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Tyrosine-130 in bullfrog thyroglobulin is a thyroid hormone generating site

Hiroaki Hayashi, Yoshihiro Ohmiya* and Shintaro Suzuki

Institute of Endocrinology, Gunma University, Maebashi, 371 Japan

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Bullfrog thyroglobulin was digested with lysyl endopeptidase, known to be highly specific to cut the C-terminal side of lysine residue in protein, after reduction and carboxymethylation. We isolated one peptide which lacked the C-terminal lysine, and which corresponds to 103–129 of bovine thyroglobulin sequence. Tyrosine 130 in the mammalian thyroglobulin molecule is known to be an iodination site. These findings suggest that tyrosine 130 in frog thyroglobulin is a thyroid hormone generating site.

Bullfrog thyroglobulin; Partial amino acid sequence; Thyroid hormone generating site

1. INTRODUCTION

Thyroid hormone is synthesized by iodination of a particular tyrosine residue and the transfer of an iodophenoxyl group from a 'donor' to an 'acceptor' iodotyrosine residue within the thyroglobulin (TG) molecule. This coupling reaction results in the conversion of an acceptor to a thyroid hormone residue, and a donor to a dehydroalanine residue [1,2]. Thus, the peptide bonds of both sides of the acceptor site should be cleaved in the process of thyroid hormone release, whereas those of the donor site remain intact [2]. Five tyrosine residues have been suggested as the acceptor site at 5, 1291, 2555, 2569 and 2748 in a few mammalian TG [3-7]. We have recently demonstrated the 4 donor tyrosine residues at 5, 926, 986 or 1008 and 1375 by the analysis of dehydroalanine containing peptide in bovine TG [8]. Tyrosine residues at 5 and 239 [9], and 130 [10], 2469 and/or 2559 [11] have also been suggested to be the donor site. In the present study, we show evidence supporting that tyrosine 130 is the 'acceptor site' in bullfrog TG.

2. MATERIALS AND METHODS

2.1. *Chemical modification and enzymatic digestion of frog TG*

TG from the thyroid glands of bullfrog tadpole (stage XVII-XX [12]), *Rana catesbeiana*, was prepared as in [13]. Frog TG was reduced and carboxy-methylated (RCM) according to Crestifield et al. [14], then dialysed against 4 M guanidine-HCl/50 mM Tris, pH 9.0 to remove excess reagents. RCM-frog TG (15 mg) was digested with 0.15 mg of lysyl endopeptidase (Wako Pure Chemicals, [EC 3.4.21.4]) at 37°C in 4 M guanidine-HCl/50 mM Tris, pH 9.0. After 3 h, 0.15 mg of the enzyme was further added and kept another 3 h, then the pH dropped to ca. 2 with 10% aqueous trifluoroacetic acid to stop the digestion.

Abbreviations: TG, thyroglobulin; RCM, reduced and carboxymethylated

**Present address:* Osaka Bio Science Institute, Suita, Osaka 565, Japan.

Correspondence address: H. Hayashi, Department of Protein Chemistry, Institute of Endocrinology, Gunma University, Maebashi 371, Japan. Fax: (81) (272) 341788.

2.2. *Separation of peptides*

The digest obtained above was diluted with 5 vols of water. After centrifugation at 15 000 rpm for 30 min to remove insoluble materials formed by dilution, the supernatant fraction was desalted through Nucleosil 5C18 column (8×250mm). Peptides were recovered by elution with 60% acetonitrile/0.1% trifluoroacetic acid. Peptides were fractionated by ion-exchange and reverse-phase HPLC.

2.2. *Amino acid analysis and sequence determination*

Peptides were hydrolyzed in the gas phase with constant boiling HCl containing 0.2% phenol at 110°C for 24 h under reduced pressure. Amino acid analysis was performed on a Hitachi 835 amino acid analyzer. Amino acid sequences of peptides were determined by a gas-phase automatic sequence analyzer (Applied Biosystems 477A with on-line 120A phenyl thiohydantoin amino acid analyzer).

3. RESULTS AND DISCUSSION

3.1. *Characterization of frog TG*

Bullfrog TG had a molecular size similar to that of bovine TG on the 8–25% SDS-polyacrylamide gel electrophoresis without the reducing reagent on the Phast System (Pharmacia). The amino acid composition is roughly similar between frog and bovine TG [13].

3.2. *Isolation of lysyl endopeptidase digested peptide*

The peptide fraction from Nucleosil 5C18 column was dried and dissolved in 10 ml of 50 mM Tris-HCl buffer, pH 9.0, and applied to a Mono Q column (Pharmacia, 5×50 mm), which was previously equilibrated with 50 mM Tris-HCl, pH 9.0. Peptides were eluted with a 60 min linear gradient (0–0.5 M) of NaCl in the same

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buffer at a flow rate of 1 ml/min and room temperature, and collected in 14 fractions (A–N by elution order). Peptides in three of those fractions (C, L and M) were further separated on a reverse-phase HPLC using a Chemco3C18 (6×100 mm) column with a 60 min linear gradient (5–60%) of acetonitrile in 0.1% aqueous trifluoroacetic acid at 40°C, and the flow rate of 1 ml/min. Fig. 1 shows a representative chromatographic elution profile.

3.3. *Amino acid sequence determination and comparison with bovine TG sequence*

The amino acid sequences of 5 peptides with high recovery (30–35%) among 20 purified peptides were determined. Considering the maximum homology, 4 peptides L44 (and M44), C19, L51 (and M51) and C45 could be located at the amino terminal, central and carboxy terminal portion in bovine TG sequence predicted from the cDNA nucleotide sequence [6], respectively, as shown in Fig. 2. In peptide C19, two X should be Thr or Tyr, judging from the amino acid composition. Possible reasons for the failure to identify these two residues are phosphorylation or *O*-linked sugar chain for Thr and iodination for Tyr. Peptide L44 (and M44), L51 (and M51) and C45 are homologous with the bovine TG sequence 103–129, 1428–1467 and 2039–2065, in 60, 40 and 50%, respectively. Peptide C19 should correspond to the bovine TG sequence 299–311, with a maximum of 38% homology. One peptide (Tyr-Phe-Cys-Val-Asp-Asn-Ser-Gly-Lys) could not be located in the bovine TG sequence with homology. All the peptides sequenced above except peptide L44 (M44), have lysine at the C-terminus, according to the specificity of the lysyl endopeptidase. This enzyme is known to be highly specific to cut the C-terminal side of the lysine residue. Our previous observations of the specificity of this enzyme applied to other proteins also show that the cutting site is strictly limited to the lysine residue [15–17]. Thus, it is highly probable that the C-terminal side of peptide L44 is already cleaved before lysyl endopeptidase digestion. This means that this is one of the naturally cleaved sites as suggested by Marriq et al. [10].

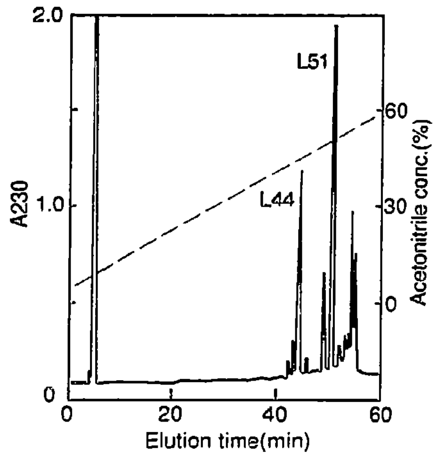

Fig. 1. Purification of Mono Q chromatography fraction on reverse-phase HPLC. Fraction L on Mono Q chromatography was adjusted to pH 2 with trifluoroacetic acid, and applied to a Chemco 3C18 column (6×100 mm), previously equilibrated with 5% acetonitrile in 0.1% aqueous trifluoroacetic acid. Peptides were eluted with a 60 min linear gradient from 5 to 60% of acetonitrile in 0.1% aqueous trifluoroacetic acid at a flow rate of 1 ml/min and 40°C. One-ml fractions were collected. (——), A_{230}; (- - - -), acetonitrile concentration.

In the bovine TG sequence, residue 130 is known to be Tyr, and one of the iodination sites. Marriq et al. [10] suggested that Tyr^{130} is the natural cleavage point in a fully iodinated bovine TG molecule, and to be one of the donor sites by assuming that the resultant dehydroalanine is unstable. However, our previous studies on bovine TG showed the dehydroalanine, resulting from the loss of iodophenoxy group at the donor site is stable [8]. Thus, natural cleavage sites should include the 'acceptor' sites. As stated in section 1, both side peptide bonds at the acceptor tyrosine should be cleaved when

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           1          103                                130
Bovine:    N - - -    - - D S G D Y S P V Q C D L R R R Q C W C V D A E G M E V Y - - -
Bullfrog:                 N • • E • E D • • • • R Q L G E • • • • • N • • • • I
                                          (L44, M44)

                          299                 311
                          - - S C H P D G E Y Q A A Q C - -
                              • V X • S A V X • • S L K
                                         (C19)

1428                                                                                  1467
- - - - R V V A A S D A S Q D A L G C V K C P E G S Y F Q D E Q C I P C P A G F Y Q E Q -
        L L I S R A • E I G N V A • • G I • • V • • E S E • G E • V • • • H • • • • Y K
                                       (L51,M51)

           2039                                                   2065       2750
           - - - T Y P F G W Y Q K P <> V S P S D A P S F C P S V A L P A - -    - - K
                 • S • • • • • • • • • G • E G • I L • G L • • T A D V L K
                                        (C45)
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Fig. 2. Alignment of bullfrog thyroglobulin fragment with deduced bovine TG sequence [6]. Number over sequence indicate the amino acid residue number of bovine TG. The peptide name is shown in parentheses. The substituted amino acid residue from bovine TG is shown by one letter. (X), unidentified (Thr or Tyr); (·), residue identical to that of bovine TG; (<>), deletion.

the thyroid hormone is generated. On the assumption that the TG sequence around those regions is conserved during the evolution, we suggest that Tyr^{130} in bullfrog TG is the acceptor site.

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