N-Glycans Modulate in Vivo and in Vitro Thyroid Hormone Synthesis

STUDY AT THE N-TERMINAL DOMAIN OF THYROGLOBULIN*

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Thyroglobulin (Tg), the prohormone of thyroxine (T4) and triiodothyronine (T3) synthesis, is a large secretory glycoprotein consisting of more than 660 amino acids and containing about 10% of carbohydrates. The carbohydrate moieties on Tg have been shown to play a major role in Tg routing through cellular compartments and recycling after endocytosis. Here we show that glycoconjugates also play a direct role in hormone biosynthesis, which requires tyrosine iodination and iodotyrosine coupling and occurs at the apical membrane of the thyrocytes. Tg glycoconjugates have been shown to play a major role in Tg routing through cellular compartments where iodination and hormone synthesis occur. In contrast, there have been no reports about a direct role of N-glycans in thyroid hormone synthesis. A major difficulty in studying Tg is its large size, which precludes detailed analysis of the various domains in terms of structure-function relationships. To circumvent this problem, we focused on the N-terminal domain (NTD) of Tg. Previously, we had separated NTD from the peptides obtained after CNBr treatment of Tg (12). NTD (Asn<sup>1</sup>–Met<sup>217</sup>) of human Tg, which bears the preferential hormone biosynthesis site, brings two N-glycans (Asn<sup>57</sup> and Asn<sup>91</sup>) into the C terminus of the molecule (1).

Human Tg is glycosylated with N-linked and O-linked oligosaccharides and sialic acid. The N-linked oligosaccharides of Tg contain 0.75 to 1.5% of carbohydrates per chain. During the past few years, numerous studies have focused on the mechanisms and the location of thyroid hormone synthesis (4). It is generally accepted that the follicular lumen is the main site of Tg iodination, although it may also take place during the intracellular transport (5). Tg is iodinated, and hormone synthesis occurs in presence of tyrosine peroxidase and the H<sub>2</sub>O<sub>2</sub> generating system. Lastly, hormone secretion requires that hormone-containing Tg be reabsorbed from the colloid by endocytosis and then degraded in the lysosomes. The hormones are finally released into the venous flow. In the intracellular movements of Tg, the physiological role of glycans is not fully understood. It has been demonstrated that in thyroid cell cultures, exocytosis of Tg is suppressed if glycosylation is totally inhibited by tunicamycin (6, 7). It has also been proposed that sialylation of Tg may operate as an export signal because certain thyroid pathologies in which sialyltransferases are lacking are associated with a defect in Tg secretion (8). However, in primocultures of porcine thyroid cells, inhibition of the formation of sialylactosaminyl structures does not impair Tg secretion (9). On the other hand, thyrocyes contain GlcNAc receptors (10) that reclyde the GlcNAc-bearing Tg back to the colloid and prevent these molecules from lysosomal degradation (11). Taken together, the data point to a major role of glycoconjugates in routing Tg to the cellular compartments where iodination and hormone synthesis occurs. In contrast, there have been no reports about a direct role of N-glycans in thyroid hormone synthesis.

Thyroglobulin (Tg).<sup>1</sup> The prohormone of thyroxine and triiodothyronine synthesis, is a large secretory glycoprotein (2 × 330 kDa) containing about 10% of carbohydrates. Among all proteins, Tg has the unique ability to form triiodothyronine (T3) and thyroxine (T4) residues by coupling the iodotyrosine residues. The iodine content of human Tg varies largely with the iodine intake: 0.05–1.1% (w/w), i.e. 2.5–55 atoms of iodine/mol of Tg. With as few as four iodine atoms, Tg can form T4, which indicates that specific mechanisms succeed in iodinating only few of the Tg molecules. Only four hormonogenic sites have been identified in human Tg. The preferential site is Tyr<sup>5</sup>, where most of the T4 is formed; the other sites are localized in the C terminus of the molecule (1).

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and Asn91) was cleaved at Met127, and a disulfide bond linked this peptide with Glu128–Met171. The apparent molecular weight of NTD varies according to the number of oligosaccharide side chains, which are known to be structurally heterogeneous (13). Note that NTD was able to form T4 in vitro after iodination and coupling of the acceptor residue (Tyr9) with the donor residue (Tyr4) (14). This indicated that NTD maintained most of its three-dimensional conformation, which was further demonstrated by surface epitope mapping (15). NTD thus offered an interesting opportunity to determine the role of N-glycans in the hormone synthesis process.

**EXPERIMENTAL PROCEDURES**

**Human Thyroglobulin Preparations—**

Thyroglobulin was purified as described previously (16) from a single colloid goiter (PI-Tg) and from the thyroid of a patient with Graves' disease (T4-Tg). PI-Tg was poorly iodinated (2.8 atoms of iodine/mole, traces of T3, 10 mM T4/mole), whereas T4-Tg was more iodinated and contained a significant level of hormone residues (6.0 atoms of iodine/mole, 85 mM T3/mole, 420 mM T4/mole). From PI-Tg we derived two other Tg preparations. The first one (PTg-I) had a high iodine content and only traces of hormones (15 atoms of iodine/mole, traces of T3, 82 mM T4/mole). It was obtained as follows. PI-Tg (1 mg/ml), dissolved in 100 ml of 50 mM Tris-HCl buffer, pH 7.2, was incubated at 37°C with 60 µm KCN, 50 mM KI, 5 mg of lactoperoxidase (Boehringer Mannheim) and an H2O2 generating system (glucose (10 mg/ml), glucose oxidase (25 µg/ml)). After 15 s of incubation, the reaction was stopped by adding 0.1 M sodium hyposulphite, and the excess of iodine was eliminated by extensive dialysis against redistilled water. The second one (PTg-T4) 12 atoms of iodine/mole, 200 mM T3/mole, 920 mM T4/mole) was obtained from the same poorly iodinated PI-Tg by using a lower iodine concentration (20 µm) and a longer incubation time (20 min) according to the method described above.

Preparation of the NTD of Tg—Native Tg (PI-Tg and T4-Tg) and Tg obtained by in vitro iodination (PI-Tg-I) and coupling (PI-Tg-T4) were treated by cyanogen bromide. The NTDs were separated by chromatography on a Sepharose G-200 column (12). This yielded four types of NTD as summarized in Fig. 1 (A and B): PI-NTD and T4-NTD corresponding to the NTD of PI-Tg and T4-Tg, respectively, and then PI-NTD-I and PI-NTD-T4 corresponding to the NTD from PI-Tg-I and PI-Tg-T4, respectively.

Two other types of NTDs were prepared from PI-NTD (Fig. 1C) after desialylation (PI-NTD-dS) or deglycosylation (PI-NTD-dG) as described below. In vitro iodination and coupling of PI-NTD, PI-NTD-dS, and PI-NTD-dG were performed according to Marrig et al. (14).

**ConA-Sepharose Chromatography—** Each type of NTD of Tg and the 3H-labeled oligosaccharide structures were submitted to affinity chromatography on ConA-Sepharose 4B (Pharmacia) at room temperature. The column (1 × 10 cm) was equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM CaCl2, 1 mM MgCl2, and 100 mM NaCl. Elution was carried out first with equilibration buffer and then successively with 10 mM α-methyl-D-glucopyranoside and 300 mM α-methyl-D-mannopyranoside in the same buffer. The fractions obtained were pooled and lyophilized. Two successive gel filtrations on a Bio-Gel P-2 column (1.5 × 50 cm) were performed to remove the sugars and salts, and then the fractions were lyophilized.

**RCA120-Sepharose Chromatography—** A column containing 1 ml of RCA120 agarose (Sigma) was equilibrated in the same buffer as the one used for ConA-Sepharose chromatography. Elution of the different preparations of the asialo- or asialo-3H-labeled oligosaccharide structures was performed with the equilibration buffer and then with the same buffer containing 0.2 M lactose.

**Preparation of 3H-labeled Oligosaccharide Structures—**

Borne by the NTDs—The different isoforms of the NTDs obtained by ConA-Sepharose chromatography were hydrolyzed and tritium-labeled according to Takasaki et al. (17). The peptides were heated with anhydrous hydrazine (Sigma) in a sealed tube at 100°C for 12 h. The residues, developed by repeated extraction with toluene, were acetylated by adding excess acetic anhydride. The mixture was applied to a column (1 × 5 cm) of Bio-Rad AG 50W (H+ form) and then washed thoroughly with distilled water. The eluate was lyophilized and chromatographed on paper by using butanol/ethanol/water (4:1:1) for 48 h to separate oligosaccharide from polypeptide moieties. The oligosaccharides larger than trisaccharides stay at the origin and are later eluted from the paper with distilled water. After elution, the residue was desalted on a Bio-Gel P-2 column (1.5 × 50 cm). The fractions containing neutral sugars were detected by sulfuric acid orcinol reaction. After reduction by NaBH4 the 3H-labeled oligosaccharides were separated from contaminants by paper chromatography for 48 h with ethyl acetate/pyridine/acetic acid/water (5:5:1:3). For the isoforms not retained on the ConA-Sepharose column, complete purification was performed by chromatography of the asialo-oligosaccharide structures on a RCA120-Sepharose (see above).

**Ammonium Bicarbonate Treatments—**

Peptide PI-NTD (300 µg) was deglycosylated by 1.5 units of peptide-N-(acyethyl-β-glucosaminyl) asparagine amidase (EC 3.5.1.52) (Boehringer Mannheim) in 400 µl of 50 mM phosphate buffer, pH 8.5, containing 10 mM Na2-EDTA according to Hirani et al. (38). Incubation was performed at 37°C and stopped after heating at 100°C for 3 min. Each sample was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

The NTDs and 3H-labeled oligosaccharide structures were desialylated by 20 units of neuraminidase (sialidase from Vibrio cholerae; Boehringer Mannheim). After incubation at 37°C overnight in 100 mM acetate buffer, pH 4.5, containing 100 mM CaCl2, samples were heated at 100°C for 3 min. Each of the asialo-peptides or asialo-oligosaccharide structures was further chromatographed on a RCA120-Sepharose column.

Tryptic hydrolysis of the PI-NTD-dG was performed with trypsin (Sigma type XXI and leucine aminopeptidase (Boehringer Mannheim) according to Roland et al. (19). Tryptic hydrolysis and coupling of PI-NTD, PI-NTD-dS, and PI-NTD-dG was performed according to CRESTFIELD et al. (18). The hydrolysate was fractionated on a Bio-Gel P-30 column (1.0 × 70 cm) in 50 mM ammonium bicarbonate.

**Hormone Residue Analysis—**

After digestion with protease type XXI and leucine aminopeptidase (Boehringer Mannheim) according to Roland et al. (19), hormone residue analysis was carried out with a radiomunnoassay (RIA) by using a solid antigen procedure. The RIA sensitivity was 5 pm. Cross-reactivity of the antibody was 0.8% for T3, 1% for L-3,3’,5’-triodothyronine, < 0.01% for L-3,5-diiodothyronine, 0.01% for diiodothyronine, and < 0.01% for moniodotyrosine. No cross-reactivity was observed with L-tyrosine.

**Other Procedures—**

Amino acid analysis was performed with a Pico-Tag system (Waters Millipore, Milford, MA) after hydrolyzing of the samples under vacuum in 6 N HCl at 110°C for 24 h. Neutral sugars were detected by sulfuric acid orcinol reaction (20).

**RESULTS**

ConA Affinity Chromatography and Characterization of NTD Isoforms—Starting with poorly iodinated Tg (PI-Tg) and T4-bearing Tg (T4-Tg), we obtained two preparations of the Tg N-terminal domain, PI-NTD and T4-NTD, respectively, by CBnBr treatment and Sephadex G-200 fractionation (see "Experimental Procedures" and Fig. 1A). Both preparations were fractionated by affinity chromatography on a ConA-Sepharose column and further characterized with regard to oligosaccharide structure and molecular weight.

The ConA affinity chromatography elution profiles showed that both PI-NTD and T4-NTD separated into three fractions (Fig. 2, A and B). The nonretained (NR) fractions contained the NTD isoforms unable to bind to ConA. The isoforms of the weakly retained (WR) fractions bound to ConA and were eluted with 10 mM α-methyl-D-glucopyranoside. The firmly retained (FR) isoforms also bound to ConA but were eluted with 300 mM α-methyl-D-mannopyranoside. The elution profiles of PI-NTD and T4-NTD were nearly identical, but the relative distribution of isoforms in the three fractions differed slightly. The relative content of the NR fraction was 2-fold lower for PI-NTD than for T4-NTD (4.9 ± 1.2% and 11.9 ± 2.4%, respectively). Conversely, the relative content of the WR fraction was higher for PI-NTD than for T4-NTD (41.7 ± 4.6% and 35.0 ± 3.8%, respectively).
respectively). Finally, the FR fractions of PI-NTD and T4-NTD did not significantly differ (53.4 ± 4.8% and 54.1 ± 6.2%, respectively).

The fractions obtained by ConA affinity chromatography of PI-NTD and T4-NTD preparations were labeled with tritium and analyzed for 3H-labeled oligosaccharide side chains. As expected (Table I), both NR fractions presented only triantennary complex type structure. The WR fractions contained both types of complex structures; the amount of bi-antennary was over 50%. This indicated that all WR isoforms presented at least one biantennary structure, which reflected the weak binding of these isoforms to ConA. On the other hand, PI-NTD and T4-NTD differed in their relative amounts of triantennary oligosaccharide side chains (46 and 25%, respectively). All the isoforms bearing high mannose type structures were found in the FR fraction, which was anticipated from ConA elution conditions. High mannose type side chains of each NTD may or may not be associated with bi- or triantennary structures. It is noteworthy that PI-NTD contained less high mannose type structure (62%) than T4-NTD did (71%). The difference can be explained by the higher amount of triantennary complex type oligosaccharides (15 and 8% for PI-NTD and T4-NTD, respectively). Taking this into account, we then calculated the overall percentage of different N-glycan structures (Table II).

Electrophoresis study of the three fractions obtained by ConA affinity chromatography showed (Fig. 2, A and B, insets) that the NR isoforms were consistently separated into two bands (lanes 2): one migrating in the 25-kDa region and the second one in the 19-kDa region. WR isoforms migrated in only one band in the 25-kDa region (lanes 3). FR isoforms showed two bands: one band in the 25-kDa region, as already observed with NR and WR isoforms, and one band in the 22-kDa region.

Taken together, these results pointed to a rather large heterogeneity of NTD isoforms. Considering that the molecular weight of NTD, deduced from its amino acid composition, was 18,000, variations in the molecular weight of NTD isoforms may be accounted for by differences in the number of oligosaccharide side chains. On the basis of the conditions of elution of ConA affinity chromatography, the data gathered by analysis of the carbohydrate structure, and the molecular weight of each isoform, we reasoned that the FR fractions contained isoforms with at least one high mannose type structure associated or not with any of the three carbohydrate structures identified. FR fractions would therefore contain four NTD isoforms: high mannose type/high mannose type, high mannose type/biantennary complex type, high mannose type/triantennary complex type, and high mannose type/O. The first three isoforms migrated in the 25-kDa region, whereas the fourth isoform migrated in the 22-kDa region. Effectively, such bands were observed. The same reasoning would apply to the other fractions. The WR fractions were thus considered to contain two 25-kDa isoforms (biantennary complex type/biantennary complex type and biantennary complex type/tri-antennary complex type), and the NR fraction as containing one 25-kDa isoform (triantennary complex type/tri-antennary complex type). Moreover, the presence of a peptide migrating in the 19-kDa region of the NR fraction suggested that unglycosylated NTD might be present. Accordingly we further separated the NR fraction and characterized the 19-kDa NTD peptide.

Purification by RCA$_{200}$ Affinity Chromatography and Characterization of the 19-kDa Isoform—To purify the 19-kDa isoform, we reasoned that the NTD fractions unable to bind to ConA (NR fractions) contained, apart from unglycosylated NTD, only one type of isoform bearing two triantennary side chains with galactose at the ultimate or penultimate position.
The three NTD fractions isolated by ConA-Sepharose chromatography were hydroxylated and tritium-labeled. After purification, the ³H-labeled oligosaccharide structures borne by each NTD fraction were chromatographed on a ConA-Sepharose column (1 × 3 cm) as described in the legend of Fig. 2. The radioactivity was calculated as a percentage.

**Table I**

| Complex type          | PI-NTD | T4-NTD |
|-----------------------|--------|--------|
| Triantennary complex  | 100    | 100    |
| Biantennary complex   | 0      | 0      |
| High mannose type     | 0      | 0      |

**Table II**

| Complex type          | PI-NTD | T4-NTD |
|-----------------------|--------|--------|
| Triantennary complex  | 29.2   | 36.3   |
| Biantennary complex   | 22.2   | 38.4   |
| High mannose type     | 34.5   | 39.4   |

The values were calculated from the results of the percentage of each fraction isolated by ConA-Sepharose chromatography and of the percentage of the ³H-labeled oligosaccharide side chain identified from each fraction (see Table I).

Analysis of Hormone Residues in the NTD Isoforms—The T4 content of the various NTD fractions obtained by ConA and RCA₁₂₀ affinity chromatography was measured by a specific RIA with an anti-T4 monoclonal antibody. As shown in Table I, the fractions derived from PI-NTD presented very few T4 residues; most were present in the FR fraction containing the isoforms with at least one high mannose type structure. The unglycosylated isoform did not have a detectable level of T4. In the T4-NTD fractions, T4 residues were also not detected in the unglycosylated isoform. The amounts of T4 residues in the other isoform preparations were similar for the NR and WR fractions containing complex type oligosaccharide structures (32.4 ± 5.8 and 38.3 ± 6 nmol T4/mol peptide, respectively) and far higher for the FR fractions containing at least one high mannose type structure (119 ± 48 mmol T4/mol peptide).

Western blot analysis with an anti-T4 monoclonal antibody was performed on the initial preparation and the ConA affinity fractions of T4-NTD. As shown in Fig. 4, most of the T4 residues were detected in the 25-kDa region whatever the sample analyzed (lanes 1–4). T4 residues, however, were also detected in the 22-kDa bands in the initial preparation of T4-NTD (lane 1) and its FR fraction (lane 4). Once again, no T4 residues were observed in the 19-kDa region of the initial preparation (lane 1) and the NR fraction (lane 2).

T4 residues were also detected in the 25-kDa region of the initial preparation of T4-NTD (lane 1) and its FR fraction (lane 4). Once again, no T4 residues were observed in the 19-kDa region of the initial preparation (lane 1) and the NR fraction (lane 2).

Taken together, our results showed the absence of T4 residues in the unglycosylated isoform and, equally intriguing, the higher amount of T4 residues in isoforms bearing at least one high mannose side chain than in those with only complex type oligosaccharide structure. To gain insight into the relationship between the presence of high mannose type side chain and the amount of T4 residues, we conducted experiments aimed at measuring T4 residues in the three different isoforms present in the FR fraction.

Analysis of Hormone Residues in the NTD Isoforms Bearing High Mannose Type Structure—The NTD isoforms present in the FR fractions could belong to three different species depending on the oligosaccharide structure(s) borne: only one high mannose type structure (22-kDa isoform), one high mannose type structure associated with a complex type structure, and finally two high mannose type structures. To study the hor-
under nonreducing conditions and then transfer to polyvinylidene difluoride. Immunoblot detection was probed with anti-T4 monoclonal antibody as described under “Experimental Procedures.” Gels were calibrated with Rainbow™ low molecular weight markers (Amersham Corp.). Lane 1, T4-NTD, lane 2, NR fraction; lane 3, WR fraction; lane 4, FR fraction.

VI. Hormone Residue Analysis

The peptides of 19 and 25 kDa belonging to the NR fractions were separated by chromatography on RCA 120-Sepharose column. T4 analysis was performed by RIA. Results are expressed in mmol of T4/mol of peptide and are the means ± S.D. Und, undetectable value.

| Prior to ConA chromatography | NR fractions | 19 kDa | 25 kDa | MR. 10^-3 |
|-----------------------------|--------------|--------|--------|------------|
| PI-NTD                      | 4.5 ± 3.1    | Und    | 0.5 ± 0.3 | 25         |
| T4-NTD                      | 100.5 ± 9.5  | Und    | 32.4 ± 5.8 | 22         |

**Fig. 4. Immunoblotting of T4-NTD and of the different fractions separated by ConA-Sepharose chromatography.** Separation was performed on SDS-PAGE (10% acrylamide) under nonreducing conditions and then transfer to polyvinylidene difluoride. Immunoblot detection was probed with anti-T4 monoclonal antibody as described under “Experimental Procedures.” Gels were calibrated with Rainbow™ low molecular weight markers (Amersham Corp.). Lane 1, T4-NTD, lane 2, NR fraction; lane 3, WR fraction; lane 4, FR fraction.

**Fig. 5. Immuno blotting of the three isoforms present in the FR fraction of the T4-NTD.** The FR fraction obtained after ConA-Sepharose chromatography of the T4-NTD was desialylated and then chromatographed on a RCA 120-Sepharose column. Isoforms bearing only high mannose type (lane 1) and the isoforms bearing high mannose type associated with a bi- or triantennary complex type (lane 2) were analyzed by immunoblotting as described in the legend of the Fig. 4.
N-Glycans and Thyroid Hormone Synthesis

Hormone residue analysis and percentage of each isoform separated on ConA-Sepharose column from PI-NTD-I and PI-NTD-T4

| Substrate | T4 residues |
|-----------|-------------|
| PI-NTD    | 185         |
| PI-NTD-dS | 182         |
| PI-NTD-dG | 92          |

After 1 h of incubation with the glycosidase, the PI-NTD preparation was separated into a major band of 25 kDa and a minor one of 22 kDa. After 24 h of incubation, the 25-kDa band was no longer visible, and the preparation resolved into one band of 22 kDa and one of 19 kDa. Densitometry of the bands showed that the material was equally distributed in the two bands (52 and 48% for the 22- and the 19-kDa bands, respectively). This indicated that only half of the peptide was completely deglycosylated. Effectively, analysis of the neutral sugar remaining in the NTD preparation after 24 h of incubation showed that about 30% of the total sugar stayed linked to the peptide. The observation that part of the peptide preparation remained glycosylated accounted for the presence of T4 after iodination and coupling. The 50% decrease in the amount of T4 correlated well with the results showing that half of the peptide was the unglycosylated peptide unable to participate in hormone formation, as was confirmed by immunoblotting (Fig. 6B).

These results confirmed that unglycosylated NTD was unable to form thyroid hormone. They also provided direct evidence for thyroid hormone synthesis in the presence of only one oligosaccharide side chain. Note that T4 formation was equally efficient in the presence of one or two chains, desialylated or not. This alluded to a conformational role of the oligosaccharides in thyroid hormone synthesis.

Localization of the Oligosaccharide Side Chain Resistant to Peptide-N4-(acetyl-β-glucosaminyl)Asparagine Amidase—The observation that one of the two oligosaccharide side chains resisted peptide-N4-(acetyl-β-glucosaminyl)asparagine amidase prompted us to identify the Asn bearing this chain. After 24 h of incubation with peptide-N4-(acetyl-β-glucosaminyl)-asparagine amidase, the PI-NTD-dG peptide was reduced, S-carboxymethylated, and then digested with trypsin. The tryptic fragments were separated by chromatography on a Bio-Gel P-30 column (Fig. 7). Neutral sugars were observed only in lane 2, which, according to a previous study (13), contains the peptide Gln92-Met127 including Asn91. We found the peptide containing Asn57, the other site of glycosylation of NTD, in peaks III and IV (Fig. 7). Fraction II, submitted to ConA affinity chromatography, segregated into three fractions: 33% NR isoforms with triantennary complex structures, 44% WR isoforms with bi- and triantennary structures, and 63% isoforms with high mannose type structure. It thus appeared that Asn91

| TABLE IV

| Prior to chromatography | NR fractions | WR fractions | FR fractions |
|-------------------------|-------------|-------------|-------------|
| PI-NTD-I                | 8.0 ± 2.1(100) | Und (3.8 ± 0.3) | Und (1.4 ± 0.3) | 3.7 ± 1.4 (40.6 ± 4.0) |
| PI-NTD-T4               | 145.8 ± 9.3(100) | Und (4.1 ± 0.4) | 12.4 ± 6.0 (1.3 ± 0.4) | 69.2 ± 7.3 (42.3 ± 3.4) |

**DISCUSSION**

Most proteins and peptides are efficient substrates for tyrosine iodination. Tg is unique in that its iodotyrosine coupling leads to thyroid hormone formation, a process that does not occur at random (1). Among the numerous tyrosines of Tg that
can be iodinated, only a few are involved in hormone synthesis. The strict specificity of the four hormone-forming sites obviously requires not only consensus sequences (21) but also stringent spatial organization of the Tg molecule. In turn, the threedimensional structure of Tg is modified during the process of tyrosine iodination and tyrosine coupling (22). Moreover, it has been demonstrated that glycosylation of Tg was also able to modify the conformational structure of this molecule (see below). Consequently, the tight relationship between the structure of Tg and its unique ability to form hormones could point to a direct role of Tg oligosaccharide moieties in hormone synthesis. Up to now, this had not been confirmed. Confirmation was offered by the observation that the N-terminal part of Tg presents up to two N-linked oligosaccharide side chains and that it is able to form T4 in vitro after being separated from the core molecule (14).

CNBr treatment and separation of the fragments of Tg and then lectin affinity chromatography of NTD provided several isoforms that differed in molecular weight and oligosaccharide composition. The oligosaccharide side chains contained three structures: biantennary and triantennary complex types as well as high mannose type structures. The peptides migrating in the 25-kDa regions all brought two oligosaccharide side chains, but they differed in chain structure. Six 25-kDa isoforms were identified depending on the combination of the three different types of oligosaccharide structures present on the two available glycosylation sites. Conversely, the 19-kDa isoform was shown to be the unglycosylated NTD isoform. Regarding the 22-kDa form, which brings only one oligosaccharide side chain, one would have expected to find three isoforms presenting high mannose, biantennary, or triantennary structure; only the 22-kDa form with one high mannose type structure was detected. These data are in general agreement with our previous results on the heterogeneity of glycosylation in this part of the human Tg molecule (13). They, however, are at variance with those of Rawitch et al. (23), who reported that high mannose type structures were limited to the C terminus in bovine Tg. The present study clearly demonstrates the existence of totally (19 kDa) or partially (22 kDa) unglycosylated isoforms. Furthermore, it provides evidence that NTD isoforms may bring only high mannose type structures, one for the 22-kDa form and two for the 25-kDa form. The presence of such isoforms was not expected because Tg prepared from human goiter issues mainly if not totally from the follicular lumen, which is expected to contain mature molecules. Indeed, it is generally accepted that a secreted protein bears mainly mature complex type structures. The presence of high mannose type structures in a mature protein may be explained by the folding of the protein before it enters the Golgi apparatus.

Many proteins possess one or more Asn-Xaa-Ser or Asn-Xaa-Thr consensus sequences, which are potential sites for N-glycosylation (24). Glycosylation at some of these sites has been demonstrated to play a role in the structure, function, expression, or stability of glycoproteins (25, 26). Regarding Tg, Nglycans have been reported to be involved in its intracellular transport and iodination in relationship with recycling after endocytosis and sialylation of the protein (8). This process was recently explained by the presence of a GlcNAc receptor in the apical membrane and also in the subapical compartments of thyroid epithelial cells (11). The GlcNAc receptor would play a major role in the processing of internalized Tg molecules bearing GlcNAc and be recycled through the Golgi apparatus via the apical membrane to the colloid. During this transit, Tg molecules would complete their glycosylation of complex type oligosaccharide side chains in the Golgi apparatus and would increase their iodination levels at the apical membrane by contacting thyroperoxidase. Glycosylation has also been shown to be modulated by TSH in cultured thyroid cells (27, 28). In primary culture of porcine thyroid cells, TSH increased the number of oligosaccharide side chains borne by Tg without modifying the relative distribution of the various types of oligosaccharide structures (29). In FRTL-5 cells it appeared rather that TSH decreased (30) or increased (31) the number of oligosaccharide side chains. For the latter group, TSH stimulation resulted in the addition of one high mannose type structure at the N-terminal part of Tg and led to maturation of pre-existing high mannose type side chains, forming complex type structures (32). Discrepancies in the data on cultured thyroid cells may be explained by differences in cultured cells and experimental conditions, notably cell culture media (33). It has also been observed that Tg obtained from thyroid tissue differed with regard to N-glycosylation from that obtained from cultured cells (34). Nevertheless, a general consensus might be derived from these and other studies: whatever the effect of TSH or modifications in experimental conditions on the number and composition of oligosaccharide side chains, the tridimensional structure and antigenic properties of Tg molecules changed, which ultimately affects the ability of Tg to form thyroid hormones (35, 36). This conclusion is in close agreement with ours. The modification in the structure of the N-terminal part of Tg induced by changes in N-glycan structure and number has an important impact on hormone formation at the major site of T4 synthesis: unglycosylated NTD does not form hormone; the presence of a single N-glycan side chain at Asn97 allows NTD to form T4; as compared with complex type structures, high mannose type structures enhance the ability of NTD to form hormones. Taking into account that TSH may modify N-glycans at the NTD, it appears that TSH may modulate thyroid hormones more directly than we think. Considering the relationship between NTD oligosaccharide structure and its ability to form T4 residues, we speculate that Tg gene abnormality involving the potential glycosylation sites of NTD might induce abnormality in the thyroid status of patients (37). The present results open a new way to apprehend the physiology and pathology of the thyroid gland. This has been made possible by the ability of the Tg NTD, separated from the core molecule, to form thyroid hormone residues. This stresses the potential interest of molecular dissection in establishing the structure-activity relationship of proteins.

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N-Glycans and Thyroid Hormone Synthesis

29888

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