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The Earliest Site of Iodination in Thyroglobulin Is Residue Number 5*

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In most highly structured native proteins, as well as in thyroglobulin, the reactivity *in vitro* of the various tyrosyl residues toward iodine is widely different. The present work demonstrates that of nearly 70 tyrosyl residues present in rat thyroglobulin, there is one, residue number 5 from the NH_2-terminal end, which has *in vivo* the highest affinity toward iodine, being the first one to be iodinated.

In fact, when 6-(*n*-propyl)-2-thiouracil (PTU)-treated, iodine-deficient animals were injected with ^{125}I and killed shortly after, we isolated from thyroid glands poorly iodinated thyroglobulin (about 1 iodine atom/thyroglobulin molecule), nearly 90% of the radioactivity of which was found as monoiodotyrosine. Although CNBr cleavage of this protein gave several fragments after gel electrophoresis only one of these, with apparent mass 27,000 Da, contained ^{125}I. This fragment was isolated and fully characterized. Twelve cycles of automated Edman degradation were performed; the sequence found, *i.e.* N-I-F-E-*X*-Q-V-*X*-A-Q-*X*-L, indicated that the 27,000-Da fragment is the NH_2 terminus of thyroglobulin. This portion of the polypeptide chain contains several tyrosyl residues which may well all be potentially involved in the early iodination of the protein. The observation that the removal of seven amino acids from the NH_2 terminus is accompanied (at the fifth step) by the total disappearance of radioactivity in the resulting shortened peptide suggested that the fifth residue was the only one iodinated under these conditions. A second, more quantitative experiment was performed on thyroglobulin obtained from 6-(*n*-propyl)-2-thiouracil-treated animals whose death was postponed 24 h after the injection of ^{125}I. In this case the radioactivity was found not only in a single CNBr fragment (27,000 Da) but also in other discrete species of lower molecular mass. The mixture of these peptides was subjected to seven steps of manual Edman degradation. Fragments before and after partial degradation were run in parallel on a polyacrylamide gel and the distribution of ^{125}I compared. Besides some change in the background, the two profiles were identical except for the absence of the 27,000-Da species. This proves that all the ^{125}I present in the 27,000-Da species was localized at the fifth residue, the same site at which the hormone molecule is preferentially synthesized under normal conditions. This result is not unexpected and is in accord with the known properties of thyroglobulin which has a polypeptide chain designed for efficient synthesis of the hormone even at low levels of iodination.

Thyroglobulin is the polypeptide matrix necessary for the synthesis of thyroid hormones. It is a glycoprotein (10% w/w in carbohydrate) of 660,000 Da having a sedimentation coefficient of 19 *s* and consists of two identical chains of 330,000 Da. Several years ago Edelhoch (1) demonstrated that thyroglobulin has two types of tyrosyl residues which behave differently on iodination. Indeed, of about 140 tyrosines in the thyroglobulin dimer only 25–30 are ordinarily iodinated and a much smaller number of the resulting iodotyrosines (to a maximum of 6–8) may undergo coupling to form 3,5,5′-triiodothyronine and thyroxine (2). The coupling reaction takes place by transfer of an iodophenyl group from a "donor" site to an "acceptor" site, where the hormone molecule actually is formed. Both iodination and coupling are catalyzed by a membrane-bound thyroid peroxidase. Although the maximum number of hormone residues is not very large, the efficiency of thyroglobulin as a hormonal precursor is remarkably high even under severe restriction of iodine intake. Indeed, under such conditions a significant part of the iodine is found in iodothyronines (3). To date various observations have suggested that (i) iodination of thyroglobulin tyrosines proceeds according to an established order (4), (ii) early iodinated tyrosyl residues are preferentially involved in thyroxine synthesis (5), and (iii) the subsequent hormone formation needs stringent steric conditions for efficient coupling of iodoaminoacids (6). This means that the 25–30 (out of about 140) residues ordinarily iodinated under physiological conditions are far from being equivalent in respect to their individual reactivity toward iodine.

So far five hormonogenetic tyrosines have been characterized within the primary structure of thyroglobulin in various animal species (7–13). These locations have been deduced from available cDNA nucleotide sequences of bovine, human, and rat thyroglobulin (14–16): they map at residues 5, 2555, 2569, and 2748 (bovine thyroglobulin); an additional site (position 1291) has more recently been described in rabbit thyroglobulin (13); the only donor iodotyrosine so far described has been localized in bovine thyroglobulin within a CNBr fragment (between residue 2451 and 2597) (17). The aim of the present work is to demonstrate the occurrence *in vivo* of a site of early iodination and to find out where within the polypeptide chain of rat thyroglobulin this site is localized.

EXPERIMENTAL PROCEDURES

Chemicals

Acrylamide monomer and *N,N′*-methylene-bis-acrylamide were from Bio-Rad; AcrylAide cross-linker and GelBond PAG were from FMC BioProducts; cyanogen bromide and 6-(*n*-propyl)-2-thiouracil were purchased from SERVA; $Na^{125}I$ was from Amersham Corp.

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Solvents for HPLC[1] were J. T. Baker Chemical Co. HPLC-grade. All other chemicals were reagent-grade, except those for Edman degradation which were obtained from Pierce Chemical Co.

19S-Thyroglobulin Purification and Iodine Determinations

Twenty-three Wistar male rats (about 400 g each) were all fed a commercial low iodine diet (Morini, Italy) for 4 weeks. 6-(*n*-Propyl)-2-thiouracil was added to the drinking water (0.02%, w/v) sweetened with a small amount of sucrose. The animals were divided into three groups named B (11 animals), C, and D (six animals each). Six additional rats, from the same source, (group A), were kept for the same period on a normal diet (controls). About 30 h before death, 6-(*n*-propyl)-2-thiouracil treatment was suspended; the following day each animal, controls included, received by intraperitoneal injection 75 μCi of $Na^{125}I$. Animals from groups A and B were killed within 90 min after the injection; animals of groups C and D were killed 24 h later.

Thyroglobulin from control and iodine-deficient rats was isolated from glands under conditions designed to minimize the autolytic breakdown of the protein. Thyroid glands were collected, cleaned of surrounding tissues, and minced with scissors. The soluble proteins were extracted three times with 0.1 M sodium phosphate buffer, pH 7.15, fractionated by ammonium sulfate precipitation (1.4–1.75 M), briefly dialyzed against the extraction buffer and finally purified by sucrose gradient centrifugation in an SW 41 rotor at 26,000 rpm for 20 h at 20 °C. Fractions containing thyroglobulin (the profiles of radioactivity and optical density being superimposable in all cases) were pooled and desalted by filtration on G-25 prepacked columns (P_D-10, Pharmacia LKB Biotechnology Inc.) equilibrated with distilled water. Finally they were freeze-dried. The resulting fluffy powders were stored at −80 °C until used. The homogeneity of each preparation was tested by analytical gel electrophoresis in sodium dodecyl sulfate (see below).

Iodine content of each preparation was determined according to Palumbo *et al.* (18).

CNBr Cleavage

Freeze-dried thyroglobulin samples (from groups A–C) were individually dissolved in 0.5–1.0 ml of 70% formic acid in a conical tube to which CNBr crystals were slowly added (10 mg/mg of protein) with continuous stirring. The tubes were capped and kept for 18 h at room temperature. Preparation D was treated similarly, but a second aliquot of CNBr crystals was added to the sample after 4 h. The ratio CNBr/protein (w/w) was in this case two times higher than usual. The solutions were then desalted on a Sephadex G-25 column (PD-10) equilibrated with distilled water, freeze-dried, and stored at −80 °C until used.

Gel Electrophoresis

Samples were denaturated and reduced in a boiling water bath in the presence of 1% SDS and 0.75 M 2-mercaptoethanol. Electrophoresis was performed in 15–20% or 17.5–23.5% gradient acrylamide gels, cast in a Bio-Rad apparatus. The acrylamide stock solution contained 30% acrylamide, 0.2% bis-acrylamide, and 1% AcrylAide. The latter reagent was used to tightly bind the polymerized gel to a sheet of polyester (GelBond PAG) which was included in the glass sandwich. When GelBond and AcrylAide were omitted, the bis-acrylamide concentration was 0.8%. Stacking gels were 3.5% acrylamide. The buffer system was the same as described by Laemmli (19). Electrophoresis was carried out in a cold room at a constant current of about 20 mA until the marking dye (bromphenol blue) reached the bottom of the gel. Molecular weight standards were from commercial sources (Bio-Rad and Sigma).

Staining was performed in 10% methanol, 50% acetic acid containing 0.25% Coomassie Brillant Blue. Destaining was obtained by soaking the stained gels in 25% isopropyl alcohol and 10% acetic acid. Normally 50–70 μg/well of sample were loaded on the gels.

When electrophoresis was used for preparative purposes, two identical gels were run in a twin chamber (standard Bio-Rad electrophoresis apparatus). Each gel was loaded with approximately 700 μg of material divided in nine wells (the tenth being used for molecular weight standards). The conditions were the same as described above. The gels, bound to the polyester support, were air-dried and autoradiographed. The peptides of interest were cut out with a razor blade, collected in a conical plastic tube, and briefly soaked in distilled water. The slices were then equilibrated for 2 h with 0.01% SDS and finally electroeluted in an Isco apparatus in 0.01 M sodium borate buffer, 0.01% SDS, pH 8.3. The electroeluted peptides (normally in 100–200 μl of solution) were precipitated according to the method of Wessel and Flugge (20). Four volumes of ice-cold methanol were mixed with 1 volume of peptide sample in an Eppendorf microcentrifuge tube. The sample was thoroughly mixed and centrifuged. Then 1 volume of cold chloroform was added. The sample was vortexed and centrifuged. Three volumes of cold water were subsequently added. The sample was again vortexed and centrifuged. The upper phase was carefully discarded, and three volumes of cold methanol were added to the lower phase. The sample was 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. The peptides obtained by this method could be used directly for automated Edman degradation or, after extensive Pronase digestion, for iodo-amino acid detection by HPLC (see below).

Autoradiography

Autoradiographs were obtained by using Kodak X-OMAT AR films in a X-Omatic cassette equipped with two intensifier screens.

Edman Degradation

Automated—The NH_2-terminal sequence of the 27,000-Da peptide (obtained by electroelution from preparation B), was determined by automated Edman degradation (12 amino acids) in the Department of Biochemistry and Biophysics, University of California, Davis. The analyses were performed on an Applied Biosystems 470 A gas-phase sequenator (sensitivity was 200–800 pmol) using 5–10 μg of sample in the presence of 1.2 mg of Polybrene. Residues were identified by two independent HPLC methods (21, 22).

Manual—After CNBr cleavage of protein (from groups B or D) the resulting fragments were simultaneously subjected to manual Edman degradation. In brief, to 100 μl of sample solution in water (about 500,000 cpm), 100 μl of 5% (v/v) phenylisothiocyanate in distilled pyridine was added in a capped tube under dry nitrogen. The mixture was kept for 1 h at 45 °C. The excess of unreacted phenylisothiocyanate was removed by extraction with five 500-μl volumes of benzene. The lower phase was dried under nitrogen, and 50 μl of trifluoroacetic acid added. The sample was reincubated for 10 min at 45 °C and dried again under nitrogen to eliminate the trifluoroacetic acid excess. One-hundred μl of 0.1 M HCl was then added to the tube and the PTH was extracted (twice) with 600 μl of 2-chlorobutane (Pierce Chemical Co.). The PTH, the benzene extract (used to eliminate the phenylisothiocyanate excess), and the resulting peptides each lacking the NH_2-terminal amino acid were counted for ^{125}I. Seven cycles of Edman degradation were performed. When this was done with the purified 27,000-Da species (from B), no significant counts survived after the seventh cycle. When the procedure was performed on all the fragments from preparation D, the material still contained a significant amount of the original radioactivity and was subjected to analytical electrophoresis. The gel was dried and sliced for counting.

Iodo-amino Acid Distribution by HPLC within the Preparations and Purified Fragments (27,000 Da)

Iodo-amino acid distribution in the various preparations of ^{125}I-thyroglobulin and in the M_r = 27,000 fragment was determined by HPLC on a Gilson model 302 apparatus equipped with a holochrome UV detector (model 116), a Data Master (model 620), and a fraction collector (model 202). Samples were digested overnight at 37 °C with 0.5% (w/v) Pronase (Behring Diagnostics) in 30 mM Tris-HCl, 110 mM NaCl, pH 8.4, in the presence of 50 mM methylmercaptoimidazole. Aliquots containing about 50,000 cpm of each sample were diluted to 70 μl with water. The volume was then brought to 100 μl by adding 30 μl of a solution containing 1 μg each of the following internal standards: KI, monoiodotyrosine, diiodotyrosine, 3,5,5′-triiodothyronine and thyroxine in methanol ammonia (99:1).

Samples were injected into a Brownlee RP-8 Spheri-5 HPLC column (4.6 × 250 mm) previously equilibrated with 10% of solvent A (acetonitrile) and 90% of solvent B (25 mM ammonium acetate, pH 4). Both solvents contained 0.1% trifluoroacetic acid. The following non-linear gradient was used: solvent A concentration was initially increased from 10 to 32% in 5 min, brought to 30% within 7

[1] The abbreviations used are: HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate; PTH, phenylthiohydantoin.

min, to 35% within 3 min, and to 38% in 5 min. The percentage of solvent A was then kept constant for 10 min and finally lowered to the initial value (10%) within 10 min. Fractions of 0.75 ml were collected at a flow rate of 1.5 ml/min and subsequently counted. The labeled iodo-amino acids were identified by comparing the retention times of the standards (optical profile at 290 nm) with those of the radioactive peaks. The relative amount of each iodo-amino acid was expressed as percent of the loaded counts.

RESULTS

Thyroglobulin

All thyroglobulin preparations showed no signs of degradation as judged by analytical SDS-gel electrophoresis. Indeed in all cases thyroglobulin appeared as two closely migrating bands of about 330,000 Da.

The sources from which the thyroglobulin preparations were obtained and the iodine contents are summarized in Table I. Animals from group A and B were killed 90 min after injection of radioiodine, and preparations C and D 24 h later. The number of iodine atoms/mol of protein was less than 1 in preparations B–D and 33 in preparation A (control).

Cyanogen Bromide Cleavage

The electrophoretic profiles of CNBr fragments obtained from thyroglobulin isolated from the three groups (A–C) were essentially indistinguishable, as judged by Coomassie Blue-stained acrylamide gradient gels. A typical pattern is shown in Fig. 1 (*right lane*). Nearly 30 fragments ranging between M_r = 45,000 and 12,000 were clearly stainable. Actually 24 fragments are to be expected by CNBr cleavage on the basis of the known sequence of the rat protein. Very likely fragments having sizes greater than 30,000 Da arise from incomplete CNBr cleavage. Fig. 1 (*left lanes*) shows autoradiography after gel electrophoresis of the peptides obtained by CNBr treatment of the various preparations (CNBr fragments from preparation D are not shown; they were obtained by a slightly modified procedure (see "Experimental Procedures") and analyzed separately. It is evident that all preparations contain a fragment with molecular mass close to 27,000 Da. This species appears to be essentially the only one in preparation B. To gain a quantitative estimate of the relative abundance of each ^{125}I-labeled fragment from every preparation (*A–C*) each slot of the gel was cut and sliced. Sixty slices were obtained (1.5 mm each) and the radioactivity counted. Fig. 2 shows the profile of the radioactivity *versus* the slice number. The slice containing the fragment of 27,000 Da is in all preparations slice 29 and represents the most abundant species in preparations A and B and a significant one in preparation C, in which the label appears to be more evenly distributed. Cleavage of thyroglobulin from animals belonging to group D was performed differently, *i.e.* by doubling the molar ratio of CNBr/protein. With this procedure all species having molecular sizes higher than 30,000 Da disappeared, while the remaining profile remained unchanged and was superimposable on the pattern obtained from preparation C.

TABLE I

Protocol for low iodine diet (LID), propylthiouracil (PTU), iodine injections, and iodine content of various thyroglobulin (Tg) preparations

	Group			
	A (6)[a]	B (11)	C (6)	D (6)
LID + PTU	−	+	+	+
^{125}I (75 μCi, intraperitoneal)	+	+	+	+
I (atoms/molecule Tg)	33.0	≃1	≃1	≃1
Elapsed time injection-death (h)	1.5	1.5	24	24

[a] Number of rats is in parentheses.

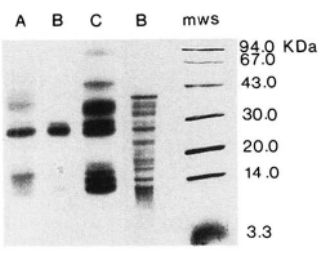

FIG. 1. **Electrophoretic and autoradiographic patterns of CNBr fragments from thyroglobulin preparations.** Electrophoresis was performed in 15–20% polyacrylamide gradient gel in the presence of 1% SDS and under reducing conditions. *Left lanes*, autoradiographic patterns of fragments from preparations A–C (see other details in Table I). *Right lanes*, a typical electrophoretic pattern after Coomassie Blue staining of the same gel whose autoradiogram is shown in the left lanes and molecular weight standards (MWS).

Purification and Analysis of the 27,000- and 15,500-Da Fragments

Variable amounts of the fragments were obtained by electroelution from several gels (see "Experimental Procedures"). The overall recovery of material was estimated consistently around 90% by ^{125}I counts. Purification is documented in Fig. 3, in which the 27,000-Da species is shown together with other purified fragments.

Analyses of the Distribution of ^{125}I Among Iodo-amino Acids by HPLC after Extensive Pronase Digestion—The distribution of ^{125}I among the amino acids was estimated by HPLC in each thyroglobulin preparation (*A–D*) after extensive Pronase digestion of samples containing about 50,000 cpm. Similarly the distribution of ^{125}I among the amino acids of the 27,000-Da species (purified from preparations A–C) was estimated by digesting enough material to contain nearly 50,000 cpm of iodine. An overview of the distribution of [^{125}I]iodo-amino acids is reported in Table II.

Determination of Sequence of the 27,000-Da Species—The

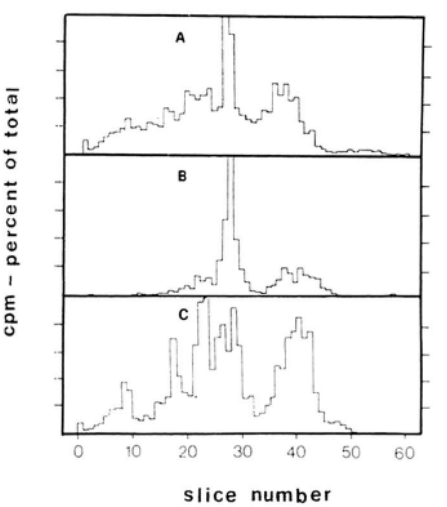

FIG. 2. **Distribution of radioactive iodine among CNBr fragments of the thyroglobulin preparations.** Electrophoresis was performed in 15–20% polyacrylamide gradient gels in the presence of 1% SDS and under reducing conditions. After electrophoresis, lanes containing different preparations were longitudinally cut, sliced (60 slices, of about 1.5 mm) and counted. *Abscissa*, slice number (from top to bottom of the gel). *Ordinate*, percent of the maximal radioactivity in each preparation. Peak radioactivity was about 170,000 cpm in preparation B, about 70,000 in C, and about 18,000 in preparation A. In all cases, about 80 μg of protein/well were loaded.

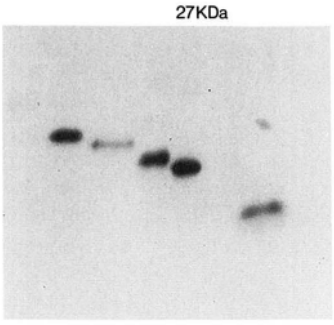

FIG. 3. **Analytical electrophoresis and autoradiography of the 27-kDa peptide and other peptides purified by preparative electrophoresis.** Bands of interest were cut from preparative gels, electroeluted, and precipitated as described under "Experimental Procedures." The picture shows an analytical 15–20% gradient gel as described under "Experimental Procedures." Peptides analyzed here were derived from preparation C, except the 27,000-Da fragment which was from preparation B and the 15,500-Da fragment from preparation D. The approximate fragment masses are (left to right): 36, 33, 29, 27, and 15.5 kDa.

TABLE II

Percent distribution of ^{125}I within the various iodotyrosine derivatives in thyroglobulin preparations (A–D) and their respective 27,000-Da fragments

Free iodine was in trace amounts and was not included in calculations. The abbreviations used are: MIT, monoiodotyrosine; DIT, diiodotyrosine; T_3, 3,5,5′-triiodothyronine; T_4, thyroxine; TA, trace amounts.

	MIT	DIT	T_3	T_4
A	45.0	47.0	2.0	6.0
A-27,000	50.0	42.0	5.0	3.0
B	89.0	11.0	TA	TA
B-27,000	87.0	13.0	TA	TA
C	83.0	16.0	TA	TA
C-27,000	83.0	17.0	TA	TA
D	81.0	18.0	TA	TA

TABLE III

Amino-terminal sequence of the 27,000-Da fragment from preparation B of rat thyroglobulin and comparison with corresponding sequences of hormone-rich peptides and cDNA-derived sequences from other animal species

Asterisk indicates the presence of ^{125}I.

	Residue no.											
	1	2	3	4	5	6	7	8	9	10	11	12
[a]N	I	F	E	X*	Q	V	X	A	Q	X	L	
[b]N	I	F	E	T_4	Q	V	D	A	Q	P	L	
[c]N	I	F	E	Y	Q	V	D	A	Q	P	L	

[a] Rat 27-kDa peptide.

[b] Five identical hormone containing peptides from rabbit, bovine, ovine, porcine, and human thyroglobulins (13–16).

[c] cDNA derived NH_2-terminal sequence of bovine, human, and rat thyroglobulins.

27,000-Da peptide, electroeluted from preparation B, was subjected to automated Edman degradation and the sequence of the first 12 amino acids determined. Table III shows a comparison between the determined sequence, the sequence of other hormone-rich peptides so far characterized by other authors and the NH_2-terminal sequences of bovine, human, and rat thyroglobulin obtained from the cDNA-deduced primary structure of the protein.

The *X* stands for ambiguous identification. It is significant that the residue at position 5 was not identified by the laboratory which performed the determination.

Partial Edman Degradation (Manual) of the Electroeluted 27,000-Da Species from Preparation B and Unfractionated CNBr Fragments from Preparation D—While automated Edman degradation was performed by a service laboratory, a manual Edman degradation was attempted by us on a small amount of the same sample. After each step, the distribution of radioactivity between the PTH and the resulting peptide was measured; in addition ^{125}I losses which accompany every step were carefully evaluated. It was observed that the fifth, and, to a much lesser extent, the sixth PTH, contained most of the original radioactivity, while the initial four and the seventh contained only trace amounts of ^{125}I. The conclusion was that most (if not all) of the radioactivity was associated with the fifth residue. Electrophoretic analysis of the resulting material was not performed, since both weight and radioactivity were estimated to be insufficient for staining or even autoradiography. To obtain better information in this regard, the fragments (without any purification) obtained by CNBr cleavage of thyroglobulin from group D were subjected to manual Edman degradation. From Table I, animals from this group were killed 24 h after ^{125}I injection. This caused the appearance of other radioactive fragments in addition to the 27,000-Da species. The two *arrows* in Fig. 4 (*upper panel*) point to the major peaks: the 27,000-Da fragment and a faster migrating one (15,000 Da, whose purification is shown in Fig. 3). This profile is identical to that obtained by electrophoresis of CNBr fragments from thyroglobulin of group C except for the lack of species larger than the 27,000-Da peptide. No attempt was made to identify PTHs, but, as previously done with the 27,000-Da species from preparation B, the mixture of PTHs resulting after every step was counted for ^{125}I. The starting material contained about 500,000 cpm. The first four

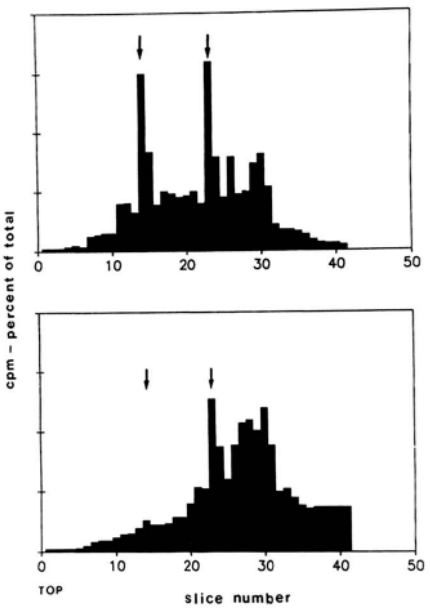

FIG. 4. **Comparative radioactive profiles after electrophoresis of CNBr fragments of thyroglobulin from animals of group D and the same material after seven steps of manual Edman degradation.** The gel (17.5–23.5% acrylamide) was longitudinally cut along the two channels and sliced. Each slice was counted and the radioactivity (percent of the total) plotted *versus* the slice number. Each division along the *y* axis corresponds to 5%. About 200,000 cpm/slot were loaded on the gel. By comparing the upper and lower panels, the total disappearance of the fragment of 27,000 Da and the retention of the other smaller peptides is evident. The *arrows* point to the 27,000 and 15,500-Da species, which are shown in their purified form in Fig. 3.

PTHs contained very little radioactivity (1,500–3,000 cpm). Each step was accompanied after washing with benzene, drying with nitrogen and warm acidic treatments (trifluoroacetic acid and HCl) by an average loss of radioactivity amounting to about 40,000 cpm. The fifth derivative contained about 65,000 cpm, *i.e.* about ⅓ of the remaining counts, the sixth again gave some radioactivity (14,000 cpm, probably carryover). Finally the seventh returned sharply to the initial levels. The resulting peptides (which contained the original fragments minus 7 residues and a significant amount of residual radioactivity), were subjected to acrylamide gel electrophoresis after solubilization with 1% SDS and reduction with 2-mercaptoethanol. The channels corresponding to the sample (CNBr fragments after Edman degradation) and control (CNBr fragments before the Edman degradation) were cut, and slices (1.5 mm) were counted. The relative radioactivity was then plotted *versus* the slice number. Fig. 4 (*lower panel*) clearly shows that, while peptides having molecular sizes lower than 15,500 Da (included) were still present, the 27,000-Da species was no longer apparent. This clearly indicates that all the ^{125}I contained within the 27,000-Da species is associated with the fifth residue from the NH_2 terminus of the fragment and thus the NH_2 terminus of the protein itself. In addition the survival of other labeled peptides indicates that the disappearance of radioactivity from the 27,000-Da species is not due solely to nonspecific losses of ^{125}I during the Edman procedure. A similar conclusion could be indirectly reached from the experiment performed on the purified 27,000-Da species (from preparation B), but since this peptide is the only one present at the start, it would be difficult to disregard the possibility that iodine was lost by nonspecific processes due to the conditions used in the Edman degradation.

DISCUSSION

Thyroglobulin is a large glycoprotein containing two identical polypeptide chains, forming a functional unity of nearly 660,000 Da. It is synthesized in the thyroid gland, where, during the process of maturation it undergoes several post-translational modifications, the most significant of which is iodination. The specific function of the thyroid gland is to actively concentrate iodine from the diet and, by a complex and still poorly understood enzymatic pathway, change tyrosines into mono and diiodo derivatives and, finally, synthesize thyroid hormones. The average number of iodine atoms normally present in a thyroglobulin molecule may vary significantly: molecules containing from 10 up to 50 iodine atoms are considered to be within the physiological range. However, the number of thyroid hormone molecules synthesized normally does not exceed 2–3 residues for subunit. Considering the large size of the molecule with respect to the small number of hormone molecules obtainable, the entire process may appear very expensive; the efficiency and the specificity of thyroglobulin, however, become evident when the intake of iodine is limited. Under these conditions the available iodine is exclusively utilized for hormone synthesis.

In the present paper we demonstrate that, at minimal levels of iodination, radioiodine given *in vivo* to iodine-deprived rats is found in a single CNBr fragment having an apparent size of about 27,000 Da, which corresponds to the NH_2 terminus of the protein. Among the several tyrosyl residues contained within the sequence of this peptide, only the first, corresponding to the fifth amino acid in the sequence, contains ^{125}I.

At the time of this report various hormone-rich fragments from mammalian thyroglobulins have been isolated and characterized (7–13). All these studies have indicated that several specific sequences designed for hormone synthesis exist in the protein. To date it is generally recognized that, among these sites, the fifth residue (from the NH_2 terminus of the protein) is a special one, since at this site the first hormone molecule is formed. The other, less efficient, hormonogenetic sites have been reported to map at positions 2555, 2569, and 2748 and, more recently, 1291 (in rabbit thyroglobulin (13)).

Indeed several observations in the past suggested that (i) the iodination of thyroglobulin does not occur at random (4), (ii) preferential synthesis of thyroxine involves the early iodinated tyrosyl residues (5), and (iii) iodination affects, proportionally to its level, the tertiary structure of the protein (23). This hypothesis implies that a newly synthesized, not yet iodinated molecule, contains in its conformation the signal for directing the first atom of iodine toward a specific tyrosyl residue. As a consequence of this initial iodination, the protein modifies its structure in such a way that new sites are exposed to facilitate and control further iodination. At the same time appropriate tyrosyl residues (acceptors and donors) are aligned into favorable steric conditions to allow hormone formation by means of the coupling reaction. In other words the whole process seems to depend on the structure of the thyroidal protein, which determines the rates of initial iodination and the subsequent coupling.

The aim of the present work was to prove the existence of a specific site of initial iodination and possibly to localize the tyrosyl residue which undergoes such transformation.

We have observed that ^{125}I administered in trace amounts to severely iodine-deprived rats (about 1 iodine atom/thyroglobulin molecule) is found mainly in a single CNBr fragment if animals are killed within 2 h after ^{125}I injection (preparation B). The sequence of the peptide indicates that it corresponds to the NH_2 terminus of the protein. In the cDNA-deduced sequence of the NH_2-terminal portion of bovine, human, and rat thyroglobulin (14–16), the first methionine residue (apart from methionine 1) is encountered at position 146. Within this stretch of amino acids not less than 5 tyrosines are present and all might be candidates for early iodination. To solve this problem we exploited the observation that if chronically iodine-deprivated animals are killed 24 h after injection of ^{125}I (preparations C and D), radioactivity is found not only in the 27,000-Da species, but also in fragments of smaller size. Thus, when the mixture of CNBr peptides from preparation D was subjected to seven steps of Edman degradation, a distinct peak of radioactivity was found at position 5; moreover, when the remaining material was electrophoresed, no residual radioactive 27,000-Da peptide was observed, whereas the label persisted in the other species (15,000 Da and lower). This is proof that residue 5 of thyroglobulin is the site of early iodination.

One point merits additional comment. The apparent mass of our fragment ($M_r = 27,000$) has been estimated by comparing its electrophoretic mobility to that of proteins of known size. However, on the basis of the primary structure of the protein and the relative positions of its methionine residues, the mass should be significantly smaller. To explain the discrepancy between the theoretical and estimated sizes, some speculations may be made. First, thyroglobulin is a glycoprotein containing a significant number of carbohydrate moieties/monomer of both high mannose and complex types (24) which contribute about 10% of the total molecular mass. Upstream of the first cleavage site for CNBr, the cDNA sequence has one (bovine and rat) or two (human) putative sites for *N*-linked glycosylation. The presence of carbohydrate may well cause significant alterations in the electrophoretic mobility, although their actual localization has not been es-

tablished so far. A second possibility is that the methionine residue at position 127 is not readily amenable to CNBr breakdown because of local structural constraints. Actually this possibility is realistic. The cysteine content of the 27,000-Da species amounts to about 10% of its total amino acids, while in the whole protein the cysteine content does not exceed 4%. It is possible that numerous S–S bridges in this region protect the above-mentioned methionyl residues. Indeed CNBr cleavage was performed on the unreduced protein and may not have occurred quantitatively, even in preparation D, where the ratio CNBr/protein (w/w) was twice the usual.

In keeping with the observation that at very low levels of iodination most of the radioiodine administered is incorporated into the 27,000-Da peptide, the distribution of the tracer among iodo-amino acids in this species is fully representative of the pattern of the parent thyroglobulin.

As to the other labeled species of 15,000 Da and lower observed in preparations C and D, they seem to indicate that a redistribution of radioiodine among various peptides occurs in time. A similar observation has already been made by Dunn *et al.* (25). After *in vivo* iodination of low iodine thyroglobulin from animals killed at various times, iodine appeared first in high molecular species and then in a much smaller peptide of about 20,000 Da. In this regard several authors (10, 26, 27) have suggested that lower molecular weight peptides may derive from larger ones as a consequence of oxidative reactions which accompany or ensue from iodination. The experiment depicted in Fig. 4 indicates that the lower molecular mass species observed by us are distinct from the 27,000-Da NH_2-terminal peptide.

A few months after we presented a preliminary report of this work (28), Rawitch *et al.* (29) presented a short note on a similar issue. Studying *in vitro* iodination of goitrous human thyroglobulin by thyroid peroxidase, they reported that early iodination is restricted to tyrosines 5, 2,520, and 2,553. In accordance with these authors and with previous observations, our data indicate that other sequences of the protein contain specific tyrosine residues which are subjected to sequential iodination. Unfortunately, in our hands all attempts to characterize such species after electrophoretic purification have failed. The electrophoretically homogeneous 15,000-Da peptide (see Fig. 3) is indeed a mixture of two or more species as indicated by the concomitant presence of more than 1 amino-terminal residue.

However, the present data, further supported by independent observations, conclusively indicate that the early iodination of rat thyroglobulin is an ordered process and that tyrosine at position 5 is *in vivo* the earliest site available to this purpose.

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