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AMINO ACID SEQUENCE AROUND A HORMONOGENIC TYROSINE RESIDUE IN THE N-TERMINAL REGION OF HUMAN THYROGLOBULIN AFTER IN VIVO AND IN VITRO IODINATION

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SUMMARY. Reduced and S-alkylated human thyroglobulin (hTgb), normally iodinated, was previously shown by SDS/PAGE to contain a small peptide (M_r 26 000) rich in thyroxine. This peptide was not found when very poorly iodinated hTgb was treated under the same conditions but was present after iodination in vitro. Peptide 26 K was purified from in vivo and in vitro iodinated hTgb. The study of these peptides shows that : 1/ at the iodination levels studied (0.18 and 0.25% iodine) one of the preferential hormonogenic sites is the same in hTgb iodinated both in vivo and in vitro ; 2/ the amino acid sequence around the thyroxine residue : Asn-Ile-Phe-Glu-T_4-Gln-Val is identical with the previously described hormonogenic site of bovine thyroglobulin. Most probably the hormone-containing peptide 26 K is the N-terminal peptide of the hTgb chain.

Thyroglobulin (Tgb) the high molecular weight glycoprotein (660 000 Da) is the macromolecular support of thyroid hormone synthesis in the thyroid gland. Only a limited fraction (about 7 %) of the tyrosine residues contained in the protein is involved in hormone formation. These are located in a structural environment in which, after iodination the iodotyrosine residues, (3-iodotyrosine or MIT and 3,5-diiodotyrosine or DIT) can couple into iodothyronine residues (3,5,3'-triiodothyronine or T_3 and thyroxine or T_4). Both iodination of tyrosine and coupling of iodotyrosine residues are catalyzed by the same membrane - bound enzyme, thyroid peroxidase (1,2). Up to now, the information available on the stucture of hormone-forming sites of Tgb was scarce (3 - 6). In contrast the in vitro iodination of poorly iodinated human Tgb by thyroid or lactoperoxidase was extensively used to study hormone synthesis in the protein. It was admitted that the in vitro iodination models reflected the main steps of hormone synthesis in vivo without, however, giving any evidence as to the processes involved at the molecular level.

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Recently Dunn et al. (7) isolated from normally iodinated human Tgb and after reduction of the protein, a small hormone - rich fragment of M_r 26 K. The origin of this fragment in relation to Tgb iodination was discussed (7,8). In addition the 26 K was shown to be absent when Tgb was very poor in iodine but appeared after *in vitro* iodination of the same Tgb preparation (8).

In this communication, we report the results of a comparative structural study of one of the hormone-forming sites of human Tgb iodinated *in vivo* and *in vitro*.

MATERIALS AND METHODS

Preparation of Tgb : Two types of Tgb were prepared from human thyroid tissue obtained by surgery : iodine - poor Tgb (4 iodine atoms/mol) from colloid goitre (Tgb A) and normally iodinated Tgb (14 iodine atoms/mol) from a Graves' goitre (Tgb B). Both were prepared under conditions avoiding protease contamination according to (9).

In vitro iodination of Tgb : In a final volume of 1 ml 0.05M Tris-Cl pH 7.2 at 37°C were added 1 nmol Tgb A, 1 mg glucose, carrier-free Na ^{125}I (N.E.N.), from 10 to 150 nmol KI and 5 µg lactoperoxidase (Boehringer, Mannheim, FRG). The reaction was started by 2.5 µg glucose oxidase (Boehringer), and stopped 30 min later by addition of 10 µl 0.1 M $NaHSO_3$. Excess iodide was then eliminated by filtration of the reaction mixture on a Sephadex G-25 column (0.9 x 15 cm) in 5 mM Tris -Cl pH 7.2. Iodine incorporated in Tgb was estimated as in (10).

Reduction and S-alkylation were performed in 0.25 M Tris-acetate, 8 M urea, pH 8.6 according to (4), then dialyzed for 48 hr against 0.05 M Tris-Cl, 8 M urea, pH 7.6.

Other techniques : Polyacrylamide gel electrophoresis, densitometric tracing of stained gels, amino acid and iodoamino acid analyses and microsequencing of peptides were carried out as previously described (5,8).

RESULTS

Purification of 26 K peptides from human Tgb.

When poorly iodinated Tgb (Tgb A) was fully reduced and electrophoresed in SDS-polyacrylamide gel, no peptide band was observed in the range of M_r 26 K whereas this species was present in *in vivo* normally iodinated Tgb (Tgb B) (Fig.1). This peptide accounted for 1.5 % of total protein material after densitometric recording of stained gels. In addition, when Tgb A was iodinated *in vitro* in order to incorporate 8 to 82 iodine atoms/mol on top of the 4 preexisting ones, the 26 K peptide appeared in maximum amount when Tgb A contained 20 iodine atoms/mol (Fig. 1). From this iodination level, the 26 K peptide progressively disappeared as two lighter species (M_r 22 K and 18 K) increased.

From these results, we selected an iodination level of 20 iodine atoms/mol Tgb A to prepare the 26 K peptide. Under these conditions, it represented

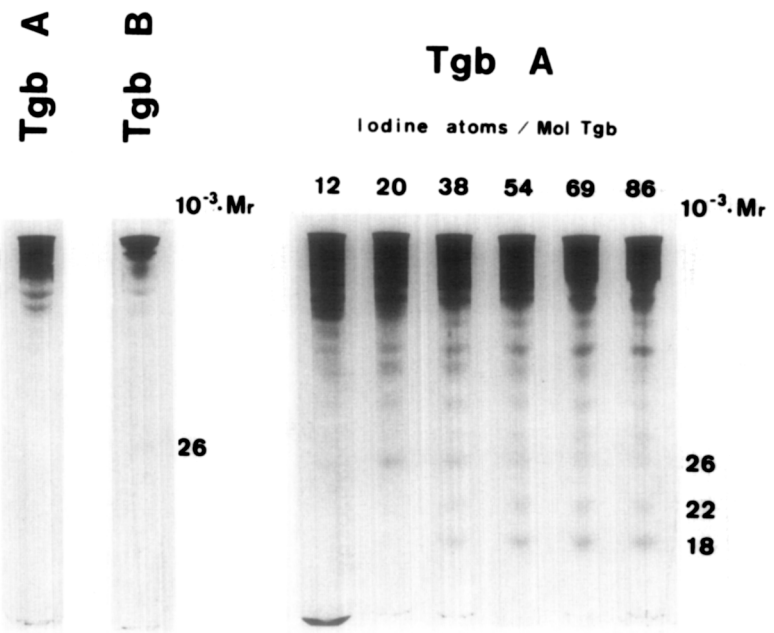


Fig. 1 - SDS-polyacrylamide gel electrophoresis of poorly iodinated (Tgb A : 4 iodine atoms/mol) and of normally iodinated human thyroglobulin (Tgb B : 14 iodine atoms/mol) and of poorly iodinated human thyroglobulin (Tgb A) after iodination in vitro with increasing amounts of ^{127}I. Fifty µg protein per gel (8 % polyacrylamide) were analyzed after reduction by 1 % dithiothreitol for 3 min at 100°C.

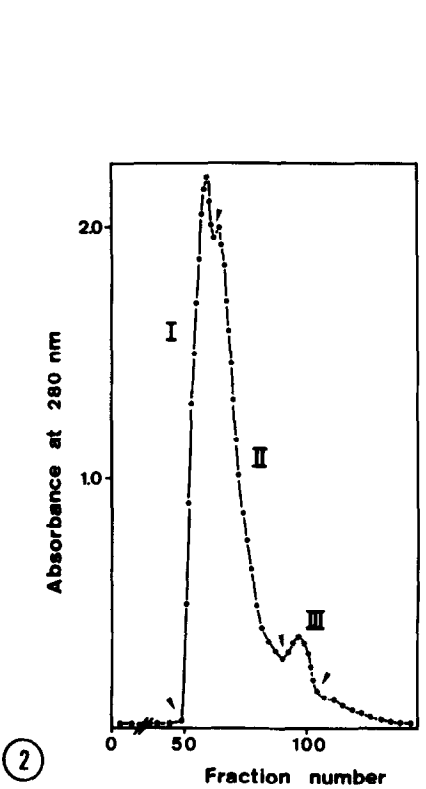
3.3 % of the total protein material whereas peptides 22 K and 18 K were present only in trace amounts.

Tgb A was iodinated in vitro to add 16 additional iodine atoms to the molecule. In Table 1, the iodoamino acid composition of Tgb before and after in vitro iodination is presented. Deprived of hormones in the native state Tgb A synthesized 1.0 mol T_4 and 0.2 mol T_3/mol Tgb after in vitro iodination (20 iodine atoms/mol).

Table 1 - Iodoamino acid composition of human Tgb B, Tgb A and Tgb A iodinated in vitro.

	MIT	DIT	T_3	T_4
	residue/mol			
Tgb B (14)	6.4	2.3	tr	0.75
Tgb A (4)	3.6	0.2	0	0
Tgb A (20) iodinated in vitro	8.8	2.6	0.2	1.0

In parentheses number of iodine atoms/mol thyroglobulin
tr = trace



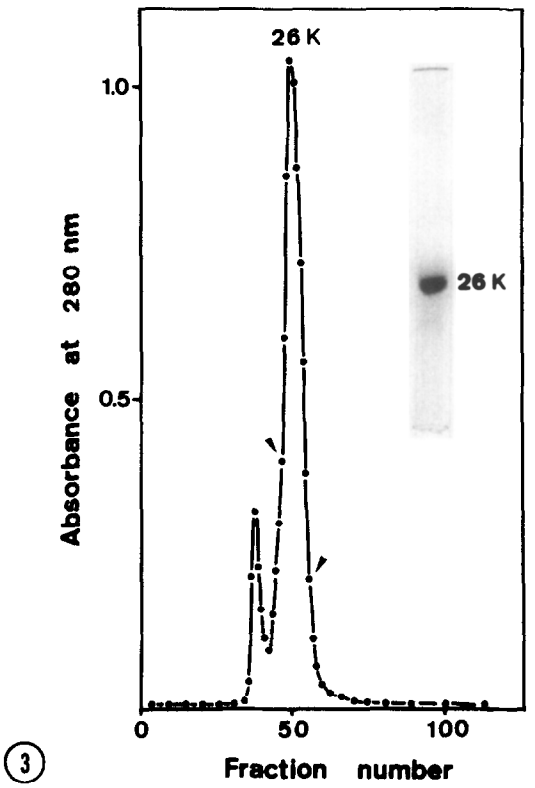


Fig. 2 - Filtration on Biogel A-5m of reduced and S-alkylated Tgb A after iodination in vitro (20 iodine atoms/mol Tgb). 150 mg protein were layered onto a 2.6 x 90 cm column equilibrated and eluted with 0.05M Tris-Cl, 8M urea, pH 7.6. Flow-rate : 10 ml/hr. Volume of fractions : 2 ml. Tubes were pooled according to arrows to give fractions I, II and III.

Fig. 3 - Filtration on Biogel A-0.5m of fraction III (see Fig. 2). Approximately 8 mg protein were layered onto a 1.5 x 60 cm column equilibrated and eluted in the same buffer as in Fig. 2. Fraction 26 K was pooled according to arrows. Inset : SDS-polyacrylamide gel electrophoresis of fraction 26 K in 8 % gel.

The purification procedure of the 26 K species followed closely that previously described (8). Tgb A iodinated in vitro (20 iodine atoms/mol) was reduced and S-alkylated, and filtered on Biogel A-5m in 0.05 M Tris-Cl, 8 M urea, pH 7.6 ; fraction III (Fig.2) was dialyzed against water and freeze-dried. After solubilization in the previous buffer and filtration on Biogel A-0.5m, the pure 26 K fraction was recovered (Fig.3).

The same procedure was used to purify the 26 K peptide from Tgb B.

Study of 26 K peptides isolated from in vivo and in vitro iodinated human Tgb.

As shown in Table 2, both 26 K peptides have the same amino acid composition, observed differences not exceeding the range of experimental errors. At

Table 2 - Amino acid and iodoamino acid compositions of 26 K iodopeptides purified from Tgb A iodinated *in vitro* and Tgb B.

	26 K iodopeptides from :	
	Tgb A[a] iodinated *in vitro*	Tgb B[a]
	Residue/mol peptide[b]	
Carboxymethylcysteine	12.6	11.2
Aspartic acid	18.0	18.2
Threonine	7.1	6.9
Serine	15.3	14.6
Glutamic acid	37.8	36.7
Proline	12.1	11.7
Glycine	16.5	15.6
Alanine	14.0	13.1
Valine	16.2	15.3
Methionine	2.2	1.9
Isoleucine	6.1	5.3
Leucine	16.3	15.5
Tyrosine	6.4	5.7
Phenylalanine	8.2	7.6
Histidine	1.9	1.7
Lysine	5.1	5.8
Arginine	8.8	8.5
Tryptophane	nd[c]	nd[c]
3-iodotyrosine	0.61	0.67
3'5-diiodotyrosine	0.18	0.23
Thyroxine	0.79	0.97
3,5,3'-triiodothyronine	0.19	0

[a] Average of 4 analyses. [b] Amino acid content was calculated on the basis of an M_r of 26 000. [c] n.d. = not determined.

similar iodination levels (14 iodine atoms/mol for Tgb B and 20 iodine atoms/mol for Tgb A) the iodoamino acid distribution in the two peptides was very similar. However small amounts of T_3 were detected in 26 K issued from *in vitro* iodinated Tgb A whereas T_3 was absent from the fragment purified from Tgb B.

The identity of the two 26 K peptides was fully confirmed by N-terminal amino acid sequence determination (Table 3). At the 5th step of recurrent

Table 3 - N-terminal sequence of 26 K iodopeptides isolated from Tgb A iodinated *in vitro* and from Tgb B.

26 K iodopeptide from :	
Tgb A iodinated *in vitro*	: Asn-Ile-Phe-Glu-T_4-Gln-Val-
Tgb B	: Asn-Ile-Phe-Glu-T_4-Gln-

Amino and iodoamino acids were identified by microsequencing (11).

degradation, T_4 was clearly identified in the two peptides whereas neither tyrosine nor MIT, DIT or T_3 were detected at this step.

DISCUSSION AND CONCLUSIONS

1. The present results show unambiguously that for the iodination levels studied (0.18 % for Tgb B and 0.25 % for Tgb A), the characterized hormone-forming site is identical both in in vivo (Tgb B) and in vitro (Tgb A) iodinated Tgb.

2. In agreement with previous observations (7) our results equally show that the 26 K peptide probably contains one of the major hormonogenic sites of human Tgb. Indeed, in our in vitro experiments, the 26 K peptide represents 3.3 % of the total protein material ; taking into account the T_4 level of Tgb A iodinated in vitro (Table 1) and that of the 26 K peptide (Table 2), one can easily calculate that the 26 K peptide contains 65 % of the T_4 synthesized in vitro. At the same iodination level, but using a different methodology, Dunn et al. (7) reached a neighbouring value of 56 %. From Tgb B (14 iodine atoms/mol), the 26 K peptide represents only 1.5 % of the protein material. Nevertheless, the preceding calculation allows to evaluate that the peptide contains 49 % of the total T_4 formed in vivo.

Whatever its origin, the 26 K peptide presents only one hormonogenic tyrosine residue located in position 5 from the N-terminal sequence (Table 3). Indeed 26 K contains a stoichiometric amount of hormone and at the 5th step of degradation neither tyrosine nor any iodoamino acid other than T_4 have been detected. Thus, MIT and DIT estimated in these peptides are probably the result of iodination of another or other residue(s) of tyrosine present in the peptide but non hormonogenic at the studied level of iodination.

Tgb B contains only traces of T_3 whereas after in vitro iodination, Tgb A (20 iodine atoms/mol) synthesizes 0.2 mol T_3/mol (Table 1), 65 % of which is recovered in the 26 K peptide. T_3 was not detected at the 5th step of recurrent degradation (Table 3) perhaps due to the small amount present. However it is not excluded, considering the equimolar sum of iodothyronines (T_4+T_3) in the 26 K peptide (Table 2) that the same site is able to synthesize both hormones. This previously suggested hypothesis (7) is fully compatible with our results on porcine Tgb showing that the same structure is capable of giving rise to T_3 or T_4 (12). Whether T_3 is synthesized only after in vitro iodination is still unknown. A possible explanation might be the different nature of the two thyroid tissues used in this experimentation.

3. The primary structure of this hormone-forming site is remarkably similar to those isolated from Tgb from other animal species. Indeed, the se-

Table 4 - Primary structure of amino-terminal portion of bovine Tgb as deduced from the sequence of its structural gene according to (13). Correspondence is shown between the sequence from residue 19-37 and those of the hormonogenic peptides identified in bovine (6), porcine (not published) and human Tgb (present work).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Met-	Pro-	Trp-	Pro-	Met-	Gly-	Leu-	Arg-	Ser-	Ala-	Gly-	Leu-	Asn-	Leu-	Leu-
	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
	Gly-	Ile-	Arg-	Asn-	Ile-	Phe-	Glu-	Tyr-	Gln-	Val-	Asp-	Ala-	Gln-	Pro-	Leu-
bovine	Asn-	Ile-	Phe-	Glu-	T_4	-Gln-	Val-	Asp-	Ala-	Gln-	Pro-	Leu-			
porcine					T_4	-Gln-	Val-	Asp-							
human	Asn-	Ile-	Phe-	Glu-	T_4	-Gln-	Val-								

	31	32	33	34	35	36	37
	Arg-	Pro-	Cys-	Glu-	Leu-	Gln-	Arg-
	Arg-	Pro-	Cys-	Gln-	Leu-	?	-Arg-

quence of 7 amino acid residues (Table 3) is identical with the N-terminal sequence of the T_4-containing peptide isolated from bovine Tgb (6). Recently, we purified a new T_4-forming site from porcine Tgb ; this tetrapeptide : T_4-Gln-Val-Asp was obtained by digestion with the Staphylococcus aureus protease of a CNBr-fraction [CB-1 (4)] from porcine Tgb (unpublished results). This sequence coincides perfectly with those of bovine and human hormone peptides.

4. In bovine Tgb, this hormone synthesis site is located in the N-terminal part of the molecule as shown by Mercken et al. (13). Indeed these authors have derived from the sequence of 350 nucleotides at the 5'-end of the bovine Tgb mRNA, the primary structure of the amino terminal portion of the protein. Thus, the T_4-containing peptide isolated by Rawitch et al. (6) from bovine Tgb appears to be located between residues 19 and 37 of this sequence (Table 4). By analogy, probably the 26 K peptide is identically located in human Tgb. As illustrated in Table 4 the hormone-containing peptide is preceded by a relatively hydrophobic sequence of 18 amino acid residues which may represent, as already suggested (13), the signal peptide for Tgb secretion. Up to now, the numerous attempts to determine the N-terminal amino acid of the Tgb chain have failed. However if the latter hypothesis could be confirmed, the cleavage of the Arg_{18}-Asn_{19} bond during or after translation should give rise to the mature human Tgb polypeptide chain. Then the 26 K peptide would represent the N-terminal peptide of human Tgb.

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REFERENCES

1. Lamas, L., Dorris, M.L. and Taurog, A. (1972) Endocrinology 90, 1417-1426.
2. Pommier, J., Deme, D. and Nunez, J. (1975) Eur.J.Biochem. 51, 329-336.
3. Dunn, J.T. (1970) J.Biol.Chem. 245, 5954-5961.
4. Marriq, C., Arnaud, C., Rolland, M. and Lissitzky, S. (1980) Eur.J.Biochem. 111, 33-47.
5. Marriq, C., Rolland, M. and Lissitzky, S. (1982) Embo J. 1, 397-401.
6. Rawitch, A.B., Chernoff, S.B., Litwer, M.R., Rouse , J.B. and Hamilton, J.W. (1983) J.Biol.Chem. 258, 2079-2082.
7. Dunn, J.T., Kim, P.S. and Dunn, A.D. (1982) J.Biol.Chem. 257, 88-94.
8. Lejeune, P.J., Marriq, C., Rolland, M. and Lissitzky, S. (1983) FEBS Lett. in press.
9. Marriq, C., Rolland, M. and Lissitzky, S. (1977) Eur.J.Biochem. 79, 143-149.
10. Maurizis, J.C., Marriq, C., Michelot, J., Rolland, M. and Lissitzky, S. (1979) FEBS Lett. 102, 82-86.
11. Marriq, C., Rolland, M. and Lissitzky, S. (1981) Anal.Biochem. 116, 89-92.
12. Marriq, C., Rolland, M. and Lissitzky, S. (1983) Biochim. Biophys. Res. Comm. in press.
13. Mercken, L., Simons, M.J. and Vassart, G. (1982) FEBS Lett. 149, 285-287.