

Hormone synthesis in human thyroglobulin: possible cleavage of the polypeptide chain at the tyrosine donor site

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At moderate iodination levels (20 iodine atoms/mol) human thyroglobulin (hTg) produces after reduction a hormone-rich peptide of 26 kDa which contains the preferential hormonogenic 'acceptor' tyrosine (Tyr 5) of the protein. The site of cleavage of the hTg chain was demonstrated by analysis of the 26 kDa tryptic hydrolysis products. It consistently yielded the peptide Gln-82–Val-129 which consequently made it possible to localize the hTg chain cleavage at tyrosine residue 130. Evidence for tyrosine involvement in hTg cleavage during thyroid hormone formation supports the hypothesis that peptide bond cleavage would occur at the 'donor' tyrosine residue and suggests that tyrosine 130 would be the donor site reacting with the major hormone-forming acceptor site (Tyr 5) of hTg.

Thyroglobulin; Hormone-forming acceptor site; Donor tyrosine residue; Peptide bond cleavage

1. INTRODUCTION

Thyroglobulin (Tg), the large dimeric iodoglycoprotein (2×330 kDa) of the thyroid gland, is the matrix within which thyroid hormones (T_4 and T_3) are synthesized. During hormone formation the intramolecular coupling reaction implies the fission of an iodotyrosyl residue (donor) next to its aromatic ring and a transfer of the iodophenolic moiety to the other iodotyrosine (acceptor). Besides coupling, other events take place in Tg molecule. In all vertebrate species studied so far, small hormone-rich peptides (10–30 kDa) are found after reduction of disulfide bridges of iodinated Tg [1–3]. It has been shown that at moderate iodination levels human Tg (hTg) produced after reduction a hormone-rich fragment

which shows an apparent molecular mass of 26 kDa in SDS-PAGE [4,5]. This hormonepeptide represents the N-terminal part of the hTg chain and contains the preferential hormonogenic acceptor tyrosine residue (Tyr 5) of the protein [6], but the location of neither the corresponding donor site nor the cleaved peptide bond has been determined.

Recent experiments [7,8] have indicated that this peptide bond cleavage might be part of the coupling reaction. So, taking into consideration that after fission of the iodotyrosine donor, the 'lost side chain' remains in the Tg polypeptide chain as a dehydroalanine residue [9,10], an unstable residue, we explored the possibility that the peptide bond cleavage would occur at the donor tyrosine residue.

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Abbreviations: hTg, human thyroglobulin; T_4 , thyroxine; T_3 , 3,5,3'-triiodothyronine; MIT, 3-iodotyrosine; DIT, 3,5-diiodotyrosine; TFA, trifluoroacetic acid

2. MATERIALS AND METHODS

2.1. 26 kDa hormonepeptide preparation

Poorly iodinated hTg (4 I atoms/mol) was purified from a human colloid goitre as in [11] and was in vitro iodinated as in [6] using the amount of KI necessary to incorporate about 20 I atoms/mol. Excess iodide was eliminated by dialysis. Iodinated

hTG was reduced and *S*-carboxymethylated [6], then filtered on a Biogel A-5 m column in 0.05 M Tris-Cl, 8 M urea, pH 7.6. The column effluent was pooled as 3 fractions I, II and III as previously described [6]. Fraction III, containing the 26 kDa species, was further purified on a Biogel P-100 column in 0.1 M NH_4HCO_3 without urea to avoid the possible coelution with contaminating species.

2.2. Tryptic digestion

26 kDa peptide (1% in 0.1 M NH_4HCO_3 , pH 8.6) was 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 filtered on a column of Biogel P-30 in 0.05 M NH_4HCO_3 . The different pools of peptides were lyophilized then dissolved in 0.05% TFA, 5% CH_3CN (solvent A) and subjected to high-performance liquid chromatography (HPLC) (Waters, Milford, USA) on a Select B reverse-phase column (4 × 250 mm, 5 μm particle size, Merck, Darmstadt, FRG). The elution was carried out for 5 min with solvent A then with a linear gradient from 0 to 55% of solvent B (0.05% TFA, CH_3CN) for 70 min at room temperature and at a flow rate of 0.8 ml/min.

2.3. Partial acid hydrolysis

It was accomplished by dissolving the peptide in 0.03 N HCl (approx. 1 mg/ml) and hydrolysing in an evacuated sealed tube at 110°C for 20 h. In these conditions aspartyl and asparaginyl bonds were cleaved on both sides and free aspartic acid was liberated [12]. The hydrolysate was desiccated, dissolved in 0.05% TFA, 5% CH_3CN and applied on a Lichrosorb RP-8 column (4 × 250 mm, 5 μm particle size, Merck). HPLC elution was carried out as above.

2.4. Carboxypeptidase P digestion

The tryptic peptide T6 was dissolved (0.5 mg/ml) in 5 M urea, pyridine-acetate buffer, pH 4.0. Carboxypeptidase P (Boehringer Mannheim, Germany) was used at an enzyme to substrate ratio of 1:20 (w/w). The reaction mixture was incubated at 25°C for 120 min, dried and subjected to amino acid analysis.

2.5. Other techniques

Amino acid analyses were performed as in [13], manual microsequencing as in [14] and iodoamino acid estimation as in [15].

3. RESULTS

Previous investigations of 26 kDa hormonepeptide had revealed that this fragment represents the N-terminal part of hTg [6] but the site of cleavage of hTg chain had not been determined since no amino acid was released after action of carboxypeptidases A, B or Y for digestion times 30 and 120 min at pH 8.6 or 6.0 [13]. Taking into account its apparent molecular mass (26 kDa) and the previous results of tryptic hydrolysis [13] it could

be supposed that the fragment contained about 220 amino acid residues; however, calculated on this basis, the amino acid composition of the peptide was not in good agreement with the composition predicted by cDNA sequence [16], especially regarding the arginine residues.

3.1. Tryptic peptide T6 from 26 kDa

Purified 26 kDa was submitted to trypsin hydrolysis and the hydrolysate was filtered on a Biogel P-30 column, the first eluted fraction (A, fig.1) appeared to contain a single peptide in microsequence analysis and was analyzed without further purification. Its N-terminal sequence, determined after 8 steps of microsequencing, agreed very well with that of tryptic peptide T6 predicted from cDNA sequence (fig.2), however, no amino acid was released after 120 min incubation.

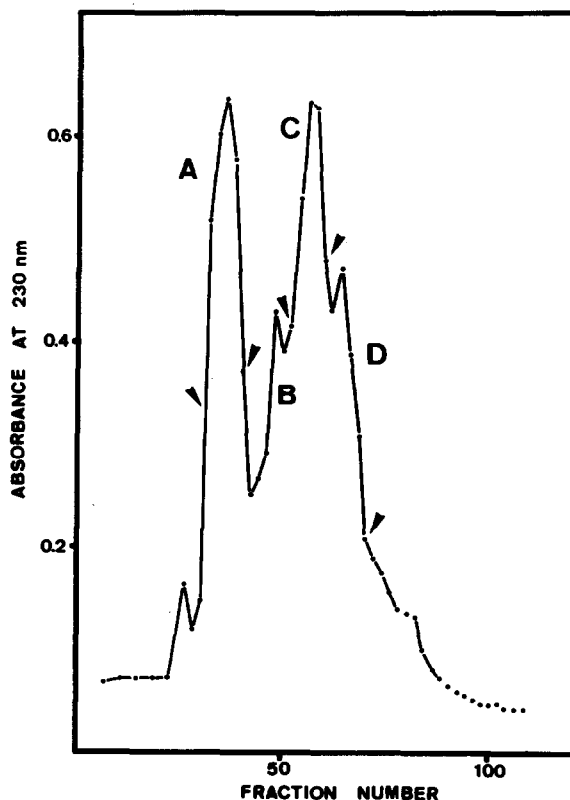


Fig.1. Filtration on Biogel P-30 of the tryptic digest of 26 kDa peptide. About 2 mg protein were applied on a 1.5 × 65 cm column equilibrated and eluted in 0.05 M NH_4HCO_3 ; fraction volume, 1 ml; flow rate, 7 ml/h. Fractions A, B, C and D were the fractions pooled between the arrowheads.

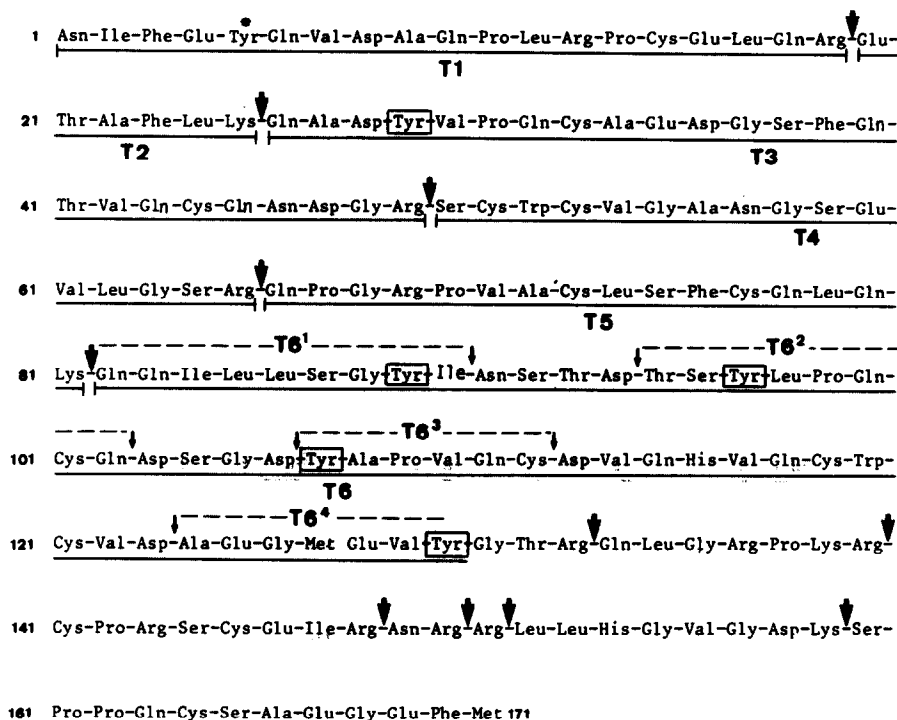


Fig.2. Sequence of the first 171 amino acid residues from the N-terminal end of hTg as deduced from the nucleotide sequence of its mRNA according to [16]. (↓) Predicted tryptic cleavages; (T1–T6) peptides purified from the 26 kDa peptide; Tyr-5 (*), identified as T4 is the preferential hormonogenic residue acceptor of hTg [6]; the other tyrosine residues are boxed.

tion with carboxypeptidase P. Furthermore, when compared with predicted T6, about 5 amino acid residues (Thr, Gly, Val, Tyr, Arg) were missing from the T6 peptide isolated from 26 kDa (table 1). Predicted T6 contained 4 tyrosine and 5 valine residues (table 1). Considering that in the course of HCl hydrolysis some valyl bonds are not totally cleaved and that iodotyrosine residues are partially destroyed, the amino acid composition analysis alone was not sufficient to prove the C-terminal sequence of tryptic peptide T6. Thus, the latter was submitted to partial acid hydrolysis to cleave only its Asp and Asn bonds and the derived acid peptides were separated by HPLC (fig.3). Their composition, when compared with corresponding predicted sequences (table 1), showed that Tyr-89, Tyr-97 and Tyr-107 were recovered respectively in peptide T6¹, T6² and T6³ and that T6⁴, the probable C-terminal peptide of T6, did not contain tyrosine at residue 130 but a valine residue. Consequently T6 purified from 26 kDa represents the hTg sequence Gln-82–Val-129 (fig.2).

3.2. C-terminal sequence of 26 kDa

If the specificity of trypsin is taken into consideration, the formation of T6 (Gln-82–Val-129) after tryptic digestion of 26 kDa could be explained by assigning to this fragment the position of the 26 kDa C-terminal peptide. However we have previously shown that some aspecific cleavages by trypsin could occur in 26 kDa purified from highly iodinated hTg [13]; so, to unambiguously locate T6 in the C-terminal position of 26 kDa, all other tryptic peptides were isolated from 26 kDa tryptic hydrolysate (fractions B, C and D, fig.1). The amino acid composition of each HPLC-purified peptide was compared with the predicted sequence from cDNA. Only the peptides representing the hTg sequence 1–81 (T1–T5, fig.2) were recovered with a yield of 20–36%. The few others that do not belong to this part of the chain were obtained with a very poor recovery (never over 5%).

These data show that the 26 kDa peptide is most likely the N-terminal fragment of hTg from Asn-1 to Val-129 (fig.2).

Table 1

Amino acid and iodoamino acid compositions of tyrosine-containing peptides purified from 26 kDa peptide

Peptide no. (see fig.2):	Experimental procedures							
	Tryptic hydrolysis of 26 kDa peptide		T6 predicted from cDNA sequence	Partial acid hydrolysis of tryptic peptide T6				T6 ⁴ predicted from cDNA sequence
	T3	T6		T6 ¹	T6 ²	T6 ³	T6 ⁴	
Amino acid residue positions (see fig.2)	26-49	82-?	82-133	82-90	95-102	107-112	124-?	124-133
Amino acid ^a								
Cys	2.2	3.7	4		0.5	0.5		
Asp	4.2	6.0	6					
Thr	1.0	2.3	3		0.8			1
Ser	1.2	4.1	4		1.0			
Glu	6.8	8.9	9	1.4	1.3	1.2	2.0	2
Pro	1.4	1.9	2		0.5			
Gly	2.2	3.2	4	1.2			0.9	2
Ala	2.3	2.0	2			1.0	0.4	1
Val	2.1	3.9	5			1.0	1.0	1
Met		0.7	1				0.4	1
Ile		1.9	2	1.6				
Leu		3.6	4	2.0	1.0			
Tyr	1.0	2.3	4	1.1	0.6	1.1		1
Phe	1.1	0.1						
His		0.6	1					
Lys								
Arg	1.0	0.2	1					1
Iodoamino acid								
MIT		0.5			0.3			
DIT		0.08			0.0			
T ₄		0.0			0.0			

^a The amino acid composition of T3, T6, T6¹, T6², T6³ and T6⁴ was normalized respectively to Arg: 1, Asp: 6, Leu: 2, Leu: 1, Ala: 1 and Glu: 2

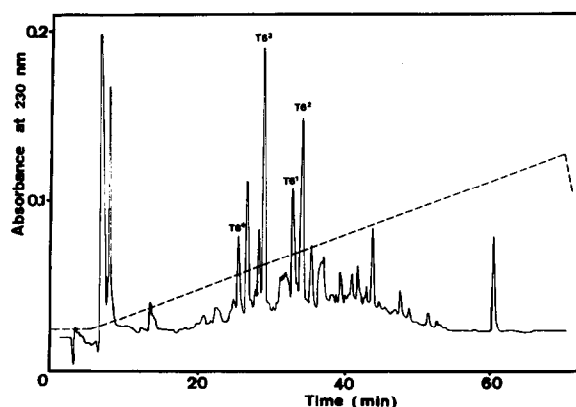


Fig.3. HPLC analysis of the acid hydrolysate of the tryptic peptide T6. Approx. 190 µg protein were injected into a Lichrosorb RP-8 column. The elution was carried out as described in section 2. The elution positions of the tyrosyl acid peptides (T6¹, T6², T6³ and T6⁴) are indicated.

4. DISCUSSION

If the 26 kDa peptide is considered as originating from the cleavage of the valyl-129-tyrosyl-130 bond, it is difficult to explain why carboxypeptidases, used herein, under many different conditions, fail to hydrolyse the C-terminal residues. On the other hand, the presence of an unusual residue at the C-terminal end of 26 kDa (or T6) may explain these results. Thus, it is tempting to suggest that the cleavage occurs at the dehydroalanine residue, the 'lost side chain' of the donor tyrosine-130.

We have recently shown that the peptide Asn-1-Met-171 isolated from a poorly iodinated hTg was able to form thyroid hormones at Tyr-5 when submitted to enzymatic iodination in vitro

[17,18]. This observation demonstrated the presence of a donor residue in the sequence 1–171. Among the 6 tyrosine residues present in this part of the molecule, 5 have been recovered from the 26 kDa hormonepeptide: Tyr-5, the preferential acceptor site of hTg [6], Tyr-29 recovered in tryptic peptide T₃, Tyr-89 in peptide T6¹, Tyr-97 (as tyrosine and MIT) in T6² and Tyr-107 in T6³ (table 1). Consequently the 6th tyrosine residue, Tyr-130, would be a very good candidate for the donor site reacting with the major hormone-forming acceptor site (Tyr-5) of hTg.

It is difficult to know if the cleavage is actually part of the coupling reaction *in vivo*. Recent results of Kondo et al. [10] suggest that dehydroalanine residues remain in the Tg chain after hormone formation: it is possible that the cleavage occurs during the *in vitro* reduction step of iodinated Tg. Why this peptide containing 129 or 130 amino acid residues behaves in SDS-PAGE like a 26 kDa peptide is still unclear. A possible dimerization or the influence of the oligosaccharide chains identified at positions 57 and 91 [19] could be evoked.

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