Precursor-product relationship between the 26-kDa and 18-kDa fragments formed by iodination of human thyroglobulin

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At moderate iodination levels (20 iodine at atoms/molecule), human thyroglobulin (hTgb) produces after reduction a thyroxinyl-peptide of 26 kDa which represents the N-terminal part of the protein. At higher iodination levels, the 26-kDa peptide is accompanied by another T_4-containing peptide of 18 kDa. A precursor-product relationship between the 26- and 18-kDa fragments was demonstrated by the study of the tryptic fragments of both hormonopeptides. In addition, comparison with the protein sequence deduced from the nucleotide sequence of the 5'-end of hTgb mRNA demonstrated that the N-terminal region of Htgb from which are issued the 26-kDa peptide and its 18-kDa derivative is especially sensitive to proteolysis. This character is possibly related with a facilitated release of thyroid hormones in vivo.

Human thyroglobulin *Thyroxine-containing peptide* *Peptide structure* *HPLC* *In vitro iodination*

1. INTRODUCTION

Thyroglobulin (Tgb), the high-molecular dimeric glycoprotein (2 × 330 kDa) of the thyroid gland, is the support of thyroid hormone synthesis, T_4 and T_3. Indeed, the hormones are formed by iodination of certain tyrosine residues of the protein and the oxidative coupling of some of the resulting iodotyrosyl residues. Iodination of Tgb provokes different modifications of the physico-chemical properties of the molecule, e.g., change in the sedimentation coefficient, decrease in the dissociability into subunits and change in its susceptibility to proteases. Recently another characteristic of Tgb in relation to iodination was evidenced: the presence of peptides of small molecular mass (9–45 kDa) rich in hormones and associated to the bulk of the molecule by disulfide bridges. This was demonstrated in the Tgb of different species iodinated in vivo or in vitro [1–7]. It was also demonstrated for some of them that they originated from the cleavage of the peptide chain, presumably in relation with iodination and/or hormone synthesis [6,8]. In hTgb, two hormonopeptides of 26 kDa and 18 kDa were characterized [4,6]. In a previous paper [6], we have demonstrated that the 26-kDa peptide represented the N-terminal part of the Tgb chain and that the T_4 residue in this fragment was located in position 5 from the N-terminal Asn residue. This T_4-forming site is extremely active since it forms approx. 50% of the total T_4 when the hTgb contains 20 I atoms/molecule. By progressive iodination of poorly iodinated hTgb in vitro, we have also demonstrated [6] that the amount of the 26-kDa peptide was maximum when the molecule had bound 20 I atoms/molecule and decreased for higher iodination levels, whereas the 18-kDa species increased in parallel to become the main component for hTgb preparations containing 60 I atoms/molecule.

To broaden our knowledge of the structure-

Abbreviations: hTgb, human thyroglobulin; MIT, 3-iodotyrosine; DIT, 3,5-diiodotyrosine; T_4, thyroxine; TFA, trifluoroacetic acid

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function relationship in hTgb, it was important to specify whether the 18-kDa hormonopeptide contained a hormone-forming site different from the site identified in the 26-kDa peptide and was only operative at high iodination levels or whether the 18-kDa species originated from the 26-kDa peptide by peptide bond cleavage and derived from the same part of the hTgb molecule.

Here, thanks to the study of the tryptic peptides of these two hormonopeptides and by comparing their amino acid sequences with that deduced from the sequencing of the hTgb mRNA [9], we demonstrate unambiguously: (1) a precursor-product relationship between the 26- and 18-kDa species; (2) that the N-terminal segment of hTgb from which these peptides originate is especially sensitive to peptide bond cleavage, a characteristic possibly related to a facilitated release of thyroid hormone in vivo.

2. MATERIALS AND METHODS

2.1. *Thyroglobulin preparation*

Undegraded poorly iodinated hTgb (4 I atoms/molecule) was purified from a human colloid goiter as in [10].

2.2. *Thyroglobulin iodination*

Poorly iodinated hTgb was iodinated as in [6] using the amount of KI necessary to incorporate about 60 I atoms/molecule. Excess iodide was eliminated by dialysis and iodine incorporated in the protein was measured by iodoamino acid estimation [11].

2.3. *Preparation of 26- and 18-kDa hormonopeptides*

After iodination to 56 I atoms/molecule, hTgb was reduced and S-carboxymethylated [6], then chromatographed on Biogel A-5m in 0.05 M Tris–Cl, 8 M urea, pH 7.6. The retarded fraction was refiltered on Ultrogel AcA 54 in 0.2 M NH_4HCO_3.

2.4. *Tryptic digestion*

The 26- and 18-kDa peptides (1% in 0.2 M NH_4HCO_3,pH 8.6) were digested for 4 h at 37°C with trypsin–TPCK (Worthington, Freehold, USA) at an enzyme to substrate ratio of 1:25 (w/w). The digest was then adjusted to pH 3.0 with acetic acid. The precipitate (acid core) was separated by centrifugation and the supernatant preserved for further analysis.

2.5. *Purification of the tryptic peptides*

The tryptic digest supernatant was filtered on Biogel P-6 in 0.2 M NH_4HCO_3 and each group of peptides was analyzed by high performance liquid chromatography (HPLC) (Waters, Milford, USA) on a Lichrosorb RP-18 column (4×250 mm, 7 μm particle size). A linear gradient was applied with an initial solvent (A) of 1% NH_4HCO_3 and a second solvent (B) of 100% CH_3CN from 5–60%(B) for 45 min at a flow-rate of 0.8 ml/min. Column

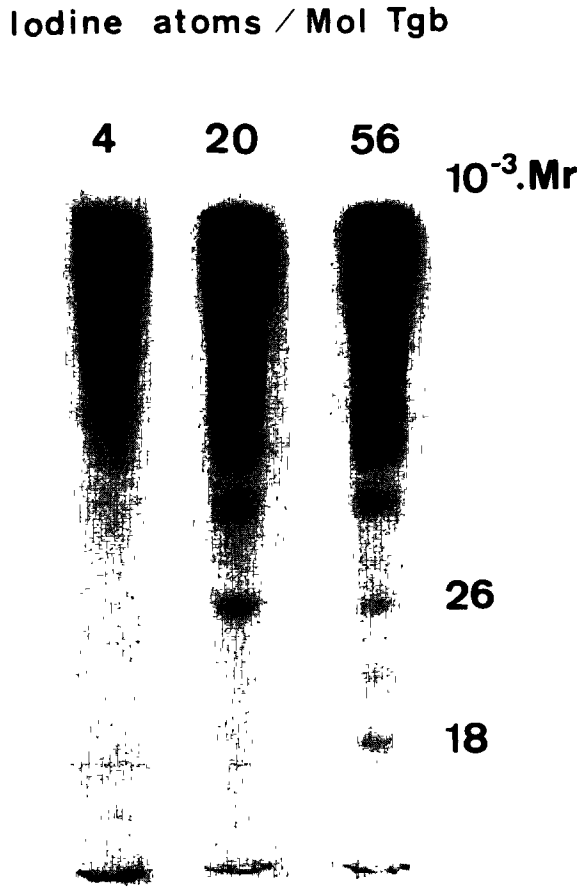

Fig. 1. SDS-PAGE of poorly iodinated (4 I atoms/molecule) before and after iodination in vitro (20 and 56 I atoms/molecule). 50 μg protein per gel (8% polyacrylamide) were analyzed after reduction by 1% dithiothreitol for 3 min at 100°C.

eluates were continuously monitored for absorbance at 230 nm. Peptides from the acid core were first dissolved in 6 M guanidine · HCl and purified on the same column using a linear gradient formed of solvent(A),0.05% aqueous TFA and solvent(B)CH_3CN,0.05% TFA for 50 min from 10–55% B at a flow rate of 1 ml/min.

2.6. *Sequence analysis*

Automated N-terminal sequencing of the 18-kDa peptide was performed in a 890 C Beckman Sequenator using the 0.1 M quadrol program. The manual microsequencing method of [12] which allows the identification of hormone residues [13] was used for N-terminal sequencing of the 18-kDa and all the purified tryptic peptides.

2.7. *Other techniques*

Amino acid analyses were performed in a model 4101 LKB amino acid analyzer after hydrolysis in 6 N HCl, 0.5% thioglycolic acid for 20 h in sealed evacuated tubes. Separation and quantitative estimation of iodoamino acids were carried out as in [11] and polyacrylamide gel electrophoresis (PAGE) as in [5].

3. RESULTS AND DISCUSSION

Analysis by SDS–PAGE of poorly iodinated hTgb after reduction and before and after the incorporation of 20 and 56 I atoms/molecule shows (fig. 1) the absence of 18- and 26-kDa fragments in poorly iodinated hTgb and their appearance after iodination of the protein. Both peptides were

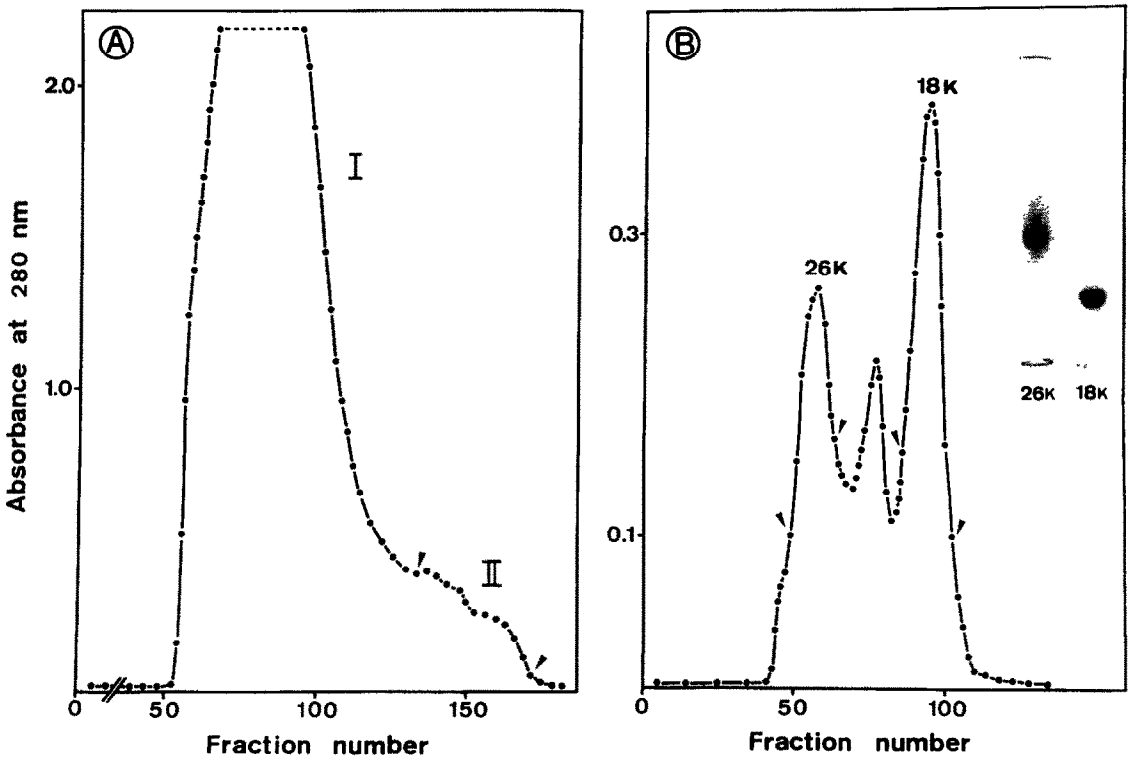

Fig. 2(A) Filtration on Biogel A-5m of reduced and S-carboxymethylated hTgb after iodination in vitro (56 I atoms/molecule). 400 mg protein were layered on a 2.6 × 90 cm column equilibrated and eluted with 0.05 M Tris–Cl, 8 M urea, pH 7.6. Flow-rate, 8 ml/h, volume of fractions, 2 ml. Tubes were pooled according to arrows to give fractions I, II.

(B) Filtration on Ultrogel AcA 54 of fraction II (see A). 30 mg protein were layered on a 2.6 × 90 cm column equilibrated and eluted with 0.2 M NH_4HCO_3. 26- and 18-kDa fractions were pooled according to arrows. Inset: SDS–PAGE of 26- and 18-kDa fractions in 8% gel.

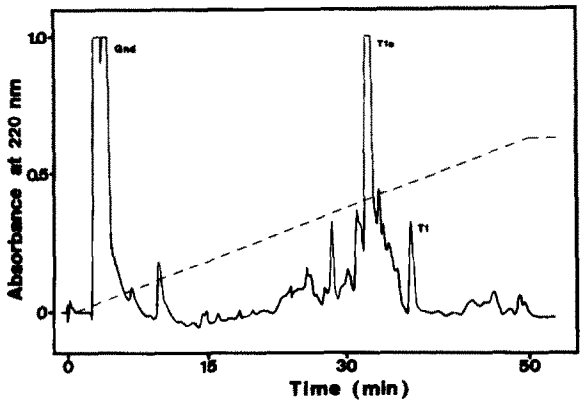

Fig. 3. HPLC analysis of the acid core of the tryptic digest of the 26 kDa peptide. Approximately 500 μg protein dissolved in 200 μl 6 M guanidine · HCl (Gnd) were injected into a Lichrosorb RP-18 column. Elution conditions: mobile phase, (A) 0.05% aqueous TFA, (B) CH_3CN, 0.05% TFA; linear 50 min gradient from 10–55% (B). Flow-rate, 1 ml/min.

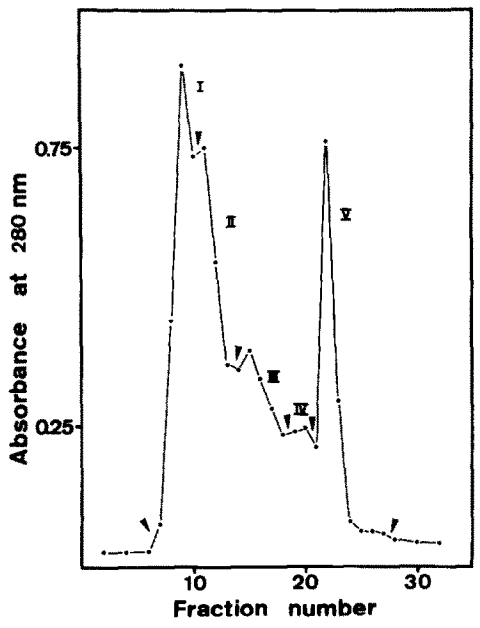

Fig. 4. Filtration on Biogel P-6 of the tryptic digest of the 26 kDa supernatant. About 2.2 mg protein was applied on a 1×30 cm column equilibrated and eluted in 0.2 M NH_4HCO_3. Fraction volume, 0.8 ml. Flow-rate, 8 ml/h. Fractions were pooled according to arrows.

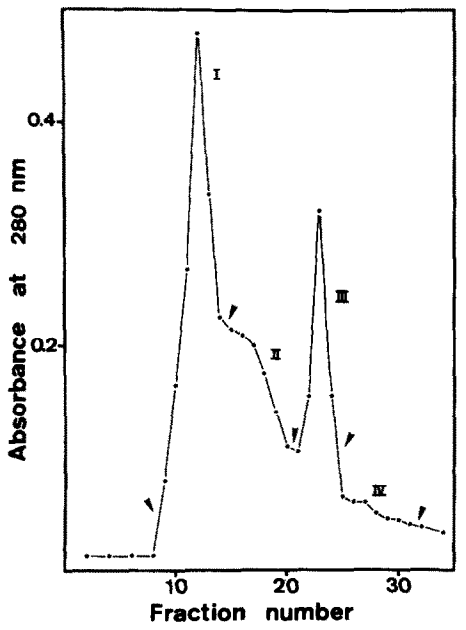

Fig. 5. Filtration on Biogel P-6 of the tryptic digest of the 18 kDa supernatant. Same conditions as in Fig. 3. 1.2 mg protein was applied to the column.

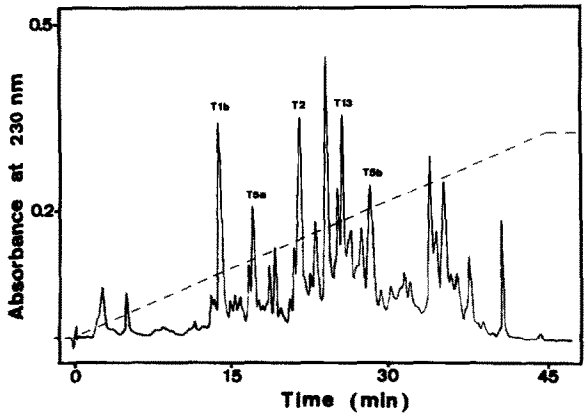

Fig. 6. HPLC analysis of fraction III of the tryptic digest of the 26 kDa peptide (see fig. 4). Approx. 250 μg protein were injected into a Lichrosorb RP-18 column. Mobile phase: (A) 1% NH_4HCO_3, (B) 100% CH_3CN, linear 45 min gradient from 5–60% (B). The elution positions of the tryptic peptides T_1^b, T_5^a, T_2, T_{13} and T_5^b are indicated by arrows.

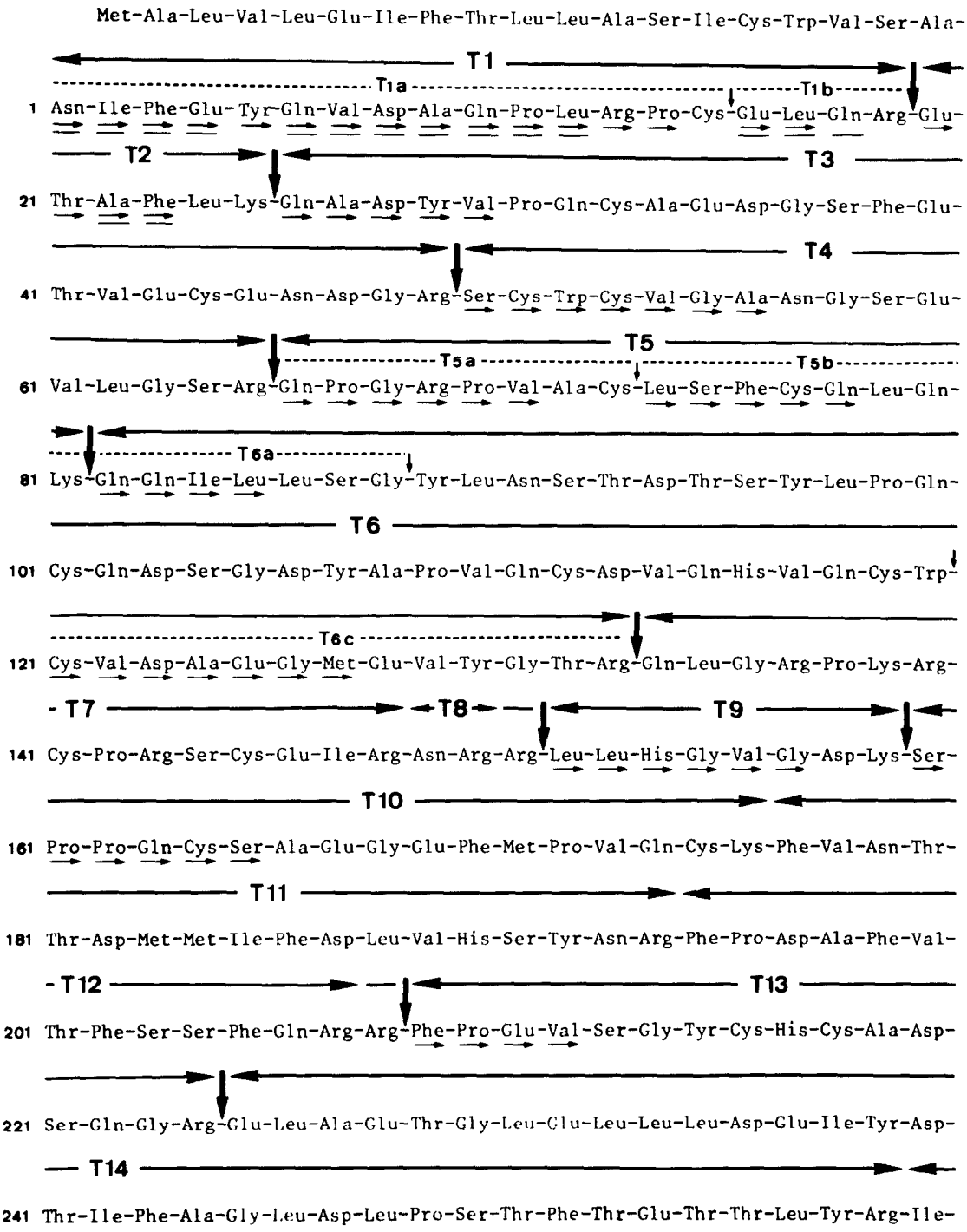

Fig. 7. Sequence of the first 260 amino acids from the N-terminal end of hTgb as deduced from the nucleotide sequence of its mRNA [9]. Residues are numbered from the N-terminal amino acid (Asn) of the 26- and 18-kDa peptides. T_1–T_{14} are the theoretical tryptic peptides taking into account the specificity of trypsin. The vertical arrows indicate the actual bonds cleaved by trypsin in the 26- and 18-kDa fragments as deduced from analysis of the purified peptides. →, amino acids identified by microsequencing; those underlined were identified by automatic sequencing of the 18-kDa peptide. T_1, T_1^a, T_1^b, T_2, T_3, T_4, T_5^a, T_5^b, T_6^a, T_6^c, T_9 were purified from the 18- and T_1, T_1^a, T_1^b, T_2, T_3, T_4, T_5^a, T_5^b, T_6, T_{10}, T_{13} from 26-kDa fragment. The tyrosine residue in position 5 has been identified as T_4 by micro-sequencing both in the 26- and 18-kDa peptide.

purified from hTgb containing 56 I atoms/molecule as described in the legend of fig. 2 and both contained only traces of MIT and DIT and close to 1 mol T_4/mol. After microsequencing of the 18-kDa fragment, T_4 was identified in the sequence: Asn-Ile-Phe-Glu-T_4-Gln-Val-, identical to the sequence determined in the 26-kDa peptide [6]. This structure was confirmed by automatic sequencing of the 18-kDa peptide. This is a first evidence for the common origin of the two peptides.

However, it has been suggested that homologous repeating segments might exist in the Tgb molecule [14–16]. Moreover, study of the N-terminal part of the hTgb chain deduced by sequencing of DNA clones complementary to hTgb mRNA showed the presence of several homologous repeats in the nucleotide sequence [17]. To confirm that the 26-kDa peptide was actually the precursor of the 18-kDa fragment, the tryptic peptides of these two fragments have been compared.

After tryptic digestion of both peptides and acidification, the whole of T_4 was recovered in the acid core as peptides T_1^a and T_1 (fig. 3). Purification on Biogel P-6 and HPLC (figs 4–6) of the acid soluble fraction of the digests of the 26- and 18-kDa fragments allowed isolation of 12 peptides (T_1^b, T_2, T_3, T_4, T_5^a, T_5^b, T_6, T_6^a, T_6^c, T_9, T_{10}, T_{13}) whose homogeneity was established by amino acid composition and micro-sequencing.

Of the 14 peptides identified, 9 (T_1^a, T_1, T_1^b, T_2, T_3, T_4, T_5^a, T_5^b, T_6) were found to be common to the hydrolysates of the 26- and 18-kDa fragments. In fig. 7, these peptides have been related to the sequence of the N-terminal part of hTgb deduced from the nucleotide sequence of the 5'-end of the hTgb mRNA as recently established by authors in [9]. Comparison with the results obtained by protein sequencing of the 26-kDa peptide confirms our previous results [6] suggesting that the latter likely represented the N-terminal segment of hTgb; indeed, the 26-kDa peptide is preceded by a peptide of 19 amino acids whose size and hydrophobicity correspond to a signal peptide of secretion.

Thus, the sequence of the first 133 amino acids of the N-terminal end of the hTgb chain is common to the two hormonopeptides, demonstrating unambiguously that the 26-kDa peptide is the precursor of the 18-kDa fragment.

However, since no amino acid was released by action of carboxypeptidases A, B and Y on the 26- or 18-kDa peptides, for digestion times of 30 and 120 min at pH 8.6 or 6.0, it is difficult to specify the points of cleavage of the hTgb chain that produce the 26-kDa segment and its derived 18-kDa product.

On the other hand, study of the tryptic peptides of both fragments revealed several aspecific bonds of tryptic cleavage especially involving carboxymethylcysteinyl bonds. Indeed: (1) 90% of the T_4-containing peptide T_1 was recovered as T_1^a in the acid core and T_1^b in the acid-soluble fraction; (2) the totality of peptide T_5 was cleaved at the carboxymethylcysteinyl bond in position 73; (3) peptide T_6 has been partially cleaved at $glycyl_{88}$ and $tryptophanyl_{120}$ bonds.

It is possible that these bonds have been made more fragile by a special secondary structure of the chain at their level. Whatever the mechanism of these aspecific cleavages, it is worth relating the great susceptibility to peptide bond cleavage of this part of the molecule, which contains a very active T_4-forming site, to the aptitude of Tgb to release the thyroid hormones easily.

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