In vitro and in vivo iodination of human thyroglobulin in relation to hormone release

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Received 21 March 1983; revision received 11 April 1983

Reduced and *S*-alkylated thyroglobulin (Tgb) from different species were shown by SDS–PAGE to contain small peptides (from 45–9 kDa) rich in thyroxine. Several hypotheses were proposed to explain their origin. The polypeptide composition of iodine-poor (Tgb A) and normally iodinated (Tgb B) human Tgb prepared by two different procedures (one minimizing and the other favoring post-mortem proteolysis) was compared in the native state and after in vitro iodination. Results show that one of the hormonogenic sites of human Tgb is part of a domain of the molecule most susceptible to proteolysis, especially when it is very iodinated.

Human thyroglobulin *Hormonogenic site* *Thyroxine-containing peptide* *Proteolysis* *Iodination in vitro*

1. INTRODUCTION

Thyroglobulin (Tgb), the specific iodoglycoprotein of the thyroid gland is the support of thyroid hormone synthesis. On the about 120 tyrosine residues contained in this large protein (660 kDa) only a limited number of sites are involved in their synthesis. Indeed, 25–30 tyrosines are available for iodination into iodotyrosine residues (3-iodotyrosine or MIT and 3,5-diiodotyrosine or DIT) and only 6–8 of the latter can couple to form the iodothyronines or hormone residues (thyroxine or T_4 and 3,5,3′-triiodothyronine or T_3).

We have described [1] the presence of 14–80 kDa fragments rich in thyroid hormones in the Tgb of several mammalian species. These fragments were obtained with or without previous reduction and alkylation or in the presence or absence of sodium dodecylsulfate (SDS) and were non-covalently associated to the bulk of the Tgb molecule. Such findings were later confirmed [2]. This tendency of T_4-rich peptides to be released from Tgb was most likely attributed to the attack of the molecule by proteases at preferential sites of cleavage.

Recently, several investigators have isolated from Tgb of several species (rabbit [3], beef [4], man [5], guinea pig [6]) and after reduction of the disulfide bonds, small peptide fragments rich in hormones (from 45–9 kDa) and representing one [7] or several undetermined hormone-forming sites of Tgb.

The origin of these fragments has been most debated and several possibilities were raised among which:

(1) The presence of a separate component linked to the 660 kDa Tgb by disulfide bridges formed during iodination [3];
(2) The post-translational cleavage of Tgb produced either by the iodination system itself (peroxidase–H_2O_2) [5] or by lysosomal enzymes [4,5], where this cleavage might be involved in the physiological processing of the Tgb peptide chains.

* In the conditions used for electrophoresis (section 2), it is not possible to detect with precision species of >120 kDa

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00145793/83/$3.00

Here, we have tried to clarify this situation and we present strong evidence favoring hypothesis (2).

2. MATERIALS AND METHODS

2.1. *Preparations of thyroglobulin*

Two types of human thyroid tissue were obtained at operation and immediately processed. The first one (A) was a colloid goitre poor in iodine (2 iodine atoms/mol) and the second (B) a Graves' goitre containing 14 iodine atoms/mol. Both were separated into 2 parts which were separately treated:

Procedure (1): one part was immersed at 0–2°C in 0.1 M Na-phosphate buffer (pH 7.2) containing 0.2% sodium azide for transport to the laboratory, sliced (~3 mm width), extracted for 20 min in the same buffer, precipitated with 1.8 M phosphate and filtered on Biogel A 5m in conditions as in [8] giving Tgb A_1 and Tgb B_1.

Procedure (2): the other halves were frozen at −20°C and stored for several days before thawing, homogenized in an Ultraturrax (speed 4) for 1 min in a (pH 6.2) Na-phosphate buffer; after centrifugation, the supernatant was fractionated by salting out between 1.5 M and 1.8 M ammonium sulfate. The 1.8 M precipitate was dissolved in 0.1 M Na-phosphate buffer (pH 7.2). Final steps followed procedure 1 giving Tgb A_2 and Tgb B_2.

2.2. *In vitro iodination of thyroglobulin*

In a final volume of 1 ml, 0.05 M Tris–HCl (pH 7.2) were dissolved 1 nmol Tgb B_1 or B_2, 1 mg glucose, carrier free $Na^{125}I$ (NEN), from 10–150 nmol KI and 5 μg lactoperoxidase (Boehringer, Mannheim). The reaction was started by glucose oxidase (2.5 μg) (Boehringer), continued for 30 min at 37°C and stopped by addition of 10 μl 0.1 M $NaHSO_3$. Excess iodide was then eliminated by Sephadex G-25 gel filtration (0.9 × 15 cm column) in 5 mM Tris–HCl (pH 7.2). Iodine incorporated in Tgb was calculated as in [9].

2.3. *Polyacrylamide gel electrophoresis*

This was performed in 8% gels in 0.05 M Tris–glycine (pH 8.6) containing 0.1% SDS as initially described in [10]. Samples (50 μg) were dissolved in 1% SDS containing 0.5 mg dithiothreitol, boiled for 3 min at 100°C and then electrophoresed for 2 h at 3.3 mA/tube. Gels were stained with Coomassie blue and destained as in [11]. Protein markers [12] were lysozyme (14 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), serum albumin (67 kDa) and its cross-linked polymers. Densitometric tracings of the stained gels and surfaces under peaks were carried out with a Cellomatic Gel Scanner (Sebia, Milan).

2.4. *Reduction and* S-*carboxymethylation*

Tgb B was reduced and alkylated in 0.25 M Tris–acetate, 8 M urea (pH 8.6) by dithiothreitol according to [13] then dialyzed for 48 h against 0.05 M Tris, 8 M urea (pH 7.6).

2.5. *Other techniques*

Amino acid analyses were performed on 200 μg samples with an Auto-analyzer Technicon according to [14] after hydrolysis in 6 N HCl at 110°C for 20 h in sealed evacuated tubes. Separation and quantitative estimation of [^{127}I]iodoamino acids were carried out by Dowex 50 × 4 chromatography on hydrolysates by pronase and leucine aminopeptidase as in [15].

3. RESULTS AND DISCUSSION

3.1. *Polypeptide composition of human thyroglobulin and iodination in vivo*

The release of thyroid hormones in blood is the consequence of Tgb hydrolysis in the phagolysosomes of the thyroid cell. In the course of Tgb purification and even when special care is taken to avoid cell damage, it is inevitable to injure some cells by slicing. Some lysosomal proteases are released and attack the Tgb more or less according to the procedure used for its purification. Freeze–thawing and homogenization of the thyroid favor at different degrees the release of lysosomal proteases [16]. Procedure 1 (section 2) minimizes Tgb proteolysis as compared to procedure 2 which brings together all the conditions favoring a greater hydrolysis. However, even when Tgb is prepared by procedure 1 and whatever its iodine content, some evidence of proteolysis exists, detectable by the presence in small amounts of 80–120 kDa species* (fig.1). These signs of proteolysis are much more obvious when Tgb was

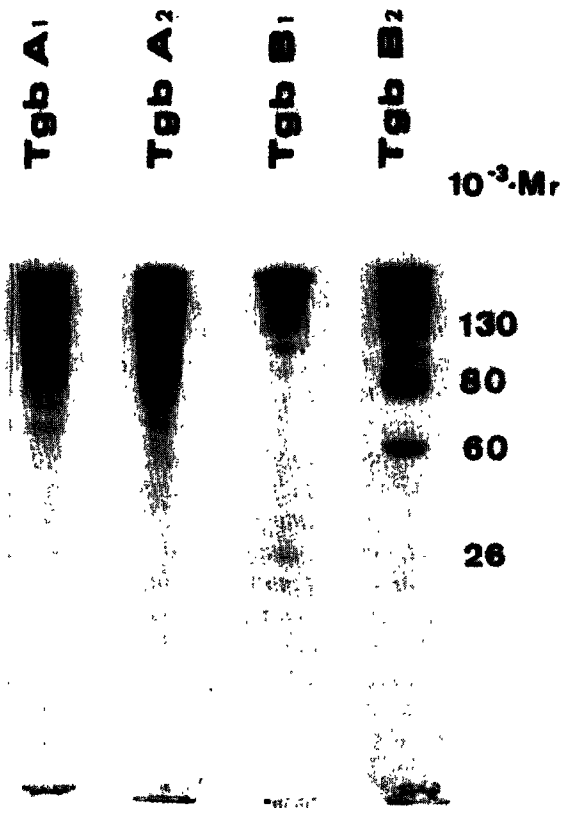

Fig.1. SDS–polyacrylamide gel electrophoresis of poorly iodinated (Tgb A: 2 iodine atoms/mol) and of normally iodinated human thyroglobulin (Tgb B: 14 iodine atoms/mol) prepared by procedure 1 (Tgb A_1, Tgb B_1) or procedure 2 (Tgb A_2, Tgb B_2): 50 μg protein/gel (8% polyacrylamide) were analyzed after reduction by 1% dithiothreitol for 3 min at 100°C.

prepared by procedure 2 (Tgb A_2 and Tgb B_2). The band at 80 kDa is the more characteristic element of proteolysis and amounts to 10–13% of the total protein material in Tgb A_2 and Tgb B_2.

On the other hand, the 26 kDa species (fig.1) described in [5], is only present in Tgb B (containing 14 iodine atoms/mol). In Tgb A of very low iodine content (2 iodine atoms/mol), this peptide is absent whatever the procedure used for its preparation. Unless large differences in lysosomal proteases activities are involved, these results provide direct evidence of a relation between the formation of 26 kDa and Tgb iodination.

To know more about the 26 kDa fragment, the latter was isolated from Tgb B_2. The reduced and *S*-alkylated Tgb B_2 was filtered on Biogel A 5m in 0.05 M Tris–HCl, 8 M urea (pH 7.6). Three fractions (I–III) (fig.2) were collected, dialyzed against water and lyophilized. Polyacrylamide gel electrophoresis in 0.1% SDS showed that I contained a material of > 100 kDa, II a mixture of the 80 and 60 kDa species and III the peptide of 26 kDa. The latter was purified by refiltration of fraction III on Biogel A 0.5 m (fig.3b). The iodioamino acid composition of fractions I, II and IIIb (table 1) shows an important enrichment of the 26 kDa peptide in all the iodoamino acids and especially in T_4 as compared to the rest of the molecule. Using a M_r of 26000 for this peptide, it can be calculated that 77% of its iodine content is represented by T_4 and that the peptide itself contains 0.97 mol T_4/mol peptide.

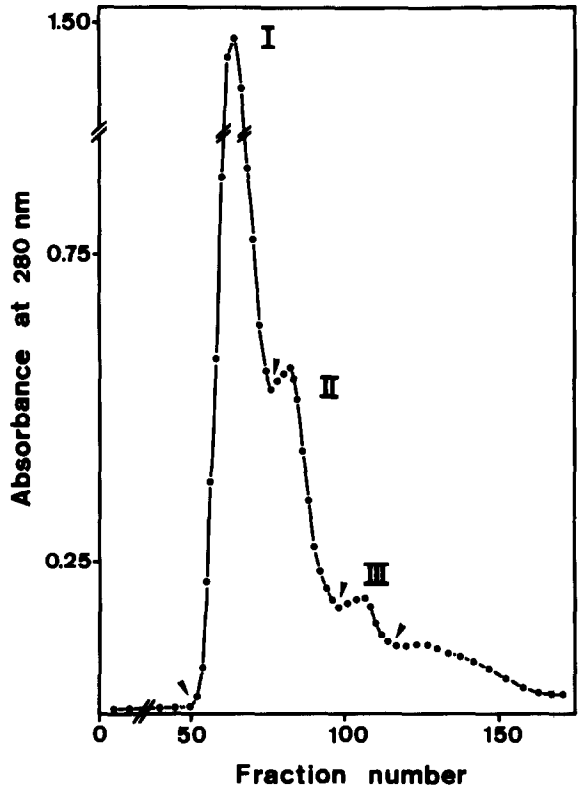

Fig.2. Filtration on Biogel A 5m of reduced and *S*-alkylated Tgb B_2. 100 mg protein were layered on a 2.6 × 90 cm column equilibrated and eluted with 0.05 M Tris–HCl, 8 M urea (pH 7.6); flowrate, 10 ml/h; fraction vol., 2 ml. Tubes were pooled according to arrows to give fractions I–III.

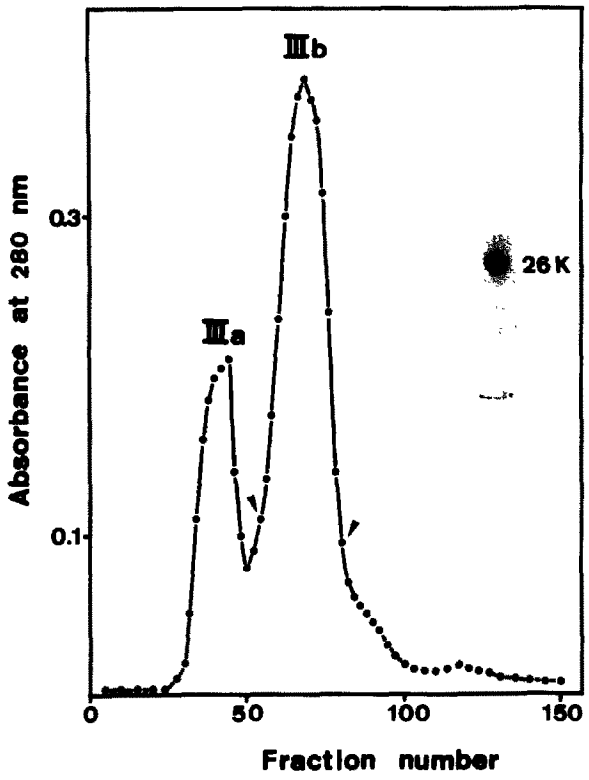

Fig.3. Filtration on Biogel A 0.5m of fraction III (see fig.2): ~5 mg protein were layered on a 1.5 × 60 cm column equilibrated and eluted in the same buffer as in fig.2. Fraction IIIb was pooled according to arrows. Inset: SDS–polyacrylamide gel electrophoresis of fraction IIIb in 8% gel.

2.2. *Polypeptide composition of human thyroglobulin and in vitro iodination*

To study the evolution of the Tgb peptide composition with increasing iodination levels, Tgb B_1 and Tgb B_2 were iodinated in vitro to add 6–69 iodine atoms in the protein to the pre-existing 14. SDS–polyacrylamide gel electrophoresis of the fully reduced Tgb B_1 and B_2 and of increasing iodine content are shown in fig.4. In the native state (14 iodine atoms/mol) Tgb B_1 contains 2-times more 26 kDa peptide (1.5% total protein) than Tgb B_2 (0.6% total protein) (fig.5).

Table 1

Iodoamino acid composition of Tgb and fraction I, II and IIIb

	MIT	DIT	T_4	T_3
	(μmol/100 g protein)[a]			
Tgb	1.06	0.38	0.12	tr
Fraction I	0.84	0.22	0.08	0
Fraction II	1.66	0.74	0.24	0
Fraction IIIb	2.58	0.88	3.74	0

[a] Calculated from amino acid composition

During progressive iodination, the amount of 26 kDa increased to reach a maximum when Tgb B_1 contained 27 iodine atoms/mol; from this value, a lighter species (18 kDa) appeared and remained constant up to 83 iodine atoms/mol. To the exception of the 18 kDa species, the polypeptide composition of Tgb B_1 was only very slightly changed at all iodine content values.

In contrast, the effect of iodination on the degradation of Tgb B_2 was obvious. The species of 80 kDa progressively disappeared whereas the fragment of 60 kDa, present in small amount in native Tgb B_2, then represented the major species. In addition, the 26 kDa species which, as for Tgb B_1, reached its maximum at about the same iodine level (~22 iodine atoms/mol) completely disappeared when the protein contained $\geqslant$42 iodine atoms/mol.

These very reproducible results (4 independent expt) show:

(1) That iodination in vitro favors the degradation of Tgb, all the more that Tgb underwent a beginning of proteolysis during its preparation;

(2) The fragment of 26 kDa which is present in lower amount in Tgb B_2 than in Tgb B_1 is especially susceptible to degradation since completely disappearing when Tgb B_2 contains 42 iodine atoms/mol.

It thus appears that peptide 26 kDa belongs to a domain of the Tgb molecule very sensitive to proteases. Despite care taken to avoid proteolysis during the preparation of Tgb B_1, signs of degradation are nevertheless present (traces of 80 kDa and 120 kDa species, fig.1); therefore peptide 26 kDa could also represent an element of this proteolytic activity. The relationship between iodination of Tgb and the presence of peptide 26 kDa can be explained because:

(i) Iodination of hormonogenic tyrosine residues in this domain followed by coupling of the formed iodotyrosines can modify the three-dimensional conformation of the molecule [17];

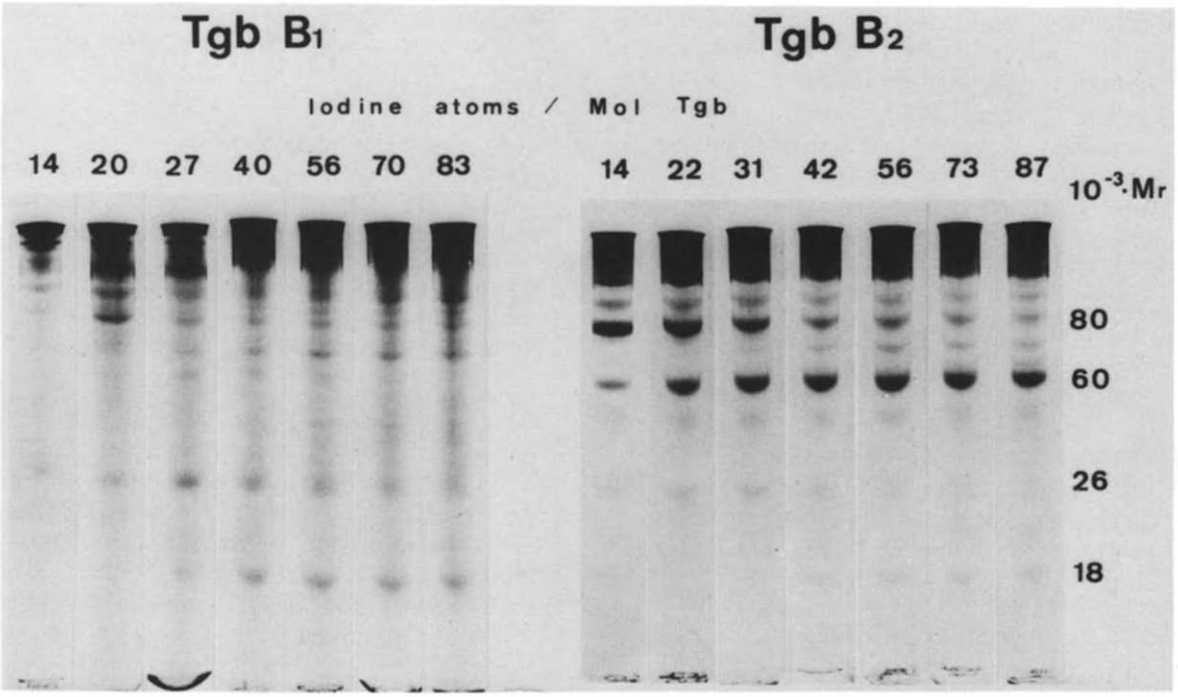

Fig.4. SDS–polyacrylamide gel electrophoresis of Tgb B after iodination in vitro with increasing amounts of ^{127}I. Tgb B (14 iodine atoms/mol) prepared by procedure 1 (Tgb B_1) and by procedure 2 (Tgb B_2).

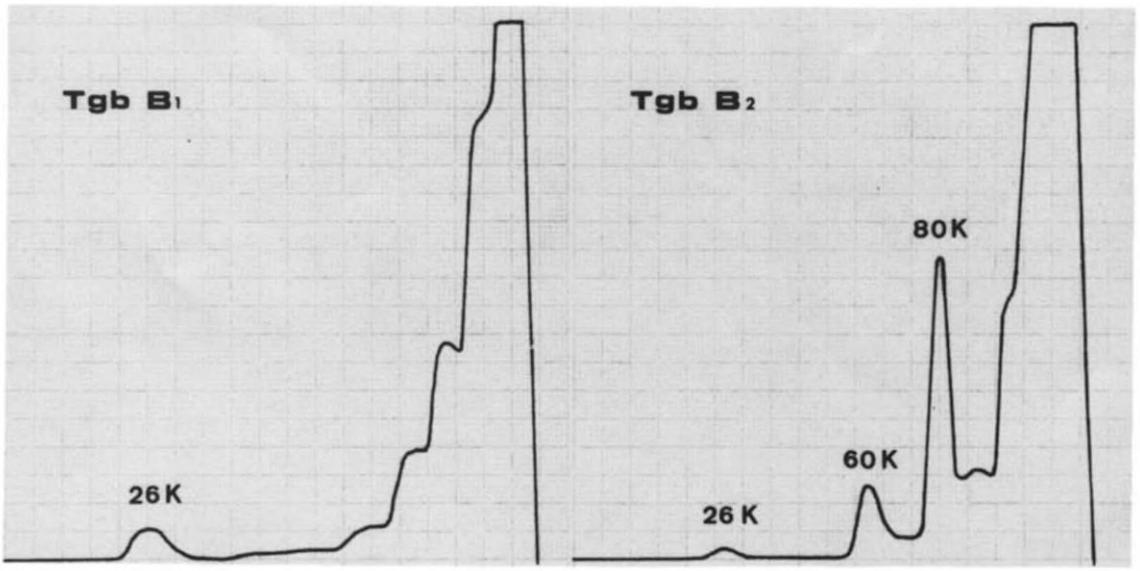

Fig.5. Densitometric recording of native Tgb B_1 and Tgb B_2 after SDS–polyacrylamide gel electrophoresis and staining with Coomassie blue (fig.1). Origin of migration is to the right.

(ii) The latter modification of conformation can offer more accessibility of sensitive peptide bonds to lysosomal proteases;
(iii) Some of these sensitive bonds could be the thyroxinyl and triiodothyronyl bonds themselves.

We have established the sequence of an hormonogenic peptide from porcine Tgb, harboring both T_3 and T_4 [18]. This study showed:

(i) The presence of sensitive bonds to trypsin-like enzymes in the vicinity of the hormonal residues;

(ii) That the triiodothyronyl and thyroxinyl bonds of the peptide were completely cleaved by several proteases and that this effect occurred only after the coupling of the hormonogenic iodotyrosines.

Our results fully agree with the hypothesis that the 26 kDa peptide is a proteolytic fragment issued from Tgb. It is likely that the proteolytic cleavage resulting in the formation of peptide 26 kDa might be a part of the normal degradative processing of the Tgb peptide chains giving rise to the free hormones.

ACKNOWLEDGEMENTS

The skillful technical assistance of Mrs S. Lasry and L. Vinet is gratefully acknowledged.

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