run and separately pooled. (b) 25 μ g of each of the hTSH subunit preparations were analysed by reverse-phase HPLC on a Vydac C₄ column (0.46 $\times$ 15 cm) using a 40 min linear gradient of acetonitrile (18–42%) in 0.1 M triethylamine phosphate buffer (pH 6.5) at a flow rate of 0.8 ml/min.

Separation of glycopeptides of hTSH α -subunit

The two glycosylation sites, Asn 52 and Asn 78, were isolated as tryptic glycopeptides by reverse-phase HPLC, following the procedures used for human lutrophin (hLH) (Weisshaar *et al.*, 1991a), except that one gel-filtration step (Sephadex G-25) was included to remove small peptides prior to reverse-phase HPLC separation. This facilitated the purification of glycopeptides of interest from the tryptic digests of hTSH α . The separation of tryptic digests of reduced and alkylated hTSH α (RCM-hTSH α) by reverse-phase HPLC is shown in Figure 2. Fractions collected as indicated in the figure were subjected to 1 H-NMR analysis, which showed on the basis of characteristic carbohydrate signals that three peaks, viz. fractions 1, 2 and 4, contained glycopeptides. It was inferred from characteristic signals of amino acid residues that fractions 1 and 2 contained glycopeptides α GPI (residues 52–63) and α GPII (residues 76–91), respectively, as described in detail elsewhere (Weisshaar *et al.*, 1991a, b). 1 H-NMR analysis of these glycopeptide fractions revealed a number of chemical shifts that distinguished α GPI from α GPII, e.g. Asn-linked GlcNAc H1 and NAc as well as aromatic protons of His and Tyr which occurred only in α GPII. The absence of these characteristic signals associated with α GPI in the spectrum of α GPII, and vice versa, indicated little or no cross-contamination. 1 H-NMR analysis also showed no evidence of peptide heterogeneity in the respective glycopeptide fractions, thus the somewhat heterogeneous peaks observed for both glycopeptides probably reflected carbohydrate heterogeneity. Another (very minor) glycopeptide fraction (fraction 4) was found to contain small amounts of an α GPII-related glycopeptide together with some peptides and was not used in the structural studies reported here. The yields of glycopeptides obtained from 47 mg hTSH α were 9.5 mg for α GPI (fraction 1) and 11.0 mg for α GPII (fraction 2). Oligosaccharides obtained by hydrazinolysis from α GPI, α GPII and the β subunit

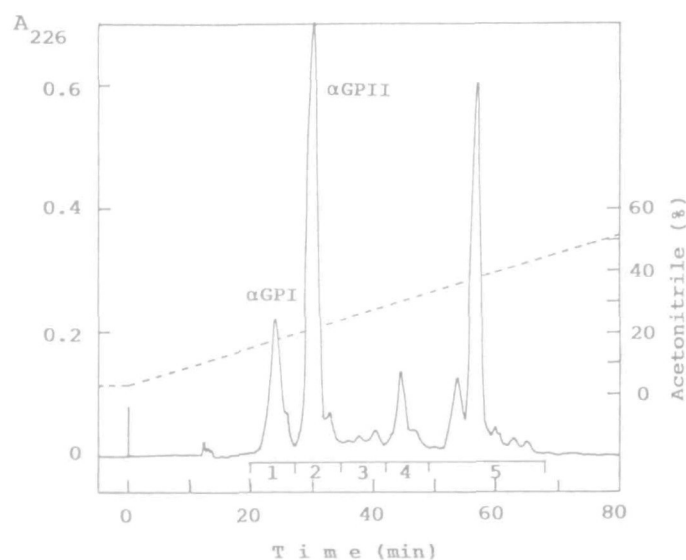


Fig. 2. Separation of tryptic glycopeptides from hTSH α by reverse-phase HPLC. After Sephadex G-25 chromatography, tryptic digests of reduced and alkylated hTSH α were applied in aliquots (10 mg) to a Vydac Protein C₄ column (1 $\times$ 25 cm) and eluted with an 80 min linear gradient of acetonitrile (3–51%) in 0.1 M triethylamine phosphate buffer (pH 6.5) at a flow rate of 1.2 ml/min. Five fractions were collected from each run as indicated and separately pooled.

which represented 18% of total oligosaccharides on hTSH β (Asn 23). S-³N was also identified in the corresponding sub-fractions of A₁-3 and A₂-3; it was found to be the major structure at both glycosylation sites of hTSH α (25 and 29% at Asn 52 and Asn 78, respectively), albeit with a low degree of core fucosylation (5–10%).

The structural element NeuAc α 2-6Gal was not found in any of the major amine-adsorption subfractions of A₁-3, A₂-3 or B-3. In the few respective minor subfractions where an ¹H-NMR spectrum was attempted, no signals around $\delta = 1.7$ p.p.m. (NeuAc α 2-6, H3a) and $\delta = 2.7$ p.p.m. (Neu α 2-6, H3e) were detected. These spectra were totally uninterpretable because of the minuscule amounts of sample (probably <50 μ g).

The ¹H-NMR spectrum of fraction B-3a is depicted in Figure 5. The major component was readily identified as the diantennary, disulphated, core-fucosylated compound S2 (see the structure on top of Figure 5) on account of its characteristic ¹H-NMR data reported earlier (Weisshaar *et al.*, 1990). The presence of a second component was particularly apparent from the additional proton resonances at $\delta = 4.339$ p.p.m. (doublet of doublets; coupling constants $J = 3.2$ and 10.0 Hz) and $\delta = 4.294$ p.p.m. ('quasi'-doublet; $J = 3.2$ and <1 Hz). These signals have recently been found to be typical of the H3 and H4 protons, respectively, in a non-reducing terminal β -galactose residue bearing a sulphate group at C3 (Kamerling *et al.*, 1988; De Waard *et al.*, 1991). The chemical shifts of the

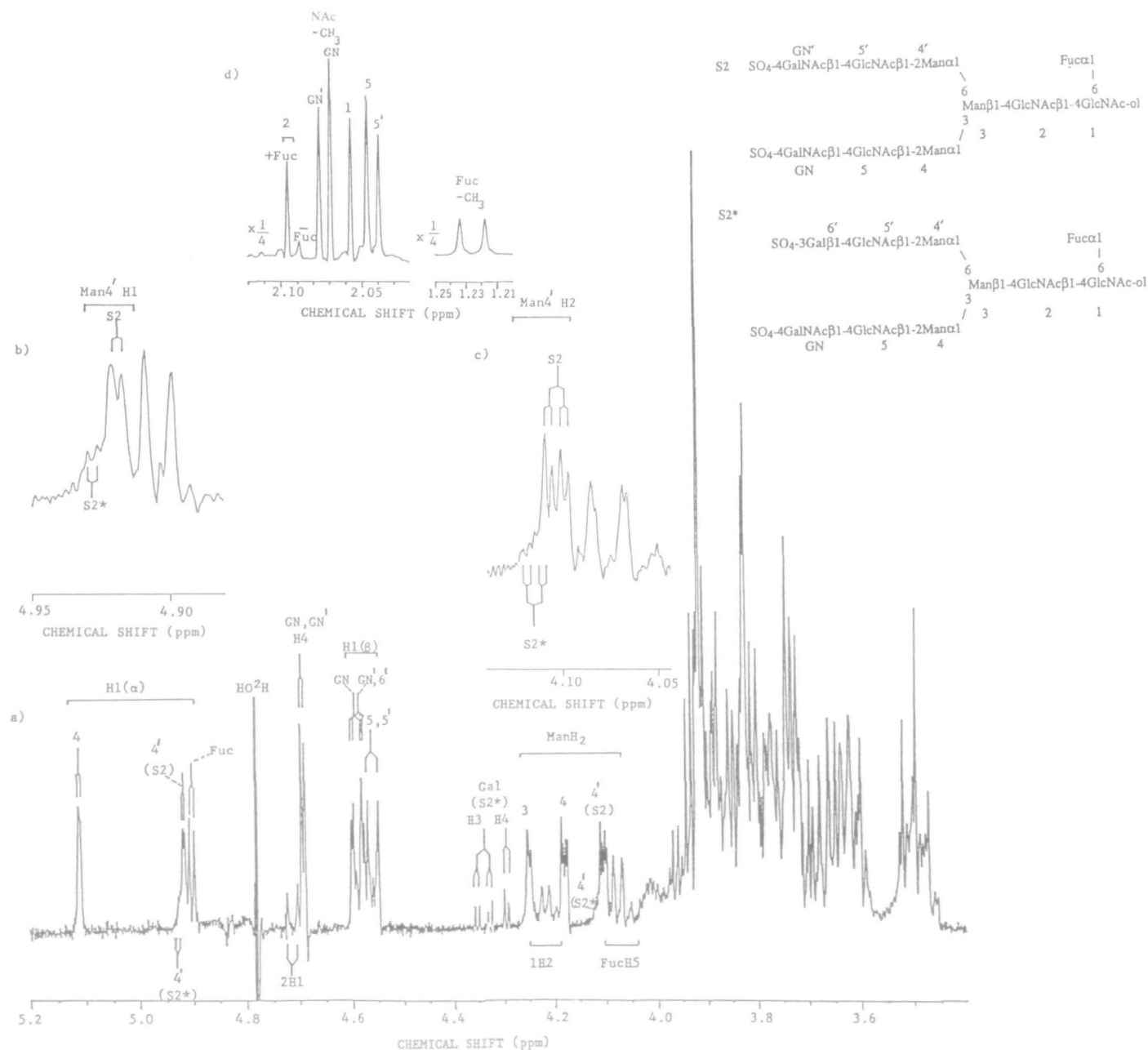


Fig. 5. The 400 MHz ¹H-NMR spectrum of fraction B-3a recorded in ²H₂O at 298°K. A survey spectrum ($\delta = 3.4$ –5.2 p.p.m.) is shown in (a) and resolution-enhanced regions of Man4' H1 ($\delta = 4.88$ –4.95 p.p.m.) and Man4' H2 ($\delta = 4.05$ –4.15 p.p.m.) are reproduced in insets (b) and (c). N-Acetyl (NAc) and FucCH₃ signals are expanded and plotted with lower intensity scale (d). Labelling of characteristic resonances refers to structures shown in the top right-hand corner.

corresponding Gal H1 ($\delta = 4.590$ p.p.m.) and H2 ($\delta = 3.683$ p.p.m.), as determined by two-dimensional scalar shift-correlated NMR spectroscopy (2D COSY) (spectrum not shown), were also in excellent agreement with the data reported by the same authors for the structural unit $\text{SO}_4\text{-3Gal}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6}$ in a diantennary oligosaccharide alditol isolated from porcine thyroglobulin. Compared with the ^1H -NMR spectrum of (pure) **S2** (Weisshaar *et al.*, 1990), the spectrum of fraction B-3a showed additional signals for H1 ($\delta = 4.928$ p.p.m.) and H2 ($\delta = 4.115$ p.p.m.) of the $\alpha\text{1-6}$ -linked Man4' residue (Table I), both with intensities comparable to those of Gal H3 and H4. On the other hand, only one set of chemical shifts for the $\alpha\text{1-3}$ -linked Man4 residue was observed. The ^1H -NMR analysis therefore suggests that the only structural difference between the major component **S2** and the minor component, denoted **S2***, lies in the replacement of $\text{SO}_4\text{-4GalNAc}$ (in **S2**) by $\text{SO}_4\text{-3Gal}$ (in **S2***) in the Man $\alpha\text{1-6}$ branch. The likely confinement of 3-*O*-sulphated galactose to the $\alpha\text{1-6}$ branch is corroborated by the fact that it was not detectable in any of the monosulphated compounds, which are mainly characterized by an $\alpha\text{1-3}$ branch terminating in $\text{SO}_4\text{-4GalNAc}$ and an incompletely processed $\alpha\text{1-6}$ branch (see below).

Taking into account two 4-*O*-sulphated GalNAc in **S2** and one each of 4-*O*-sulphated GalNAc and 3-*O*-sulphated Gal in **S2***, the ratio of the signal intensities of GalNAc H4 ($\delta = 4.692$ p.p.m.) and Gal H4 ($\delta = 4.294$ p.p.m.) of 7:1 indicated that the molar ratio **S2**:**S2*** is 3:1. This is consistent with the relative intensities of the GalNAc *N*-acetyl peaks at $\delta = 2.069$ p.p.m. ($\alpha\text{1-3}$ branch; **S2** and **S2***) and $\delta = 2.076$ p.p.m. ($\alpha\text{1-6}$ branch; **S2** only), determined to be 4:3.

Approximately 85 mol % of the formerly Asn-bound GlcNAc carried an $\alpha\text{1-6}$ -linked fucose residue, as calculated from the NAc signal intensities of the respective GlcNAc2 in the fucosylated ($\delta = 2.097$ p.p.m.) and non-fucosylated ($\delta = 2.090$ p.p.m.) components (Weisshaar *et al.*, 1990).

The established chemical shifts of the sequence $\text{SO}_4\text{-3Gal}\beta\text{1-4GlcNAc}\beta\text{1-2Man4'}$ are compiled in Table I. Comparison

of **S2** and **S2*** reveals that replacement of $\text{SO}_4\text{-4GalNAc}$ by $\text{SO}_4\text{-3Gal}$ does not apparently alter the chemical shifts of the neighbouring GlcNAc residue, but leads to significant down-field shifts for Man4' H1 ($\Delta\delta = 0.010$ p.p.m.) and H2 ($\Delta\delta = 0.011$ p.p.m.); the resonances of the anomeric protons of GalNAc (GN') and Gal (6') coincide at $\delta = 4.590$ p.p.m. All remaining characteristic chemical shifts of both compounds [compiled for **S2** in Weisshaar *et al.* (1990)] were found to be identical (within the limits of experimental error). Compounds **S2** and **S2*** (approximate ratio of 3:1) were also identified in the corresponding fractions obtained from both glycosylation sites of hTSH α . In contrast to hTSH β , only 5–10% of the reduced GlcNAc carried a fucose residue.

Oligosaccharides with a single negative charge were eluted in the anion-exchange fractions A₁₋₂, A₂₋₂ and B-2, respectively (Figure 3). They constituted 36, 44 and 28% of total oligosaccharides formerly attached to Asn 52, Asn 78 and Asn 23, respectively. Subfractionation by ion-suppression amine-adsorption HPLC gave rise to three main fractions. Reference to ^1H -NMR data previously reported on a number of monosulphated oligosaccharides (Weisshaar *et al.*, 1990) greatly facilitated structural determination of the constituent oligosaccharides by inspection of the ^1H -NMR spectra of the respective fractions, the results of which are included in Table II.

The oligosaccharides were characterized by a Man $\alpha\text{1-3}$ branch that terminated in 4-*O*-sulphated GalNAc and a

Table II. Structures and relative amounts of the majority of N-linked oligosaccharides at individual glycosylation sites of human thyrotrophin. Relative abundances are expressed in mol % of total oligosaccharides at each site. The core structure (in bold type), common to all identified components, is extended by the listed sequences; minor (e.g. monosialylated) oligosaccharides make up the total of 100%

R		Relative abundance (%) in		
		hTSH α		hTSH β
		Asn 52	Asn 78	Asn 23
SO ₄ -4GalNAc β 1-4GlcNAc β 1-2	S2	15	6	24
SO ₄ -3Gal β 1-4GlcNAc β 1-2	S2*	5	2	8
NeuAc α 2-3Gal β 1-4GlcNAc β 1-2	S-3'N	25	29	18
Gal β 1-4GlcNAc β 1-2	S1-6'^b	10	11	10
GlcNAc β 1-2	S1-5'	4	9	3
H	S1-4'	11	9	8
Man α 1-3	S1-A	5	4	3
[Man α 1-6]Man α 1-3	S1-AB	3	2	< 2
Tri-/tetra-charged oligosaccharides		12	15	18
Neutral oligosaccharides		2	2	3
		92	89	97

^a5–10% of structures on hTSH α (Asn 52 and Asn 78) and 85–90% of structures on hTSH β (Asn 23) carry a fucose residue $\alpha\text{1-6}$ -linked to the proximal *N*-acetylglucosamine.

^bApproximately one-third of **S-6'** carries a (peripheral) fucose $\alpha\text{1-3}$ -linked to GlcNAc5' in the Man $\alpha\text{1-6}$ branch.

Table I. ^1H -Chemical shifts for constituent sugar residues within the Man $\alpha\text{1-6}$ branch of compound **S2***. Chemical shifts were measured in $^2\text{H}_2\text{O}$ at 298°K and referenced to internal acetone ($\delta = 2.225$ p.p.m.). The remaining proton resonances of **S2*** (complete structure in Figure 5) are essentially identical to those of compound **S2** reported in Weisshaar *et al.* (1990)

Residue	Proton	Chemical shift (δ) in		$\Delta\delta$ (p.p.m.)
		S2* $\text{SO}_4\text{-3Gal}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6}$	S2 $\text{SO}_4\text{-4GalNAc}\beta\text{1-4GlcNAc}\beta\text{1-2Man}\alpha\text{1-6}$	
Gal	H1	4.590	—	
	H2	3.683	—	
	H3	4.339	—	
	H4	4.294	—	
GalNAc	H1	—	4.590	
	H2	—	3.938	
	H3	—	3.902	
	H4	—	4.692	
GlcNAc	H1	4.558	4.558	0
	H2	3.738	3.738	0
Man	H1	4.928	4.918	0.010
	H2	4.115	4.104	0.011

Man α 1-6 branch that terminated in either Gal6' (compound denoted **S1-6'**), GlcNAc5' (**S1-5'**) or Man4' (**S1-4'**), along with small amounts of the hybrid-type structures **S1-A** (eluted with **S1-5'**) and **S1-AB** (eluted with **S1-6'**). In addition, roughly one-third of compound **S1-6'** (at all three sites) carried a fucose residue in α 1-3-linkage to GlcNAc5', as indicated by the characteristic resonances (Vliegthart *et al.*, 1983) of Fuc H1 (δ = 5.127 p.p.m.) and CH₃ (δ = 1.180 p.p.m.), Gal6' H1 (δ = 4.448 p.p.m.) and GlcNAc5' NAc (δ = 2.042 p.p.m.). The Fuc H2 signal was found by 2D COSY at δ = 3.692 p.p.m., whereas the Fuc H5 resonance (expected around 4.83 p.p.m.) could not be determined.

Furthermore, trace amounts of (mainly α 2-3-) mono-sialylated compounds were discernible in the spectra of the respective anion-exchange fractions, but could not be located in any of the minor amine-adsorption subfractions due to their low incidence.

As mentioned above, no monosulphated compounds that contained 3-O-sulphated Gal were present, as concluded from the lack of the Gal H3 and H4 signals, characteristic of this structural feature.

No information about the neutral oligosaccharides (fraction 1, Figure 3) could be obtained; the minute amount of material (2–3% of total) was insufficient for ¹H-NMR analysis.

Only limited structural information could be extracted from the ¹H-NMR spectra of the respective anion-exchange fractions 4 and 5 (Figure 3). The spectrum of fraction B-4 (hTSH β) indicated the presence of (mainly core-fucosylated) triantennary sugar chains, as inferred from the Man H2 signal pattern (Vliegthart *et al.*, 1983); they contained the peripheral units SO₄-4GalNAc, SO₄-3Gal, NeuAc α 2-3Gal and little NeuAc α 2-6Gal, recognized by their characteristic ¹H-NMR resonances. The same structural features were found in the spectrum of fraction B-5; in addition, appreciable amounts of the sequence NeuAc α 2-3Gal β 1-3(NeuAc α 2-6)GlcNAc β appeared to be present, as suggested by the occurrence of the typical Gal H1 doublet at δ = 4.499 p.p.m. (Green *et al.*, 1988) and the corresponding H2 at δ = 3.52 p.p.m. Unfortunately, owing to structural heterogeneity and low amounts of sample, no detailed structures could be established. The ¹H-NMR spectra of the corresponding fractions derived from hTSH α were of even more inferior quality, but suggested the presence of structures similar to those on the β -subunit; however, they were nearly devoid of α 1-6-linked fucose.

Discussion

The Asn-linked oligosaccharides of hTSH were structurally characterized by HPLC, endo- and exoglycosidase digestion, and lectin-affinity chromatography, and a variety of sulphated and sialylated oligosaccharides, including a unique sulphated/sialylated diantennary oligosaccharide, were identified (Green and Baenziger, 1988a, b). In these studies, however, the structural details at specific glycosylation sites were not examined, although these are important in understanding oligosaccharide processing which is implicated in the biological function of TSH (Weintraub *et al.*, 1985). Several studies have demonstrated the importance of N-glycans in TSH in the expression of intrinsic biological activity and metabolic clearance (Amr *et al.*, 1985; Amir *et al.*, 1987; Thotakura *et al.*, 1990). In addition, available evidence suggests that the presence of carbohydrates in the common α -subunit, particularly those at Asn 52,

is essential for signal transduction (Sairam, 1989), although it remains unclear whether or not specific oligosaccharide processing is necessary. In the case of hTSH, it has been reported that the N-glycans at its three glycosylation sites differ with respect to their branching structures (Ronin *et al.*, 1987). However, since these conclusions were solely derived from experiments with endoglycosidases, more precise structural characterization of oligosaccharides was needed.

In the present study, we have sought to define the oligosaccharide structures of native hTSH at individual glycosylation sites using 1D and 2D ¹H-NMR spectroscopy. To achieve this goal, oligosaccharides were prepared from each glycosylation site in quantities of 2–3 μ mol, which from our previous studies allowed the detailed characterization of most individual structures. Moreover, since hTSH exists in multiple forms that differ in charge and glycosylation (Sergi *et al.*, 1991), hydrophobic chromatography was used for the isolation of the hormone to obviate artefactual modification of its intrapituitary forms.

The N-glycans isolated from each glycosylation site of hTSH were highly heterogeneous with a spectrum of structures that ranged from hybrid type to mono-, di-, tri- and possibly tetra-antennary complex type. The vast majority of oligosaccharides had a common Man α 1-3 branch that terminated in 4-O-sulphated GalNAc, but differed widely in the peripheral structure of the Man α 1-6 branch (see Table II). Diantennary complex-type structures comprised ~60% of the oligosaccharides at each site and, on a mol % basis, the sulphated/sialylated (**S-³N**) and the disulphated (**S2**) diantennary oligosaccharides were most abundant, accounting for 24 and 15% of total oligosaccharides on hTSH, respectively. Apart from core fucosylation, the structures and overall amounts of these major diantennary oligosaccharides were very similar to those reported by Green and Baenziger (1988a, b); however, those of other components differed significantly in several aspects. Disulphated **S2*** and monosulphated **S1-5'** and **S1-6'** oligosaccharides, which together accounted for 20% of total oligosaccharides on hTSH, were not detected in their studies. Moreover, the presence of reported levels of mono- and disialylated oligosaccharides was not confirmed by our results. In addition, we found only small amounts of neutral but appreciable amounts of tri- (and possibly tetra-) charged oligosaccharides in our hormone preparations. Quantitative differences and the discrepancies mentioned above may be partly due to the different modes of hormone purification; however, since some oligosaccharides identified by ¹H-NMR could not be completely purified by HPLC, their unambiguous identification by other analytical methods may be difficult.

While the oligosaccharides present at each glycosylation site of hTSH were mainly diantennary structures, relative amounts of the major complete diantennary structures (**S-³N** and **S2**) and their core fucosylation were noticeably different between the two subunits. At both sites on the α -subunit, the sulphated/sialylated component **S-³N** was more abundant whereas the disulphated, core-fucosylated **S2** was more plentiful at the single glycosylation site in the β -subunit.

The distinct distribution of the **S-³N** and **S2** structures, and their different degrees of core fucosylation in hTSH α (5–10%) and hTSH β (85–90%) may reflect the influence of surrounding polypeptide chains on the glycosylation of these subunits. The markedly limited core fucosylation of oligosaccharides in hTSH α appears to correlate with the observation of Ronin *et al.* (1987) that the α -subunit in the intact hormone was resistant to

Endo F digestion, whereas the β -subunit was sensitive and both subunits were readily deglycosylated following dissociation. These results suggested that the two N-linkage sites on hTSH α are inaccessible to this endoglycosidase due to subunit association. Such steric hindrance may also affect the access of α 1-6 fucosyltransferase to the Asn-linked GlcNAc residues on this subunit.

In the oligosaccharide processing of hTSH, since the Man α 1-3 branch of the oligosaccharides was exclusively terminated with 4-O-sulphated GalNAc at all three sites, it is apparent that local protein structures exert few constraints on the action of GalNAc- and sulphotransferases on this branch. In contrast, processing of the Man α 1-6 branch by GalNAc transferase appears to be markedly limited at all three sites, particularly at those on the α -subunit, since the Gal residue was mainly transferred to this branch at each site (Table II). The preferential action of GalNAc transferase on the Man α 1-3 branch may be closely related to the substrate specificity and properties of this unique processing enzyme, which requires recognition of a tripeptide for its action (Smith and Baenziger, 1992). GalNAc transferase present in bovine pituitary membranes has been shown to act specifically on and with high affinity for the agalacto-oligosaccharides in some glycoprotein hormones (Smith and Baenziger, 1988). On the other hand, the apparently predominant addition of Gal to the Man α 1-6 branch appears to reflect the relatively high activity of the Gal transferase compared with the GalNAc transferase in human thyrotroph cells, since cell-free sulphation of hTSH with bovine pituitary membranes showed that synthesis of the disulphated oligosaccharides was preponderant (Green *et al.*, 1985).

In an earlier investigation of the biosynthesis of sulphated oligosaccharides on the pituitary glycoprotein hormones, the activities of GalNAc- and Gal transferases were thought to be key factors in regulating the biosynthesis of the disulphated and sulphated/sialylated diantennary complex oligosaccharides, since GalNAc and Gal residues added to GlcNAc β 1-2Man α 1-6 are usually terminated with sulphate and sialic acid, respectively (Green *et al.*, 1986). In the case of hTSH, however, chain termination of the Man α 1-6 branch of oligosaccharides appears to be more complex, because it involves sulphation of Gal and peripheral fucosylation of GlcNAc, in addition to the more dominant sialylation of Gal and sulphation of GalNAc. It was shown that the terminal glycosylation of the Gal β 1-4-GlcNAc sequence in the complex Asn-linked oligosaccharides by Gal- α 2-6-sialyltransferase and GlcNAc- α 1-3-fucosyltransferase is mutually exclusive (Paulson *et al.*, 1978). It has also been described that Gal-3-O-sulphotransferase and sialyltransferases compete for the same primary substrate (De Waard *et al.*, 1991). Our structural data indicate that S1-6', in which Gal is added to GlcNAc β 1-2Man α 1-6, is the major synthetic intermediate at each site in hTSH, although S2 was more abundant than S-3N on the β -subunit. This observation suggests that, in some instances at least, there may be competition between these sialyl-, sulfo- and fucosyltransferases in the terminal glycosylation of human thyrotrophin.

As mentioned above, two new structures in the N-glycans of hTSH have been identified in the present study. One is a disulphated structure S2* that bears two distinct sulphated terminal sequences SO₄-3Gal (Man α 1-6 branch) and SO₄-4-GalNAc (Man α 1-3 branch), and the other is a monosulphated structure S1-6' with a peripheral fucose α 1-3-linked to GlcNAc5'. The sequence SO₄-3Gal β 1-4GlcNAc has recently been described as a novel feature of the N-glycans of porcine,

calf and human thyroglobulin (Kamerling *et al.*, 1988; Spiro and Bhoyroo, 1988; Spiro and Spiro, 1988; De Waard *et al.*, 1991). Moreover, a galactose-3-O-sulphotransferase has been identified and characterized in the Golgi compartment of calf thyroid microsomes (Kato and Spiro, 1989). Our findings suggest that such a sulphotransferase is also present in the thyrotroph cells of human pituitary glands, together with the known GalNAc-4-O-sulphotransferase (Baenziger and Green, 1988). The relatively low incidence of chain-terminating SO₄-3Gal in the carbohydrate structures of hTSH could well reflect low activity of this enzyme, especially in comparison with (mainly α 2-3) sialyltransferase.

Materials and methods

Biologically active hTSH was prepared as previously described (Hiyama *et al.*, 1990).

Isolation of hTSH subunits

Purified hTSH (145 mg) was dissolved to 10 mg/ml in 0.1 M sodium phosphate buffer (pH 7.0) that contained 6 M guanidine HCl and 0.02% sodium azide, and was incubated for 20 h at 37°C. The treated hormone was applied in aliquots (1 ml) to a Vydac Protein C₄ column (1 × 25 cm, Separations Group, Hesperia, CA) and eluted with a linear gradient of acetonitrile in 0.1 M triethylamine phosphate (TEAP) buffer (pH 6.5). The 45 min linear gradient from 40–70% mobile phase B was applied at a flow rate of 1.6 ml/min, with mobile phases A (0.1 M TEAP, pH 6.5) and B (0.1 M TEAP in 60% (v/v) acetonitrile, pH 6.5). Fractions that contained the subunits were collected, desalted on a Sephadex G-25 column (1.6 × 55 cm) in 0.5% ammonium bicarbonate and freeze-dried. Subunits were identified by amino acid and partial N-terminal sequence analyses, and their purity was checked by SDS-PAGE on 15% slab gels (Segrest and Jackson, 1972). Analytical reverse-phase HPLC on a Vydac C₄ column (0.46 × 15 cm) was carried out as described by Hiyama *et al.* (1990), except that a 40 min linear acetonitrile gradient from 30 to 70% mobile phase B was used with the systems given above.

Separation of glycopeptides of hTSH α -subunit

Glycopeptides that included Asn 52 (α GPI) and Asn 78 (α GPII), respectively, were separated by reverse-phase HPLC after tryptic digestion. hTSH α -subunit (47 mg) was dissolved in 9 ml of 1 M Tris-HCl buffer (pH 8.2) that contained 6 M guanidine HCl and 1 mM EDTA. After the vessel was purged with N₂, dithiothreitol (45 mg) was added and the mixture was incubated for 4 h at 37°C. Following reduction, iodoacetic acid (135 mg) was added dropwise with stirring in 500 μ l of 0.5 N NaOH; alkylation was carried out in the dark for 30 min at room temperature. The RCM-hTSH α was desalted on a Sephadex G-25 column (1.6 × 55 cm) in 0.5% ammonium bicarbonate and freeze-dried. The RCM-hTSH α (45 mg) was dissolved in 0.2 M ammonium bicarbonate (5 mg/ml) and digested with L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Sigma type XIII, 900 μ g/90 μ l 1 mM HCl) at 37°C for 2 h. The tryptic digests were applied to a Sephadex G-25 column (1.6 × 55 cm) in water, and the mixture of glycopeptides and peptides eluted in the void volume were separated by reverse-phase HPLC on a Vydac C₄ column (1 × 25 cm) in 0.1 M TEAP buffer (pH 6.5). An 80 min linear gradient from 5 to 85% mobile phase B was used at a flow rate of 1.2 ml/min, with mobile phases A (0.1 M TEAP, pH 6.5) and B (0.1 M TEAP in 60% (v/v) acetonitrile, pH 6.5). After desalting on a Sephadex G-15 column (1.6 × 55 cm) in water and lyophilization, the HPLC fractions were analysed by ¹H-NMR for the presence of carbohydrates and for peptide homogeneity; the glycopeptides were identified by amino acid sequence analysis.

Generation and fractionation of oligosaccharide alditols

The glycopeptides α GPI (9.0 mg) and α GPII (10.5 mg) from hTSH α and hTSH β (39 mg) were each treated with anhydrous hydrazine (1.5 ml) at 100°C for 10 h, and the released oligosaccharides were recovered by cation-exchange chromatography on an AG 50W-X4 column (1.6 × 55 cm) in water after re-N-acetylation with acetic anhydride (500 μ l). The oligosaccharides were reduced with sodium borohydride (10 mg) in 0.2 M sodium borate buffer, pH 9.8 (6 ml), at 30°C for 3 h and freed from reaction products by gel filtration on a Sephadex G-15 column (1.6 × 55 cm) in water as described previously (Weisshaar *et al.*, 1990). The oligosaccharide alditol mixture obtained from

each sample was fractionated by anion-exchange (Baenziger and Natowicz, 1981) and ion-suppression amine-adsorption HPLC (Mellis and Baenziger, 1983) on a MicroPak AX-5 column (0.4 × 30 cm) as described previously (Weisshaar *et al.*, 1991a). Each oligosaccharide fraction was desalted on Sephadex G-15 in water prior to structural analysis.

For quantitative analysis, 5% of oligosaccharides were radiolabelled by reduction with [³H]sodium borohydride (600 mCi/mmol, New England Nuclear) in 0.2 M sodium borate buffer (pH 9.8), following the procedures described by Takasaki *et al.* (1982). The labelled oligosaccharide alditols (1.2–2.3 × 10⁵ c.p.m.) were subjected to anion-exchange and amine-adsorption HPLC under conditions identical to those used for preparative separations. Fractions (300 µl) were collected and the radioactivity of aliquots was determined on an LKB 1209 Rackbeta liquid scintillation counter. The recovery of labelled oligosaccharides after HPLC separation was 88–100%.

¹H-NMR spectroscopy

The 400 MHz ¹H-NMR spectra of the desalted samples, that were exchanged in ²H₂O and lyophilized repeatedly, were recorded on a Bruker AM-400 spectrometer at a probe temperature of 298°K and a pD of ~5.0 (determined in several but not all fractions). Chemical shifts refer to internal acetone (δ = 2.225 p.p.m.). Two-dimensional COSY experiments were performed as described earlier (Weisshaar *et al.*, 1990, 1991a).

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Abbreviations

AUFS, absorbance unit full scale; COSY, scalar shift-correlated NMR spectroscopy; 2D, two-dimensional; hLH, human lutropin; hTSH (hTSHα, hTSHβ), (α, β subunits of) human thyrotrophin; RCM-hTSHα, reduced and alkylated hTSHα; SDS-PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TEAP, triethylamine phosphate; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

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