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The Combined Action of Two Thyroidal Proteases Releases T_4 from the Dominant Hormone-Forming Site of Thyroglobulin*

ANN D. DUNN, HELEN E. MYERS, AND JOHN T. DUNN

Department of Medicine, Division of Endocrinology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

ABSTRACT

Thyroid hormones are synthesized within the thyroglobulin (Tg) molecule and must be released to reach the circulation and exert their metabolic effect. We have previously shown that three lysosomal endopeptidases, cathepsin B, D, and L, are active in the early stages of intrathyroidal degradation of Tg but do not themselves release free hormone. The current study examines the role of exopeptidases as the next step in thyroid hormone release. Human thyroidal cathepsin B and two partially purified exopeptidases, dipeptidyl peptidase II (DPPII) and lysosomal dipeptidase I (LDPI), were used to digest the 20-kDa N-terminal peptide of rabbit Tg, which contains the dominant T_4 site of Tg at residue 5. Cathepsin B acted as an endopeptidase initially, producing small T_4-containing peptides. After more extended digestion, it also acted as an exopeptidase, producing the dipeptide T_4-Gln, corresponding to residues 5 and 6 of Tg. Lysosomal dipeptidase I alone had no effect on 20 kDa but acted in combination with cathepsin B to release T_4 from the T_4-Gln dipeptide. Although addition of DPPII increased the release of hormone from ^{125}I-Tg by an extract of DPPII-deficient lysosomes, it had no apparent effect on the degradation of the 20-kDa peptide, either alone or in combination with cathepsin B or LDPI. Thus DPPII may act in synergy with some other endopeptidase, or alternatively, may play a role in the release of hormone from other sites in Tg. We conclude that the N-terminus of Tg, which contains its major hormonogenic site, is particularly susceptible to hydrolysis by the endopeptidase cathepsin B and that cathepsin B additionally has an important exopeptidase action that allows it to release a T_4 dipeptide that is then further degraded by LDPI to release free T_4. (*Endocrinology* **137:** 3279–3285, 1996)

THE THYROID hormones are synthesized and stored at specific sites within the prohormone thyroglobulin (Tg), a 660-kDa glycoprotein (1). Proteolytic processing of Tg and the attendant release of its hormones are believed to occur within the endosome-lysosomal compartment of the thyroid cell (2, 3). Our earlier work suggests that, in the initial stage of intrathyroidal digestion, Tg is cleaved at discrete sites by three endopeptidases, cathepsins B, D, and L, to release hormone-enriched peptides from both the amino and carboxy-termini (2, 4). Subsequently, exopeptidases are thought to free T_4 and T_3 from these intermediates, but there has been little information about the enzymes involved in this process. One potential candidate is a cathepsin H-like enzyme, TP-1, which has been isolated from hog thyroids and found to slowly remove T_4 from intact Tg, and more rapidly from a 15-kDa peptide derived from Tg (5).

Indirect evidence suggests that two additional exopeptidases, dipeptidyl peptidase II (DPPII) and lysosomal dipeptidase I (LDPI), could also play a role in thyroid hormone release. Dipeptidyl peptidase II is a serine protease that cleaves N-terminal dipeptides from larger peptides. It is widely distributed among mammalian tissues but occurs in especially high levels in the thyroid, where it has been localized to lysosomes (6). Lysosomal dipeptidase I, a less well characterized enzyme, appears to be a metalloprotease, based on its sensitivity to chelating agents, and is capable of cleaving a variety of dipeptides (7). Loughlin and Trikojus (8) partially purified a zinc-activated enzyme with LDPI-like activity from beef thyroids but could not relate its activity to proteolytic processing of Tg. More recently, we have shown that release of hormone from Tg by lysosomal extracts from human thyroids can be partially blocked by EDTA, suggesting the involvement of a LDPI-like protease (2). An additional enzyme with potential exopeptidase activity in the thyroid is cathepsin B (CB). We have previously shown that CB acts as an endopeptidase on Tg with a preference for N-terminal sites (4), but this enzyme isolated from extrathyroidal tissue has been reported to also have dipeptidylcarboxypeptidase and carboxypeptidase activities with a variety of substrates (9, 10).

In the present studies, we investigated the potential roles of DPPII and LDPI together with CB in releasing thyroid hormones, using the N-terminal 20-kDa peptide of rabbit Tg as substrate to examine their action. As described before (4), we used rabbit Tg, as substrate, in the absence of available ^{125}I-labeled human Tg. We have found rabbit Tg closely homologous to that of human Tg, a relationship supported by recent analysis of proteins in lagomorphs and primates (11). The 20 kDa is cleaved from the bulk of Tg during the process of iodination *in vivo* (12). Although this peptide varies in size among species, its fifth residue represents the most important hormone-forming site of Tg in all species thus far examined, and its initial amino acid sequence has remained invariant among all mammals including humans (1).

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Address all correspondence and request for reprints to: Dr. Ann D. Dunn, University of Virginia Health Sciences Center, Box 511, Charlottesville, Virginia 22908. E-mail: add7K@virginia.edu.

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Materials and Methods

Enzyme assays

Protease activities were measured fluorometrically with benzoyl-arginine-2-naphthylamide for CB, glycyl-phenyl alanine-2-napthylamide for DPPI, and L-lysyl-alanine-2-napthylamide or lysyl-alanine 7-amido-4-methylcoumarin for DPPII, as substrates, as previously described (13). One unit of enzyme activity is equivalent to 1 nmol of 2-naphthylamine or amido-4-methylcoumarin released/min. Lysosomal dipeptidase I activity was measured with seryl-methionine as substrate (10 mM) in an assay modified from McDonald *et al.* (14). Samples were preincubated in 0.05 M sodium acetate buffer, pH 5.5, containing 0.3 mM zinc acetate at 37 C for 15 min, at which time the substrate was added and the incubation continued for 30 min. Samples (50 μl) were taken after 0 and 30 min incubation and the release of amino acids determined colorimetrically using 2,4,6-trinitrobenzene sulfonic acid. One unit of activity is defined as 1 nmol dipeptide hydrolyzed/min.

Isolation of lysosomes from human thyroid glands

Normal-appearing thyroid tissue (2–6 g) was obtained from patients undergoing thyroidectomy for various medical indications. This use has been approved by the University of Virginia Human Investigations Committee. Lysosomes were prepared by differential centrifugation followed by Percoll density centrifugation as previously described (2). A soluble extract was obtained from frozen-thawed material by centrifugation.

Purification of substrate and enzymes

In vivo labeled ^{125}I-Tg was prepared from rabbit thyroids as previously described (4, 15). The 20-kDa peptide was released from the N-terminus of Tg by reduction with 2-mercaptoethanol in the presence of 8 M urea at pH 8.5. The reduced protein was alkylated with acrylonitrile. The alkylation reaction was terminated after 10 min by an excess of 2-mercaptoethanol to avoid modification of lysine residues. The sample was immediately placed on a Sephacryl S300 column equilibrated in 0.1 M sodium phosphate buffer, pH 7.3, containing 6 M urea at 22 C to separate the 20-kDa peptide from the bulk of Tg. Final purification of 20-kDa was by HPLC on a C_3 column with a gradient of NH_4HCO_3: CH_3CN (15). Purified 20-kDa contained 71% of its ^{125}I as T_4 and 5% as T_3. Cathepsin B was isolated from human thyroid glands obtained at autopsy as described earlier (15).

Purified preparations of both LDPI and DPPII were obtained by two different procedures as outlined in Fig. 1. In Procedure 1, 34 g of thyroid tissue from a patient with Graves' disease were extracted in 2% 1-butanol containing 1 mM EDTA and 0.3 M NaCl, and the extract then precipitated in 80% saturated $(NH_4)_2SO_4$. An aqueous solution of the precipitate was introduced onto a column (2.5 × 40 cm) of hydroxylapatite. A linear gradient of 0.05–0.5 M potassium phosphate buffer, pH 5.9, in 0.15 M NaCl eluted DPPII activity at 0.1 M potassium phosphate, followed by LDPI in a broad peak between 0.1–0.3 M potassium phosphate. After ultrafiltration and further purification on a Bio-Gel A 0.5 M column equilibrated in 0.05 M sodium phosphate buffer, pH 6.9, fractions with LDPI activity and those with DPPII activity from hydroxylapatite chromatography were each equilibrated in 0.02 M bis Tris propane buffer, pH 6.5, and further purified by FPLC anion exchange chromatography (Mono Q, HR5/5, Pharmacia Biotech Inc., Piscataway, NJ) with a linear gradient of NaCl (0–0.5 M). Lysosomal dipeptidase I activity eluted in the void volume under these conditions and DPPII activity eluted with 0.1 M NaCl. Fractions containing each enzyme were pooled and concentrated by ultrafiltration, then passed through an affinity column of pepstatin immobilized on Sepharose 6B to remove contaminating cathepsin D (16). Active fractions from the LDPI preparation were equilibrated in 0.1 M sodium succinate buffer, pH 4.5, and subjected to FPLC cation exchange chromatography (Mono S, HR 5/5 column) with a linear gradient of 0–0.5 M NaCl. Lysosomal dipeptidase I activity, eluting at 0.18 M NaCl, was concentrated and used to digest Tgs 20-kDa peptide. The final purification step for DPPII was by FPLC size exclusion (Superose 12, HR 10/30). The column was calibrated with the molecular mass markers alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa). The active fraction was concentrated and used to digest Tg and its 20 kDa peptide.

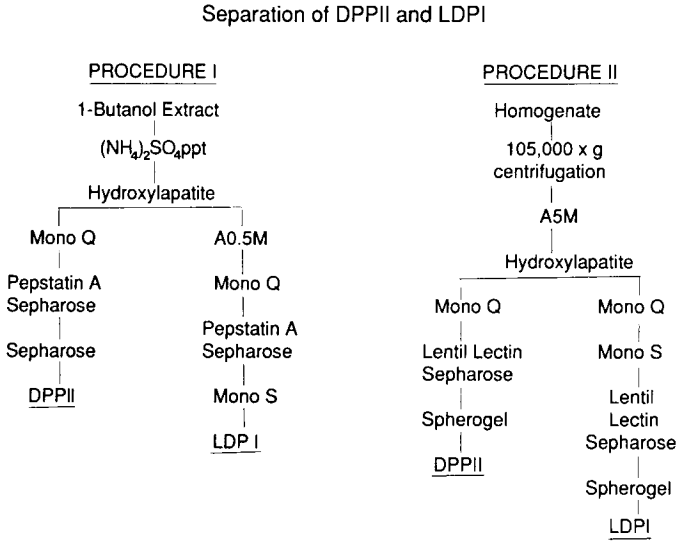

FIG. 1. Separatory steps taken in the purification of DPPII and LDPI by Procedures 1 and 2.

In an attempt to improve recovery, a second purification scheme was adopted. In Procedure 2, 24 g of thyroid tissue from a multinodular gland were frozen and thawed, then homogenized in 0.05 M sodium phosphate buffer, pH 6.9, and centrifuged at 105,000 × *g*. Proteases in the soluble fraction were separated from Tg on a column (2.5 × 100 cm) of Bio-Gel A5 M equilibrated in 0.05 M sodium phosphate buffer, pH 6.5, then partially purified by sequential chromatographic steps using hydroxylapatite, Mono Q and Mono S (for LDPI) under the conditions described above. Further purification was by affinity chromatography on a lentil lectin-Sepharose 4B column (0.9 × 5 cm) equilibrated in 0.01 M potassium phosphate buffer, pH 7.0, with each enzyme eluted by 0.5 M α-methyl-D-mannoside. Final purification for each enzyme was by HPLC size exclusion (Spherogel 3000; Beckman, Fullerton, CA) equilibrated in 0.05 M sodium phosphate buffer, pH 7.0.

Active fractions of DPPII and LDPI were concentrated and used for enzyme analysis. Approximately 5 μg of the DPPII preparation were treated with 2.5 μCi of ^{3}H-labeled-diisopropyl fluorophosphate (DFP), an affinity site inhibitor of serine proteases, (specific activity 8.4 μCi/μmol) for 1 h at 37 C. The ^{3}H-labeled DPPII and a sample of the LDPI preparation were each reduced in 2-mercaptoethanol then run on SDS-PAGE using Minigel slabs (Bio-Rad, Hercules, CA) in a discontinuous system (17). The gels were stained with Coomassie blue, and the ^{3}H-DFP-DPPII gel was then treated with EN^{3}HANCE (New England Nuclear, Boston, MA) before drying and exposure to x-ray film. These enzyme preparations were used to determine pH optima with standard synthetic substrates and sensitivity to protease inhibitors. Protein determinations were by the method of Lowry *et al.* (18) using BSA as standard.

Hydrolysis of Tg by a DPPII-deficient lysosomal extract

A lysosomal extract of normal-appearing thyroid tissue, well removed from any pathologic lesion, was obtained from a patient undergoing total thyroidectomy for medullary thyroid carcinoma. This extract had generally low levels of protease activities, but barely detectable levels of DPPII activity (1.5 U DPPII enzyme activity/mg lysosomal protein compared with 112 ± 94 U/mg in lysosomal extracts of six thyroids obtained at surgery under similar conditions). The DPPII-deficient extract (22 μg) was incubated with ^{125}I-Tg (35 μg) ± 1.2 U of DPPII, purified by Procedure 1, in sodium acetate buffer pH 5.0 and 4.6 mM dithiothreitol at 37 C for 20 h. The digestion products were analyzed both by one-dimensional paper chromatography and by 2-dimensional TLC (4).

Limited hydrolysis of Tg's 20-kDa peptide by CB

Tg's 20-kDa peptide (200 μg) was incubated with or without CB (5.6 U) in 0.15 M sodium phosphate buffer, pH 4.5 and 2.5 mM dithiothreitol

in a total volume of 275 μl at 37 C for 60 min and the reaction stopped by the addition of 8 μl of 1 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64). The ^{125}I-labeled digestion products were analyzed by SDS-PAGE on tube gels in a continuous system (19) and compared with mol wt standards run simultaneously. Fractions of interest were analyzed for ^{125}I-iodo amino acid content after Pronase digestion as described above. The remainder of the incubate was placed on a reverse phase C_3 column for separation on HPLC using the system described above. After determination of ^{125}I, selected fractions were dried under N_2 then directly sequenced by automated Edman degradation as previously described (4).

Extensive hydrolysis by CB ± LDPI or DPPII

The 20-kDa peptide of Tg (20 μg) was incubated with CB (110 U) with or without DPPII (92 U) and LDPI (44 U) in the same medium as for the limited digestion in a total volume of 500 μl at 37 C for 16 h, followed by immediate separation on HPLC in the NH_3HCO_3:CH_3CN system. Fractions of interest were sequenced by Edman degradation and their ^{125}I-iodoamino acid content determined before and after Pronase digestion. To determine if DPPII or LDPI was able to release T_4 from 20-kDa in the absence of CB, 2 μg of 20-kDa were incubated with DPPII (9 U) or LDPI (11 U) under the same conditions and digestion products were examined for release of free iodoamino acids by paper chromatography.

Results

Characterization of exopeptidases

Dipeptidyl peptidase II and LDPI as prepared by Procedure 1 were free of contamination by other proteases including DPPI. The latter finding was of particular note because LDPI from beef spleen was reported to copurify with DPPI (14). The two exopeptidases obtained by Procedure 1 were used to digest Tg and its 20-kDa peptide. Lack of material prevented their analysis by SDS-PAGE. Dipeptidyl peptidase II from Procedure 2 was also free of contaminating proteases, whereas LDPI from Procedure 2, although free of CB and DPPI, activity contained trace amounts of DPPII activity. Enzymes obtained by Procedure 2 were analyzed by SDS-PAGE under reducing conditions (Fig. 2). Three peptide bands were visible in the LDPI sample (Fig. 2, lane 2) with apparent molecular masses of approximately 60 kDa, 53 kDa, and 50 kDa. Attempts to identify LDPI among these bands by ^{65}Zn-affinity labeling were unsuccessful. Their relationship to LDPI, therefore, remains to be determined. The ^{3}H-DFP-treated DPPII was located by autoradiography as a band corresponding to a molecular mass of approximately 56 kDa (Fig. 2, lane 5). A faint stained band at the same position was seen on the original gel but is not obvious in the photograph (Fig. 2, lane 4). The several stained bands visible on the gel (Fig. 2, lane 4) of approximately 60–73 kDa are presumed to represent contaminants of the preparation. Efforts to further purify either enzyme resulted in significant losses of activity. These partially purified preparations were therefore used to characterize enzyme activity. The specific activities of LDPI and DPPII obtained from each purification procedure are given in Table 1.

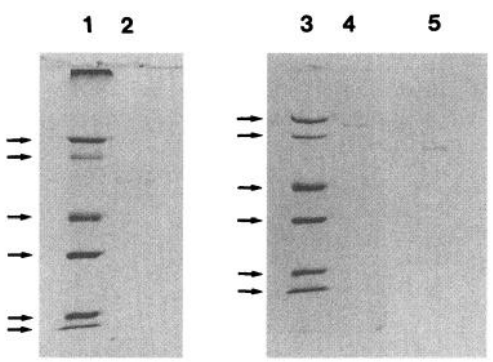

FIG. 2 Preparations of LDPI and ^{3}H-DFP-treated DPPII (Procedure 2 purification) analyzed by SDS-PAGE on 9% gels under reducing conditions. Lanes 1 and 3, Molecular mass markers identified by *arrows*: phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa). The origin material in lane 1 is an artifact. Lane 2, LDPI, Coomassie blue stained. Lane 4, ^{3}H-DFP-treated DPPII, Coomassie blue stained. Lane 5, autoradiogram of 4.

The properties of thyroidal DPPII (Table 1) resemble those described for this enzyme in other tissues (6, 20, 21), including its apparent molecular mass by size exclusion chromatography under nondenaturing conditions and its subunit size by SDS-PAGE, pH optimum with a synthetic substrate, and sensitivity to both a serine protease inhibitor [phenylmethylsulfonoyl fluoride (PMSF)] and a large cation (puromycin).

A lysosomal localization for LDPI activity was confirmed by Percoll gradient centrifugation of unfrozen surgical thyroid tissue. As anticipated from its subcellular location the enzyme had an acidic pH optimum (Table 1). Its apparent molecular mass of 100 kDa by size exclusion chromatography was smaller than the 180 kDa reported for LDPI from beef spleen (14). The properties of LDPI from thyroid tissue confirm its designation as a metalloendopeptidase: its activity against the synthetic substrate seryl-methionine was increased by 70% in the presence of added 0.15 mM Zn^{++}, was completely inhibited by 0.3 mM EDTA, and this inhibition was overcome by a 2-fold molar excess of Zn^{++}. The activity profile of this enzyme with other protease inhibitors was also consistent with that of a metalloprotease (Table 1). It was sensitive to the chelating agent 1,10-phenanthroline but showed little or no sensitivity to PMSF or the cysteine protease inhibitor E64.

TABLE 1. Properties of exopeptidases

	DPPII	LDPI
Specific activity (U/μg)[a]		
Procedure 1	1.1	9
Procedure 2	0.4	26
Molecular mass (kDa) by size exclusion[b]		
Procedure 1	110	
Procedure 2	120	100
Molecular mass (kDa) by SDS-PAGE	56	
pH optimum	5.5	5.0
Percent inhibition		
1 mM PMSF	85	9
1 mM Puromycin	74	0
1 mM Phenanthroline	0	94
1 mM E64	0	0

Except as noted, data are for enzymes obtained by Procedure 2.

[a] Enzyme activity was measured using Lys-Ala-2-napthalamide (DPPII) or seryl-methionine (LDPI) as substrate. Enzymes were incubated with individual inhibitors for 15 min before the introduction of the substrate.

[b] Molecular mass determinations by size exclusion for DPPII, Procedure 1, were on a Superose 12 column; those for LDPI and DPPII, Procedure 2, were on a Spherogel 3000 column.

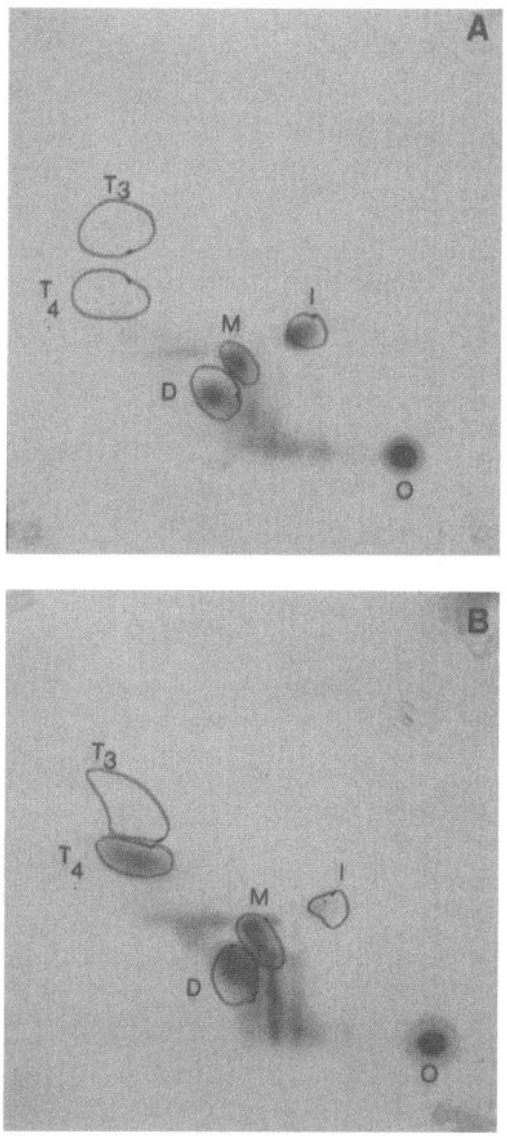

FIG. 3. Autoradiogram of ^{125}I-Tg digested by an extract of DPPII-deficient lysosomes. Separation was by two-dimensional TLC. Vertical dimension:1-butanol-ethanol-1N ammonia (5:1:2); horizontal dimension, 1-butanol-2N acetic acid (1:1). Iodoamino acid markers were cochromatographed and their positions identified under UV light. O, origin; M, 3-iodotyrosine; D, 3,5 diiodotyrosine. A, Lysosomal extract; B, Lysosomal extract plus added DPPII.

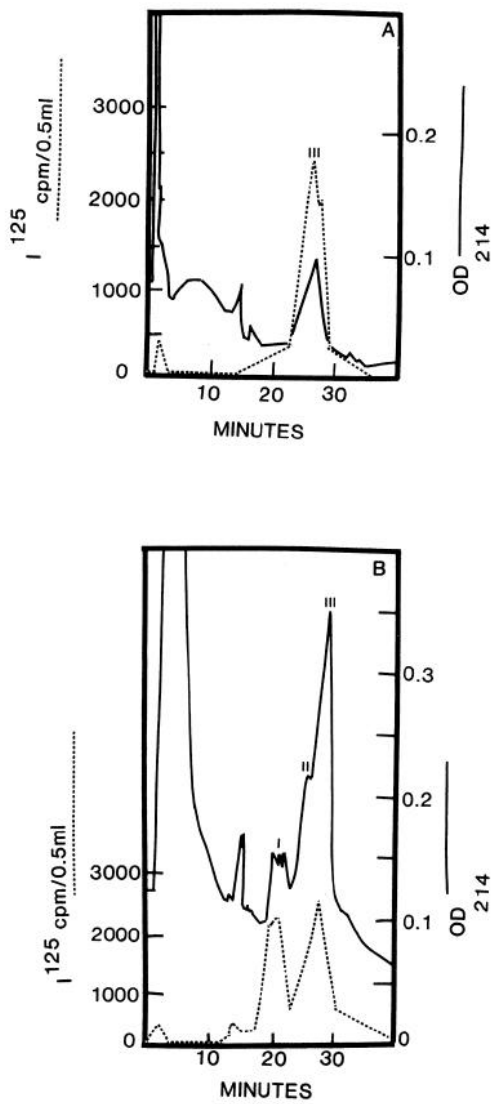

FIG. 4. HPLC separation of products from limited digestion (1 h incubation) of Tg's 20-kDa peptide (200 μg) by CB (5.6 U). A, Control, no enzyme; B, CB.

Digestion of Tg by a DPPII deficient-lysosomal extract

Digestion of ^{125}I-Tg by an extract of DPPII-deficient lysosomes for 20 h resulted in 48% of Tg's ^{125}I remaining in peptide linkage as assessed by 1-dimensional paper chromatography. Addition of purified DPPII to the incubation decreased peptide- bound ^{125}I to 26% and increased free ^{125}I-labeled thyroid hormone from 4% to 9%. The increase in T_4 release with DPPII was confirmed by 2-dimensional TLC (Fig. 3).

Digestion of 20 kDa by purified proteases

Limited digestion of Tg's 20-kDa peptide by CB produced several fragments which were separated on HPLC (Fig. 4B). Peak I in Fig. 4B contained 28% of the ^{125}I present in intact 20 kDa and, according to its optical density profile, was composed of at least three peptides with apparent mol mass of <5 kDa on SDS-PAGE and containing 85% or more of their ^{125}I as T_4, suggesting that they originated from the N terminus of 20kDa and included residue 5 (site A). Peak II in Fig. 4B had only 22% of its ^{125}I as thyroid hormone, compared with 76% in the intact 20-kDa peptide. Amino acid sequencing of Peak II identified 2 peptides in approximately equal molar amounts; one corresponding to N-terminal 20 kDa and a second peptide whose N-terminal sequence was A–TEVPGS-QPG. This sequence has an 80% homology with human Tg starting at residue 56, and a 55% homology starting with residue 124 (22), suggesting proteolytic cleavage at one of these sites within 20 kDa. Peak III in Fig. 4B probably represented unhydrolyzed 20 kDa because its mobility on HPLC and SDS-PAGE and its hormone content were identical to those of the intact peptide.

Extensive exposure to LDPI or DPPII, alone or in combination, had no demonstrable effects on Tg's 20-kDa peptide. In contrast extended digestion of this peptide by CB resulted in the disappearance of the intact peptide (Peak 5 in Fig. 5A) and the appearance of three ^{125}I products (Peaks 1, 2, and 4 in Fig. 5B). Table 2 shows the distribution of 20 kDa's ^{125}I among the peaks in Fig. 5 and the iodoamino acid content and peptide sequence for each. Peak 1 contained free ^{125}I and ^{125}I-iodopeptides but no hormone. Its dominant sequence had a 60% homology with the peptide identified from limited digestion of 20 kDa by CB and a 60% homology with human Tg starting at either residue 56 or 124. Peak 2 was identified as the T_4-dipeptide corresponding to residues 5 and 6 of Tg. Peak 4 appeared as a doublet by its optical density. It was identified as the N-terminus of 20 kDa but had a shorter retention time on HPLC than the intact peptide (Fig. 5A, peak 5). Of interest was the identification of both aspartic acid and asparagine at residue 8 (underlined in Table 2), corresponding to the two halves of the doublet which had been sequenced separately. Only aspartic acid has been identified at this site in all mammalian species thus far examined (1). Addition of DPPII to CB's incubation with 20 kDa had no apparent effect on the digestion products (Fig. 5C), whereas the inclusion of LDPI to the digestion, with or without added DPPII, resulted in a loss of T_4 in dipeptide form (Peak 2) and the appearance of free T_4 (Peak 3, Fig. 5D and Table 2).

Discussion

The current studies indicate that CB can act as both an endopeptidase and an exopeptidase in the processing of Tg. We identified an early and perhaps primary cleavage site in rabbit Tg that could correspond to either residue 56 or 124 of human Tg. Cathepsin B also produced small T_4-containing

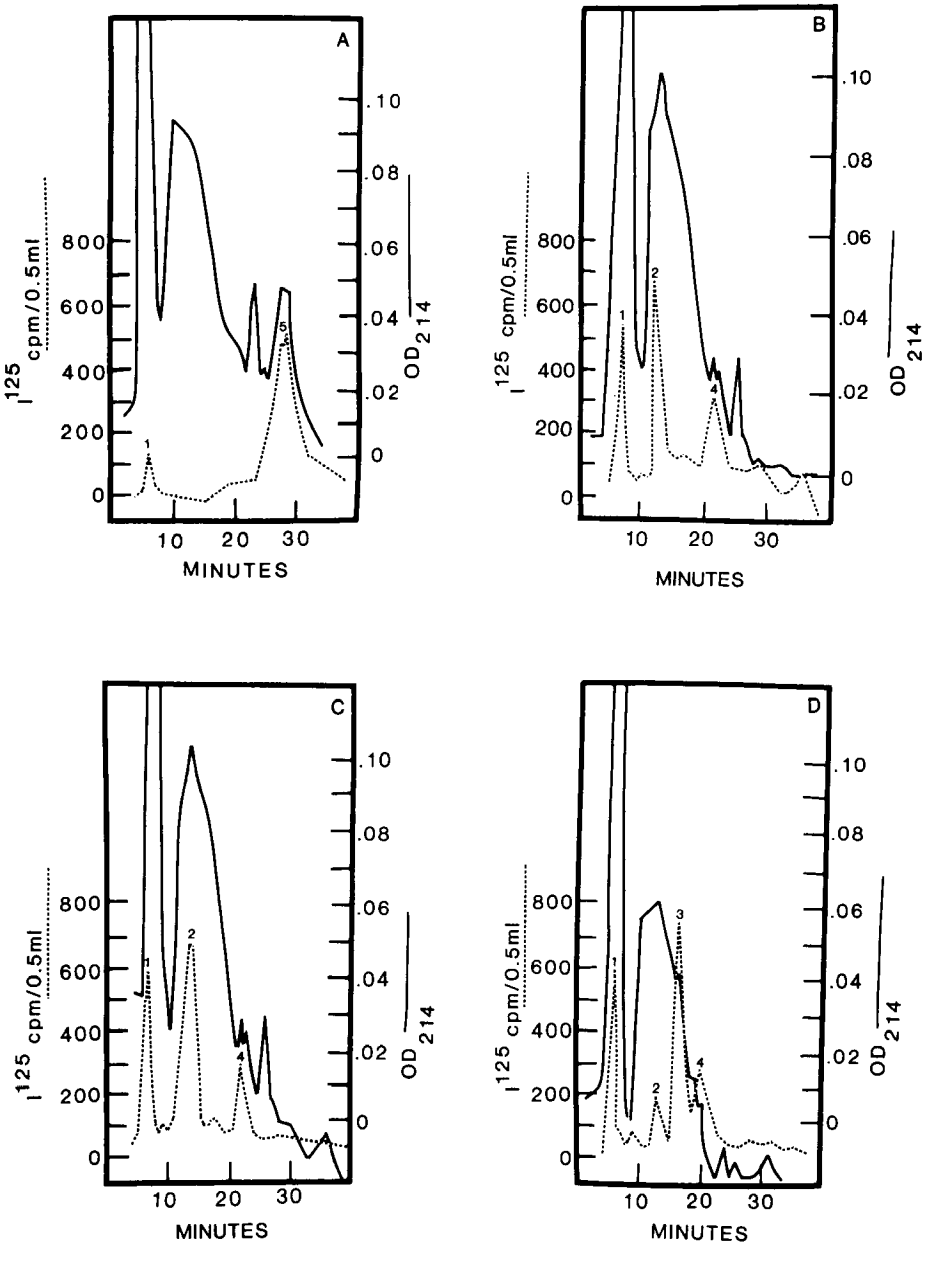

FIG. 5. HPLC separation of products from extensive digestion (16 h incubation) of Tg's 20-kDa peptide (20 μg). A, Control, no enzyme; B, CB (110 U); C, CB (110 U) plus DPPII (92 U); D, CB (110 U) plus DPPII (92 U) plus LDPI (44 U).

TABLE 2. Digestion of the 20-kDa peptide pf Tg by CB with and without dipeptidyl peptidase II and lysosomal dipeptidase I

HPLC peak[a]	% of 20 kDa's total ^{125}I in peak				^{125}I-Iodoamino acid content of peak	N-terminal sequence[b]
	CB	CB+DPPII	CB+LDPI	CB+DPPII+LDPI		
1	15	14	14	16	DIT/MIT	(56, 124) ADGMPVQGSR
2	28	30	7	7	T_4	(5)T_4Q
3	5	5	29	32	T_4	(5)T_4
4	11	11	13	14	T_4	(1)NIFE*QVDAQPLR[c] (1)-IFE*QVNAQ

[a] Refers to peaks in Fig. 5, A–D.
[b] Numbers in *parentheses* refer to location of N-terminal residue based on the cDNA-derived sequence of human Tg (22).
[c] *Asterisk* denotes radioactive residue, identified as T_4 by paper chromatography.

peptides under limited digestion conditions; whether from a combination of endopeptidase and exopeptidase activities by CB or directly from endopeptidase cleavages remains to be determined. In any event, the rapid formation of the N-terminal oligopeptides by CB from Tg's 20-kDa peptide suggests that this important hormone-containing region is very susceptible to proteolysis, a prerequisite for rapid release of hormone. Of additional interest was the production of the T_4 dipeptide by CB, thus confirming this enzyme's dipeptidyl peptidase action and suggesting its importance in Tg processing. The earlier studies of Nakagawa and Ohtaki (5) also suggested a potential role for CB in thyroid hormone release. They isolated a CB-like enzyme (TP-2) from hog thyroids which synergized with TP-1 (a cathepsin H-like enzyme) to release hormone at an unidentified site or sites within Tg.

The present paper establishes both the presence of LDPI in the human thyroid and a function for it in the release of thyroid hormone. The enzyme shares characteristics with LDPI in other tissues (7), including its lysosomal localization, an acid pH optimum for activity, and its identification as a metalloprotease. The apparent molecular mass of the thyroidal enzyme was 100 kDa in the present experiments, whereas LDPI from beef spleen has been reported to be 180 kDa, based on its copurification with DPPI (14). In our experience with thyroid extracts from a variety of sources, LDPI may coelute either with DPPI or DPPII on size exclusion chromatography in an unpredictable manner unrelated to either the initial extraction procedure or to the animal species source, perhaps reflecting variable subunit association (unpublished observations). Additional work is needed to identify the subunit size of LDPI. The ability of thyroidal LDPI to act together with CB in freeing T_4 from Tg's 20-kDa peptide suggests that this exopeptidase plays an important role *in vivo* in the release of hormone from its major storage site in Tg. Our earlier finding that EDTA partially inhibits the release of hormone from Tg by lysosomal extracts is consistent with such a role (2).

The abundance of DPPII in thyroid tissue (6), combined with its ability to significantly increase hormone release by a DPPII-deficient extract of thyroid lysosomes as reported here, makes this exopeptidase an attractive candidate for further studies of Tg processing. However, we found that under *in vitro* conditions using Tg's 20-kDa peptide as substrate, DPPII had no apparent effect on hormone release from the major T_4 site (residue 5), either alone or in combination with CB or LDPI. These results do not preclude a physiological role for DPPII in thyroid hormone release because under *in vivo* conditions it may synergize with endopeptidases other than CB, such as cathepsins L or D, or it may play a role in releasing hormone from alternate sites in Tg. In addition to residue 5, we have described three other hormonogenic sites within rabbit Tg labeled *in vivo* with ^{125}I which together accounted for 32% and 41% of Tg's ^{125} I-labeled T_4 and T_3, respectively (23).

Two incidental findings relate to our continuing interest in Tg structure and hormone synthesis. One is the isolation of approximately equal amounts of two N-terminal peptides, one with asparagine and the other with aspartate at residue 8. Every other mammalian species examined so far has had aspartate at this position, and asparagine is unlikely to be an isolation artifact. We suggest this may represent heterogeneity in the message for Tg's N-terminus, a new finding. The second point of interest for peptide structure is the several sequences we report that are homologous to human TG beginning at residue 56 (ANGSEVLGSRQPG) and 124 (AEGMEVYGTRQLG). Indirect evidence from several authors including us has pointed to the Y at residue 130 (*underlined* in the latter sequence) as a donor of T_4's outer ring at residue 5 (24–26). The absence of a Y or of ^{125}I at this position in the peptides we describe here suggests that the outer ring donor may come from elsewhere, at least in rabbit Tg (which nevertheless has vigorous T_4 formation at residue 5) (23).

References

1. **Dunn JT** 1991 Thyroglobulin: chemistry and biosynthesis. In: Braverman LE, Utiger RD (eds) The Thyroid. JB Lippincott Co, Philadelphia, pp 98–110
2. **Dunn AD, Crutchfield HE, Dunn JT** 1991 Proteolytic processing of thyroglobulin by extracts of thyroid lysosomes. Endocrinology 128:3073–3080
3. **Kostrouch Z, Munari-Silem Y, Rajas F, Bernier-Valentin F, Rousset B** 1991 Thyroglobulin internalized by thyrocytes passes through early and late endosomes. Endocrinology 129:2202–2211
4. **Dunn AD, Crutchfield HE, Dunn JT** 1991 Thyroglobulin processing by thyroidal proteases. Major sites of cleavage by cathepsins B, D, and L. J Biol Chem 266:20198–20204
5. **Nakagawa H, Ohtaki S** 1985 Thyroxine (T_4) release from thyroglobulin and its T_4-containing peptide by thyroid thiol proteases. Endocrinology 116:1433–1439
6. **McDonald JK, Callahan PX, Ellis S** 1971 Polypeptide degradation by dipeptidyl aminopeptidase I (cathepsin C) and related peptidases. In: Barrett AJ, Dingle JT (eds) Tissue Proteinases. North-Holland Publishing Co, Amsterdam, pp 69–107
7. **McDonald JK, Barrett AJ** 1986 Mammalian Proteases: A Glossary and Bibliography. Academic Press, New York, vol 2:298–300
8. **Loughlin RE, Trikojus VM** 1964 A metal-dependent peptidase from thyroid glands. Biochim Biophys Acta 92:529–542
9. **Aronson NN Jr, Barrett AJ** 1978 The specificity of cathepsin B. Biochem J 171:759–765

10. **Takahashi T, Dehdarani AH, Yonezawa S, Tang J** 1986 Porcine spleen cathepsin B is an exopeptidase. J Biol Chem 261:9375–9381
11. **Graur D, Duret L, Gouy M** 1996 Phylogenetic position of the order Lagomorpha (rabbits, hares and allies). Nature 379:333–335
12. **Dunn JT, Dunn AD, Heppner DG Jr, Kim PS** 1981 A discrete thyroxine-rich iodopeptide of 20,000 daltons from rabbit thyroglobulin. J Biol Chem 256:942–947
13. **Dunn AD** 1984 Stimulation of thyroidal thiol endopeptidases by thyrotropin. Endocrinology 114:375–382
14. **McDonald JK, Zeitman BB, Ellis S** 1972 Detection of a lysosomal carboxypeptidase and a lysosomal dipeptidase in highly-purified dipeptidyl aminopeptidase I (cathepsin C) and the elimination of their activities from preparations used to sequence peptides. Biochem Biophys Res Commun 46:62–70
15. **Dunn AD, Dunn JT** 1988 Cysteine proteinases from human thyroids and their actions on thyroglobulin. Endocrinology 123:1089–1097
16. **Dunn AD, Dunn JT** 1982 Thyroglobulin degradation by thyroidal proteases: action of purified cathepsin D. Endocrinology 111:280–289
17. **Laemmli UK** 1970 Cleavage of structural proteins during assembly of the head of bacteriophage T_4. Nature 227:680–685
18. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275
19. **Weber K, Osborn M** 1969 The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 224:4406–4412
20. **Fukasawa K, Fukasawa KM, Hiraoka BY, Harada M** 1983 Purification and properties of dipeptidyl peptidase II from rat kidney. Biochim Biophys Acta 745:6–11
21. **Eisenhauer DA, McDonald JK** 1986 A novel dipeptidyl peptidase II from the porcine ovary. J Biol Chem 261:8859–8865
22. **Malthiery Y, Lissitzky S** 1987 Primary structure of human thyroglobulin deduced from the sequence of its 8448-base complementary DNA. Eur J Biochem 165:491–498
23. **Dunn JT, Anderson PC, Fox JW, Fassler CA, Dunn AD, Hite LA, Moore RC** 1987 The sites of thyroid hormone formation in rabbit thyroglobulin. J Biol Chem 262:16948–16952
24. **Marriq C, Lejeune P-J, Venot N, Vinet L** 1991 Hormone formation in the isolated fragment 1–171 of human thyroglobulin involves the couple tyrosine 5 and tyrosine 130. Mol Cell Endocrinol 81:155–164
25. **Ieiri T, Cochaux P, Targovnik HM, Suzuki M, Shimoda S, Perret J, Vassart G** 1991 A 3′ splice site mutation in the thyroglobulin gene responsible for congenital goiter with hypothyroidism. J Clin Invest 88:1901–1905
26. **Lamas L, Anderson PC, Fox JW, Dunn JT** 1989 Consensus sequences for early iodination and hormonogenesis in human thyroglobulin. J Biol Chem 264: 13541–13545