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Hormonogenic donor Tyr2522 of bovine thyroglobulin. Insight into preferential T3 formation at thyroglobulin carboxyl terminus at low iodination level

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ABSTRACT

A tryptic fragment ($b5_{TR,NR}$), encompassing residues 2515–2750, was isolated from a low-iodine (0.26% by mass) bovine thyroglobulin, by limited proteolysis with trypsin and preparative, continuous-elution SDS–PAGE. The fragment was digested with Asp-N endoproteinase and analyzed by reverse-phase HPLC electrospray ionization quadrupole time-of-flight mass spectrometry, revealing the formation of: 3-monoiodotyrosine and dehydroalanine from Tyr2522; 3-monoiodotyrosine from Tyr2555 and Tyr2569; 3-monoiodotyrosine and 3,5-diiodotyrosine from Tyr2748. The data presented document, by direct mass spectrometric identifications, efficient iodophenoxyl ring transfer from monoiodinated hormonogenic donor Tyr2522 and efficient mono- and diiodination of hormonogenic acceptor Tyr2748, under conditions which permitted only limited iodination of Tyr2555 and Tyr2569, in low-iodine bovine thyroglobulin. The present study thereby provides: (1) a rationale for the preferential synthesis of T3 at the carboxy-terminal end of thyroglobulin, at low iodination level; (2) confirmation for the presence of an interspecifically conserved hormonogenic donor site in the carboxy-terminal domain of thyroglobulin; (3) solution for a previous uncertainty, concerning the precise location of such donor site in bovine thyroglobulin.

1. Introduction

Thyroid hormones are synthesized via the iodination and "coupling" of a small subset of tyrosyl residues in the polypeptide chains of thyroglobulin (Tg), a 660-kDa homodimeric glycoprotein. Coupling entails the transfer of an iodophenoxyl ring from a "donor" 3-monoiodotyrosine (MIT) or 3,5-diiodotyrosine (DIT) to an "acceptor" DIT, yielding 3,5,3′-triiodothyronine (T3) or 3,5,3′,5′-tetraiodothyronine (T4), respectively, at the hormonogenic acceptor site and dehydroalanine (DHA) at the donor site [1]. Both iodination and coupling are catalyzed by thyroid peroxidase. Different tyrosyl residues show different reactivities towards iodine [2], while coupling has stringent steric requirements [3]. Out of 72 tyrosyl residues per bovine Tg (bTg) monomer, only 15 were iodinated and 6–8 underwent coupling to form T3 and T4 [4,5]. Four major hormonogenic acceptor sites have been identified in Tg. Tyr5 was most favored for T4 formation in several animal species studied [6–11]. Conserved T4-forming sites included Tyr2554 (human Tg numbering) [9–13] and Tyr1291 [9–11,14], while Tyr2747 was a site of preferential T3 synthesis [9,10,13,15]. Acceptor sites identified in single species include

Abbreviations: AChE, acetylcholinesterase; bTg, bovine thyroglobulin; CAD/MS, collisionally activated dissociation mass spectrometry; DABITC, dimethylaminoazobenzene-isothiocyanate; DIT, 3,5-diiodotyrosine; ES/MS, electrospray mass spectrometry; HPLC-ESI-Q-TOF/MS, HPLC electrospray ionization quadrupole time-of-flight mass spectrometry; hTg, human thyroglobulin; MIT, 3-monoiodotyrosine; MS, mass spectrometry; mTg, mouse thyroglobulin; SDS–PAGE, polyacrylamide gel electrophoresis in SDS; S-(4-APC), S-(4-aminophenyl)cysteine; T3, 3,5,3′-triiodothyronine; T4, 3,5,3′,5′-tetraiodothyronine; Tg, thyroglobulin; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl ketone; TR, Trypsin.

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Tyr2569 in hog Tg [12], Tyr685 in human Tg (hTg) [13] and Tyr973 in mouse Tg (mTg) [11].

The inventory of donor sites in Tg is far less complete. Indirect early studies, exploiting the conversion of DHA to S-(4-aminophenyl)cysteine, indicated the possible presence of donor Tyr5, Tyr926, Tyr986 or Tyr1008, and Tyr1375 [16]. Another donor site was identified as one of two tyrosyl residues (Tyr2469 or Tyr2522) in fragment 2462–2544 of bTg, after chemical conversion of DHA to labeled [^{3}H]alanine or [^{14}C]cyanoalanine [17]. Alanine recovery at position 130 of hTg peptide 1–171 was traced back to DHA partly by speculation [18]. The use of MS permitted the identification of donor Tyr1375 [14] and Tyr130 [19] in bTg, and donor Tyr239 and Tyr2519 in mTg [11], in addition to various acceptor sites. The latter three donor residues were in the form of pyruvic acid, likely due to peptide bond hydrolysis, upon DHA formation, as suggested by Gavaret [20].

We aimed at localizing hormonogenic donor tyrosyl residue(s) in a tryptic fragment of bTg ($b5_{TR,NR}$) [21,22], encompassing the carboxy-terminal 41% of the acetylcholinesterase (AChE)-homologous domain of bTg (residues 2515–2750). We focused on this fragment because of its limited size and expected content of hormonogenic tyrosyl residues, already detected in the Tgs of other animal species [9–13,15]. Because hormones were efficiently formed upon *in vitro* iodination of a corresponding peptide, comprising the 224 COOH-terminal amino acids of rat Tg [23], it probably contained hormonogenic donor site(s) too. Fragment $b5_{TR,NR}$, purified by preparative, continuous-elution SDS–PAGE in non-reducing conditions and further digested with Asp-N endoproteinase, was analyzed by reverse-phase HPLC electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF/MS), which yielded an informative peptide map, covering 59% of $b5_{TR,NR}$ length. The post-translational status of eight out of fifteen tyrosyl residues was assessed. Detection of DHA in correspondence of Tyr2522 confirmed the presence of an interspecifically conserved donor site in the AChE-homologous bTg domain [11]. Moreover, efficient mono- and diiodination of Tyr2746, concomitant with limited iodination of Tyr2555, Tyr2569 and Tyr2522 to MIT, might provide a rationale for the preferential synthesis of T3 at the carboxyl terminus of bTg, upon limitation of the iodine supply.

2. Material and methods

2.1. Preparation of bTg

bTg was extracted twice on ice from fresh, finely minced thyroid tissue in 0.1 M sodium phosphate, pH 7.4 and purified by fractional precipitation in 1.4–1.8 M $(NH_4)_2SO_4$ at 4 °C and gel filtration on Sephacryl S-300 HR in 0.13 M NaCl, 0.05 M Tris/HCl, pH 7.8, at 4 °C.

2.2. Limited proteolysis of bTg with trypsin and separation of the products

Limited digestion of bTg in 0.13 M NaCl, 0.05 M Tris/HCl, pH 8.2 with TosPheCH$_2$Cl-treated trypsin (henceforth trypsin) and analysis of the digestion products by SDS–PAGE, with or without reduction, were conducted as detailed in Supplementary Data.

2.3. Preparation of purified bTg fragment $b5_{TR,NR}$ by continuous-elution SDS–PAGE

The products of digestion of 25 mg of bTg with trypsin at the enzyme:substrate ratio of 1:100 (w/w), at 30 °C for 80 min, under non-reducing conditions, were separated by preparative, continuous-elution SDS–PAGE [14], as detailed in Supplementary Data. Fractions were analyzed by SDS–PAGE and those containing fragment $b5_{TR,NR}$ were pooled and concentrated by lyophilization, freed from Tris/HCl and glycine, lyophilized and stored at −20 °C until further use.

2.4. Analysis of fragment $b5_{TR,NR}$ by reverse-phase HPLC-ESI-Q-TOF/MS

Purified fragment $b5_{TR,NR}$ was hydrolyzed with Asp-N endoproteinase at the enzyme:substrate ratio of 1:100 (w/w) in 0.05 M NH_4HCO_3, 10% (v/v) acetonitrile, pH 8.5, at 37 °C for 18 h. Digestion products were immediately lyophilized. Analysis of the Asp-N endoproteinase digest by HPLC-ESI-Q-TOF/MS was performed with a quadrupole-time-of-flight (Q-TOF) Ultima hybrid mass spectrometer (Waters, Manchester, UK), equipped with an electrospray ion source (ESI). Multiply charged ions generated were separated on a 15-cm × 100-μm i.d. Atlantis C18 capillary column (Waters), with a linear gradient of aqueous acetonitrile in 0.1% TFA (see Supplementary Data for details). Spectral mass signals were associated with peptides on the base of expected molecular masses, in accordance with the cDNA-derived bTg sequence (UniProt KB/Swiss-Prot P01267) [24], allowing for the dynamic modification of oxidized Met (Δm = +15.99) and the conversion of Tyr to MIT (Δm = +125.90), DIT (Δm = +251.79), T3 (Δm = +469.72), T4 (Δm = +595.61), DHA (Δm = −94.04) and pyruvic acid (Δm = −93.03).

3. Results

3.1. Purification of fragment $b5_{TR,NR}$ by continuous-elution SDS–PAGE

Because T3 formation was favored over T4 at low level of iodination [10] and we were interested in donor site(s) supporting T3 formation from Tyr2748, we selected a low-iodine bTg preparation (0.26% by mass) for the isolation and analysis of fragment $b5_{TR,NR}$. Eventually, we recovered 0.45 mg of purified fragment $b5_{TR,NR}$. As the latter was 8% of total fragments by gel densitometry and these were 80% of starting bTg (25 mg), the yield was 28%.

3.2. Fragment $b5_{TR,NR}$ characterization by HPLC-ESI-Q-TOF/MS

The HPLC-ESI-Q-TOF/MS map of fragment $b5_{TR,NR}$ digested with Asp-N endoproteinase (Table 1 and Fig. 1) covered 59% (139 out of 236 residues) of the primary sequence of fragment $b5_{TR,NR}$, which was identical with the cDNA-derived sequence [24]. The MS map included eight of fifteen tyrosyl residues, disclosing post-translational modifications of four of them (Tyr2522, Tyr2555, Tyr2569 and Tyr2748). Three aspecific cleavages occurred at the amino side of Glu2662, Glu2681 and Glu2723. Lys2750 was identified as the carboxy-terminal residue of fragment $b5_{TR,NR}$, which extended through the C-terminal bTg extremity. A disulfide bond linking Cys 2573 and Cys2697 was evidenced by the mass signals associated with peaks 11 (2912.36 Da) and 12 (3038.35 Da) of the HPLC-ESI-Q-TOF/MS total ion chromatogram, which were compatible with the combined masses expected for peptides 2568–2576 and 2696–2711, as such and with the addition of one iodine atom (Δm = +125.90), respectively. Isolated peptide 2568–2576 was also recovered, both unmodified and modified by the conversion of Tyr2569 to MIT, as judged from the mass signals respectively associated with peaks 14 and 15. This finding was compatible with the fragmentation of the S–S bond linking peptides 2568–2576 and 2696–2711, during the MS analysis. The detection of MIT2569 permitted to rule out the iodination of Tyr2703, in peptide 2696–2711 linked with peptide 2568–2576.

Table 1
Reverse-phase HPLC-ESI-Q-TOF/MS analysis of the digestion products of purified bTg fragment $b5_{TR,NR}$ with Asp-N endoproteinase.

Peak number[a]	Measured mass[b] (Da)	Peptide[c]	Theoretical mass[d] (Da)	Post-translational modifications
1	1854.84	2553–2567	1854.81	MIT2555
2	768.30	2674–2680	768.33	
3	632.39	2669–2673	632.33	
4	1360.51	2723–2735	1360.54	
5	1948.94	2515–2534	1948.99	DHA2522
6	3691.76	2577–2608	3691.73	
7	2169.99	2515–2534	2168.89	MIT2522
8	1633.86	2736–2750	1633.89	(Tyr2748)
9	1759.87	2736–2750	1759.80	MIT2748
10	1885.69	2736–2750	1885.71	DIT2748
11	2912.36	2568–2576 + 2696–2711	2912.41	S-S 2573-2697
12	3038.35	2568–2576 + 2696–2711	3038.31	S-S 2573-2697 MIT2569
13	816.41	2662–2668	816.38	
14	1081.56	2568–2576	1081.55	(Tyr2569)
15	1207.50	2568–2576	1207.47	MIT2569

[a] Peaks of the HPLC-ESI-Q-TOF/MS total ion chromatogram.
[b] Average molecular masses in Da obtained by integrating multiple peaks for each molecular species, differing only in the number of charges.
[c] Amino acid residues at peptide extremities, assigned by comparison with the cDNA-derived sequence of bTg (UniProtKB/Swiss-Prot P01267) [24], *minus* the 19-residue leader peptide.
[d] Masses calculated on the base of the cDNA-derived sequence of bTg (UniProtKB/Swiss-Prot P01267) [24], allowing for the post-translational modifications indicated.

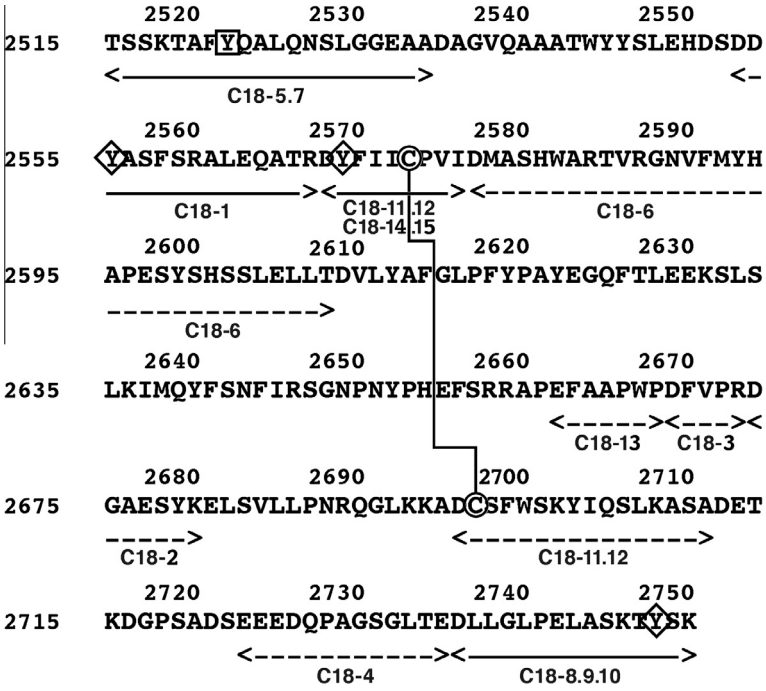

Fig. 1. MS peptide map of fragment $b5_{TR,NR}$ digested with Asp-N endoproteinase. Molecular masses measured by HPLC-ESI-Q-TOF/MS (Table 1) were associated with peptides along the cDNA-derived bTg sequence [24]. Arrowheads mark peptide extremities. Continuous lines mark peptides containing post-translationally modified Tyr residues: donor Tyr2522 is inscribed in a *square*; acceptor Tyr2555 and Tyr2569 (both modified into MIT) and Tyr2748 (modified into MIT and DIT) in *diamonds*. Dashed lines mark the other peptides. Numbers below the lines refer to peaks of the total ion chromatogram. The disulfide bond linking Cys2573 and Cys2697 is indicated.

3.3. Tyr2522 is a hormonogenic donor residue

Two peaks yielded mass signals related with peptide 2515–2534 (Table 1 and Fig. 1). The mass signal of 2169.99 Da, in peak 7, was compatible with peptide 2515–2534, containing MIT2522, while the signal of 1948.94 Da, in peak 5, could be accounted for by the same peptide, in which Tyr2522 had been converted to DHA ($\Delta m = -94.04$). No mass signals compatible with peptide 2515–2534 or overlapping peptides with unmodified Tyr2522 or DIT2522 were detected in any other peak. Thus, Tyr2522 was a hormonogenic donor residue and, in our low-iodine bTg preparation, its quantitative monoiodination was ensued by the transfer of its monoiodophenoxyl group to some acceptor site.

3.4. Tyr2555, Tyr2569 and Tyr2748 were monoiodinated, but only Tyr2748 proceeded to form DIT

Peak 1 revealed a mass signal of 1854.84 Da, in agreement with the mass value expected for peptide 2553–2567, allowing for the addition of one iodine atom ($\Delta m = +125.90$) to Tyr2555 (Table 1

and Fig. 1). No traces of peptide 2553–2567 or overlapping peptides with Tyr2555 or DIT2555 were found in any other peak. Four peaks revealed mass signals related with peptide 2568–2576. The mass signal associated with peak 14 (1081.56 Da) was compatible with the mass value expected for the unmodified peptide, while the signal detected in peak 15 (1207.50 Da) was compatible with the same peptide, after conversion of Tyr2569 to MIT (Δm = +125.90). No traces of peptide 2568–2576 or overlapping peptides with DIT2569 were detected. Signals compatible with the mass values expected for peptide 2568–2576, containing Tyr2569 and MIT2569 in disulfide linkage with peptide 2696–2711, were recorded in peaks 11 and 12, respectively, as noted above. Three peaks yielded mass signals related with peptide 2736–2750. The signal associated with peak 8 (1633.86 Da) was compatible with the unmodified peptide, while those detected in peaks 9 (1759.87 Da) and 10 (1885.69 Da) were compatible with the same peptide, after Tyr2748 conversion to MIT (Δm = +125.90) and DIT (Δm = +251.79), respectively. Thus, in bTg containing 0.26% iodine by mass, 3-monoiodination appeared to be quantitative for Tyr2555 and partial for Tyr2569, but neither residue was iodinated further, whereas iodination of Tyr2748, even though not quantitative, proceeded via successive 3-monoiodination and 3,5-diiodination.

4. Discussion

We report a HPLC-ESI-Q-TOF/MS analysis of a tryptic fragment ($b5_{TR,NR}$), comprising 41% of the AChE-homologous domain of bTg at its carboxyl end (residues 2515–2750). We assessed the post-translational status of eight out of fifteen tyrosyl residues within fragment $b5_{TR,NR}$, detecting iodination- or coupling-dependent modifications of four of them: (1) quantitative modification of Tyr2522 into MIT and DHA; (2) quantitative, although limited modification of Tyr2555 into MIT; (3) partial, limited modification of Tyr2569 into MIT; (4) partial conversion of Tyr2748 to MIT and DIT. The disulfide bond linking Cys2573 with Cys2697 was in keeping with the pattern determined in various members of the esterase/lipase family [25–27]. Our data highlight the conservation of donor Tyr2522 between bTg and mTg [11] and settle the location of this site, which previous data assigned to either bTg Tyr2469 or Tyr2522 [17]. Such finding is not merely confirmatory. The inventory of hormone-forming sites in Tg has regularly benefited from comparative data. As not all sites were found in most animal species, repeated detections proved essential for bearing testimony to the interspecific conservation and functional importance of the major sites. Tyr2522 is the sole donor site reported so far in the AChE-homologous domain of Tg and the sole of both kinds directly identified in this bTg domain, known to harbor three acceptor sites in other species [9–13,15] (Table 2 and Fig. 2).

Known aspects of the structure–function relationships that make Tg uniquely suited for thyroid hormonogenesis include the preferential synthesis of T4 from early iodinated tyrosyl residues [28] and the optimal iodine usage for iodothyronine formation [29]. The data reported here enlarge this perspective, to include the regulation of the T3:T4 biosynthetic ratio in the carboxy-terminal domain of Tg. Increased serum TSH levels and T3:T4 ratio in thyroid are among the main metabolic effects of chronic iodine deficiency [30,31]. In guinea pig and rabbit Tg, TSH stimulated T3 formation from Tyr2746 and decreased T4 synthesis from Tyr5 [10], via the maturation of the Asn91-linked oligosaccharide chain from the high-mannose to the complex type [32]. TSH also stimulated T3 release from Tg, by activating cathepsin B [33], and intrathyroidal T4-to-T3 conversion [30]. Our data indicate that, besides TSH, intrinsic Tg structural determinants may favor T3 over T4 formation at its carboxyl end, upon iodine shortage. The amount of MIT formed in Tg was much less than expected from the kinetic studies with free N-acetyltyrosine and N-acetyl-monoiodotyrosine [34], as though the relative amounts of MIT and DIT synthesized were controlled more by the local environment of tyrosyl groups, i.e., the native Tg conformation, than by the ratio between the respective rate constants of iodination [35]. Difference spectroscopy in water and urea of native and iodinated Tg and the effects of solvent polarity on the hydroxyl pK and the rates of iodination of tyrosine and MIT indicated that a nonpolar environment might favor DIT relative to MIT formation [35,36].

In keeping with its preferential T3-forming ability [9,10,13,15], Tyr2748 was most liable, among tyrosyl residues in fragment $b5_{TR,NR}$, to 3,5-diiodination, at low level of iodination, which might favor its early involvement in T3 formation as an acceptor residue. Under the same conditions which permitted DIT formation from Tyr2748, only MIT was formed from Tyr2555, Tyr2569 and Tyr2522. On one hand, this might prevent Tyr2555 and Tyr2569 from competing with Tyr2748 as acceptor T4-forming sites, upon iodine shortage. On the other hand, the factors which limited further iodination of MIT2522, before the transfer of its iodophenoxyl ring (or favored coupling in advance of DIT formation), are expected to favor T3 over T4 formation from acceptor Tyr2748. We deem this realistic, even though we did not detect T3 at position 2748. However, in the porcine homologous of bTg peptide 2743–2750, T3 and T4 synthesis from Tyr2748, upon increasing iodination of Tg, was associated with marked increases in the proteolytic susceptibilities of the peptide bonds at the carboxyl side of Tyr2748 and between Lys2746 and Ser2747, so that all the T3 and T4 were detected in Ser-T3/T4 dipeptides [15]. Thus, it is likely that a tetrapeptide with T3 at position 2748 was released upon tryptic

Table 2
Hormonogenic donor tyrosines in the Tgs of various animal species.

Tyr	Modification	Species	Technique	References
Tyr5[a]	DHA	Cow	DHA conversion to S-(4-APC)[c]	[16]
Tyr130[a]	Pyr	Cow	CAD/MS	[19]
Tyr239[b]	Pyr	Mouse	Nano-LC-MS/MS	[11]
Tyr926[a]	DHA	Cow	DHA conversion to S-(4-APC) [c]	[16]
Tyr986/1008[a]	DHA	Cow	DHA conversion to S-(4-APC) [c]	[16]
Tyr1375[a]	DHA	Cow	DHA conversion to S-(4-APC) [c]	[16]
Tyr1375[a]	DHA	Cow	ES/MS	[14]
Tyr2519 [b]	Pyr	Mouse	Nano-LC-MS/MS	[11]
Tyr2469/2522[a]	DHA	Cow	DHA conversion to [^{3}H]alanine or [^{14}C]cyanoalanine	[17]
Tyr2522[a]	DHA	Cow	HPLC-ESI-Q-TOF/MS	Present work

[a] bTg numbering (UniProtKB/Swiss-Prot P01267) [24], *minus* the 19-residue leader peptide.
[b] mTg numbering (UniProtKB/Swiss-Prot O08710), *minus* the 20-residue leader peptide.
[c] S-(4-APC), S-(4-aminophenyl)cysteine.

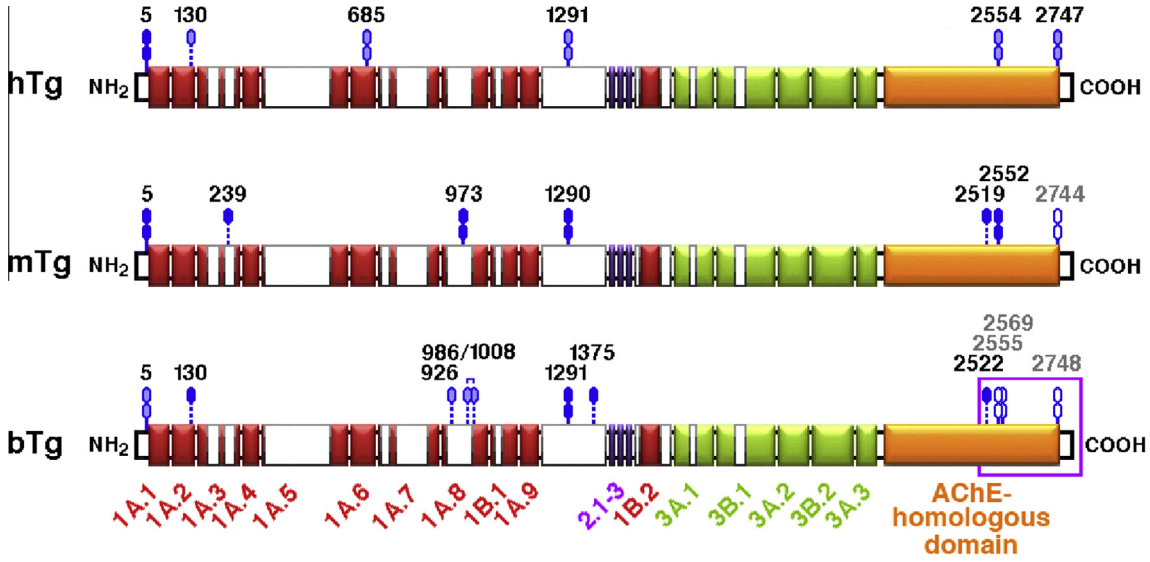

Fig. 2. Localization of hormonogenic tyrosines in the monomeric subunits of bTg, mTg and hTg. Tandemly repeated and other Tg regions are represented by color-coded boxes: *red*, type-1 repeats; *violet*, type-2 repeats; *green*, type-3 repeats; *orange*, AChE-homologous domain; *white*, inserts interrupting Tg-like repeats and spacer regions separating 1A.9 from type-2 repeats and 1B.2 from type-3 repeats. Spaces between boxes bear no relationship to gene introns. Fragment $b5_{TR,NR}$ is framed in *violet*. Double hexagons and hexagons with dashed stalks mark hormonogenic acceptor and donor Tyr residues, respectively. *Solid blue* hexagons mark hormonogenic sites identified by manual peptide sequencing with DABITC chemistry or by MS, *light blue-cored* hexagons mark those identified by more indirect techniques (Table 2 and Supplementary Table 1S); empty hexagonal rings with dimmed numbers mark hormonogenic sites identified in Tgs of animal species different from the one shown.

hydrolysis of the Lys2746-Ser2747 bond and was lost, during fragment $b5_{TR,NR}$ preparation.

In conclusion, the data presented suggest that the reported differences in reactivities of different tyrosyl residues towards iodination [2,4,5,13] might be reflected also in their differential susceptibilities to graded steps of iodination and contribute to regulate the T3:T4 biosynthetic ratio in Tg. In particular, the observed propensity of Tyr2522 to transfer its iodophenoxyl ring, even in the form of MIT, and the differential amenabilities of Tyr2555 and Tyr2569 to 3-monoiodination and of Tyr2748 to 3,5-diiodination might contribute to optimize the T3 yield from Tyr2748 in bTg, at low iodination level.

5. Animal rights

The work described was carried out in accordance with EU Directive 2010/63/EU for animal experiments.

6. Conflict of interests

The authors declare no conflict of interests regarding the publication of this article.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.05.144.

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