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 (T₃), and tetraiodothyronine (thyroxine, T₄) from tyrosine 1291; and 3) monoiodotyrosine, diiodotyrosine, dehydroalanine from tyrosine 1375. Iodothyronine formation from tyrosine 1291 accounted for 10% of total T₄ of thyroglobulin (0.30 mol of T₄/mol of 660-kDa thyroglobulin), and 8% of total T₃ (0.08 mol of T₄/mol of thyroglobulin). This is the first documentation of the hormonogenic nature of tyrosine 1291 of bovine thyroglobulin, as tyroxine 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), a homodimeric glycoprotein with a molecular mass of 660 kDa, is the site of the biosynthesis of 3,5,3'-triiodothyronine (T₃) and 3,5,3',5'-tetraiodothyronine (thyroxine, T₄) (reviewed in Ref. 1). T₃ and T₄ 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 T₃ or T₄, 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 T₃ and T₄ (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 T₄ 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), Tg-2553 (human Tg numbering) was the second most efficient T₄-forming residue, whereas Tg-2746 was a site of preferential synthesis of T₃ (15, 16, 18, 19). Another T₄-forming site found in rabbit and guinea pig Tg corresponded to human Tg-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 T₄ 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.
EXPERIMENTAL PROCEDURES

Materials—Thermolysin from Bacillus thermo-protéolyticus rokkō (EC 3.4.24.4) and t-1-lysotidyl-2-phenylethylchloromethyl-treated bovine pancreatic trypsin (EC 3.4.21.4), diithiothreitol, iodoacetic acid, glycerol, thioglycerol, 3-iodo-t-tyrosine (MIT), 3,5-diido-t-tyrosine (DTT), 3,5,3'-triiodothyronine (3,5',3,5'-tetraiodothyronine (thyroxine, T4)) were from Sigma Chimica (Milan, Italy); endoprotease Asp-N from Pseudomonas fragi (EC 3.4.24.33) and endoprotease 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). Phenylsothiocyanate and EDTA were from Fluka Chimica (Milan, Italy). AcrylAlde-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 from resin and binchoninic acid Protein Assay Reagent were from Pierce (Rockford, IL). HPLC grade solvents were obtained from Carlo Erba (Milan, Italy). The Vydec 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 Biosys (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 abattoir. 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 bicarbonate (0.1 ml of resin every 50 ml of the original pool) in a pool was concentrated by lyophilization, freed from Tris/HCl, and glycerol, thioglycerol, 3-iodo- L-tyrosine (MIT), 3,5-diiodo- L-tyrosine (DTT), 3,5,3'-triiodothyronine (T3), 3,5,3'-triiodothyronine (thyroxine, T4) were from Sigma Chimica (Milan, Italy); endoprotease Asp-N from Pseudomonas fragi (EC 3.4.24.33) and endoprotease 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). Phenylsothiocyanate and EDTA were from Fluka Chimica (Milan, Italy). AcrylAlde-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 from resin and binchoninic acid Protein Assay Reagent were from Pierce (Rockford, IL). HPLC grade solvents were obtained from Carlo Erba (Milan, Italy). The Vydec 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 Biosys (Santa Clara, CA); PD-10 Sephadex G-25 cartridges and Sephacryl S-300 HR were from Pharmacia Biotech (Uppsala, Sweden).

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Preparation of Tg—Bovine Tg was prepared from fresh bovine thyroids from the local abattoir. 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 bicarbonate (0.1 ml of resin every 50 ml of the original pool) in a pool was concentrated by lyophilization, freed from Tris/HCl, and glycerol, thioglycerol, 3-iodo- L-tyrosine (MIT), 3,5-diiodo- L-tyrosine (DTT), 3,5,3'-triiodothyronine (T3), 3,5,3'-triiodothyronine (thyroxine, T4) were from Sigma Chimica (Milan, Italy); endoprotease Asp-N from Pseudomonas fragi (EC 3.4.24.33) and endoprotease 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). Phenylsothiocyanate and EDTA were from Fluka Chimica (Milan, Italy). AcrylAlde-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 from resin and binchoninic acid Protein Assay Reagent were from Pierce (Rockford, IL). HPLC grade solvents were obtained from Carlo Erba (Milan, Italy). The Vydec 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 Biosys (Santa Clara, CA); PD-10 Sephadex G-25 cartridges and Sephacryl S-300 HR were from Pharmacia Biotech (Uppsala, Sweden).
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 b6TL, 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 b6TL 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 b6TL by Mass Spectrometry—A 50-kDa peptide starting at residue 1291 (peptide b6TL) (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 b6TL, which was identical to the cDNA-derived sequence (10). Ala-1591 was identified as the COOH-terminal residue of peptide b6TL. 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
fractions of peptide b6\textsubscript{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.

**Hormonogenic Tyrosines in Fragment 1218–1591 of Thyroglobulin**

**FIG. 2.** Reverse-phase HPLC chromatography monitored at 220 nm of the products of digestion of peptide b6\textsubscript{TL}, of bovine Tg with endoproteinase Asp-N. Reduction, carboxymethylation, and hydrolysis of peptide b6\textsubscript{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) trifluoroacetic acid in acetonitrile (solvent B). The percentage of B was held at 4% for the next 45 min. All the main peaks are numbered.

**TABLE I**

| 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                    |                  |
| 6               | 766.3 ± 0.2                          | 1355–1362    | 766.8                    |                  |
| 7               | 527.5 ± 0.2                          | 1540–1544    | 527.6                    |                  |
| 8               | 1732.6 ± 0.3                         | 1438–1452    | 1732.9                   | Tyr-1450         |
| 9               | 1141.8 ± 0.3                         | 1518–1527    | 1141.2                   | Tyr-1512         |
| 10              | 1354.3 ± 0.8                         | 1502–1512    | 1353.8                   |                  |
| 11              | 1386.2 ± 0.2                         | 1528–1539    | 1386.4                   |                  |
| 12              | 2316.1 ± 1.0                         | 1336–1354    | 2315.6                   |                  |
| 13              | 1347.4 ± 0.3                         | 1555–1566    | 1347.4                   |                  |
| 14              | 1036.9 ± 0.2                         | 1401–1409    | 1036.1                   |                  |
| 15              | 1268.5 ± 0.2                         | 1545–1554    | 1268.5                   |                  |
| 16              | 1615.8 ± 0.2                         | 1366–1381    | 1615.8                   | DHA 1375         |
| 17              | 1587.5 ± 0.4                         | 1454–1465    | 1586.8                   | Tyr-1464         |
| 18              | 1724.9 ± 0.2                         | 1290–1303    | 1724.8                   | MET 1291         |
| 19              | 1850.2 ± 0.2                         | 1296–1303    | 1850.7                   | DIT 1291         |
| 20              | 2965.2 ± 0.2                         | 1304–1329    | 2965.2                   |                  |
| 21              | 4075.1 ± 0.4                         | 1355–1393    | 4074.4                   | DHA 1375         |
| 22              | 4169.0 ± 0.2                         | 1355–1393    | 4168.4                   | Tyr-1375         |
| 23              | 4293.4 ± 0.6                         | 1355–1393    | 4294.4                   | MET 1375         |
| 24              | 4420.1 ± 0.6                         | 1355–1393    | 4420.3                   | DIT 1375         |
| 25              | 2616.9 ± 0.2                         | 1410–1433    | 2616.8                   |                  |
| 26              | 1895.7 ± 0.5                         | 1528–1544    | 1896.0                   |                  |
| 27              | 2931.9 ± 0.8                         | 1330–1354    | 2931.3                   |                  |
| 28              | 1709.4 ± 0.2                         | 1366–1381    | 1709.8                   | Tyr-1375         |
| 29              | 3018.0 ± 0.7                         | 1410–1437    | 3018.2                   |                  |
| 30              | 2998.3 ± 0.1                         | 1545–1566    | 2998.3                   |                  |
| 31              | 2714.2 ± 0.5                         | 1567–1591    | 2714.0                   |                  |
| 32              | 2108.3 ± 0.2                         | 1382–1400    | 2108.2                   |                  |
| 33              | 4316.3 ± 0.4                         | 1218–1252    | 4316.7                   | Tyr-1234         |
| 34              | 4292.9 ± 0.4                         | 1218–1252    | 4292.8                   | MET 1234         |
| 35              | 3371.6 ± 0.3                         | 1382–1393    | 3371.5                   |                  |
| 36              | 1836.3 ± 1.0                         | 1366–1381    | 1835.8                   | MET 1375         |
| 37              | 1961.6 ± 0.1                         | 1366–1381    | 1961.7                   | DIT 1375         |
| 38              | 3257.8 ± 0.7                         | 1466–1495    | 3258.6                   | Tyr-1484         |
| 39              | 2066.5 ± 0.5                         | 1290–1303    | 2066.7                   | T\(_2\) 1291     |
| 40              | 2192.5 ± 0.6                         | 1290–1303    | 2192.7                   | T\(_2\) 1291     |
| 41              | 34252.9 ± 0.2                        | 1293–1289    | 34252.7                  |                  |

\(a\) 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.

\[\Delta m = -415, T_3 (fraction 29, \Delta m = -631), \text{and } T_4 (fraction 29, \Delta m = -757), \text{respectively, from position 1291. This experiment demonstrated the presence of all the molecular species involved in the pathway of } T_3 \text{ and } T_4 \text{ synthesis at position 1291, with the exception of unmodified Tyr.}\]

**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 (\(\Delta m = -94\), 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
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, after one step of Edman degradation (Fig. 6), the four digests had in common the MH ion at m/z 1961.6 (fraction 27), DIT 1291, m/z 1912.5 (0.6), and T4 1291, m/z 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-1234, DIT 1375, panel C (fraction 13), DHA 1375, m/z 1615.8 ± 0.2; panel D (fraction 19), Tyr-1375, m/z 1709.4 ± 0.2; panel E (fraction 26), MIT 1375, m/z 1836.3 ± 1.0; panel F (fraction 27), DIT 1375, m/z 1961.6 ± 0.1.

FIG. 4. ES/MS spectra of the HPLC fractions of the endoproteinase Asp-N digest of peptide b6TL, 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/z 1724.9 ± 0.1, and DIT 1291, m/z 1850.3 ± 0.2; panel B (fraction 29), T3 1291, m/z 2066.5 ± 0.5, and T4 1291, m/z 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/z 1615.8 ± 0.2; panel D (fraction 19), Tyr-1375, m/z 1709.4 ± 0.2; panel E (fraction 26), MIT 1375, m/z 1836.3 ± 1.0; panel F (fraction 27), DIT 1375, m/z 1961.6 ± 0.1.

linked glycosylation of peptide b6TL, 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 T4- and T3-forming Site—The data of Table II indicate that the iodine content of peptide b6TL (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 b6TL/mole 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 b6TL, was found in T4 and 3% in T3, as opposed to 21 and 5%, respectively, in bovine Tg. Tyr-1291 contributed 10% of the T3 and 8% of the T4 content of Tg. The relative amounts of iodine incorporated into iodothyronines and iodo-tyrosines were 1 versus 5 in peptide b6TL, 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 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 b6TL 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

Asn-1346 Is Not Glycosylated—The sole putative site of N-
FIG. 5. Combined analysis by FAB/MS and Edman degradation of the HPLC fractions of the endoproteinase Asp-N digest of peptide b6TL, 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⁺ and DHA ions expected for peptide 1290–1303 containing the already mentioned modifications of Tyr-1291, and of Tyr-1291, m/z = 1724, and DIT 1291, m/z = 1850; panel B (fraction 29), Tg 1291, m/z = 2066, and Tg 1291, m/z = 2192. The FAB mass spectra collected after one cycle of Edman degradation (middle part of panels A and B) revealed the same 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 MIT 1291, m/z = 1735; panel B (fraction 29), Tg 1291, m/z = 1951, and Tg 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.

peptide b6TL, 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 as, or greater than, the amount of DHA formed at Tyr-1375. Modification of Tyr-1375 was inferred from the lack of known phenylthiohydantoin-derivatives of iodinated Tyr-1375, and of MIT and DHA from Tg and DHA from Tg. 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 dimethylaminobenzenselenoic acid (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).

FIG. 6. Combined analysis by FAB/MS, endoproteinase Lys-C digestion, and Edman degradation of the HPLC fractions of the endoproteinase Lys-C digest of peptide b6TL, 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 already mentioned modifications of Tyr-1375; panel A (fraction 13), Tg 1375, m/z = 1615; panel B (fraction 19), Tg 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.

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 NH₂-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 ¹²⁵I (16), and in Tg from human goiters subjected to low-level iodination in vivo with ¹²⁵I (18), was based on the monitoring of the contents of ¹²⁵I or ¹²³I in the automated sequencing cycles, and the determination of the distribution of iodoamino acids. In this regard, although ¹²⁵I 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)lysine, the presence of the latter at positions 5, 926, 986 or 1008, and 1029 was inferred from the lack of known phenylthiohydantoin-derivatives in sequencing cycles when tyrosine was expected, and from differences between the actual and expected tyrosine content of the peptides (20). In another study, the labeling of dehydroalanine residues of bovine Tg with Na⁺¹¹⁷I, and their conversion to labeled aspartic acid with Na⁺¹⁴CN 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
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.

| b6TL | Bovine Tg |
|------|-----------|
| Molar ratio to Tg (660 kDa) | 2 | 1 |
| Fraction of Tg mass | 0.15 | 1.00 |
| Iodine content (% of mass)* | 1.11 | 1.00 |
| Fraction of iodine in Tg (660 kDa) | 0.16 | 1.00 |
| Moles of iodoamino acids/mole of Tg (660 kDa)* | 3.0 | 10.82 |
| 3-Iodotyrosine | 2.53 | 10.82 |
| 3,5-Diiodotyrosine | 2.56 | 14.70 |
| T₄ | 0.08 | 0.98 |
| T₃ | 0.30 | 2.85 |
| Fraction of Tg’s T₄ | 0.08 | 1.00 |
| Fraction of Tg’s T₃ | 0.10 | 1.00 |
| Fractional iodine distribution among iodoamino acids* | 0.28 | 0.20 |
| 3-Iodotyrosine | 0.56 | 0.54 |
| T₃ | 0.03 | 0.05 |
| T₄ | 0.10 | 0.21 |
| Iodine in T₄ + T₃/Iodine in MIT + DIT | 0.19 | 0.36 |

* Averages of at least three determinations.

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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

Fabrizio Gentile, Pasquale Ferranti, Gianfranco Mamone, Antonio Malorni and Gaetano Salvatore

J. Biol. Chem. 1997, 272:639-646. doi: 10.1074/jbc.272.1.639

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