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Identification of Hormonogenic Tyrosines in Fragment 1218–1591 of Bovine Thyroglobulin by Mass Spectrometry

HORMONOGENIC ACCEPTOR TYR-1291 AND DONOR TYR-1375*

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A fragment of bovine thyroglobulin encompassing residues 1218–1591 was prepared by limited proteolysis with thermolysin and continuous-elution polyacrylamide gel electrophoresis in SDS. The reduced and carboxymethylated peptide was digested with endoproteinase Asp-N and fractionated by reverse-phase high performance liquid chromatography. The fractions were analyzed by electrospray and fast atom bombardment mass spectrometry in combination with Edman degradation. The post-translational modifications of all seven tyrosyl residues of the fragment were characterized at an unprecedented level of definition. The analysis revealed the formation of: 1) monoiodotyrosine from tyrosine 1234; 2) monoiodotyrosine, diiodotyrosine, triiodothyronine (T_3), and tetraiodothyronine (thyroxine, T_4) from tyrosine 1291; and 3) monoiodotyrosine, diiodotyrosine, and dehydroalanine from tyrosine 1375. Iodothyronine formation from tyrosine 1291 accounted for 10% of total T_4 of thyroglobulin (0.30 mol of T_4/mol of 660-kDa thyroglobulin), and 8% of total T_3 (0.08 mol of T_3/mol of thyroglobulin). This is the first documentation of the hormonogenic nature of tyrosine 1291 of bovine thyroglobulin, as thyroxine formation at a corresponding site was so far reported only in rabbit, guinea pig, and turtle thyroglobulin. This is also the first direct identification of tyrosine 1375 of bovine thyroglobulin as a donor residue. It is suggested that tyrosyl residues 1291 and 1375 may support together the function of an independent hormonogenic domain in the mid-portion of the polypeptide chain of thyroglobulin.

Thyroglobulin (Tg),[1] a homodimeric glycoprotein with a molecular mass of 660 kDa, is the site of the biosynthesis of 3,5,3′-triiodothyronine (T_3) and 3,5,3′,5′-tetraiodothyronine (thyroxine, T_4) (reviewed in Ref. 1). T_3 and T_4 are synthesized via the iodination and coupling of a small subset of tyrosyl residues within the polypeptide chains of Tg. The coupling reaction takes place by the transfer of an iodophenyl group from a donor 3-monoiodotyrosine or 3,5-diiodotyrosine to an acceptor 3,5-diiodotyrosine. This causes the formation of T_3 or T_4, respectively, at the acceptor site and dehydroalanine at the donor site (2, 3). Both reactions are catalyzed by thyroid peroxidase. Different tyrosyl residues have different reactivities toward iodine, so that iodination proceeds in a sequential order, which is controlled by the native structure of Tg (4, 5). Early iodinated tyrosyl residues are preferentially involved in iodothyronine synthesis (6); the coupling of iodotyrosines, in turn, has stringent steric requirements (7). In fact, out of 72 tyrosyl residues per bovine Tg monomer, only 15 are iodinated and a maximum of 6–8 of them undergo coupling to form T_3 and T_4 (8, 9).

So far, four major hormonogenic tyrosines have been identified, by the isolation and sequencing of hormone-rich peptides from Tgs of various animal species and comparison of their sequences with the cDNA-deduced sequences of bovine (10) and human Tg (11). Tyr-5 was the most favored site for T_4 formation in most species studied, including humans (12), calf (13), sheep, hog (14), rabbit (15), and guinea pig (16). In hog (17), rabbit (15), guinea pig (16), and human Tg subjected *in vitro* to low-level iodination (18), Tyr-2553 (human Tg numbering) was the second most efficient T_4-forming residue, whereas Tyr-2746 was a site of preferential synthesis of T_3 (15, 16, 18, 19). Another T_4-forming site found in rabbit and guinea pig Tg corresponded to human Tyr-1290: in those species this site was third in ranking order of hormonogenic efficiency and its function was greatly enhanced by TSH (15, 16). Nevertheless, so far it has received little attention in the bovine and human species. Tyrosines reported as possible donor sites include Tyr-5, -926, -986 or -1008, -1375 (20), -2469 and/or -2522 of bovine Tg (21), and Tyr-130 of human Tg (22).

The main goal of this work was to establish whether Tyr-1291 of bovine Tg is also a site of T_4 formation. To this purpose, a preparation of bovine Tg containing 1.05% iodine by mass was subjected to limited proteolysis with thermolysin and the products were separated by preparative SDS-PAGE. A thorough mass spectrometric analysis of a peptide spanning residues 1218–1591, together with an analysis of its iodine and iodoamino acid content, were performed. Post-translational modifications of three out of seven tyrosyl residues were documented at an unprecedented level of definition: in particular, we report the first direct evidence of the entire spectrum of modifications typical of a hormonogenic acceptor and a hormonogenic donor site at residues 1291 and 1375, respectively, of bovine Tg.

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[1] The abbreviations used are: Tg, thyroglobulin; ES/MS, electrospray mass spectrometry; FAB/MS, fast atom bombardment mass spectrometry; HPLC, high performance liquid chromatography; MH^+, protonated molecular ion; MIT, monoiodotyrosine; DIT, diiodotyrosine; T_3, 3,3′,5-triiodothyronine; T_4, 3,5,3′,5′-tetraiodothyronine; PAGE, polyacrylamide gel electrophoresis; TL, thermolysin from *Bacillus thermoproteolyticus rokko*; TSH, thyrotropin; DHA, dehydroalanine.

EXPERIMENTAL PROCEDURES

Materials—Thermolysin from *Bacillus thermo-proteolyticus rokko* (EC 3.4.24.4) and L-1-tosylamide-2-phenylethylchloromethyl-treated bovine pancreatic trypsin (EC 3.4.21.4), dithiothreitol, iodoacetic acid, glycerol, thioglycerol, 3-iodo-L-tyrosine (MIT), 3,5-diiodo-L-tyrosine (DIT), 3,5,3′-triiodothyronine (T_3), 3,5,3′,5′-tetraiodothyronine (thyroxine, T_4) were from Sigma Chimica (Milan, Italy); endoproteinase Asp-N from *Pseudomonas fragi* (EC 3.4.24.33) and endoproteinase Lys-C from *Lysobacter enzymogenes* (EC 3.4.21.50) were from Boehringer Mannheim Italia (Milan, Italy). Aminopeptidase M from porcine kidney (EC 3.4.11.2) and Pronase from *Streptomyces griseus* were from Calbiochem (San Diego, CA). Phenylisothiocyanate and EDTA were from Fluka Chimica (Milan, Italy). AcrylAide cross-linker and GelBond PAG film were from FMC BioProducts (Rockland, ME), other products for electrophoresis were from Bio-Rad Laboratories (Milan, Italy). Extracti-gel resin and bicinchoninic acid Protein Assay Reagent were from Pierce (Rockford, IL). HPLC grade solvents were obtained from Carlo Erba (Milan, Italy). The Vydac C-18 column (250 × 4.6 mm, 5 μm) was from The Separation Group (Hesperia, CA) and the Brownlee C-8 column (250 × 4.6 mm, 5 μm) from Applied Biosystems (Santa Clara, CA); PD-10 Sephadex G-25 cartridges and Sephacryl S-300 HR were from Pharmacia Biotech (Uppsala, Sweden).

Preparation of Tg—Bovine Tg was prepared from fresh bovine thyroids from the local abbattoir. The tissue was finely minced with scissors and Tg extracted briefly on ice in 0.1 M sodium phosphate, pH 7.2, and purified by fractional precipitation with 1.4–1.8 M ammonium sulfate, 50 mM Tris/HCl, pH 7.2, and gel filtration on Sephacryl S-300 HR in 130 mM NaCl, 50 mM Tris/HCl, pH 7.2, at 4 °C.

Limited Proteolysis of Tg—Limited proteolysis of Tg with thermolysin was carried out as described previously (23). Tg at the concentration of 1 mg/ml in 130 mM NaCl, 50 mM Tris/HCl, pH 8.0, was incubated with thermolysin at the enzyme/substrate ratio of 1/1000 or 1/100 (w/w) at 30 °C for the time indicated. The digestion was stopped by adding EDTA to a final concentration of 10 mM and concentrated SDS-PAGE sample buffer to a concentration of 10 mM Tris/HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 1.36 M glycerol, 0.0025% bromphenol blue, and by heating the samples in a boiling water bath for 1.5 min.

Separation and Identification of the Products of the Limited Proteolysis of Tg—Analytical SDS-PAGE in reducing conditions of the digestion products was performed according to Laemmli (24) on 4–16% total acrylamide gradient gels polymerized on GelBond PAG plastic backing (FMC BioProducts). The gels were stained with 0.1% Coomassie Brilliant Blue R-250 in 25% 2-propanol (v/v), 10% acetic acid (v/v), destained in 25% methanol (v/v), 10% acetic acid, soaked in 0.7 M glycerol, and air dried. The main peptides produced were identified on the basis of their mobility, according to detailed characterization of their NH_2-terminal peptide sequences provided in a previous study (23).

Purification of Peptide $b6_{TL}$—Bovine Tg was digested for 80 min with thermolysin at the enzyme/substrate ratio of 1/100 (w/w) and the digestion stopped as described above. The fragments were precipitated in chloroform/methanol (25), redissolved in SDS-PAGE sample buffer, and separated by preparative continuous-elution SDS-PAGE, using an electrophoresis chamber Bio-Rad model 491. A discontinuous gel with an annular cross-section was prepared according to Laemmli (24) in a cylindrical assembly having a diameter of 3.5 cm, whose center was occupied by a cooling core having a diameter of 1.5 cm. The 50-ml separating gel contained 12% total acrylamide and was 6.3 cm high; it was topped with a 10-ml 1.3-cm stacking gel containing 3.75% total acrylamide. The products of digestion of 25 mg of Tg with thermolysin were loaded onto a single gel. The electrode buffer contained 0.025 M Tris, 0.19 M glycine, 0.1% SDS, pH 8.2. The apparatus was designed so that, as soon as the migrating bands reached the lower extremity of the gel, they were conveyed by a stream of electrode buffer, aspirated by a peristaltic pump from a reservoir, to a fraction collector. Electrophoresis was carried at 20 mA. Collection was started as soon as the tracking dye began to exit from the gel (in 16 h); 4 fractions per hour were collected, with the pump flow rate set at 20 ml/h, for 24 h. The fractions were analyzed by SDS-PAGE. Those of interest were pooled and the pool was concentrated by lyophilization, freed from Tris/HCl, and glycine by filtration through PD-10 Sephadex G-25 cartridges (Pharmacia Biotech) in distilled water, and from SDS by filtration through Extracti-gel resin (Pierce) (1 ml of resin every 50 ml of the original pool) in distilled water. The sample was finally lyophilized and stored at −20 °C.

Reduction and Carboxymethylation of Peptide $b6_{TL}$—Purified peptide $b6_{TL}$ was dissolved in 300 μl of 0.3 M Tris/HCl, pH 8.0, containing 6 M guanidine/HCl, 1 mM EDTA, and treated with dithiothreitol (10/1 molar excess with respect to cysteinyl residues) at 37 °C for 2 h. The reduced peptide was carboxymethylated by reaction with a 5/1 molar excess of iodoacetic acid, with respect to total -SH groups, at pH 8.0 at room temperature for 30 min in the dark. The sample was freed from low molecular weight compounds by filtration through a PD-10 G-25 column in 50 mM ammonium bicarbonate, pH 8.5, and lyophilized.

Enzymatic Digests—The reduced and carboxymethylated peptide $b6_{TL}$ was hydrolyzed with endoproteinase Asp-N at the enzyme/substrate ratio of 1/100 (w/w) in 50 mM ammonium bicarbonate, 10% (v/v) acetonitrile, pH 8.5, at 37 °C for 18 h. Hydrolyses of HPLC-purified peptides with trypsin and endoproteinase Lys-C were carried out in 50 mM ammonium bicarbonate, pH 8.5, at 37 °C, using an enzyme/substrate ratio of 1/50 (w/w), for 4 and 20 h, respectively. All the reactions were immediately followed by lyophilization.

Separation of Peptides Obtained by Hydrolysis with Endoproteinase Asp-N—The peptides obtained by hydrolysis of 0.5 mg of peptide $b6_{TL}$ with endoproteinase Asp-N were fractionated by HPLC with a Vydac C-18 column (250 × 4.6 mm, 5 μm) equilibrated in 0.1% (v/v) trifluoroacetic acid in water (solvent A), containing 4% of 0.07% trifluoroacetic acid in acetonitrile (solvent B). After 5 min at 4% of solvent B, elution was performed by a two-step linear gradient of solvent B percentage from 4 to 25% over 25 min, and from 25 to 60% over the following 45 min. The flow rate was 1 ml/min.

Electrospray Mass Spectrometry (ES/MS)—ES mass spectra of the peptides produced by hydrolysis of peptide $b6_{TL}$ with endoproteinase Asp-N were recorded with a PLATFORM mass spectrometer (Fisons, Manchester, United Kingdom) equipped with an electrospray ion source. Samples from the HPLC separation (10 μl, 50 pmol) were injected into the ion source at a flow rate of 10 μl/min; the spectra were scanned from 2000 to 400 at the speed of 10 s/scan. Mass calibration was carried out using the multiple charged ions from a separate introduction of horse heart myoglobin (average molecular mass 16, 950.5 Da). The quantitative analysis was performed by integration of the multiple charged ions of the single species. Molecular masses are reported as average values.

Fast Atom Bombardment Mass Spectrometry (FAB/MS)—FAB mass spectra were recorded with a VG Analytical ZAB-2SE double-focusing mass spectrometer fitted with a VG caesium gun operating at 25 kV. Samples (0.1 nmol) were dissolved in 5% acetic acid and loaded onto a glycerol-coated probe tip; thioglycerol was added to the matrix just before introducing the probe into the ion source. The amplification of the electric signal was reduced during the magnet scan, according to the intensity of the mass signals observed on the oscilloscope. The values correspond to the monoisotopic masses of the protonated molecular ions of the peptides and are reported as integer numbers.

Peptide Recognition—The mass signals recorded in the spectra were associated with the corresponding peptides, on the basis of the expected molecular masses, using a computer program (26). Edman degradation steps were performed on HPLC-purified peptides, and were followed by the mass spectrometric analysis of the truncated peptides, in order to confirm the assignments, as already described (27).

Analytical Techniques—Iodine determinations were performed as described (28). The concentration of Tg was estimated by the absorbance at 280 nm, using a percentual extinction coefficient of 10.5 (29). The concentration of peptide samples was assayed using a bicinchoninic acid Protein Assay Reagent (Pierce) and bovine Tg as the standard. For the analysis of iodoamino acids, triplicate samples were hydrolyzed by a modification of a method already described (30): 0.4-mg aliquots of Tg and purified peptide $b6_{TL}$ were incubated at 37 °C with Pronase at the enzyme/substrate weight ratio of 1/1 in 0.5 ml of 0.1 M Tris/HCl, 50 mM 2-mercapto-1-methylimidazole, pH 8.0, to which 10 μl of toluene were added; after 24 h, aminopeptidase M at the enzyme/substrate ratio of 1/10 was added and digestion prolonged for another 24 h at 37 °C. Iodoamino acids were separated by reverse-phase HPLC in a Kontron HPLC equipped with a Brownlee C-8 column (250 × 4.6 mm, 5 μm), as already described (31). Iodoamino acid peaks were identified by comparison with iodoamino acid standards: the contents of iodoamino acids were calculated from the iodine contents of the respective peaks. Since 2-mercapto-1-methylimidazole co-eluted with MIT and interfered in the iodine assay, its contribution was determined in triplicate samples of bovine serum albumin which were subjected to the identical treatment.

RESULTS

Limited Proteolysis of Bovine Tg and Purification of Proteolysis Products—A detailed analysis of the products of the limited proteolysis of bovine Tg with thermolysin has been reported (23). Typical time courses and a flow-diagram of the

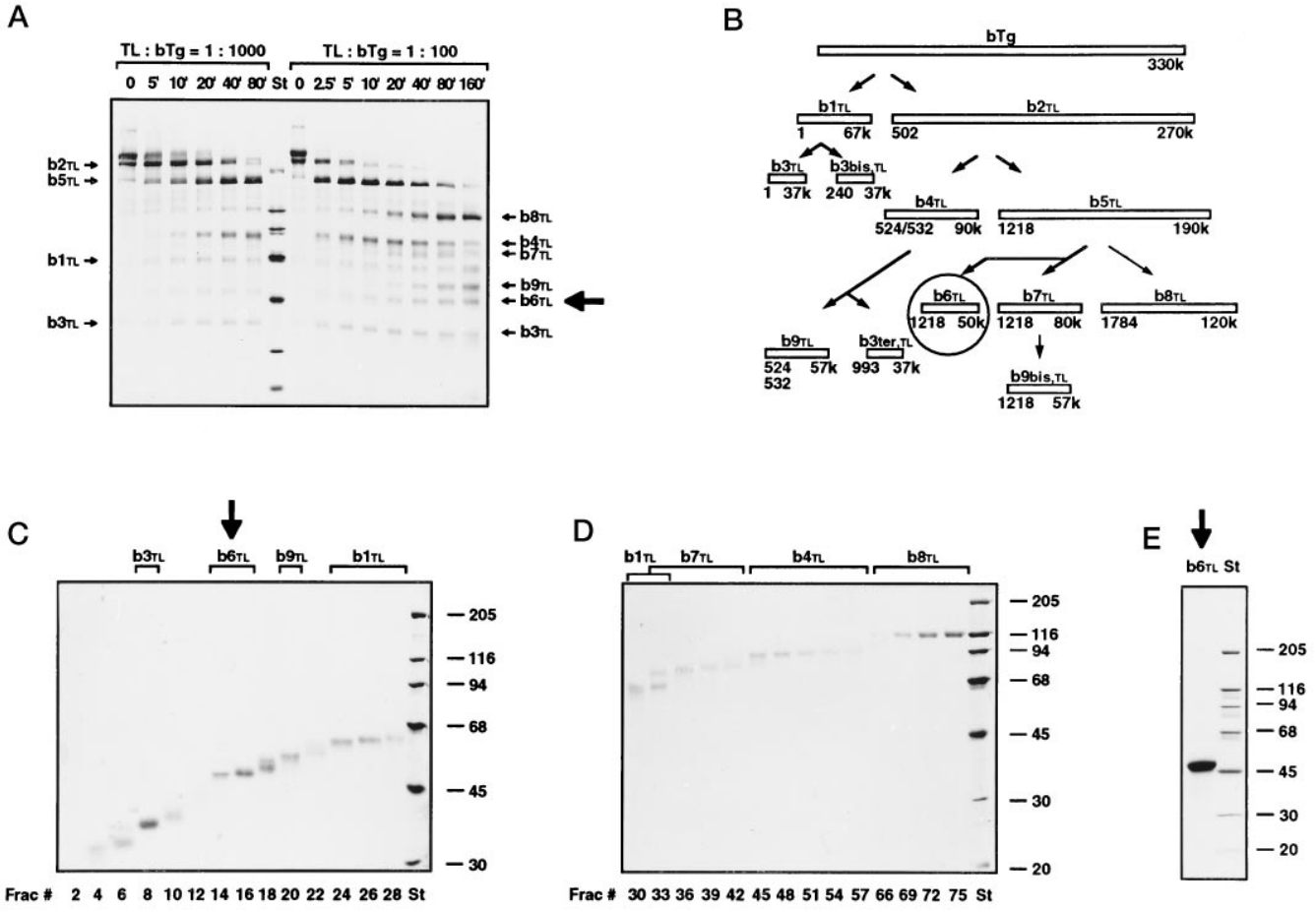

FIG. 1. **Limited proteolysis of bovine Tg with thermolysin and purification of the fragments by continuous-elution SDS-PAGE.** *Panel A*, reducing SDS-PAGE of the products of digestion of bovine Tg with thermolysin (TL) in a 4–16% acrylamide gradient gel. Tg in 130 mM NaCl, 50 mM Tris/HCl, pH 8.2, was hydrolyzed with TL at 30 °C, at the TL/Tg ratios indicated at the top of the gel. 0 indicates undigested Tg (25 μg), the other numbers indicate time points (min) of incubation. *St*, molecular mass standards: myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa). The peptides are marked on both sides of the gel, in accordance with Ref. 23. *Panel B*, flow-diagram of the cleavages: each *bar* represents a peptide, with the peptide number *above* the *bar*; the apparent mass in kDa is shown *below* the *bar* at the right and the NH_2-terminal residue number (23) below at the *left*. *Panels C* and *D*, SDS-PAGE in 10% total acrylamide gels of the fractions from the continuous-elution preparative SDS-PAGE of the products of digestion of 25 mg of Tg with TL (TL/Tg ratio of 1/100, pH 8.0, 30 °C for 80 min). Eighty 5-ml fractions were collected at a flow-rate of 20 ml/h, starting at the exit of the dye from the gel. The peptides contained in 0.15 ml of each fraction were precipitated (25) and analyzed. Fraction numbers are indicated at the bottom of the gels. *Panel E*, analysis by reducing SDS-PAGE in a 4–16% acrylamide gradient gel of peptide $b6_{TL}$ (10 μg) purified by five preparative electrophoretic separations. Peptide $b6_{TL}$ is highlighted throughout the figure.

proteolysis at pH 8.0 at 30 °C are shown in *panels A* and *B*, respectively, of Fig. 1. The proteolytic peptides corresponded exactly to those which were previously observed and characterized by amino-terminal sequencing (23). Therefore, in the present work the proteolytic peptides were identified according to their electrophoretic mobilities, on the basis of the data already reported (23).

For the preparation of peptide $b6_{TL}$, five 25-mg aliquots of a bovine Tg containing 1.05% iodine by mass were hydrolyzed with thermolysin at the enzyme/substrate ratio of 1/100 at pH 8.0 at 30 °C for 80 min. The fragments were separated by preparative continuous-elution SDS-PAGE, concentrated, further purified, and lyophilized as described under "Experimental Procedures." The analysis by SDS-PAGE of the fractions of a typical preparation is shown in *panels C* and *D* of Fig. 1. In the end, 2.2 mg of pure peptide were obtained (Fig. 1, *panel E*). Because peptide $b6_{TL}$ represented 10% of the peptides detected by densitometry of the gel (Fig. 1, *panel A*) (23) and these were 80% of the starting protein material, the yield of the purification procedure was 22%.

Analysis of Peptide $b6_{TL}$ by Mass Spectrometry—A 50-kDa peptide starting at residue 1291 (peptide $b6_{TL}$) (Fig. 1, *panels A* and *B*) was reduced and carboxymethylated, digested with endoproteinase Asp-N, and the digest was fractionated by reverse-phase HPLC on a Vydac C-18 column (250 × 4.6 mm, 5 μm). The chromatogram is shown in Fig. 2. All fractions were directly analyzed by ES/MS, and some were freeze-dried and analyzed also by FAB/MS. The results of the analysis by ES/MS are reported in Table I. The mass signals in the spectra were associated with the corresponding peptides along the sequence of bovine Tg, between residues 1200 and 1630, using a suitable computer program (26) (Fig. 3). Several cleavage sites were only partially hydrolyzed during the digestion, which yielded several overlapping peptides. A few aspecific cleavages occurred at the amino side of glutamic acid residues. However, the data permitted verification of the entire amino acid sequence of peptide $b6_{TL}$, which was identical to the cDNA-derived sequence (10). Ala-1591 was identified as the COOH-terminal residue of peptide $b6_{TL}$. In fact, two peptides, spanning residues 1567–1591 and 1580–1591, both ended at Ala-1591 and, therefore, were not expected on the basis of the enzymatic specificity of endoproteinase Asp-N. Moreover, no peptide was detected whose sequence matched Tg sequence beyond Ala-1591. The mass spectrometric analysis of the HPLC

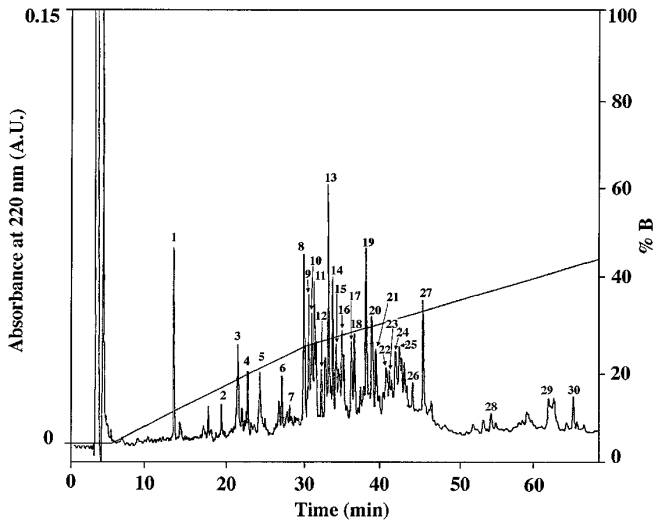

FIG. 2. **Reverse-phase HPLC chromatography monitored at 220 nm of the products of digestion of peptide b6$_{TL}$ of bovine Tg with endoproteinase Asp-N.** Reduction, carboxymethylation, and hydrolysis of peptide b6$_{TL}$ with endoproteinase Asp-N were carried out as described under "Experimental Procedures." The products of digestion of 0.5 mg of the peptide were fractionated with a Vydac C18 column (250 × 4.6 mm, 5 μm) equilibrated in 0.1% (v/v) trifluoroacetic acid in water (solvent A) containing 4% (v/v) of 0.07% trifluoroacetic acid in acetonitrile (solvent B). The percentage of B was held at 4% for the first 5 min, then raised to 25% over the following 25 min, and to 60% over the next 45 min. All the main peaks are numbered.

fractions of peptide b6$_{TL}$ (Table I) permitted characterization of its seven tyrosyl residues at positions 1234, 1291, 1375, 1450, 1464, 1484, and 1512, identifying post-translational modifications of Tyr-1234, Tyr-1291, and Tyr-1375.

Tyr-1234 Is Partially Converted to MIT—Two molecular species, having mass values of 4136.3 ± 0.4 Da and 4262.9 ± 0.4 Da, were detected by ES/MS in fractions 23 and 24, respectively. The first value was in perfect agreement with that expected for peptide 1218–1252, produced by endoproteinase Asp-N by aspecific cleavage at Glu-1253 (4136.7 Da, see Table I); the second value was compatible with that expected for the same peptide in which an iodine atom had been added to Tyr-1234 (Δm = +126). To verify this, fractions 23 and 24 were digested with trypsin and the products analyzed by FAB/MS. The spectrum of fraction 23 showed a protonated molecular ion (MH^+) at m/z = 771, corresponding to unmodified peptide 1230–1235, while that of fraction 24 contained a MH^+ ion at m/z = 897 (Δm = +126), confirming the conversion of Tyr-1234 to MIT. No other forms of this peptide were found, which restricts the modifications of Tyr-1234 to the formation of MIT.

Tyr-1291 Is a Hormonogenic Acceptor Residue—Among the peptides expected from the digestion with endoproteinase Asp-N, peptide 1290–1303 DYSGLLLAFQVFLL, containing a single Tyr residue at position 1291 and having an expected mass value of 1598.8 Da, was absent in the peptide map. However, mass values corresponding to this peptide having MIT, DIT, T_3, and T_4 at position 1291 were found in HPLC fractions 15 (1724.9 ± 0.1 Da for MIT; 1850.3 ± 0.2 Da for DIT) and 29 (2066.5 ± 0.5 Da for T_3; 2192.5 ± 0.6 Da for T_4) (Table I and Fig. 4). These assignments were confirmed by submitting the above fractions to FAB/MS followed by two manual Edman degradation steps, after which the m/z values of the truncated peptides were measured again by FAB/MS (Fig. 5). After the first Edman cycle, all peptides showed a shift of −115 mass units, corresponding to the loss of Asp-1290. After the second step, all peptides collapsed to the same m/z value of 1320, due to the loss of MIT (fraction 15, Δm = −289), DIT (fraction 15, Δm = −415), T_3 (fraction 29, Δm = −631), and T_4 (fraction 29, Δm = −757), respectively, from position 1291. This experiment demonstrated the presence of all the molecular species involved in the pathway of T_3 and T_4 synthesis at position 1291, with the exception of unmodified Tyr.

TABLE I

Analysis by ES/MS of the products of digestion of reduced and alkylated peptide b6$_{TL}$ (1218–1591) with endoproteinase Asp-N

Peptide b6$_{TL}$ was reduced and carboxymethylated; then, the peptide was hydrolyzed with endoproteinase Asp-N, the digest was fractionated by reverse-phase HPLC with a Vydac C-18 column (250 × 4.6 mm, 5 μm) and individual fractions were analyzed by ES/MS, as described in detail under "Experimental Procedures."

HPLC peak[a]	Measured mass[b] (Da, mean ± S.D.)	Peptide[c]	Theoretical mass[d] (Da)	Status of tyrosines
1	1109.8 ± 0.4	1509–1517	1110.1	
2	755.0 ± 0.2	1394–1400	754.7	
3	1386.9 ± 0.9	1580–1591	1386.6	
4	1351.7 ± 0.6	1496–1506	1352.5	
5	876.0 ± 0.1	1567–1574	876.0	
	766.3 ± 0.2	1355–1362	766.8	
	527.5 ± 0.2	1540–1544	527.6	
6	1732.6 ± 0.3	1438–1452	1732.9	Tyr-1450
7	1141.8 ± 0.3	1518–1527	1141.2	
8	1354.3 ± 0.8	1507–1517	1353.3	Tyr-1512
9	1386.2 ± 0.2	1528–1539	1386.4	
10	2316.1 ± 1.0	1336–1354	2315.6	
11	1347.4 ± 0.3	1555–1566	1347.4	
	1036.3 ± 0.2	1401–1409	1036.1	
12	1268.5 ± 0.2	1545–1554	1268.5	
13	1615.8 ± 0.2	1366–1381	1615.8	DHA 1375
14	1587.5 ± 0.4	1454–1465	1586.8	Tyr-1464
15	1724.9 ± 0.1	1290–1303	1724.8	MIT 1291
	1850.3 ± 0.2	1290–1303	1850.7	DIT 1291
16	2865.2 ± 0.2	1304–1329	2865.2	
	4075.1 ± 0.4	1355–1393	4074.4	DHA 1375
	4169.0 ± 0.2	1355–1393	4168.4	Tyr-1375
	4293.4 ± 0.6	1355–1393	4294.4	MIT 1375
	4421.0 ± 0.6	1355–1393	4420.3	DIT 1375
17	2616.9 ± 0.2	1410–1433	2616.8	
	1895.7 ± 0.5	1528–1544	1896.0	
18	2931.9 ± 0.8	1330–1354	2931.3	
19	1709.4 ± 0.2	1366–1381	1709.8	Tyr-1375
20	3018.0 ± 0.7	1410–1437	3018.2	
21	2598.3 ± 0.1	1545–1566	2597.9	
	2714.2 ± 0.5	1567–1591	2714.0	
22	2108.3 ± 0.2	1382–1400	2108.2	
23	4136.3 ± 0.4	1218–1252	4136.7	Tyr-1234
24	4262.9 ± 0.4	1218–1252	4262.6	MIT 1234
25	1371.6 ± 0.3	1382–1393	1371.5	
26	1836.3 ± 1.0	1366–1381	1835.8	MIT 1375
27	1961.6 ± 0.1	1366–1381	1961.7	DIT 1375
28	3257.8 ± 0.7	1466–1495	3258.6	Tyr-1484
29	2066.5 ± 0.5	1290–1303	2066.7	T_3 1291
	2192.5 ± 0.6	1290–1303	2192.7	T_4 1291
30	4252.9 ± 0.9	1253–1289	4252.7	

[a] Numbers refer to the peaks of the chromatogram shown in Fig. 2.
[b] Average molecular masses in Da (mean ± S.D.) obtained by integrating the multiple peaks corresponding to each molecular species, differing only in the total number of charges, measured by ES/MS.
[c] Numbers indicate the amino acid residues at the extremities of each peptide.
[d] Masses calculated on the basis of the cDNA-derived sequence of bovine Tg (10), taking into account the modifications of tyrosyl residues indicated.

Tyr-1375 Is a Hormonogenic Donor Residue—The chromatogram of Fig. 2 contained four peaks (13, 19, 26, and 27), whose analysis by ES/MS revealed mass signals related to peptide 1366–1381, containing one Tyr residue at position 1375 (Table I and Fig. 4). The mass value of 1709.4 ± 0.2 Da, in fraction 19, corresponded to peptide 1366–1381 DVEEALAGKYLAGRFA, with unmodified Tyr-1375. The mass value of 1615.8 ± 0.2 (Δm = −94), in fraction 13, could be accounted for by a form of peptide 1366–1381 in which Tyr-1375 had been converted to dehydroalanine. The mass values of 1836.3 ± 1.0, in fraction 26, and 1961.6 ± 0.1, in fraction 27, were compatible with the

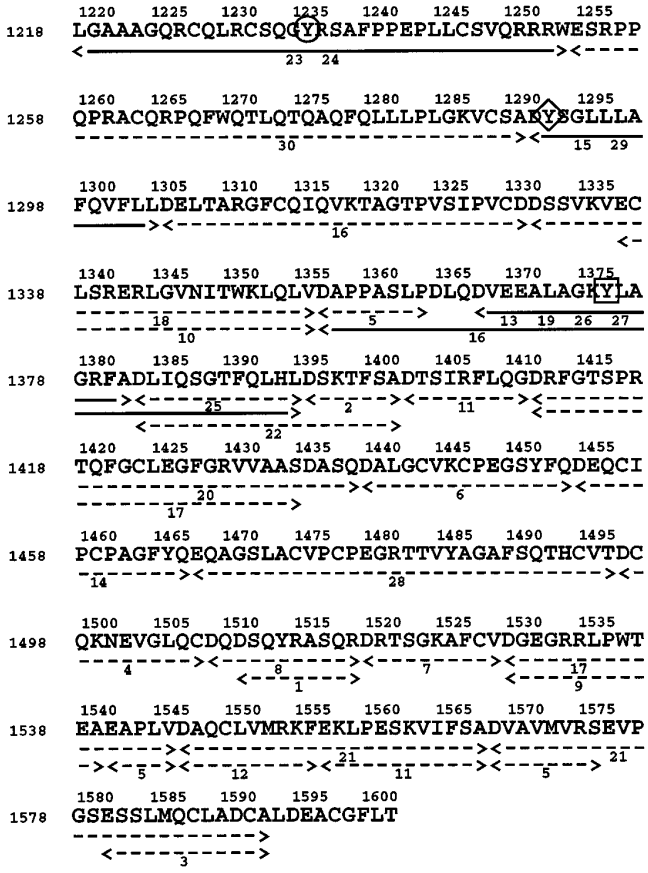

FIG. 3. **Peptide map determined by ES/MS of peptide b6$_{TL}$ of bovine Tg digested with endoproteinase Asp-N.** The peaks from the HPLC separation of Fig. 2 were analyzed by ES/MS. The measured molecular masses (Table I) were associated with the corresponding peptides along the cDNA-derived sequence of bovine Tg (10), on the basis of the theoretical masses, with the aid of a computer program (26). The extremities of individual peptides are marked by *arrowheads. Continuous lines* mark the peptides containing tyrosyl residues which underwent post-translational modifications. These are highlighted as follows: iodinated Tyr-1234 (modified to MIT) is *circled*; hormonogenic acceptor Tyr-1291 (modified to MIT, DIT, T_3, and T_4), *diamond*; donor Tyr-1375 (modified to MIT, DIT, and DHA), *square. Dashed lines* mark the other peptides. *Numbers* below the lines that underscore the peptides indicate the fractions in which these were detected.

addition of one and two iodine atoms to Tyr-1375, respectively. These identifications were confirmed by incubating the four fractions with endoproteinase Lys-C, to cleave peptide 1366–1381 into peptides 1366–1374 and 1375–1381. When analyzed by FAB/MS (Fig. 6), the four digests had in common the MH^+ ion at m/z = 931 predicted for peptide 1366–1374 DVEEALAGK, whereas they differed in the MH^+ ions corresponding to peptide 1375–1381. In fact, the spectrum of fraction 19 showed the MH^+ ion at m/z = 797 predicted for the unmodified peptide 1375–1381 YLAGRFA, while the spectra of fractions 26, 27, and 13 showed MH^+ ions at m/z = 923, 1049, and 703, corresponding to peptide 1375–1381, in which Tyr-1375 had been converted to MIT, DIT, and DHA, respectively. The analysis by FAB/MS, after one step of Edman degradation (Fig. 6), showed the expected mass shifts, by which all four peptides moved to m/z = 634, due to the loss of Tyr (fraction 19, $\Delta m = -163$), MIT (fraction 26, $\Delta m = -289$), DIT (fraction 27, $\Delta m = -415$), and DHA (fraction 13, $\Delta m = -69$). These data proved that Tyr-1375 is an iodophenyl donor residue. Mass signals corresponding to various forms of peptide 1355–1393, in which Tyr-1375 was present as such or had been modified to MIT, DIT, and DHA were detected also in fraction 16 (Table I).

Asn-1346 Is Not Glycosylated—The sole putative site of *N*-

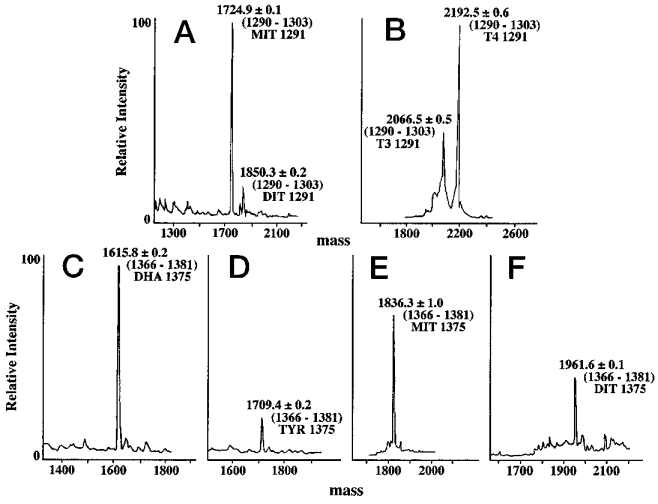

FIG. 4. **ES/MS spectra of the HPLC fractions of the endoproteinase Asp-N digest of peptide b6$_{TL}$ that contained mass signals associated with modifications of Tyr-1291 and -1375.** The fractions of the reverse-phase chromatography shown in Fig. 2 were analyzed by ES/MS. Mass spectra were transformed on a real mass scale. Average molecular masses in Da (mean ± S.D.) were obtained by integration of the multiple peaks, differing only in the total number of charges, associated with each molecular species. Each mass signal was associated with the corresponding peptide along the sequence of bovine Tg, between residues 1200 and 1630, on the basis of the theoretical mass, with the aid of a computer program (31). Those spectra are shown in which there were mass signals associated with peptides containing Tyr-1291 and -1375 and their modifications. The mass signals in the spectra in *panels A* and *B* corresponded to peptide 1290–1303 containing the following modifications of Tyr-1291: *panel A* (fraction 15), MIT 1291, m = 1724.9 ± 0.1, and DIT 1291, m = 1850.3 ± 0.2; *panel B* (fraction 29), T_3 1291, m = 2066.5 ± 0.5, and T_4 1291, m = 2192.5 ± 0.6. The mass signals in the spectra in *panels C, D, E*, and *F* corresponded to peptide 1366–1381 containing the following modifications of Tyr-1375: *panel C* (fraction 13), DHA 1375, m = 1615.8 ± 0.2; *panel D* (fraction 19), Tyr-1375, m = 1709.4 ± 0.2; *panel E* (fraction 26), MIT 1375, m = 1836.3 ± 1.0; *panel F* (fraction 27), DIT 1375, m = 1961.6 ± 0.1.

linked glycosylation of peptide b6$_{TL}$, corresponding to Asn-1346 (within the consensus sequence Asn-Ile-Thr) (10), was unmodified. In fact, peptides 1330–1354 (fraction 18) and 1336–1354 (fraction 10) had mass values typical of the non-glycosylated species (Table I), and no evidence was found of glycosylated forms of the above peptides.

Efficiency of Tyr-1375 as a T_4- and T_3-forming Site—The data of Table II indicate that the iodine content of peptide b6$_{TL}$ (1.11% by mass) exceeded slightly the average iodine content of the parent bovine Tg (1.05% by mass). Thus, the fraction of total Tg iodine contained in 2 mol of peptide b6$_{TL}$/mol of Tg dimer (0.16) was only slightly higher than the fraction of Tg mass that they accounted for (0.15). In particular, 13% of total iodine in peptide b6$_{TL}$ was found in T_4 and 3% in T_3, as opposed to 21 and 5%, respectively, in bovine Tg. Tyr-1291 contributed 10% of the T_4 and 8% of the T_3 content of Tg. The relative amounts of iodine incorporated into iodothyronines and iodotyrosines were 1 *versus* 5 in peptide b6$_{TL}$, and 1 *versus* 3 in Tg. On the basis of the moles of iodoamino acids formed per mole of Tg, the overall extent of modification of Tyr-1234, -1291, and -1375 appeared to be quite large, considering that the other 4 Tyr residues were unmodified (see Table I). Because 0.4 mol of iodothyronines were formed per mole of 660-kDa Tg (*i.e.* per 2 mol of Tyr-1291), the efficiency of hormone formation at this site, at this level of Tg iodination, was 20%. The 2.6 mol of DIT per mole of Tg in peptide b6$_{TL}$ accounted for another 65% of the combined 4 mol of Tyr-1291 and -1375 per mole of Tg dimer, considering that the modification of Tyr-1234 was restricted to formation of MIT. Out of 2.5 mol of MIT per mole of Tg found in

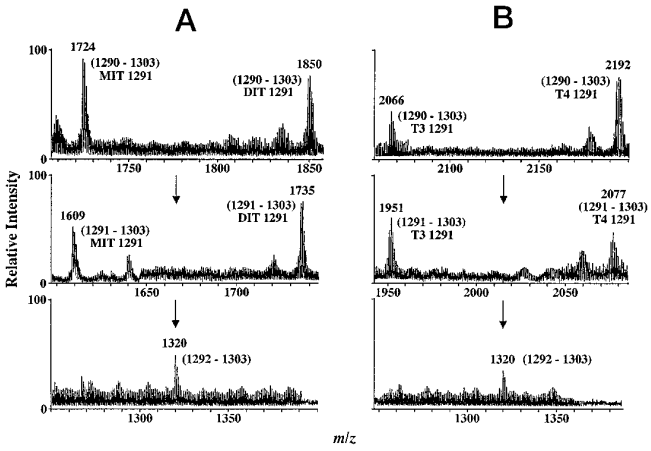

FIG. 5. **Combined analysis by FAB/MS and Edman degradation of the HPLC fractions of the endoproteinase Asp-N digest of peptide b6$_{TL}$, whose ES/MS spectra contained mass signals associated with modifications of Tyr-1291.** Two fractions, whose analysis by ES/MS revealed signals associated with various forms of peptide 1290–1303, containing Tyr-1291 and its modifications (Table I and Fig. 4), were further analyzed by FAB/MS, in combination with Edman degradation. The monoisotopic masses of the MH^+ ions of the peptides are reported as integer numbers. The MH^+ ions in the spectra shown in the *upper part* of *panels A* and *B* corresponded to peptide 1290–1303 containing the following modifications of Tyr-1291: *panel A* (fraction 15), MIT 1291, m/z = 1724, and DIT 1291, m/z = 1850; *panel B* (fraction 29), T_3 1291, m/z = 2066, and T_4 1291, m/z = 2192. The FAB mass spectra collected after one cycle of Edman degradation (*middle part* of *panels A* and *B*) revealed the MH^+ ions expected for peptide 1291–1303 containing the same modifications of Tyr-1291 mentioned above: *panel A* (fraction 15), MIT 1291, m/z = 1609, and DIT 1291, m/z = 1735; *panel B* (fraction 29), T_3 1291, m/z = 1951, and T_4 1291, m/z = 2077. The FAB mass spectra collected after two steps of manual Edman degradation (*lower* part of *panels A* and *B*) revealed the same MH^+ ion at m/z = 1320 expected for peptide 1292–1303 in both fractions, thus confirming that the mass heterogeneity of peptide 1290–1303 was due to modifications of Tyr-1291.

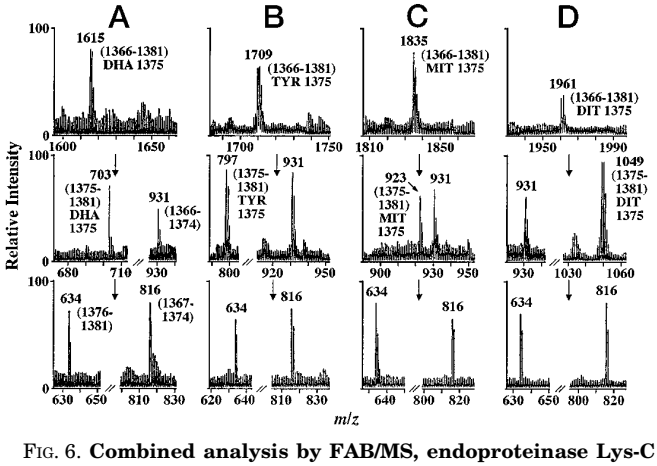

FIG. 6. **Combined analysis by FAB/MS, endoproteinase Lys-C digestion, and Edman degradation of the HPLC fractions of the endoproteinase Asp-N digest of peptide b6$_{TL}$, whose ES/MS spectra contained mass signals associated with modifications of Tyr-1375.** Four fractions, whose analysis by ES/MS revealed signals associated with various forms of peptide 1366–1381, containing Tyr-1375 and its modifications (Table I and Fig. 4), were further analyzed by FAB/MS, in combination with endoproteinase Lys-C digestion and Edman degradation. The monoisotopic masses of the MH^+ ions of the peptides are reported as integer numbers. The MH^+ ions in the spectra shown in the upper part of *panels A–D* corresponded to peptide 1366–1381 containing the following modifications of Tyr-1375: *panel A* (fraction 13), DHA 1375, m/z = 1615; *panel B* (fraction 19), Tyr-1375, m/z = 1709; *panel C* (fraction 26), MIT 1375, m/z = 1835; *panel D* (fraction 27), DIT 1375, m/z = 1961. The FAB mass spectra of the four peptides digested with endoproteinase Lys-C (*middle* part of *panels A–D*) showed a common MH^+ ion at m/z = 931, corresponding to peptide 1366–1374, together with different MH^+ ions corresponding to peptide 1375–1381 containing the already mentioned modifications of Tyr-1375: *panel A*, DHA 1375, m/z = 703; *panel B*, Tyr-1375, m/z = 797; *panel C*, MIT 1375, m/z = 923; *panel D*, DIT 1375, m/z = 1049. The FAB mass spectra collected after one step of manual Edman degradation (*lower* part of *panels A–D*) showed two common MH^+ ions, one at m/z = 816, corresponding to peptide 1367–1374, and the other at m/z = 634, corresponding to peptide 1376–1381, thus proving that the mass heterogeneity of peptide 1366–1381 was due to modifications of Tyr-1375.

peptide b6$_{TL}$, more than 0.5 mol had to be formed in correspondence of Tyr-1291 and -1375, and less than 2.0 by the iodination of the 2 mol of Tyr-1234 per mole of Tg dimer, as the ES/MS spectrum of peak 23 revealed the presence of some unmodified Tyr-1234 (see Table I). This makes it probable that the amount of DHA formed at Tyr-1375 was of the same order of magnitude of the amount of iodothyronines formed at Tyr-1291 and leaves room for only a small amount of unmodified Tyr at positions 1291 and 1375. In fact, no unmodified Tyr-1291 was found in the mass spectra.

DISCUSSION

We report a detailed analysis of the post-translational modifications of seven tyrosyl residues comprised in fragment 1218–1591 of bovine thyroglobulin. In particular, we demonstrate the formation of MIT, DIT, T_3, and T_4 from Tyr-1291, and of MIT, DIT, and DHA from Tyr-1375. Modification of Tyr-1234 was restricted to formation of MIT, while Tyr-1450, -1464, -1484, and -1512 were unmodified.

Mass spectrometry is widely employed for the analysis of post-translational modifications of proteins (32). However, it has been used here for the first time to identify iodinated tyrosyl residues in Tg, and has proved extremely valuable as a source of primary structure data not available from earlier use of Edman degradation. In the past, the identification of hormonogenic sites by the sequencing of hormone-rich peptides of Tg was not always as direct. The only iodotyrosines and iodothyronines directly identified, by the manual method of sequencing with dimethylaminoazobenzeneisothiocyanate (35, 36), were those located at positions 2553, 2567, and 2746 of hog Tg (human Tg numbering) (17, 19), and 5 of human Tg (12, 33, 34). On the other hand, the phenylthiohydantoin-derivatives of iodoamino acids, in iodopeptides subjected to automated sequencing, were generally not identified by comparison with proper standards. The localization of hormonogenic sites in the NH_2-terminal peptides of calf (13), sheep and hog Tg (14), in the tryptic peptides of rabbit (15) and guinea pig Tg labeled *in vivo* with ^{125}I (16), and in Tg from human goiters subjected to low-level iodination *in vitro* with ^{125}I (18), was based on the monitoring of the contents of ^{127}I or ^{125}I in the automated sequencing cycles, and the determination of the distribution of iodoamino acids. In this regard, although ^{125}I labeling provides an easy way to trace Tg iodopeptides and iodination sites and study hormonal turnover, it is not suited for the study of physiologically iodinated Tg of humans and other large animals.

The identification of donor tyrosyl residues was also indirect in all cases reported so far. In one study of bovine Tg, in which the separation of dehydroalanine-containing peptides exploited the conversion of dehydroalanine to *S*-(4-aminophenyl)cysteine, the presence of the latter at positions 5, 926, 986 or 1008, and 1375 was inferred from the lack of known phenylthiohydantoin-derivatives in sequencing cycles where tyrosine was expected, and from differences between the actual and expected tyrosine content of the peptides (20). In another study, the labeling of dehydroalanyl residues of bovine Tg with NaB^3H_4 and their conversion to labeled aspartic acid with $Na^{14}CN$ revealed a small labeled CNBr peptide containing possible donor Tyr-2469 and Tyr-2522, and a larger CNBr peptide, spanning residues 785-1551, possibly harboring other donor

TABLE II

Iodine and iodoamino acid content of peptide $b6_{TL}$ and its parent Tg

Iodine and protein determinations and digestions with Pronase and aminopeptidase M were performed as described under "Experimental Procedures." Iodoamino acids were separated by reverse-phase HPLC with a Brownlee C-8 column (250 × 4.6 mm, 5 μm), as reported (31). Iodoamino acid contents were calculated from the iodine contents of the respective peaks.

	$b6_{TL}$	Bovine Tg
Molar ratio to Tg (660 kDa)	2	1
Fraction of Tg mass	0.15	1.00
Iodine content (% of mass)[a]	1.11	1.05
Fraction of iodine in Tg (660 kDa)	0.16	1.00
Moles of iodoamino acids/mole of Tg (660 kDa)[a]		
3-Iodotyrosine	2.53	10.82
3,5-Diiodotyrosine	2.56	14.70
T_3	0.08	0.98
T_4	0.30	2.85
Fraction of Tg's T_3	0.08	1.00
Fraction of Tg's T_4	0.10	1.00
Fractional iodine distribution among iodoamino acids[a]		
3-Iodotyrosine	0.28	0.20
3,5-Diiodotyrosine	0.56	0.54
T_3	0.03	0.05
T_4	0.13	0.21
Iodine in T_3 + T_4/iodine in MIT + DIT	0.19	0.36

[a] Averages of at least three determinations.

residues (21). Finally, the proposal that alanine recovered at position 130 of peptide 1–171 of human Tg derived, in fact, from the conversion of dehydroalanine was largely based on speculation (22).

On the other hand, in the present study, mass spectrometry allowed the direct, unambiguous characterization of the entire spectrum of modifications of every tyrosyl residue within a large fragment of Tg. Not only the identification of T_4 and T_3 in correspondence of Tyr-1291 of bovine Tg is unprecedented, but also the localization of a donor site at position 1375 cannot be considered merely confirmatory, as it is based, for the first time, on a direct demonstration. By using the combination of limited proteolysis, preparative electrophoresis and mass spectrometry employed here, we project to extend our analysis to other still unsettled aspects of hormonogenesis in Tg, including: 1) the localization of the hormonogenic donor tyrosines of human Tg; 2) the identification of acceptor tyrosines other than tyrosine number 5 in physiologically iodinated human Tg; 3) the resolution of the uncertainties about donor sites at positions 986, 1008 (20), 2469 and 2522 (21) of bovine Tg.

The formation of T_4 at a site corresponding to residue 1291 of bovine Tg was already reported in rabbit (15, 16) and guinea pig Tg (16), in which it contributed 17 and 11%, respectively, of Tg's T_4. From the data reported in Table II, it appears that also in bovine Tg, Tyr-1291 contributed an appreciable amount of T_4, together with a small amount of T_3. It also appears that both Tyr-1291 and Tyr-1375 were to a large extent modified, mostly to DIT; however, only in one-fifth of the cases modification proceeded and iodothyronines were formed. On one hand, this probably reflects a high degree of accessibility of both residues. In this regard, the hydrophilicity plot of this region was not particularly informative (not shown); however, it is noteworthy that Tyr-1291 is located 70 residues apart from a cluster of protease-sensitive sites encompassing residues 1142, 1184, and 1218 (23). On the other hand, the prevalence of iodotyrosines at these sites raises interesting questions concerning the factors of steric hindrance that may limit the efficiency of a hormonogenic site, and the structural requirements that must be satisfied for efficient coupling to occur. In rabbit and guinea pig Tg labeled *in vivo* with ^{125}I, Tyr-1290 (human Tg numbering) contributed greatly to Tg's flexibility in meeting varying demands for hormone formation, as TSH enhanced T_4 formation at residue 1290, at the expense of T_4 formation at residue number 5, while increasing T_3 formation at residue 2746 (16). Under TSH stimulation, the percentage of T_4 neosynthesized at residue 1290 changed from 10 to 14% in rabbit and from 13 to 24% in guinea pig. In guinea pig, tyrosine 1290 was the most active site for new T_4 formation even in the presence of basal TSH levels (16). It is possible that also in bovine Tg the formation of T_4 at Tyr-1291 increase under TSH stimulation, *e.g.* as a consequence of iodide shortage. In this regard, it would be interesting to measure the share of Tg's total T_4 formed at Tyr-1291 at increasing levels of Tg iodination. On the other hand, in turtle Tg labeled *in vivo* with ^{125}I, only 5% of T_4 and 11% of T_3 were newly formed at Tyr-1290 (human Tg numbering) (37), while in human Tg iodinated *in vitro* with 7.8 atoms of iodine/Tg molecule, only traces of iodothyronines were found at residue 1290 (18). Only further work may establish whether this reflected the low level of Tg iodination, or the low efficiency of Tyr-1290 in human Tg. Interestingly, in human Tg, aspartic acid substitutes for tyrosine at position 1375. In addition, human Tyr-1447 was proposed to be a possible donor site, because it was iodinated early but did not provide inner iodothyronyl rings upon further iodination (18), whereas the corresponding Tyr-1450 of bovine Tg was unmodified in the present study. Although there is no indication that acceptor and donor residues need to be contiguous in the Tg sequence, the apparently low hormonogenic potential of human Tyr-1290 might indicate that, in bovine Tg, T_4 formation at Tyr-1291 depends on the presence of donor Tyr-1375, whereas, in human Tg, Tyr-1447 is not as good a donor site.

It was proposed that different hormonogenic sites of Tg evolved independently, and may also function independently from each other and the rest of the Tg molecule (38). Various observations support this hypothesis. Thyroid hormone formation within truncated NH_2-terminal Tg fragments, derived from the abortive translation of normal-sized mRNAs, was probably responsible for the correction of hypothyroidism, by iodide supplementation, in a strain of Dutch goats with congenital goiter (39–41), and for euthyroidism in Afrikander cattle (42–44). Efficient T_4 formation was demonstrated in isolated fragment 1–171 of human Tg (22, 34). Thyroid hormones were also formed upon *in vitro* iodination of a fragment comprising the 224 COOH-terminal amino acids of rat Tg (45). It would be interesting to test the ability of peptide $b6_{TL}$, isolated from low-iodine bovine Tg, to sustain T_4 (and T_3) formation at Tyr-1291 upon peroxidase-catalyzed iodination *in vitro*. Should T_4 be formed, peptide $b6_{TL}$ could represent an interesting model for the study of the minimal structural requirements of the hormonogenic function.

REFERENCES

1. Gentile, F., Di Lauro, R., and Salvatore, G. (1995) in *Endocrinology* (DeGroot, L. J., ed) 3rd Ed., Vol. 1, pp. 517–542, W. B. Saunders Co., Philadelphia, PA
2. Gavaret, J.-M., Nunez, J., and Cahnmann, H. J. (1980) *J. Biol. Chem.* **255,** 5281–5285
3. Gavaret, J.-M., Cahnmann, H. J., and Nunez, J. (1981) *J. Biol. Chem.* **256,** 9167–9173
4. Edelhoch, H. (1962) *J. Biol. Chem.* **237,** 2778–2787
5. Gavaret, J. M., Dème, D., Nunez, J., and Salvatore, G. (1977) *J. Biol. Chem.* **252,** 3281–3285
6. Lamas, L., Taurog, A., Salvatore, G., and Edelhoch, H. (1974) *J. Biol. Chem.* **249,** 2732–2737
7. Cahnmann, H. J., Pommier, J., and Nunez, J. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5333–5335
8. Ogawara, H., Bilstad, J. M., and Cahnmann, H. J. (1972) *Biochim. Biophys. Acta* **257,** 339–349
9. Izumi, M., and Larsen, P. R. (1977) *J. Clin. Invest.* **59,** 1105–1112
10. Mercken, L., Simons, M.-J., Swillens, S., Massaer, M., and Vassart, G. (1985) *Nature* **316,** 647–651
11. Malthiéry, Y., and Lissitzky, S. (1987) *Eur. J. Biochem.* **165,** 491–498
12. Lejeune, P.-J., Marriq, C., Rolland, M., and Lissitzky, S. (1983) *Biochem. Biophys. Res. Commun.* **114,** 73–80
13. Rawitch, A. B., Chernoff, S. B., Litwer, M. R., Rouse, J. B., and Hamilton, J. W.

(1983) *J. Biol. Chem.* **258,** 2079–2082
14. Rawitch, A. B., Litwer, M. R., Gregg, J., Dziadik Turner, C., Rouse, J. B., and Hamilton, J. W. (1984) *Biochem. Biophys. Res. Commun.* **118,** 423–429
15. Dunn, J. T., Anderson, P. C., Fox, J. W., Fassler, C. A., Dunn, A. D., Hite, L. A., and Moore, R. C. (1987) *J. Biol. Chem.* **262,** 16948–16952
16. Fassler, C. A., Dunn, J. T., Anderson, P. C., Fox, J. W., Dunn, A. D., Hite, L. A., Moore, R. C., and Kim, P. S. (1988) *J. Biol. Chem.* **263,** 17366–17371
17. Marriq, C., Rolland, M., and Lissitzky, S. (1982) *EMBO J.* **1,** 397–401
18. Lamas, L., Anderson, P. C., Fox, J. W., and Dunn, J. T. (1989) *J. Biol. Chem.* **264,** 13541–13545
19. Marriq, C., Rolland, M., and Lissitzky, S. (1983) *Biochem. Biophys. Res. Commun.* **112,** 206–213
20. Ohmiya, Y., Hayashi, H., Kondo, T., and Kondo, Y. (1990) *J. Biol. Chem.* **265,** 9066–9071
21. Palumbo, G. (1987) *J. Biol. Chem.* **262,** 17182–17188
22. Marriq, C., Lejeune, P.-J., Venot, N., and Vinet, L. (1991) *Mol. Cell. Endocrinol.* **81,** 155–164
23. Gentile, F., and Salvatore, G. (1993) *Eur. J. Biochem.* **218,** 603–621
24. Laemmli, U. K. (1970) *Nature* **227,** 680–685
25. Wessel, D., and Flügge, U. I. (1984) *Anal. Biochem.* **138,** 141–143
26. Pucci, P., and Sepe, C. (1990) *Biomed. Environ. Mass Spectrom.* **17,** 287–291
27. Pucci, P., Carestia, C., Fioretti, G., Mastrobuoni, A. M., and Pagano, L. (1985) *Biochem. Biophys. Res. Commun.* **130,** 84–90
28. Palumbo, G., Tecce, M. F., and Ambrosio, G. (1982) *Anal. Biochem.* **123,** 183–189
29. Edelhoch, H., and Lippoldt, R. E. (1962) *J. Biol. Chem.* **237,** 2788–2794
30. Sorimachi, K., and Ui, N. (1974) *J. Biochem.* (*Tokyo*) **76,** 39–45
31. Palumbo, G., Gentile, F., Condorelli, G. L., and Salvatore, G. (1990) *J. Biol. Chem.* **265,** 8887–8892
32. Burlingame, A. L., Boyd, R. K., and Gaskell, S. J. (1994) *Anal. Chem.* **66,** 634R-683R
33. Marriq, C., Lejeune, P.-J., Malthiéry, Y., Rolland, M., and Lissitzky, S. (1984) *FEBS Lett.* **175,** 140–146
34. Marriq, C., Lejeune, P.-J., Venot, N., Rolland, M., and Lissitzky, S. (1986) *FEBS Lett.* **207,** 302–306
35. Chang, J. Y., Brauer, D., and Wittman-Liebold, B. (1978) *FEBS Lett.* **93,** 205–214
36. Marriq, C., Rolland, M., and Lissitzky, S. (1981) *Anal. Biochem.* **116,** 89–92
37. Roe, M. T., Anderson, P. C., Dunn, A. D., and Dunn, J. T. (1989) *Endocrinology* **124,** 1327–1332
38. Musti, A. M., Avvedimento, E. V., Polistina, C., Ursini, V. M., Obici, S., Nitsch, L., Cocozza, S., and Di Lauro, R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 323–327
39. Sterk, A., van Dijk J. E., Veenboer, G. J. M., Moorman, A. F. M., and de Vijlder, J. J. M. (1989) *Endocrinology* **124,** 477–483
40. Van Voorthuizen, W. F., de Vijlder, J. J. M., van Dijk, J. E., and Tegelaers, W. H. H. (1978) *Endocrinology* **103,** 2105–2111
41. Veenboer, G. J. M., and de Vijlder, J. J. M. (1993) *Endocrinology* **132,** 377–381
42. Tassi, V. P. N., Di Lauro, R., Van Jaarsveld, P., and Alvino, C. G. (1984) *J. Biol. Chem.* **259,** 10507–10510
43. Ricketts, M. H., Pohl, V., de Martynoff, G., Boyd, C. D., Bester, A. J., Van Jaarsveld, P. P., and Vassart, G. (1985) *EMBO J.* **4,** 731–737
44. Ricketts, M. H., Simons, M. J., Parma, J., Mercken, L., Dong, Q., and Vassart, G. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 3181–3184
45. Asunción, M., Ingrassia, R., Escribano, J., Martin, U., Méndez, E., Di Lauro, R., and Lamas, L. (1992) *FEBS Lett.* **297,** 266–270

Identification of Hormonogenic Tyrosines in Fragment 1218-1591 of Bovine Thyroglobulin by Mass Spectrometry: HORMONOGENIC ACCEPTOR TYR-1291 AND DONOR TYR-1375

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