Thyroid Hormonogenesis

IDENTIFICATION OF A SEQUENCE CONTAINING IODOPHENYL DONOR SITES(S) IN Calf Thryoglobulin*

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The formation of dehydroalanine in thyroglobulin is the result of the side chain elimination of an idophenyl group during the thyroid hormone formation from two iodothyrosyl residues. This amino acid is easily converted to labeled alanine (upon reduction with [3H] borohydride) or changed to labeled aspartic acid (upon addition of Na14CN and subsequent acid hydrolysis). The cleavage of the protein by CNBr produced many stainable electrophoretic bands, but the autoradiography indicated the presence of a much smaller number of radioactive species. Although three major species raised attention, because they could be all jointly labeled and were present in all preparations, only a species of 15,900 Da was fully studied. It was isolated and its sequence partially determined by Edman degradation. It was established that this species corresponded to the thyroglobulin fragment between methionines 2,432 and 2,578. This peptide contains two hormonogenic sites (positions 2,555 and 2,569) which are either tyrosyl residues or hormone residues arising from them, and five additional tyrosines all potentially involved as donor sites in the hormonogenesis. Upon treatment with N-chlorosuccinimide, the fragment was split into three smaller peptides of about 2,900, 8,500, and 4,800 Da containing 1, 2, and 2 tyrosyl residues, respectively. Only the 8,500-Da subfragment contained [3H]Ala. This finding strongly suggests that at least some of the tyrosines involved as donor sites in thyroid hormonogenesis are within this peptide and possibly map at positions 2,469 and/or 2,522. Moreover, at minimum levels of iodination, when thyroglobulin contains the lowest number of hormone molecules, dehydroalanine is mostly found in the 15,900-Da peptide.

Thyroid hormones are synthesized with high efficiency within the polypeptide chain of thyroglobulin, the major thyroid protein. This molecule, whose primary structure has recently been determined partially in the rat (1) and completely in bovine and in man (2, 3), consists of two identical subunits of 330,000 Da. Besides glycosylation, amounting to about 10% of the molecule, thyroglobulin undergoes a number of posttranslational modifications (4), the most remarkable of which is iodination. Thyroglobulin contains four major types of iodoaminoacids. The hormones tri- and tetrasidothryonine (T3 and T4) and their precursors, mono- and diiodothyroline. Although calf thyroglobulin contains 73 tyrosyl residues per subunit (2), fewer than one-third are actually iodinated in vivo, and no more than 4-5 will normally couple to form the hormones in the nature protein (5).

In recent years, while the primary sequence of thyroglobulin was actively and successfully investigated by DNA sequencing, other authors from different laboratories have reported the isolation and characterization of some thyroxine rich peptides (6-12). These findings were consistent with the hypothesis, now widely accepted, that thyroglobulin contains only a limited number of acceptor sites for hormone synthesis. Indeed, these sites have been localized at positions 5, 2555, and 2569 for T4 and 2748 for T3 (2). The formation of a hormone molecule, however, involves the coupling of two iodoaminoacids and the formation of a dehydroalanyl residue. This fact raises the question as to whether the donor sites are widely and randomly distributed or, as the acceptor sites, are localized at limited and fixed positions.

MATERIALS AND METHODS

Thyroglobulin Purification

Thyroglobulin was isolated from a single bovine thyroid gland according to a procedure designed to minimize hydrolytic degradation, with no freezing and thawing. The gland, rapidly chilled on ice and transferred from the local slaughterhouse to the laboratory, was cleared of the surrounding tissue and minced into small pieces with scissors. The soluble proteins were extracted three times with 0.1 M sodium phosphate buffer, pH 7.15, and filtered on a Sephacryl S-300 column (1.5 × 250 cm) equilibrated with the latter buffer. Fractions containing thyroglobulin were pooled, dialyzed against distilled water, and freeze-dried. The resulting fluffy powder was stored at -70 °C until used. About 200 mg of electrophoretically homogeneous thyroglobulin were obtained by this procedure from a single gland.

CNBr Cleavage

Thirty-five mg of freeze-dried thyroglobulin were dissolved in 3.0 ml of 70% formic acid in a conical tube to which CNBr crystals (about 700 mg) were slowly added with continuous stirring. The tube was capped, and kept for 18 h at room temperature. The solution (3 ml) was then desalted on a Sephadex G-25 column (PD-10) equilibrated with the latter buffer. Fractions containing thyroglobulin were pooled, dialyzed against distilled water, and freeze-dried. The resulting fluffy powder was stored at -70 °C until used.

Labeling Procedures

Sodium [3H]borohydride (Amersham Corp. 5 mCi) had a specific activity of 16.0 Ci/mmol and sodium [14C]cyanide (Amersham Corp. 500 mCi), 50 mCi/mmol. The former was supplied in alkaline solution (0.1 M NaOH). The latter, obtained in the solid state, was dissolved in 100 ml of anhydrous methanol; appropriate volumes were used for labeling and the remainder was dried under a stream of dry nitrogen.

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Printed in U.S.A.
and stored according to the manufacturer's recommendations.

**Labeling of Native Thyroglobulin**—Ten mg of freeze-dried thyroglobulin were dissolved in 1.5 ml of 0.01 M Tris, pH 8.0. The sample was divided into two identical aliquots. Labeling with $^{14}C$ and $^3H$ was performed by simply adding the labeled compounds (0.2 and 0.8 mCi, respectively) and allowing the solutions to react overnight in capped tubes under nitrogen with constant agitation. Reactions were stopped by addition of 10 µl of acetonitrile and filtration through Sephadex PD-10 columns. After lyophilization, the two labeled thyroglobulin samples were individually digested with CNBr according to the method described above. These samples were stored as powder at −70 °C.

**Labeling of Thyroglobulin CNBr Fragments**—About 10 mg of freeze-dried CNBr fragments were dissolved in 2 ml of a denaturing solvent consisting of 0.01 M Tris, 10% SDS, pH 8.8. The solution was divided into two identical aliquots. Labeling of fragments with $^{14}C$ (0.2 mCi) was performed according to Conslevage and Phillips (14) after full reduction in 0.7 M 2-mercaptoethanol. Labeling with $^3H$ was achieved by adding 0.8 mCi of Na$^3H_2$O in that case reduction with 2-mercaptoethanol was omitted. The solutions were kept in capped tubes under nitrogen overnight at room temperature with constant agitation. Reactions were stopped by addition of 10 µl of acetonitrile and filtration through SephadeX PD-10 columns. The samples were stored in freeze-dried state at −70 °C.

**Gel Electrophoresis**

Two different protocols for SDS-polyacrylamide gel electrophoresis were used: (a) according to Laemmli (15) or (b) as described by Anderson et al. (16), when peptides of low molecular weight were analyzed. Samples were routinely denatured and reduced according to the respective procedures and heated (2 min) in a boiling water bath. In procedure a, electrophoreses in SDS were performed in 20 or 17.5% acrylamide gels cast in the standard Bio-Rad apparatus. Stacking gels were 3.5% acrylamide. In procedure b, 10% acrylamide gels were used throughout. To this purpose the following stock solutions were prepared: (i) separation gel acrylamide 36% T, 5% C (8.2 g of acrylamide, 1.8 g of bis in 100 ml of water); (ii) separation buffer and lower vessel buffer, 1 M Tris, 0.2% SDS adjusted to pH 7.8 with NaOH (upper vessel buffer 0.074 M Tris, 0.1% SDS adjusted to pH 7.8 with HCl). Sample buffer was constituted by 0.139 M Tris, 0.5% SDS, 5 mM dithiothreitol, 20% sucrose. The peptide samples were directly suspended in the sample buffer, in this case, stacking gels (5%) were prepared from a stock acrylamide solution containing 5 g of acrylamide and 1.25 g of bis in 100 ml of water.

Electrophoresis were carried out in a cold room at constant current of about 20 mA until the marking dye (bromphenol blue or Pyronine Y) reached the bottom of the gel. Molecular weight standards were phosphorylase b (M, 92,000), bovine serum albumin (M, 67,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 31,000), soy bean trypsin inhibitor (M, 21,000), lysozyme (M, 14,400), aprotinin (M, 6,500), and glucagon (M, 3,200). Staining was performed in 10% methanol, 50% acetic acid containing 0.25% Coomassie Brilliant Blue (procedure a) or in 25% ethanol, 0.5% formaldehyde, and 0.25% Coomassie Brilliant Blue (procedure b). Staining intensity was monitored by continuous visual inspection. Destaining was obtained by soaking the stained gels in 5% acetic acid and 5% methanol (procedure a) or 25% ethanol, 1% formaldehyde (procedure b) until a clear background was observed. Formaldehyde fixation was performed to prevent the possible loss of low molecular weight peptides (17).

Gels containing radioactive samples were fixed, stained, soaked for 30–45 min in an autoradiography enhancer (Enlightning, Du Pont), dried and finally autoradiographed at −70 °C. Scans of autoradiographs were obtained by using an LKB 2202 Laser ultrascan.

Thin layer chromatography and amino acid analysis procedure was used from preparative purposes, two identical gels were run in a twin chamber (standard Bio-Rad electrophoresis apparatus). Each gel was loaded with approximately 1 mg of material divided in 9 wells (the tenth being used for molecular weight standards). Usually 20–30 min of staining, followed by 1 h of destaining with frequent changes of solvents, were sufficient for a clear visualization of the major bands. The peptides of interest were cut out with a razor blade, collected in a conical plastic tube, and soaked in distilled water. The slices were then equilibrated for 2 h with 0.01% SDS and finally electroeluted in a lace apparatus in 0.01 M sodium phosphate buffer, 0.01% SDS, pH 8.0. The electroeluted peptides (normally 100–200 µl of solution) were precipitated according to the method of Wessel and Flugge (18). Four volumes of ice-cold methanol were mixed with one volume of peptide sample in an Eppendorf microcentrifuge tube. The sample was thoroughly mixed and centrifuged. Then one volume of cold chloroform was added. The tube was vortexed and centrifuged. Three volumes of cold water were subsequently added. The sample was again vortexed and centrifuged. The upper phase was carefully decanted, and three volumes of cold methanol were added to the lower phase. The sample is vortexed once again and centrifuged. The supernatant was removed, and the protein pellet was dried under a stream of nitrogen. The purity of this material was checked by SDS-gel electrophoresis (procedure a). The peptides obtained by this method could be used directly for amino acid analysis after hydrolysis or for sequencing studies by automated Edman degradation. Although the overall recovery of material by this procedure appears to be excellent, nevertheless it was impossible to perform accurate estimation of the real amount of peptides recovered by traditional methods of determination of protein concentration.

Automated Edman Degradation

The N-terminal sequence of the 15,900-Da peptide was partially determined by automated Edman degradation (16 amino acids) in the Department of Biochemistry and Biophysics, University of California, Davis, CA.

The analyses were performed on Applied Biosystem 470-A gas-phase sequencer (separativity was 200–800 pmol) using 5–10 µg of samples in presence of 1.2 mg of Polybrene. Residues were identified by two independent HPLC methods (19, 20).

**Hydrolysis in Constant Boiling Hydrochloric Acid**

The 15,900-Da peptide and the mixture of peptides ranging from 19,000 to about 21,000 Da were transferred into two glass ampoules and dried under a stream of air. The ampoules were filled with 1.5 ml of constant boiling HCl (Pierce), sealed under vacuum, and heated in a dry oven at 100 °C for 24 h. The ampoules were then opened, dried in a Speed-vac (Savant) evaporator, and the resulting residues dissolved in citrate buffer (0.2 M, pH 2.2). The hydrolysate of the 19,000–21,000-Da mixture was used to detect the presence of labeled alanine or aspartic acid by TLC (see below). The hydrolysate of the 15,900-Da peptide was used for amino acid analysis.

**Thin Layer Chromatography and Amino Acid Analysis**

Two-dimensional chromatography was performed on Alttech, cellulose MN300 plates (250 µm, 20 × 20 cm). Approximately 10 µg of alanine or aspartic acid were added to the purified 19,000–21,000 Da species which had been labeled with $^3H$ or $^{14}C$, respectively. The solvent system used for the first and second dimension were n-butanol/acetone/water (200:100:50 v/v), respectively. After drying, the plates were sprayed with ninhydrin. The stained spots corresponding to alanine and aspartic acid were scraped off the plate, suspended in scintillation liquid (Ultrafluor, National Diagnostics), and counted in a Packard (model 3390) liquid scintillation spectrometer.

The amino acid analysis was performed on a Beckman 6300 amino acid analyzer. Fractions were collected every 2 min and counted as above.

**Cleavage of the 15,900-Da Peptide**

The cleavage of the 15,900-Da peptide at the tryptophanyl residues was accomplished according to Lischwe and Ochs (21). The products of this cleavage were analyzed by acrylamide gel electrophoresis for low molecular weight peptides (procedure b). After staining and drying, the gels were autoradiographed as described.

**Isoeicynic Centrifugation**

Fifty-two mg of lyophilized $[^3H]$thyroglobulin (about 1 × 10$^6$ cpm) and 22 g of RBCl were dissolved in 12.3 ml of Tris-HCl 0.01 M, pH 7.2, plus 30 ml of water to give a final density of 1.34 g/ml. The solution was divided in 8 aliquots of 6 ml each which were loaded into polycarbonate tubes. The isoeicynic centrifugation was carried out according to Curtiss et al. (22) in a 70 Ti rotor (Beckman) for 3 days at 34,000 rpm and 20 °C. At the end of the run, gradients were gently aspirated from the bottom with a peristaltic pump connected to a fraction collector. Corresponding fractions (200 µl) from each tube were pooled to give 38 fractions of 1.6 ml each. Protein concentration was estimated spectrophotometrically using an E$^2$ of 10 at 280 nm, or when more precise values were required, according to Lowry et al. (23).
Iodine Determination

Fractions 1–7, 12–13, and 20–23 were pooled and dialyzed against 0.01 M Tris-HCl buffer, pH 7.2. The protein concentration of each pool was adjusted to 0.8 mg/ml and iodine determination carried out as previously described (23). Iodine content was 1.12, 0.56, and 0.68% (w/w) for the three pools and 0.66% for the unfractinated thyroglobulin, respectively.

RESULTS

Occurrence of Dehydroalanine in Bovine Thyroglobulin—

Native thyroglobulin or its CNBr fragments were reacted with NaB$_3$H$_4$ or NaI$_4$CN to convert dehydroalanine residues (13) into [3H]alanine or [14C]cyanoalanine, respectively. Since, upon acid hydrolysis [14C]cyanoalanine is converted to aspartic acid (24), the hydrolysate should contain radioactive alanine and aspartic acid, respectively, which can be identified into [3H]alanine or [14C]cyanoalanine, respectively. Since, parallel by SDS-electrophoresis under reducing conditions upon acid hydrolysis [14C]cyanoalanine is converted to aspartic acid by about 90,000, 21,000 and 16,000 Da, respectively, suggests the occurrence of dehydroalanine within their polypeptide chains. However, isolation and characterization of these fragments was necessary to confirm that dehydroalanine was the material being modified by NaB$_3$H$_4$ and NaI$_4$CN. The polypeptide of about 90,000 Da was judged not suitable for further analysis on account of its large size, its high content of tyrosines (19), and low specific activity.

Identification of Dehydroalanine Containing Sequences in Thyroglobulin—

Fig. 3 depicts several CNBr fragments (from 14C- (left panel), and 3H-labeled thyroglobulin (right panel), respectively), which were purified by electrophoresis from preparative gels. Although the two panels of this figure show several fragments after their purification by electrophoresis from preparative gels, only the fragments having molecular masses of 15,900 and about 21,000 Da were further purified by quantitative precipitation by organic solvents (see “Materials and Methods”). It is evident that, while the 15,900-Da species obtained from either [14C] or [3H]thyroglobulin appears as a single electrophoretic band (lane 1, left panel, and lane 6, right panel, of Fig. 3), the 21,000-Da fragment appears rather broad, (lane 2, left panel) or even resolved into three species (lane 5, right panel of Fig. 3). Interesting was the finding that, following a second CNBr cleavage, the 21,000-Da species substantially disappeared (lane 2, Fig. 4). Despite some increase in background, presumably due to degradation which may have occurred because of the prolonged exposure of the peptides to 70% formic acid, the relative amount of the 15,900-Da species remains essentially unchanged. A major species of about 17,000 appears together with a faint band of about 19,000 Da. Moreover, although the Edman degradation analysis of this material was two times hampered by the simultaneous presence of several N-terminals (in accord with

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**Fig. 1.** Left panel, electrophoretic profile (Coomassie Blue staining) in SDS of peptides obtained by CNBr cleavage of calf thyroglobulin on a 17–22.5% acrylamide gel. From left: molecular weight standards (Bio-Rad); CNBr fragments labeled with NaB$_3$H$_4$ (lane 1); CNBr fragments labeled with NaI$_4$CN (lane 2); unlabelled fragments (lane 3, control). Each lane contained 80 µg of fragments. Right panel, autoradiography of lanes 1 and 2. A much higher activity was attained using NaB$_3$H$_4$. The major bands in common are peptides having molecular mass of 90,000, about 21,000 and 16,900 Da. Peptides of molecular weights higher than 24,000 are products of incomplete cleavage of methionines.

**Fig. 2.** Left panel, electrophoretic profile (Coomassie Blue staining) in SDS of peptides obtained by CNBr cleavage of calf thyroglobulin on a 20% acrylamide gel. In this case labeling with [3H] or [14C] was performed after CNBr cleavage. From left: molecular mass standards from 92,000 to 14,500 Da (Bio-Rad); CNBr fragments (100 µg) labeled with NaB$_3$H$_4$; CNBr fragments (80 µg) labeled with NaI$_4$CN; CNBr fragments (100 µg) labeled with NaI$_4$CN; unlabelled fragments (100 µg, control). Right panel, autoradiograph of lanes 2 and 3. Peptides of 15,900 and about 21,000 daltons are clearly visible in both lanes.
The peptide having a mass of about 21,000 Da was analyzed by two-dimensional TLC. About 5,500 cpm of $^3$H and 2,800 cpm of $^{14}$C were applied to the plates. Before chromatography, 10 $\mu$g of cold alanine or aspartic acid were added to the appropriate hydrolysate. After chromatography, the plates were dried and stained with ninhydrin. The spots, relative to alanine and aspartic acid, were subsequently scraped off the plates and counted. The distribution of radioactivity was as follows: 11% at the origin and 41% in the alanine spot, 16% at the origin, and 51% in the aspartic acid spot.

Both sets of data are in accord with the presence of dehydroalanine residues in the respective polypeptide chains.

Localization of the 15,900-Da Peptide within the Primary Structure of Bovine Thyroglobulin—This peptide was obtained by the usual electrophoretic technique from unlabeled CNBr-cleaved thyroglobulin and was further purified by precipitation with organic solvents (see “Materials and Methods”). The precipitate, which was devoid of detectable amounts of glycine was subjected to Edman degradation (see “Materials and Methods”). The N-terminal sequence of 16 amino acid residues was found to be V-S-(C)-L-R-Q-E-P-A-R-I-L-N-D-A-Q. This is identical with the calf thyroglobulin sequence following methionine 2,432, as determined by nucleic acid sequence (2). The entire peptide (between methionines 2,432 and 2,578) contains 7 tyrosyl residues; they map at positions 2,460, 2,469, 2,522, 2,545, 2,546, 2,555, and 2,569.

Identification of “Possible” Donor Tyrosines—The identification of the donor tyrosines was made possible by the fortuitous occurrence of 2 tryptophan residues within the 15,900-Da peptide at positions 2,461 and 2,544. Upon the reaction of the $^3$H-labeled 15,900-Da peptide with N-chlorosuccinimide in urea-acetic acid solution, the possible fission of these two tryptophanyl bonds should yield to three peptides of 28, 82,
and 33 amino acids, containing 1, 2, and 2 tyrosyl residues, respectively. If any of the tyrosines has been changed to a dehydroalanine residue as a consequence of the coupling reaction, after labeling with \(^{3}H\)NaBH\(_{4}\), the corresponding peptide should be radioactive. Fig. 6 shows that the splitting of the 15,900-Da band resulted in the appearance of a new radioactive peptide, whose mass, estimated by relative migration in SDS-gel electrophoresis, was close to 8500 Da. Except for this species and some uncleaved material (which, as expected (20), amounts to about 50% of the total), no other smaller radioactive fragment could be detected. This finding strongly suggests that tyrosine 2,469 and/or 2,522 are donor tyrosines, since only these 2 residues lie in the 8500-Da peptide fragment.

Fig. 7 shows schematically the position of the two tryptophans (closed triangles), tyrosines (vertical bars), and methionines 2,432 and 2,578 (closed circles).

The "Possible" Favored Donor Site(s)—The foregoing results indicated the presence of dehydroalanine in the 15,900-Da peptide but could not rule out the existence of other donor sites in thyroglobulin. In particular, besides the 21,000-Da species which appeared to arise from partial cleavage of methionyl bonds, the peptide of 90,000 Da may contain dehydroalanine residues as well. In fact, this species which is generated by the splitting at methionines 785 and 1551, could be labeled with \(^{3}C\) or \(^{1}H\) (see right panels of Figs. 1 and 2). Purification of this peptide by preparative electrophoresis followed by electrophoresis was not particularly difficult (Fig. 3, right panel, lane 1), but its large size and the number of tyrosyl residues (19) contained within its polypeptide chain, discouraged any effort to accomplish its full characterization.

Indeed, other two peptides of about 31,000 and <14,000 daltons could apparently fit the criteria chosen to select the fragments of “interest.” Nevertheless, it is clear that the species of 31,000 daltons derives from incomplete CNBr cleavage, since from the protein sequence and relative position of methionines (2), this peptide should not be found. On the other hand the smaller fragment (<14,000 daltons) is not always present. In Fig. 8, for instance, this peptide is not apparent.

For this purpose, native \(^{3}H\)-labeled thyroglobulin was fractionated according to its iodine content by isopycnic gradients on RbCl (Fig. 8, left panel). Fractions 1–7, 12–13, and 20–23 were pooled to constitute three homogenous samples whose concentration was adjusted exactly to 0.80 mg/ml. The iodine content of these three thyroglobulin samples was 1.12, 0.56, and 0.08% w/w, respectively. Each of these samples was then subjected to CNBr cleavage and the products analyzed by SDS-gel electrophoresis (method a, “Materials and Methods”) followed by autoradiography (right panel of Fig. 8) and scanning of the autoradiograph. Although the total amount of protein loaded in each lane was the same, both a visual inspection and a quantitative estimation (obtained through the scanning of the autoradiograph) indicated a remarkable decrease in the relative amount of radioactivity of the 90,000-Da species as compared to the 15,900-Da peptide. In fact, at high levels of iodine the radioactivity in the 90,000-Da peptide amounts to about 30–35% of the total (left channel of right panel in Fig. 8); at low levels of iodination the amount of radioactivity in the same species is about 5% of the total (right lane of right panel of Fig. 8). The relative amount of radioactivity in the 15,900-Da species is about 20% in the iodine-rich fragments and about 35% in the iodine-poor material. This indicates that when the iodine available is low, and consequently, a limited number of dehydroalanine residues have been formed as a consequence of the synthesis of thyronines in thyroglobulin, the donor tyrosines are those contained within the 15,900-Da peptide and, on the basis of the previous observations, are localized at positions 2,469 and/or 2,522.

**DISCUSSION**

Normal thyroglobulin contains between 10–50 iodine atoms per molecule. Less than one-third of its tyrosines are iodinated.
to form monoiodotyrosines (MIT) and diiodotyrosines (DIT), while a much smaller number of these residues is set apart for the coupling reaction to synthesize the thyroid hormones (T₄ and T₃). Considering the large size of the protein, which is composed by two identical half-sized subunits of 330,000 Da, the entire process appears to be wasteful. However, the efficiency of thyroglobulin becomes evident when the supply of the halogen is scarce. Although at iodine content below 0.05% w/w, no hormone is found (25, 26), at slightly higher levels, i.e. between 0.2-1.0% w/w iodine, the number of hormone residues increases linearly with the halogen content (27, 28).

To date four hormonogenic tyrosines (acceptor sites) have been precisely localized within the polypeptide chain of calf thyroglobulin; these sites map at positions 5, 2555, 2569, and 2748. The two subterminal positions (5 and 2748), which are believed to be the favored sites for T₄ and T₃, respectively, appear to have different evolutionary origins, since they do not share any structural similarity (2). The two intermediate positions (2555 and 2569) lie in a region of the molecule that contains an unusually high concentration of tyrosine residues and apparently possess a well-organized structure. In fact, the equivalent region of rat thyroglobulin, which has an 80% sequence homology with bovine thyroglobulin, displays an α-helix content of about 50%, that is well above the average of about 22% (35). These findings suggest that this part of the molecule may play a special role in thyroglobulin structure-function relationship.

Although the hormone-containing sites (acceptor sites) have been clearly identified, it is not known yet whether the donor sites are predetermined by the tertiary structure of the protein or, alternatively, any iodotyrosine, at random, may become the donor site.

The intramolecular-coupling reaction implies the fission of an iodotyrosyl residue next to its aromatic ring, leading to the formation, in addition to the hormone, of a modified residue at the donor site. Various hypotheses have been devised to describe the molecular rearrangements necessary to assemble two iodotyrosines into iodothyronine and how the "lost side chain" is eliminated (29-33). However, while the detailed mechanism by which hormone formation takes place is still awaiting clarification, dehydroalanine has been clearly identified as the product of the lost side chain (13, 29, 32, 34).

The present work exploits the presence of dehydroalaninyl residues in thyroglobulin to localize the donor sites in its polypeptide chain. The identification and isolation of a dehydroalanine-containing sequence in a CNBr fragment of thyroglobulin has been made possible by taking advantage of its activated olefinic bond which can be labeled with NaB₃H₄ or Na¹⁴CN. Since the reaction of these compounds with a dehydroalanine-containing sequence in a CNBr fragment of thyroglobulin, is concrete.

To show that dehydroalanine was the material being specifically modified, the matched peptides, i.e. those having the same size and labeled by H as well as C, were isolated and individually hydrolyzed and the presence of dehydroalanine residues was confirmed by the presence in the hydrolysates of [¹³H]alanine and [¹⁴C]aspartic acid, respectively.

Twenty-five CNBr fragments are theoretically expected upon cleavage of calf thyroglobulin, since 24 methionyl residues are present within its subunit polypeptide chain. However, several methionines are very close to each other, in some cases even contiguous; for this reason the number of electrophoretic bands is significantly smaller than predicted from the number of methionyl residues present in the protein. Besides a large fragment of about 90,000 Da, all the other theoretically expected fragments (from the known sequence (2)) should have molecular weights lower than 24,000. Indeed, electrophoretic patterns obtained from fully denatured and reduced thyroglobulin revealed that some methionines survived the CNBr treatment, since some stainable peptides had molecular mass higher than 24,000 Da (see Figs. 1 and 2).

The largest of the three polypeptide species potentially capable of containing dehydroalanine, i.e. the species derived from the cleavage of methionines 788 and 1,550, was not investigated because its size and its 19 tyrosyl residues made this very difficult. The remaining two peptides had masses of 15,900 and about 21,000 Da, respectively, and their amino acid analysis confirmed the presence of dehydroalanine. The smaller peptide was homogeneous, since determination of the sequence of 16 amino acids was easily accomplished. The analysis of the 21,000-Da peptide, in contrast, was hampered by its heterogeneity. There is an indication, however, that the 21,000- and 15,900-Da peptides are related, since in one partially successful Edman degradation, seven cycles were performed on the 21,000-Da peptide. Residues 1, 2, 4, 6, and 7 were clearly identified. They were identical to the corresponding amino acids in the 15,900-Da peptide. Moreover, when thyroglobulin fragments were subjected to a second CNBr cleavage and analyzed by electrophoresis, the 21,000 Da substantially disappeared giving rise to a new peptide of about 17,000 and faint species of 19,000 Da, respectively. An interpretation of this finding is that there are flanking regions, of appropriate size, at both ends of the 15,900-Da peptide with methionyl residues only partially susceptible to CNBr cleavage. Unfortunately no direct data on this issue are available to date. However, since this region of the thyroglobulin molecule appears to be highly folded (35), the possibility that methionines 2,431 and/or 2,586 were only partially accessible to CNBr, is concrete.

The 15,900-Da peptide was located between methionines 2431 and 2588. It contains 2 hormonogenic acceptor tyrosines, residues 2,555 and 2,569, and 5 additional tyrosines (2,460, 2,469, 2,522, 2,545, and 2,546). After cleavage at the 2 tryptophans of the 15,900-Da peptide, it appeared that the residues involved in hormonogenesis were residues 2,469 and/or 2,522 since only the fragment containing these residues was labeled by H, and therefore contained dehydroalanine. Due to the low recovery of the 15,900-Da peptide, stoichiometry could not be precisely determined nor could it be determined whether one or two iodophenyl groups were involved in the process.
One or both of the donor residues, 2,469 and 2,522, are implicated in the "coupling" at an early stage of thyroglobulin iodination, i.e., when the number of thyroid hormone residues per molecule does not exceed 1 or 2. Under these conditions, the only peptide containing a dehydroalanine residue is the 15,900-Da peptide. Since it has been reported that the two subterminal positions of thyroglobulin are the favored acceptor sites for hormone synthesis (2), it could be inferred that there is a preferential coupling between the subterminal acceptor residues 5 and 2,748 and the donor tyrosines 2,469 and 2,522.

Acknowledgments—I wish to express my gratitude to Dr. Jacob Robbins, Dr. Hans Cahnmann, and R. Jan Wolff for critical discussions, continuous advice and support and to Robert Boykins for the amino acid analysis. Finally, I wish to express my gratitude to Professor Gaetano Salvatore who first raised my interest for thyroperoxidase residues implicated in the "coupling" at an early stage of thyroglobulin iodination, for his unforgettable scientific and human instruction.

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