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AMINO ACID SEQUENCE OF THE UNIQUE 3,5,3'-TRIIODOTHYRONINE-CONTAINING SEQUENCE FROM PORCINE THYROGLOBULIN

Claudine Marriq, Marcel Rolland and Serge Lissitzky

Laboratoire de Biochimie Médicale associé au CNRS (LA 178) et U 38 INSERM, Faculté de Médecine, 27 Bd Jean Moulin, 13385 Marseille Cédex 05, France

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SUMMARY. A unique T_3-containing peptide was isolated by chemical and enzyme treatments from pTgb of different iodine contents and microsequenced : Leu-Ala-Ser-Lys-Ser-T_3. This site can also form T_4 to a small extent. It contains all the T_3 of the protein and presents a special susceptibility to proteolysis thus suggesting a likely mechanism to explain that the thyroid secretes T_3 preferentially to T_4 in vivo.

Thyroglobulin (Tgb), the specific iodinated glycoprotein of the thyroid gland is formed of 2 identical chains of Mr 330 000 and is the macromolecular support of thyroid hormone biosynthesis. The latter occurs on a few hormone-forming sites (1) in which the iodination of some tyrosine residues is followed by their coupling into iodothyronines (thyroxine or T_4 ; 3,5,3'-triiodothyronine or T_3). Knowledge of the Tgb chain structure is eagerly needed to reveal the molecular mechanism of this coupling : it is on the verge of being determined by sequencing of the cloned DNA complementary to the Tgb mRNA (2,3). Additional knowledge of the sequences around the hormone residues by the classical methods of protein sequencing is needed to select among the approximately 130 tyrosine residues of the chains, those involved in thyroid hormone biosynthesis.

Previous studies from this laboratory showed the presence in porcine Tgb (pTgb) of definite hormone containing peptides in limited number (1), the sequences of two of which have been established (4). The results showed that the sequences were different and that in addition the sites had different aptitude for iodotyrosine coupling, giving evidence at the molecular level, of distinct sites implied in T_4 hormonogenesis at low or high iodine level. However none of them contained T_3.

In this communication, we describe the sequence of the unique structure of pTgb containing T_3. In addition, we give evidence of the unquestionable

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sensitivity of this site to proteolysis, thus showing a new property of hormone-forming sites of Tgb, i.e. a special aptitude to release the hormone(s) from peptide linkage.

MATERIALS AND METHODS

All pTgb preparations were carried out according to Marriq et al. (5). $CNBrb_3b_4$ was purified as described in (1). The dipeptides ser-T_3 and ser-T_4 were obtained from tryptic digests of $CNBrb_3b_4$ (1).
All the techniques used were described previously (1,4,6). Filtrations on Biogels P_6 and P_2 were performed in 0.2M ammonium bicarbonate and the elutions were pursued with 50 ml 0.8N ammonium hydroxide in 30 % ethanol to eliminate the possible presence of adsorbed iodinated material.

RESULTS

Three CNBr peptides of Mr 15000 ($CNBrb_1$, b_3b_4 and e_2e_3) were previously purified from the CNBr treatment of pTgb which contained 50-55 % of the T_4 of the protein. In addition, we showed that almost all the T_3 of the protein was comprised in $CNBrb_3b_4$ whatever the iodine content of the protein (1) and that small amounts of T_4 were associated with this T_3-containing fraction.

Our studies were carried out on 3 preparations of $CNBrb_3b_4$ issued from batches of pTgb of different iodine content and numbered n°1, 2 and 3 (Table 1). To know more about these sites, $CNBrb_3b_4$ was cleaved by Staphylococcus aureus V8 protease with different E/S ratios and digestion times.

1. Hormone-forming peptides obtained from $CNBrb_3b_4$ n° 1. This peptide was obtained from the less iodinated batch of pTgb (0.65 % iodine, Table 1). After S. aureus protease digestion for 6h (E/S=1:44), the fully soluble digest was filtered on Biogel P_6 (Fig.1). Six fractions (d_1 to d_6) were obtained and lyophilized. Iodoamino acid estimation showed that 90 % of T_3 was in fraction d_6 and 10 % in d_5. Thyroxine was distributed in d_5 and d_6 and traces in d_1. Iodotyrosines were located in d_4 and d_5.

Table 1. Iodoamino acid composition of the peptide $CNBrb_3b_4$ isolated from porcine thyroglobulin of increasing iodine content

iodine in Tgb (%)[a]	0.65	1.00	1.10
$CNBrb_3b_4$ n°	1	2	3
3-iodotyrosine	0.53[b]	0.22	0.30
3,5-diiodotyrosine	0.28	0.21	0.23
thyroxine	0.15	0.45	0.33
3,5,3'-triiodothyronine	0.53	1.06	1.00

a,g iodine/100g Tgb ; b, mol. iodoamino acid/mol peptide

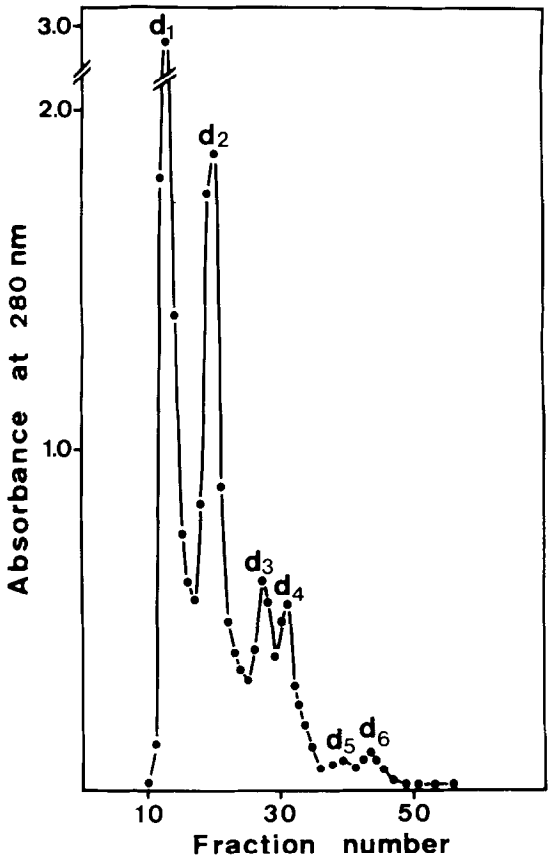

Fig. 1. Filtration on Biogel P_6 in 0.2 M ammonium bicarbonate of the digest of CNBrb$_3$b$_4$n°1 by S. aureus protease. About 7 mg protein layered onto a 1.5x 25 cm column. Volume of fractions, 1ml ; flow-rate, 12ml/hr. Tubes were pooled to produce fractions d_1 (t.12-15), d_2 (t.18-25), d_3 (t.26-29), d_4 (t.30-33), d_5 (t.36-40), d_6 (t.42-49).

1.1 Fraction d_6. Purity of fraction d_6 was confirmed by refiltration on Biogel P_2 which gave a single peak of the same composition (Table 2). This table also shows that d_6 contained 1 mole T_3/mole peptide and a trave of T_4. Its sequence agreed perfectly with the amino acid composition and clearly demonstrated that d_6 contained a single peptide, $b_3b_4d_6$. The sequence was performed with the method of Chang et al. which allows determination of both free and peptide-linked iodothyronines (6). During this study, aqueous and organic phases were controlled at all degradation steps and T_3 was identified only at the 6th step. It is thus clear that T_3 is C-terminal in $b_3b_4d_6$. Carboxypeptidase B was unable to release other amino acids from the peptide after 30 and 90 min. of action. Finally, $b_3b_4d_6$ was directly analyzed on the anion exchange resin used for iodoamino acid analysis (7). Only one peak was eluted with 0.8M ammonium hydroxide in 30 % ethanol after the peak of free T_3 (Fig. 2B) and

Table 2. Amino acid and iodoamino acid compositions of hormonopeptides isolated from differently iodinated $CNBrb_3b_4$ peptides

$CNBrb_3b_4n$°	1			2	3
peptides	$b_3b_4d_6$	$b_3b_4d_5$	$b_3b_4d_4$	$b_3b_4d_6$	$b_3b_4d_6$
amino acids	residue/mol peptide[a]				
carboxymethylcysteine	-	-	-	-	-
aspartic acid	0.3	1.0	3.2	-	-
threonine	-	-	-	-	-
serine	1.9	2.0	2.8	2.2	2.4
glutamic acid	0.2	1.1	1.8	-	-
proline	-	-	1.8	-	-
glycine	0.3	0.3	1.0	0.4	-
alanine	1.0	0.9	0.8	0.8	1.0
valine	0.2	0.8	1.0	0.2	0.3
isoleucine	-	-	-	-	-
leucine	1.0	0.8	0.7	1.0	1.0
tyrosine	-	1.2	2.1	-	-
phenylalanine	-	1.2	1.9	-	-
histidine	-	-	1.0	-	-
lysine	1.0	1.0	1.0	1.0	1.0
arginine	0.2	0.6	0.9	0.2	0.2
tryptophane	nd[b]	nd	nd	nd	nd
3-iodotyrosine	-	-	0.29	-	-
3,5-diiodotyrosine	-	0.16	0.07	-	-
thyroxine	tr	0.10	tr	0.15	0.27
3,5,3'-triiodothyronine	0.96	0.13	tr	0.84	0.70
yield in peptides[c]	0.19	0.16	1.18	0.54	0.72

[a]taking lysine = 1 ; [b]nd = not determined ; [c]expressed as mol/mol $CNBrb_3b_4$ peptide subjected to hydrolysis ; tr = trace

accompanied by a minor fraction X eluting before the iodothyronines. Absence of free T_3 was confirmed by paper chromatography in t-amylol-2N ammonia which disclosed a single iodinated spot of R_f = 0.31 with no free T_3 (R_f= 0.65).

1.2. Fractions d_4 and d_5. As shown by their amino acid and iodoamino acid compositions (Table 2), these fractions are less iodinated than d_6. Fraction d_5 contains T_3, T_4 and DIT (3,5-diiodotyrosine) and fraction d_4 only iodotyrosines. Recurrent amino acid degradation showed (Table 3) homogeneous peptides with N-terminal sequences identical to that of $b_3b_4d_6$, demonstrating that they represent the same peptide $b_3b_4d_6$ inserted in 2 longer sequences : $b_3b_4d_4$ and $b_3b_4d_5$. At the 6th degradation step Tyr and MIT (3-iodotyrosine) were characterized in the former and T_3, T_4 and DIT in the latter, in agreement with the iodoamino acid composition. However, stepwise degradation beyond the 6th step was unsuccessful due to absence of remaining peptide or to low coupling yield after this step.

Accordingly, we tried to establish the complete sequence of $b_3b_4d_4$ by digesting the peptide with trypsin (E/S=1:25) for 6hr at 37°C. Two peptides

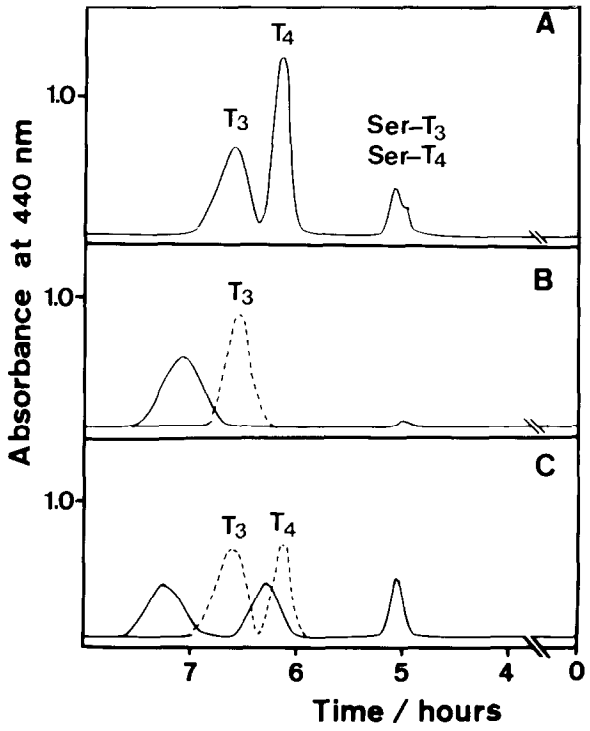

Fig. 2. Elution profiles (composite) obtained after ion-exchange chromatography of : A, mixture of T_3 and T_4 (2nmol each) + dipeptides Ser-T_3 and Ser-T_4 (about 1nmol each) ; B, $b_3b_4d_6$ (3nmol) (from $CNBrb_3n°1$) and C, $b_3b_4d_6$ (3nmol) (from $CNBrb_3b_4n°3$), before (——) and after (---) total hydrolysis by pronase and leucine aminopeptidase (7). Chromatography as in (7) with a flow-rate of 0.45 ml/min. For B and C, the elution was continued for 30 min. with 0.8N ammonium hydroxide in 30 % ethanol.

were separated by chromatoelectrophoresis of the digest : Leu-Ala-Ser-Lys corresponding to the N-terminal sequence of $b_3b_4d_4$ and Ser-Gly-Asn-Pro-Asn-Tyr-Pro-His-Glu likely corresponding to its C-terminal end. Despite careful

Table 3. Amino acid sequences of the hormonopeptides purified from $CNBrb_3b_4$ peptides isolated from pTgb of different iodine content

Origin of $CNBrb_3b_4$	peptides	
n°1	$b_3b_4d_6$	Leu-Ala-Ser-Lys-Ser-T_3
	$b_3b_4d_5$	Leu-Ala-Ser-Lys-Ser(T_3/T_4/DIT)-(Asx,Glx,Val,Phe,Arg)
	$b_3b_4d_4$	Leu-Ala-Ser-Lys-Ser-(MIT/Tyr)-(Asx_3, Ser, Glx_2, Pro_2, Gly, Val, Tyr, Phe_2, His, Arg)
n°2	$b_3b_4d_6$	Leu-Ala-Ser-Lys-Ser-(T_3/T_4)
n°3	$b_3b_4d_6$	Leu-Ala-Ser-Lys-Ser-(T_3/T_4)

All the amino and iodoamino acids have been identified by micro-sequencing (6). The C-terminal parentheses in $b_3b_4d_5$ and $b_3b_4d_4$ indicate amino acid residues deduced from amino acid composition of the peptides (Table 2).

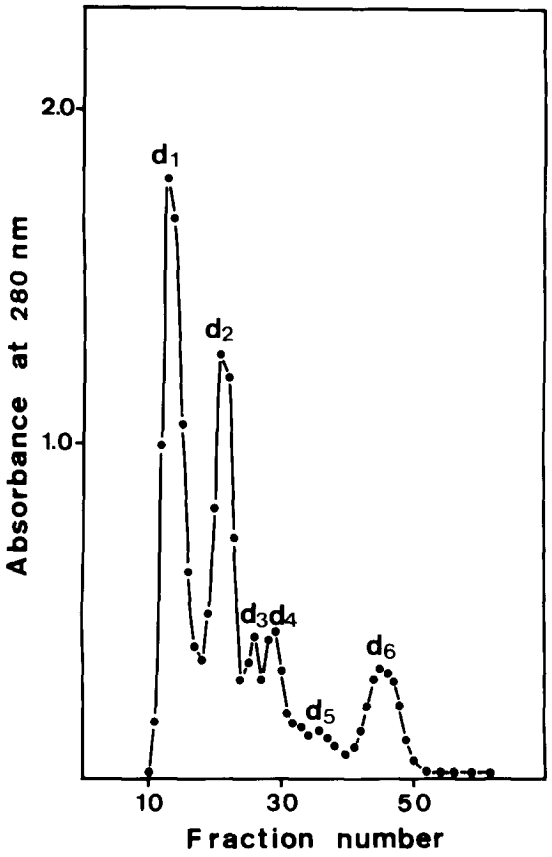

Fig. 3. Filtration on Biogel P_6 in 0.2M ammonium bicarbonate of $CNBrb_3b_4$ n°3 (10 mg) digested with S. aureus V8 protease. Column 1.5x40cm. Otherwise same conditions as in Fig. 1. Fractions were pooled as follows : d_1 (t.11-16), d_2 (t.19-24), d_3 (t.25-27), d_4 (t.28-32), d_5 (t.34-39), d_6 (t.41-50).

and repeated analyses we were not able to characterize the central region of the peptide. Only the N-terminal sequence of $b_3b_4d_4$ is shown in Table 3.

2. Hormonopeptides isolated from $CNBrb_3b_4$ n°2 and 3. To determine whether other hormone-forming sites are present in $CNBrb_3b_4$, 2 preparations of this peptide were purified from 2 batches of pTgb very rich in iodine (n°2 and 3, Table 1). $CNBrb_3b_4$ n° 2 was digested with S. aureus protease for 23hr (ES=1:44) and the same peptide from batch n°3 for 3hr with E/S=1:80. Both fully soluble digests were filtered on Biogel P_6 giving similar profiles with 6 fractions (d_1 to d_6, Fig. 3). Obviously in both chromatograms d_6 is more abundant that in the less iodinated batch (Fig.1). Amino acid and iodoamino acid compositions (Table 2) and sequence (Table 3) demonstrate that they contain the same peptide $b_3b_4d_6$ as in batch n°1. As compared to the preparation from $CNBrb_3b_4$ n°1, $b_3b_4d_6$ is recovered with a higher yield (0.54 mol peptide/mol

$CNBrb_3b_4$ n°2 and 0.72 mol/mol $CNBrb_3b_4$ n°3, Table 2). Both d_6 fractions contained T_3 and T_4. Indeed, they comprised almost all (91-96 %) of the T_3 and 60-70 % of the T_4 recovered after filtration. The molar ratio T_3+T_4/peptide was very close to 1 whereas the T_4/T_3 ratio varied from 0.18 for $b_3b_4d_6$ n°2 to 0.38 for $b_3b_4d_6$ n°3. No T_4 was found either at the first step of microsequencing or at the 6th step where T_3 was characterized.

However, bearing in mind that T_4 was detected at the 6th degradation step of $b_3b_4d_5$ (Table 3), d_6 isolated from the 2 preparations richer in iodine may contain a mixture of 2 peptides of the same primary structure, one containing T_3 and the other one T_4 (in lesser amount) in C-terminal position. This interpretation agrees with results of ion exchange chromatography of d_6 fractions. The elution profiles (Fig. 2C) can be compared with that of d_6 issued from $CNBrb_3b_4$ n°1 (Fig. 2B). Two iodinated peaks were eluted at the end of the gradient, the more retarded one corresponding to $b_3b_4d_6$ containing T_3, the other one eluting between controls T_3 and T_4 and representing the T_4-containing species. A third peak (X) was detected : it eluted before the hormones and was more abundant in the preparations richer in iodine. It can be a deiodination product of $b_3b_4d_6$ or the result of its cleavage during chromatography. The latter possibility agrees with the finding that authentic Ser-T_3 and Ser-T_4 dipeptides elute from the column in the same volume as X.

DISCUSSION

As already described (1) and confirmed in this paper T_3 was shown to be located in a very limited area of pTgb, corresponding to the $CNBrb_3b_4$ peptide producing upon digestion with S. aureus protease the unique T_3-containing peptide, $b_3b_4d_6$: Leu-Ala-Ser-Lys-Ser-T_3. This site for T_3 synthesis is also able to form T_4 but only when very iodinated (up to 0.3 mol/mol peptide for the more iodinated peptide). However, the molar ratio T_3+T_4/peptide is always of one suggesting that the more iodinated peptides $b_3b_4d_6$ are a mixture of the same peptide at different levels of coupling.

This possibility agrees with the results of $CNBrb_3b_4$ digestion with trypsin under various conditions [(1) and unpublished results] which showed the majority of T_3 and T_4 in Ser-T_3 and Ser-T_4, these 2 peptides being formed by the tryptic cleavage of the Lys-Ser bond of $b_3b_4d_6$.

Fraction X from ion-exchange chromatography of $b_3b_4d_6$ eluted in the same volume as controls Ser-T_3 and Ser-T_4. $CNBrb_3b_4$ is unlikely to contain another structure producing the 2 dipeptides after tryptic or S. aureus protease hydrolysis.

Another special feature revealed by our studies concerns the high sensitivity of the triiodothyronyl as well as thyroxinyl bonds to trypsin and S. aureus protease. These bonds were completely cleaved by these enzymes. On the other hand this sensitivity to the enzymes is absent when the hormone is not yet formed by coupling e.g. when the site contains Tyr, MIT or DIT (b_3b_4 d_4, $b_3b_4d_5$). Susceptibility to the proteases used seems specific to this site. Indeed, in the known T_4-peptides already isolated by trypsin (8) or trypsin + S. aureus protease (4) treatment, the thyroxinyl bond was not cleaved by these endopeptidases. It seems most probable that this T_3-hormone forming site corresponds to a region of the molecule especially sensitive to hydrolysis not only due to the presence of the T_3 (or T_4) bond but also to the presence of a lysyl bond sensitive to enzymes with trypsin-like activity.

This agrees with the observation that the thyroid secretes T_3 preferentially to T_4 in vivo (9-10). Indeed, the T_4/T_3 ratio in thyroid effluent is lower than in hydrolysates of the same thyroid. A mechanism behind this preferential secretion of T_3 could well be related to the facts reported in this paper that the unique T_3-hormone forming site of Tgb has a greater sensitivity to proteolysis than the already known T_4-forming sites.

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