Identification of Iodinated Proteins in Cultured Thyrocytes and Their Possible Significance for Thyroid Hormone Formation*

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ABSTRACT

The thyroid gland is known to generate the iodinated hormones T₃ and T₄ from the prohormone thyroglobulin. In this report we examined whether polypeptides other than thyroglobulin are iodinated and hormonogenic in thyrocytes and the prerequisites for their iodination. In primary cultures of porcine thyrocytes, a substantial portion of organified radioiodine was incorporated into cellular proteins other than thyroglobulin. Some of these were identified by immunoprecipitation. They included proteins of the extracellular matrix, plasma membrane proteins, and lysosomal enzymes, which follow in part a secretion and reuptake pathway. All of these proteins come into contact with the iodinating system of thyrocytes located on the apical plasma membrane and possess iodination consensus sequences. Immunoprecipitation with T₃- or T₄-specific antibodies showed that thyroid hormones were detectable only within thyroglobulin. This was confirmed by an analysis of the iodoamino acids of thyroglobulin, cathepsin-D (representing a secretory protein), and aminopeptidase-N (a membrane-integrated protein) by two-dimensional TLC, which revealed the presence of T₃ and T₄ only within the polypeptide chain of thyroglobulin. These results indicate that iodoproteins other than thyroglobulin do not participate in the generation of thyroid hormones in situ. (Endocrinology 135: 1566-1575, 1994)

** is well established that thyroid hormones are produced from the macromolecular precursor protein thyroglobulin (Tg), which is secreted by thyrocytes and stored in the follicle lumen (1). Iodination of Tg and generation of thyroid hormones are accomplished through the concerted action of several enzymes, among which the plasma membrane-bound thyroid peroxidase (TPO) is the key enzyme. It iodinates certain tyrosyl residues within the polypeptide chain of Tg and performs the coupling of iodotyrosyls to yield proteinbound T₃ and T₄ (2, 3). Storage of Tg at concentrations of at least 100 mg/ml (4) is in part accompanied by the formation of a solid luminal content consisting of covalently cross-linked Tg molecules (5). Upon TSH-signaled hormone demand, Tg is mobilized and recaptured by follicle cells. During its transport to lysosomes, degradation of Tg commences, and T₃ and T₄ are liberated from its polypeptide chain (6, 7).

So far Tg was considered to be the only specialized molecule to function as thyroid prohormone. The pioneering work on the substrate specificity of isolated TPO by Taurog's group as early as 1967 (8) questions this view, because it showed that the thyroid hormone T₄ was generated by in vitro iodination from protein substrates as different as insulin, lysozyme, or BSA. Moreover, the observation that a rat thyroid cell line (FRTL-5) incorporated radioactive iodine into proteins other than Tg (9) also raises the possibility that thyrocytes may be able to produce hormones from proteins other than Tg. However, the nature and identity of these proteins remained obscure, except for the identification of iodinated BSA present in the culture medium. We, therefore, analyzed the iodination process in more detail, i.e. we examined which proteins, other than Tg, were iodinated in cultured thyrocytes and whether intramolecular coupling of iodotyrosyls would occur within their polypeptide chains. The experiments showed that in primary cultures of porcine thyrocytes, a considerable proportion of the organified iodide was incorporated into secretory, lysosomal, and plasma membrane-bound proteins. However, thyroid hormones were absent from polypeptides other than Tg, pointing to unique structural features in which Tg differs from other proteins. We show that protein iodination is a general phenomenon in thyrocytes, which presumably represents a by-product of thyroid hormone formation. Therefore, we propose to call the iodination of proteins other than Tg a default iodination.

**Materials and Methods**

**Cell culture**

Human thyrocytes were isolated from biopsies, whereas porcine thyrocytes were obtained from glands of freshly slaughtered pigs. Follicle fragments were prepared according to a method described previously (10) and were used either directly for experimentation or after establishing two different systems of cultured thyrocytes. As known from previous studies (11), iodination rates of freshly prepared follicle segments were generally much higher than those of cultured cells. Cell culture systems of primary thyrocytes included 1) monolayers grown on Cyclopon membranes (Becton Dickinson, Heidelberg, Germany) and 2) inside-out follicles kept as suspension culture in Petriperm dishes with hydrophobic bottom (Bachofer, Reutlingen, Germany). In these two systems, thyrocytes are highly polarized, with distinct apical and baso-
were specific for human cathepsin-D (CD; obtained from Dr. A. Hasilik, Biotinylution Biosynthetic labeling many). Acrylamide (research grade) was obtained from Serva. Aprotinin and thrombospordin (TSP; obtained from Dr. P. Vischer, Institut für Arterioskleroseforschung, Munster, Germany). Protein-G-Sepharose specific for T3 and T4 were purchased from ICN (Meckenheim, Germany) and porcine major histocompatibility complex (MHC) class II (14) was obtained from E. Merck (Darmstadt, Germany). The permanent rat thyroid cell line FRTL-5 was kept in F-12 medium supplemented with six hormones and 5% calf serum according to the method of Ambesi-Impimbiotta et al. (13) on culture dishes or coverslips coated with Matrigel (Serva, Heidelberg, Germany). Supplementation of antibiotics to the culture medium and maintenance of FRTL-5 cells were the same as described for primary cultures of thyrocytes.

Materials

L-[45]-Leucine, L-[35]-methionine (in vivo cell labeling grade), carrier-free [125]I-Na, [35]C-methylated Rainbow protein molecular mass standards, and the detection kit for enhanced chemiluminescence on Western blots were purchased from Amersham Buchler (Braunschweig, Germany). Polyclonal rabbit antiserum against porcine Tg and aminopeptidase-N (APN) were raised in our own laboratory. Other rabbit antiserum was specific for human cathepsin-D (CD; obtained from Dr. A. Hasilik, Physiologisch-chemisches Institut, Munster, Germany) and porcine thrombospordin (TSP; obtained from Dr. P. Vischer, Institut für Arterioskleroseforschung, Munster, Germany).

(NH4)2SO4-precipitated immunoglobulins of the G-type (IgGs) from rabbit antiserum specific for human TPO were a generous gift from Dr. P. Carayon (Institut National de la Sante et de la Recherche Medicale, Laboratoire des Proteines Hoteques, Marseille, France). Rabbit antiserum specific for T3 and T4 were purchased from ICN (Meckenheim, Germany). The monoclonal antibody MRC OX 6, specific for RT1 B [rat major histocompatibility complex (MHC) class II] (14) was obtained from Camden Labor Service (Wiesbaden, Germany), and 5-(4,6-dichlorotiazin-2-yl)amino-fluorescein hydrochloride (DTAF)-conjugated goat antiserum. Protein-A-Sepharose Cl-4B was purchased from Pharmacia (Freiburg, Germany). Acrylamide (research grade) was obtained from Serva. Aprotinin was bought from Bayer (Leverkusen, Germany). Protein-G-Sepharose, α-dianisidin, streptavidin coupled to horseradish peroxidase, TSH, and o-dianisidin were dissolved in the same salt solution from the apical or basolateral side. The reaction was stopped by adding 1.5 vol Eagle’s Minimum Essential Medium. Cells were then washed three times with ice-cold PBS containing 1.5 mM MgCl2 and 0.1 mM CaCl2 before they were lysed.

Immuno precipitation

After labeling, cells were washed twice with PBS and lysed with 0.3–0.8 ml 50 mM Tris-Cl (pH 7.4), 0.9% NaCl, 0.5% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 5 mM iodoacetamide, and 1 trypsin inhibiting unit/ml eprotinin (lysis buffer) for 30 min at room temperature, followed by centrifugation at 15,000 × g for 5 min. The supernatant was divided into several aliquots according to the number of antigens to be immunoprecipitated. The volume of each aliquot was then raised to at least 0.4 ml with lysis buffer.

Five microliters of control serum or antiserum were added to the cell lysates, mixed, and left at room temperature for 30 min. The immune reaction was allowed to proceed overnight at 4°C. Immunocomplexes were then collected by adding 30 μl of a 1:2 suspension of protein-A-Sepharose Cl-4B in lysis buffer and shaking for 1 h at 4°C. Protein-A-Sepharose pellets were then washed twice with 0.8 ml PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, and 20 mg/ml RSA (buffer A); once with 0.8 ml buffer A and 2 M KCl; once with 0.8 ml 10 mM Tris-Cl (pH 8.5) containing 0.6 μl NaCl, 0.1% sodium dodecyl sulfate (SDS), and 0.08% Nonidet P 40; and once with 10 fold diluted PBS.

Finally, protein-A-Sepharose pellets were boiled in 30 μl 1.5-fold concentrated sample buffer under reducing conditions for 5 min at 100°C. After a 2-min centrifugation at 15,000 × g, the supernatants were applied to slots of a polyacrylamide gel for further analysis. When cell lysates were to be analyzed by gel electrophoresis, 5 μl of a 5-fold concentrated reducing sample buffer were mixed with 20 μl of the lysate and boiled for 5 min at 100°C before loading onto a gel.

In the experiment aiming to show depletion of [125]ICD from cell homogenates by immunoprecipitation, the antiserum or a control serum was reacted with protein-A-Sepharose Cl-4B, washed three times with lysis buffer, and then incubated with the [125]I-labeled cell homogenate for at least 8 h at 4°C while shaking. This procedure was employed to avoid disruption of gel electrophoretic bands by accumulated serum proteins and was repeated twice because of the low titer of the CD antiserum.

In one experiment the immunoprecipitation of [125]I-Tg was followed by a second immunoprecipitation. For this reason [125]I-Tg polypeptides contained in 5 μl sample buffer were dissolved in 469 μl lysis buffer, centrifuged for 5 min at 15,000 × g, and processed for immunoprecipitation as described above.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography were performed according to the method of Laemmli (17) with 12.5% polyacrylamide gels followed by fluorography (18). Gels containing [125]I-labeled polypeptides were fluorographed with intensifying screens.

Differential extraction with saponin and Triton X-100

To differentiate between soluble and membrane-attached polypeptides, 0.1% saponin was used to replace Triton X-100 in the lysis buffer (see above). Saponin is known to complex cholesterol, thereby perforating biomembranes without disrupting them (19, 20). Extraction of cells proceeded for 10 min at room temperature in a volume of 0.8 ml, followed by a 5-min centrifugation step at 15,000 × g. The cell pellet was extracted two more times with saponin and finally with regular lysis buffer containing 0.5% Triton X-100. Triton X-100 disintegrates membranes and is able to solubilize membrane-attached and -integrated polypeptides.

Enhanced chemiluminescence detection of biotinylated proteins on Western blots

After gel electrophoretic separation of biotinylated polypeptides on 12.5% polyacrylamide gels, the gels were equilibrated in transfer buffer (48 μM Tris-39 mM glycine (pH 7.3), 0.04% SDS, and 20% methanol) for 15 min at room temperature. Polypeptides were then transferred to nitrocellulose sheets for approximately 1 h at 25 V in a Trans-Blot semidry transfer cell (Bio-Rad, Munich, Germany). Nitrocellulose sheets...
Two-dimensional TLC analysis

After fluorography of polyacrylamide gels, regions containing $^{125}$I-labeled Tg, APN, or CD were excised from the gels and rehydrated for 1 h. Diphenoxyazol was extracted from the gel pieces by six subsequent incubations in dimethylsulfoxide. Each incubation was performed for 30 min at room temperature on a rotator. Gel pieces were then equilibrated six times for 10 min with 10% Tris-Cl, pH 6.8, at room temperature.

Alkaline hydrolysis was performed in 100 μl 1 N NaOH supplemented with 10 μg unlabeled Tg and Tg standards overnight at 110°C in a sealed Eppendorf cap. After acidifying samples with 55 μl 2 N HCl, hydrophobic amino acids were extracted with butanol and run on silica gel 60 high performance TLC plates (Merck, Darmstadt, Germany). The first dimension was developed with butanol-20% ammonium hydroxide-methanol (80:20:20, vol/vol/vol). After drying the plates, unlabeled standards for the second dimension were 5 μg monoiodotyrosine, 5 μg diiodotyrosine, 5 μg Tg, and 2.5 μg Tg. Standards were visualized by spraying plates with an aqueous 20% solution of Na2CO3 (w/vol) and, after drying, with a 1:3 aqueous dilution of the Folin Ciocalteau reagent. $^{125}$I-labeled Tg and $^{125}$I-labeled APN were spotted onto the plates. After the final wash, biotinylated polypeptides were visualized by incubation in streptavidin. All incubations were performed on a rocking platform.

Immunofluorescence detection of cell surface MHC class II molecules

FRTL-5 cells were cultured on Matrigel-coated coverslips in the absence and presence of 100 U/ml γ-interferon. They were blocked with 1% BSA-PBS, washed, and incubated with a monoclonal antibody specific for RT1 B (rat MHC class II) for 2 h at 4°C. Cells were then washed three times with ice-cold 0.1% BSA-PBS and probed with DTAF-conjugated goat F(ab)₂ directed against the Fc fragment of antibodies. The cells were fixed with 4% paraformaldehyde for 10 min at room temperature, washed again, and examined by fluorescence and phase contrast microscopy.

Results

Identification of radioiodinated polypeptides from thyrocytes by immunoprecipitation

Freshly isolated human or porcine follicle segments were labeled for up to 4 h with 5.6–18.5 mBq $^{125}$I-NaI, whereas porcine inside-out follicles were labeled with 6.8–11.1 mBq $^{125}$I-NaI for 16–62 h in the presence of 20–50 μU/ml TSH. Cells were then washed with PBS and lysed, and Tg, TPO, CD, APN, and TSP were immunoprecipitated from aliquots of the lysates. Immunoprecipitates were analyzed by SDS-PAGE under reducing conditions and fluorography; the results are shown in Fig. 1. In addition, lysates from which Tg or CD had been previously immunoprecipitated were analyzed together with control lysates from porcine follicle segments.

Tg is a homodimeric glycoprotein and the major iodinated polypeptide in the lysates of porcine thyrocytes. Besides the intact Tg subunit with a molecular mass of 330 kilodaltons (kDa; TG, i in Fig. 1A), proteolytic fragments of Tg could be detected after immunoprecipitation; the major fragment possessed a molecular mass of approximately 69 kDa. The radioactive signal of these polypeptides was greatly diminished in cell homogenates from which Tg had previously been immunoprecipitated. On the other hand, many other polypeptides remained unaffected by Tg immunoprecipitation, indicating that they are not related to Tg but represent proteins that were iodinated together with Tg. Immunoprecipitation of CD identified one of these polypeptides as being the heavy chain of mature CD (CD). C, APN and TPO were weakly radiiodinated and could not be aligned with bands of the cell lysate. They only became visible after prolonged exposure times. All forms (from top to bottom: α-, β-, and γ-chains) of APN show incorporation of radioiodide. Besides the intact TSP polypeptide (upper band), a partially degraded TSP polypeptide was immunoprecipitated (lower band). One peculiarity of the iodination process is that the thyroid peroxidase iodinates itself, as shown by immunoprecipitation of TPO from the cell homogenate of $^{125}$I-labeled human thyrocytes. The positions of molecular mass standards are given in the right margin of each panel.

![Fig. 1. Identification of iodoproteins other than Tg.](image)
Except for Tg and CD, incorporation of radioiodine into other immunoprecipitated polypeptides was so weak that they could only be detected after prolonged exposures of x-ray films. These polypeptides could not be aligned with iodoproteins from the cell homogenate. Therefore, they are shown separately in Fig. 1C.

APN was synthesized as a 160-kDa polypeptide (α-chain, Fig. 1, uppermost band), which was cleaved to yield the β- and γ-chains (22). Incorporation of iodine could be observed for all polypeptides of the enzyme (see Fig. 1C). Although numerous iodination consensus sequences (23) were contained within the β-chain (middle band), it was only weakly labeled with radioiodine. Presumably, this can be attributed to the fact that the β-chain contains the membrane-spanning domain leading to a steric hindrance of iodination by a too close apposition of the P-chain to the plasma membrane.

Two labeled TSP species were detected after labeling with [125I]Nai. Upon omission of dithiothreitol from the sample buffer, only the upper band shifted into the stacking gel (not shown). This is characteristic of the intact TSP trimer, which is held together by disulfide bonds (24). The lower band was probably a degradation product of TSP because it expressed a somewhat lower molecular mass than the intact monomer and was not linked to any other polypeptide by disulfide bridges. The iodination of TSP shows that iodination is also possible with the plasma membrane-associated polypeptides of the extracellular matrix. For further analysis of porcine cellular iodoproteins, see Fig. 4.

It is noteworthy that the thyroid peroxidase iodinates itself, as could be verified by immunoprecipitation from cell homogenates of 125I-labeled human thyrocytes. As shown in Fig. 1, TPO was the only antigen immunoprecipitated from human thyrocytes. TPO is a fairly strong iodinated polypeptide, which showed an apparent molecular mass of approximately 100 kDa. [35S]Labeled TPO, immunoprecipitated from human thyrocytes that were labeled for 2 h with [35S]methionine, had a higher molecular mass than its [125I]-labeled counterpart (not shown). The difference in molecular mass was in the range of 2–10 kDa, indicating that the TPO was subject to some posttranslational modification on its way to the plasma membrane.

Iodination of MHC class II polypeptides and their immunofluorescence detection on the plasma membrane of FRTL-5 cells

Monolayers of the rat thyroid cell line FRTL-5 were labeled with 11.1 mBq [125I]Nai or 15.9 mBq [35S]methionine in the presence or absence of 100 U/ml γ-interferon. For labeling in the presence of γ-interferon, FRTL-5 cells were preincubated with 100 U/ml γ-interferon for 48 h. [125I]Nai-labeled cells were supplemented with 10 mU/ml TSH throughout the pulse to stimulate iodination of proteins.

After the labeling period cells were washed with PBS and lysed, and the lysate was divided into two aliquots. One aliquot received nonimmune murine IgGs, and the other received monoclonal IgGs specific for RTL B (rat MHC class II polypeptides). The immunocomplexes were collected with protein-G-Sepharose Cl-4B, washed, and analyzed by SDS-PAGE and fluorography.

Expression of the MHC class II antigen could only be observed after γ-interferon stimulation (see upper panel of Fig. 2, [35S]methionine section), which confirms results from other workers (15). Both the α- and β-chains of the heterogeneous MHC class II antigen were labeled with radioactive iodine (see upper panel of Fig. 2, [125I]Nai section), indicating that they contain tyrosyl residues within iodination consensus sequences (23). Although numerous iodination consensus sequences (23) were contained within the γ-chain, only the upper band shifted into the stacking gel (not shown). This is characteristic of the intact MHC class II dimer, which was cleaved to yield the α- and β-chains of the MHC class II antigen were also radioiodinated, indicating that they had contact with the plasma membrane-inserted TPO of FRTL-5 cells. The positions of molecular mass standards are indicated in the margin. Lower panel, FRTL-5 cells were cultured on Matrigel-coated coverslips in the presence (a) or absence (b) of γ-interferon. After labeling with [35S]methionine, MHC class II polypeptides could only be observed upon γ-interferon treatment. Both the α- and β-chains of the MHC class II antigen were also radioiodinated, indicating that they had contact with the plasma membrane-inserted TPO of FRTL-5 cells. The positions of molecular mass standards are indicated in the margin. Lower panel, FRTL-5 cells were cultured on Matrigel-coated coverslips in the presence (a) or absence (b) of γ-interferon. After incubation with a monoclonal antibody specific for MHC class II molecules for 2 h on ice, cells were washed and treated with DTAF-conjugated goat F(ab′)2 specific for mouse IgGs for another 2-h period. Cells were then washed, fixed with paraformaldehyde, and examined by fluorescence microscopy. Only after γ-interferon stimulation could cell surface expression of MHC class II molecules be observed. Bars = 10 μm.

Fig. 2. Induction and iodination of MHC class II polypeptides in FRTL-5 cells. Upper panel, FRTL-5 cells were labeled with [35S]methionine ([35S]Meth) or [125I]Nai overnight in the absence or presence of 100 U/ml γ-interferon (IFN-γ). Cells were pretreated with 100 U/ml γ-interferon for 48 h when a pulse in the presence of γ-interferon was to be performed. After labeling cells, half of the cell homogenates were supplemented with either nonimmune IgGs (N) or monoclonal antibodies directed against rat MHC class II polypeptides (M). The immunocomplexes were analyzed by SDS-PAGE and fluorography. After labeling with [35S]methionine, MHC class II polypeptides could only be observed upon γ-interferon treatment. Both the α- and β-chains of the MHC class II antigen were also radioiodinated, indicating that they had contact with the plasma membrane-inserted TPO of FRTL-5 cells. The positions of molecular mass standards are indicated in the margin. Lower panel, FRTL-5 cells were cultured on Matrigel-coated coverslips in the presence (a) or absence (b) of γ-interferon. After labeling with [35S]methionine, MHC class II polypeptides could only be observed upon γ-interferon treatment. Both the α- and β-chains of the MHC class II antigen were also radioiodinated, indicating that they had contact with the plasma membrane-inserted TPO of FRTL-5 cells. The positions of molecular mass standards are indicated in the margin. Lower panel, FRTL-5 cells were cultured on Matrigel-coated coverslips in the presence (a) or absence (b) of γ-interferon. After labeling with [35S]methionine, MHC class II polypeptides could only be observed upon γ-interferon treatment. Both the α- and β-chains of the MHC class II antigen were also radioiodinated, indicating that they had contact with the plasma membrane-inserted TPO of FRTL-5 cells. The positions of molecular mass standards are indicated in the margin.
sus sequences that are accessible to TPO (23). This should be true for all polypeptides that are iodinated in thyrocytes.

For immunofluorescence examinations, FRTL-5 cells were grown on Matrigel-coated coverslips. After treatment of one culture with 100 U/ml γ-interferon for 48 h, cells were incubated with a monoclonal antibody directed against RT1 B (rat MHC class II- molecule) for 2 h on ice, followed by an incubation of DTAF-conjugated goat F(ab')2 specific for mouse lgGs for 2 h on ice. Cells were then fixed with 4% paraformaldehyde and examined by fluorescence or phase contrast microscopy; the results are shown in the lower panel of Fig. 2.

Phase contrast images of γ-interferon-treated FRTL-5 cells (Fig. 2a) as well as control cells (Fig. 2b) showed a round appearance, confirming results from Nitsch et al. (25) that FRTL-5 cells have lost the ability to grow as a continuous epithelial monolayer. Upon γ-interferon induction, a bright cell surface staining of the MHC class II antigen was observed (Fig. 2a), which was totally absent from control cells (Fig. 2b). The cell surface appearance of MHC class II molecules in FRTL-5 cells demonstrates that MHC class II molecules reach the plasma membrane-inserted TPO, which provides the basis for them to be iodinated (see upper panel of Fig. 2).

Proteins need to contact the apical plasma membrane to be iodinated

Filter-grown polarized porcine thyrocytes from either the apical or the basolateral side were biotinylated for 30 min on ice with long chain biotin. Cells were washed twice with PBS and lysed, and APN or TSP was immunoprecipitated from aliquots of the cell lysate. The immunoprecipitates were run on a SDS-polyacrylamide gel and blotted onto nitrocellulose. Nitrocellulose sheets were probed for biotinylated polypeptides with streptavidin conjugated to horseradish peroxidase, followed by an enhanced chemiluminescence detection procedure.

Only the uncleaved α-chain of the APN could be detected in this experiment, with more than 95% being expressed at the apical plasma membrane domain (see Fig. 3). This classifies APN as being a bona fide marker protein of the apical plasma membrane. In contrast, TSP was expressed on both plasma membrane domains, with a predominance for the basolateral side (24, 26). Taken together, both polypeptides were attached to or integrated into the apical plasma membrane, which allowed them to contact the TPO and to be iodinated, as shown in Fig. 1.

Two groups of iodoproteins can be distinguished by differential extraction with saponin and Triton X-100

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Follicle sheets were kept on ice for 1 h before they were labeled with 10.2 mBq [125I]NaI for 2 h on ice. After two washes with PBS, they were extracted three times with 0.1% saponin (Fig. 4, S1-S3) and once with 0.5% Triton X-100 (Fig. 4, TX). Each extraction was performed for 10 min at room temperature. Immunoprecipitations of Tg and aliquots of the extracts were analyzed by SDS-PAGE and fluorography. Before loading onto the gel, Tg immunoprecipitates were counted in a γ-counter to determine the efficiency of each extraction.

Immunoprecipitation of Tg from the extracts confirmed the absence of major proteolytic fragments of radioiodinated Tg from the cell lysate (not shown); therefore, mainly intact Tg was visible in the polypeptide pattern of the extracts. A total of 93.5% of the labeled Tg could be extracted with saponin. The remaining 6.5% of Tg was not extractable with saponin, which could reflect membrane interaction of Tg (27), but most likely can be attributed to the inability of saponin to completely release soluble proteins from perforated cells and organelles, as reported by Wassler et al. (20).

Ekholm and Björkman (3) and Rousset et al. (28) demonstrated through electron microscopic autoradiography that in

![Fig. 3](image-url)  
**Fig. 3.** Polarized cell surface biotinylation of TSP and APN. Confluent monolayers of porcine thyrocytes were biotinylated from either the apical side (a) or the basolateral side (b). Immunoprecipitation of TSP and APN, with subsequent analysis by SDS-PAGE, Western blotting, and detection by a streptavidin/horseradish peroxidase-based chemiluminescence kit, revealed the apical localization of APN and a 1:2 distribution of TSP on the apical vs. basolateral plasma membrane. Both polypeptides had in common that they were expressed on the apical plasma membrane to some extent, which is a prerequisite to iodination.

![Fig. 4](image-url)  
**Fig. 4.** Differential extraction of soluble and membrane-associated iodoproteins. Porcine follicle sheets, labeled for 2 h with [125I]NaI on ice, were extracted three times with 0.1% saponin (S1-S3) and once with 0.5% Triton X-100 (TX). Corresponding aliquots of the extracts were analyzed by SDS-PAGE and fluorography. A distinct pattern of soluble saponin-extractable polypeptides (a) and membrane-attached, Triton X-100-extractable polypeptides (b) can be distinguished. For molecular mass standards, see right margin. The position of Tg is given in the left margin.
freshly prepared follicle segments or isolated thyrocytes intracellularly in the newly formed intracellular lumina. We think that the saponin-extractable portion of radioiodinated Tg in this experiment derives from these intracellular lumina.

Besides Tg, several other radioiodinated polypeptides were detected, as shown in Fig. 4; some of them were soluble proteins (Δ), and others were membrane associated (▲). Figure 4 gives an impression of the size and diversity of these two groups of iodoproteins. The iodination of polypeptides resisting saponin extraction demonstrates that the iodination process is not restricted to secretory proteins such as Tg and lysosomal enzymes, but includes membrane-associated or integrated proteins neighboring the TPO, as demonstrated for APN (see Fig. 1) and MHC II polypeptides (see Fig. 2).

Strong iodination of porcine cathepsin-D depends on one exposed iodination consensus sequence close to the targeting region.

Human or porcine follicle segments were labeled with 9-19 mBq [125I]NaI for 2-5 h in the presence of up to 100 mU/ml TSH. Tg and CD were immunoprecipitated from the cell extracts and analyzed by SDS-PAGE and fluorography. The results are shown in Fig. 5. Because the polypeptide pattern of porcine Tg is shown in Fig. 1, and the immunoprecipitation of human CD resulted in the same polypeptide pattern as that of porcine CD, these immunoprecipitations were omitted from Fig. 5.

Comparison of the 125I-labeled polypeptide pattern from human and porcine cell homogenates showed strong similarities, with a few exceptions. Some of these exceptions were caused by the different degradation patterns of Tg in these cells. In porcine thyrocytes, the major degradation intermediate of Tg possessed an apparent molecular mass of approximately 69 kDa (see Fig. 1), whereas in human thyrocytes, the Tg degradation intermediates had apparent molecular masses of about 120, 90, and 28 kDa (see Fig. 5).

Another obvious difference in the polypeptide pattern of iodoproteins is caused by a relatively strong labeling of CD in porcine thyrocytes, which permits identification of one of the labeled bands as being the heavy chain of CD (arrowhead in Fig. 5). In the gel electrophoretic banding pattern of human iodoproteins, this band is absent, indicating that incorporation of iodine into human CD is much weaker than that into porcine CD. Porcine and human CD have been sequenced (29, 30), which allows a search for iodination consensus sequences within their polypeptide chains. The mature enzymes show 87% identity in their amino acid sequence (30). In the heavy chain of both polypeptides, one Glu-Tyr and one Glu-X-Tyr iodination consensus sequence can be found at identical positions, whereas an additional Asp-Tyr iodination consensus sequence is found in the heavy chain of porcine CD, suggesting that this specific site is responsible for the strong iodination of porcine CD.

This is supported from structural data available from the human and bovine enzymes (31, 32), which show that the Asp-Tyr consensus sequence (Asp260-Tyr261, according to the numbering of the human enzyme) lies on the surface of the protein within four amino acids distance to the targeting region of CD, which is recognized by the phosphotransferase. This enzyme is responsible for the construction of the lysosomal targeting signal mannose-6-phosphate.

A further search for iodination consensus sequences within the primary sequence of CD indicates that there are no iodination sites within the light chain of human or porcine CD. In fact, in no iodination experiment did we observe the light chain being iodinated, demonstrating the necessity of iodination consensus sequences for the iodination of proteins.

**Immunoprecipitation with T3/T4-specific antibodies reveal the presence of thyroid hormones only within Tg**

Porcine follicle sheets were labeled with 14.8 mBq [125I] NaI for 2.5 h in the presence of 100 mU/ml TSH. Cells were then washed with PBS and lysed, and aliquots of the lysate were supplemented with antisera specific for Tg, T3, T4, and CD. The immunoprecipitation was performed as described in Materials and Methods. Two microliters of the cell homogenate were analyzed together with the immunoprecipitates by SDS-PAGE and fluorography. The results are shown in Fig. 6A.

Comparison of the polypeptide pattern of the T3/T4 im-
Immunoprecipitation with the polypeptide pattern of the total cell homogenate or the CD immunoprecipitate does not allow for the alignment of polypeptides, except for Tg polypeptides. There is a striking similarity between immunoprecipitated Tg polypeptides and the polypeptide pattern generated by the T3/T4 immunoprecipitation, indicating that Tg polypeptides were recognized and precipitated by T3/T4 antibodies. A 23-kDa polypeptide was weakly precipitated by Tg antibodies, whereas a group of polypeptides with approximately the same molecular mass was strongly immunoprecipitated by T3/T4-specific antibodies. This raises the possibility that the 23-kDa group of polypeptides represents fragments of Tg with a high hormone to polypeptide ratio. On the other hand, this group of polypeptides could represent proteins unrelated to Tg. To resolve this issue, 125I-labeled Tg was immunoprecipitated, dissolved in lysis buffer, and subjected to a secondary immunoprecipitation with antiserum specific for Tg or T4. The original Tg immunoprecipitate and the secondary immunoprecipitates were analyzed by SDS-PAGE and fluorography (see Fig. 6B).

The reimmunoprecipitation of [125I]Tg with T4-specific antibodies resulted in the reproduction of the Tg polypeptide pattern, with a marked accumulation of a 23- to 28-kDa group of polypeptides and other polypeptides migrating close to the gel electrophoretic front. This proves that the 23-kDa group of polypeptides indeed consists of Tg fragments.

A Two-dimensional TLC analysis of the iodoamino acids of [125I] Tg, [125I]CD, and [125I]APN

Intact [125I]Tg (46,000 cpm), the γ-chain of [125I]APN (1,000 cpm), and [125I]CD (700 cpm) were excised from gels after immunoprecipitation, SDS-PAGE, and fluorography. Alkaline total hydrolysis of iodinated polypeptides within the gel was followed by butanol extraction of radioactive apolar iodoamino acids, two-dimensional TLC, and autoradiography. The exposure times of x-ray films were chosen to yield autoradiographs with approximately the same radioactive signal.

In the first dimension, labeled oligopeptides were separated from smaller components, such as single amino acids. The separation of the latter was achieved in the second dimension, which was developed twice with the same solvent. The positions of the iodo tyrosine, diiodotyrosine, T3, and T4 standards that were run in the second dimension are given in the left margin of Fig. 7. In contrast to [125I]CD and [125I]APN, only [125I]Tg yielded radioactive spots comigrating with the T3/T4 standards, which were added before the

FIG. 6. Immunoprecipitation with T3- and T4-specific antibodies. A, Immunoprecipitation (Ippt) with T3/T4-specific antibodies from cell homogenates of 125I-labeled porcine thyrocytes (iodoproteins) resulted in immunoprecipitation of Tg polypeptides, with accumulation of a 23-kDa group of polypeptides (arrowhead). CD was immunoprecipitated as a control. B, 125I-labeled immunoprecipitated Tg ([125I]-Tg) was reimmunoprecipitated with antibodies specific for T3 (Ippt/T3) and T4 (Ippt/T4). Immunoprecipitation of T3 yielded a reproduction of the Tg polypeptide pattern, with accumulation of a 23- to 28-kDa group of polypeptides (arrowhead) and fragments migrating close to the front. This identifies the 23-kDa group of polypeptides as being Tg fragments enriched in hormonogenic sites.
Fig. 7. Two-dimensional TLC of $^{125}$I-labeled iodoamino acids derived from APN, CD, and Tg. Regions containing $^{125}$I-labeled Tg, CD, or APN were excised from polyacrylamide gels and subjected to total alkaline hydrolysis at 110°C overnight within the gel. Butanol-extracted iodoamino acids were analyzed by two-dimensional TLC and autoradiography. Only $[^{125}]$I$^\text{Tg}$ contained iodoamino acids that comigrated with the unlabeled T$_3$ and T$_4$ standards, which were added before alkaline hydrolysis and were present throughout the extractions and TLC runs. The positions of these standards are indicated by arrows. The positions of the standards that were run only in the second dimension are given in the left margin.

alkaline hydrolysis (arrows in Fig. 7). This finding confirms the finding of the preceding experiment, that thyroid hormones are only constructed within the polypeptide chain of Tg.

**Discussion**

Based on the observation that Tg is not the only protein that can be used as iodine acceptor by TPO (8, 9), we became interested in the identification of proteins that are iodinated together with Tg in thyrocytes, in analyzing the factors that regulate their iodination, and in evaluating the physiological significance of this process. In this report we analyzed two parameters that appear to be essential for the iodination of polypeptides. The results show that the same cellular and protein structural prerequisites are necessary for protein iodination as have been described for Tg (1, 3, 23). These proteins differ, however, in their inability to form thyroid hormones in situ.

The interaction of a polypeptide with the apical plasma membrane is necessary for its iodination

The localization of the iodinating system to the apical plasma membrane of thyrocytes lining the follicle lumen represents a compartmentation that prevents many polypeptides from reaching the site of iodination. Nevertheless, this report shows that a wide spectrum of polypeptides becomes iodinated. All iodoproteins reach the apical plasma membrane during their cellular transport. With regard to membrane interaction, two groups of polypeptides can be distinguished: 1) plasma membrane-inserted polypeptides (TPO, APN, and MHC class II polypeptides), and 2) soluble polypeptides (Tg, TSP, and CD). Some members of the second group interact with constituents of the plasma membrane, e.g. TSP and Tg. The latter has been reported to associate with a low affinity binding site on the apical plasma membrane during endocytosis (27).

In cultured follicle sheets that do not possess a Tg-filled luminal space, the apical plasma membrane is not saturated with Tg because of the low concentration of Tg in the culture medium and the low affinity characteristics of the Tg receptor. Nevertheless, the majority of the iodine label was incorporated into Tg. This implies that there are still other reasons for the strong iodination of Tg besides a high specific concentration at the apical plasma membrane.

Significance of consensus sequences for the iodination of a polypeptide

A computer search of an amino acid sequence data base (Prosis) showed that there were few polypeptides that did not possess at least one of the iodination consensus sequences. This can be expected because some of the consensus sequences are composed of two amino acids only (Glu-Tyr and Asp-Tyr). Others are the three-amino acid consensus sequences (Ser/Thr-Tyr-Ser and Glu-X-Tyr) (23). This argues for a broad substrate specificity of TPO, which is documented by a relatively large number of iodinated polypeptides in thyrocytes.

The strong iodination of Tg has been explained in part by the presence and accessibility of these consensus sequences (16 subunit of human Tg according to Ref. 23). We have shown the importance of the positioning of iodination consensus sequences within a polypeptide by comparing the iodination rate of porcine to human CD. The results suggest that the presence of one additional consensus sequence of the Asp-Tyr type on the surface of porcine CD was responsible for a much stronger iodination compared to that of human CD, which lacks this motif.

This report presents data suggesting that Tg is not only an ideal iodine acceptor, but the only polypeptide from which thyroid hormones are produced by cultured thyrocytes. Apparently, the mere appearance of iodination consensus sequences on the surface of a protein is necessary, but not sufficient, for hormonogenesis in situ.

The in vitro generation of T$_4$ within insulin, BSA, and lysozyme, as reported by Coval and Taurog (8), may be due to high concentrations of the analyzed polypeptide during the iodination procedure and to the lack of competing Tg.
High concentrations of a specific polypeptide may also allow for intermolecular coupling of iodotyrosyls (as opposed to intramolecular coupling in Tg), which would explain why insulin, with one iodination consensus sequence, served as a substrate to generate T4.

The iodination of proteins other than Tg may be a default iodination

The relatively large number of iodinated polypeptides other than Tg in thyocytes led us to speculate on their possible function. One role could be to increase the capacity of iodine storage in times of ample iodine supply. Iodine bound to these polypeptides could then be released during the course of their natural turnover, which results in a retarded liberation and availability of free iodide. The incorporation of iodine into polypeptides other than Tg would render this process independent of Tg biosynthesis, which can be rather low at times, depending on the degree of TSH stimulation of the gland (9, 33).

On the other hand, it is well documented that Tg is the most potent iodine storage molecule of the thyroid gland, receiving 80–90% of the organified iodine (34). In times of rich iodine supply, the majority of iodine bound to Tg is present as mono- and diiodotyrosine (34) and can be stored for many days and weeks (35, 36) depending on the hormone requirements of the organism. Because of the low iodination rate of polypeptides other than Tg and their inability to form thyroid hormones in situ, we propose that the iodination of these proteins represents a default iodination that functionally does not affect the central role of Tg in thyocytes. The default iodination may include other constituents of the cell surface, e.g. membrane lipids. In fact, the iodination of lipids has been shown previously (37). The chemical nature of such iodolipids, however, is still unknown. The biologically interesting aspect of our finding is the ability of thyocytes in special culture systems to efficiently iodinate proteins of the apical plasma membrane, which makes them an ideal system to examine the vectorial transport of polypeptides in polarized cells.

Acknowledgments

We thank E. Kraemer for photographic work, and acknowledge the skilled technical assistance of S. Asselborn, B. Baumann, K. Bois, and K. Kretschmann. Special thanks to Drs. K. Brix and W. Neumuller for their critical comments and helpful discussions.

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