Dityrosine bridge formation and thyroid hormone synthesis are tightly linked and are both dependent on *N*-glycans

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Abstract Formation of dityrosine bridges is a ubiquitous process mainly attributed to oxidative stress leading to protein degradation and cellular damages. Here we show that dityrosine formation is involved in a physiological process, thyroid hormone synthesis, and is strictly dependent on structural characteristics, namely *N*-glycans, presented by the protein acting as the prothyroid hormone. We used two isoforms of the N-terminal thyroid hormone forming domain (NTD) of human thyroglobulin: one without *N*-glycan (19 kDa isoform) and the other with high mannose type structures (25 kDa isoform). Both isoforms were able to form iodotyrosines after in vitro iodination. However, iodotyrosine coupling to form thyroxine did not occur with the unglycosylated 19 kDa NTD. In contrast, the 25 kDa isoform formed thyroxine. Strikingly, thyroxine synthesis was accompanied by dimerization of the 25 kDa isoform and formation of a dityrosine bridge; none of this was observed with the 19 kDa isoform. Taken as a whole, our results indicate that dimerization through dityrosine bridging accompanies and could have a role in thyroid hormone synthesis.

Key words: Thyroid; Hormone synthesis; Glycosylation; Dityrosine

1. Introduction

Thyroglobulin (Tg) is a large dimeric glycoprotein ($2\times330\,000$ Da) that is the substrate for the synthesis of the thyroid hormones triiodothyronine (T3) and thyroxine (T4). Synthesis requires the initial iodination of specific tyrosines, yielding either monoiodotyrosine (MIT) or diiodotyrosine (DIT). The possible mechanism for iodination of tyrosine residues and coupling of certain iodotyrosyl residues to form hormone is not fully elucidated but it is clear that in vivo coupling is catalyzed by thyroperoxidase (TPO) [1]. It has been demonstrated that this enzyme also plays a role in the choice of the tyrosine residues of Tg which can be iodinated and/or coupled to form iodotyrosines and/or thyroid hormones [2]. Leonardi et al. [3] showed that, in the case of bovine Tg, during this reaction of coupling, some molecules of Tg were dimerized by formation of dityrosine bridges and that this process required the presence of iodide.

A major difficulty in studying Tg is its large size and molecular heterogeneity. This precludes detailed analysis of the various domains in terms of structure-activity relationship. To circumvent this problem, we focused on the N-terminal domain (NTD) of human Tg, which contains the preferential site of hormone synthesis located at Tyr^5 [4]. This peptide, obtained after CNBr treatment of a poorly iodinated Tg, is able to form hormone (T4) by in vitro iodination and iodotyrosine coupling. It has an apparent molecular mass ranging from 19 to 25 kDa according to the number of oligosaccharide side chains linked at Asn^{57} and Asn^{91} [5]. The large variability of the *N*-glycan structures borne by the NTD offered an interesting opportunity to determine the role of *N*-glycans in thyroid hormone synthesis and their possible association with the dimerization process through formation of dityrosine bridges. We selected two types of NTD: the first was unglycosylated and the second was rich in high mannose structures. We observed that dityrosine bridge formation and thyroid hormone synthesis are both dependent on the *N*-glycan structures and both the results of the same oxidative process under the action of TPO and the H_2O_2 generating system.

2. Materials and methods

2.1. Preparation of the NTD of human Tg

Tg was purified as described previously [6] from a single euthyroid goiter and was then treated with CNBr. The NTD issued from this Tg was separated by chromatography on a Sephadex G-200 column (Pharmacia Biotech, Uppsala, Sweden) in 1 M propionic acid [7].

2.2. ConA-Sepharose chromatography

The NTD was subjected to affinity chromatography on a ConA-Sepharose 4B column (Pharmacia Biotech) at room temperature. The column (1×10 cm) was equilibrated with 50 mM Tris-HCl, pH 7.4 buffer containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 100 mM NaCl. Previously [5], we showed that the non-retained fraction was a mixture of unglycosylated NTD (19 kDa) and fully glycosylated NTD (25 kDa) bearing two triantennary complex type oligosaccharides and that the fraction was eluted with an equilibration buffer. The fraction weakly retained by the lectin was constituted of isoforms bearing both bi- and triantennary complex type structures and was eluted with 10 mM α-methyl-D-glucopyranoside. The firmly retained fraction (25 kDa) was essentially composed of isoforms bearing high mannose type structures possibly associated with tri- (15%) or biantennary (23%) complex type structures and was eluted with 300 mM α-methyl-D-mannopyranoside.

2.3. RCA_{120}-Sepharose chromatography

The two isoforms of the NTD non-retained on ConA-Sepharose were separated on RCA_{120}-Sepharose column after desialylation of these isoforms. A column containing 1 ml of RCA_{120} agarose (Sigma) was equilibrated in the same buffer as used for ConA-Sepharose chromatography. The desialylated fractions were first eluted with the equilibration buffer and then with the same buffer containing 20 mM lactose. The unglycosylated isoform was eluted in the void volume whereas the NTD isoforms bearing asialo-triantennary complex type

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Abbreviations: Tg, thyroglobulin; T3, triiodothyronine; T4, thyroxine; MIT, monoiodotyrosine; DIT, diiodotyrosine; TPO, thyroperoxidase; LPO, lactoperoxidase; ConA, concanavalin A; RCA_{120}, *Ricinus communis* agglutinin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

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structures were retained on RCA_{120} and then eluted with 20 mM lactose.

2.4. In vitro iodination and coupling

In a final volume of 1 ml of 50 mM Tris-HCl, pH 7.2 at 37°C was added 10 nmol of each fraction separated by affinity chromatography, 1 mg glucose, 13.6 μg KI and 10 μg LPO or TPO. The reaction was initiated by 2.5 μg glucose oxidase and stopped 30 min later by the addition of 0.1 M sodium hyposulfite. Excess iodide was eliminated by dialysis against distilled water.

In vitro coupling was performed as described above but without addition of iodide in the medium.

2.5. Iodoamino acid analysis

After digestion with protease type XXI and leucine aminopeptidase (Boehringer Mannheim), iodoamino acid residues were separated by high-performance liquid chromatography (HPLC) on a Waters Associated chromatograph (Milford, MA, USA) with a Vydac C18 column (201 TP 54; 5 mm-300 A; 4.6×250 mm). The solvent used was water:acetic acid (99:1) as solvent A and acetonitrile:acetic acid (99:1) as solvent B. After injection, the elution was performed with a linear gradient for 20 min. The eluate was collected (0.4 ml/tube) in microtubes and iodoamino acid quantification was performed according to O'Kennedy et al. [8]. The sensitivity of the method was 0.5 pmol for each iodoamino acid.

2.6. Isolation of the dimer of the NTD

About 500 mg of NTD iodinated and coupled in vitro was chromatographed by HPLC with a Zorbax GF-250 size exclusion column (9.4×250 mm, 4 mm; Interchim, Rockland Technologies, Inc.). The elution was performed with a 0.05 M phosphate buffer pH 7.0 containing 150 mM NaCl. The flow rate was 0.6 ml/min. The elution was monitored at 280 nm and 315 nm respectively, and the eluate was collected in microtubes (0.2 ml/tube).

2.7. Preparation of dityrosine standard

Dityrosine was synthesized by oxidation of L-tyrosine (Sigma) with LPO (Sigma) according to Andersen [9]. The yield was around 2.5%. When dityrosine was prepared from DIT or MIT, the yield drastically decreased to 0.12% and a notable amount of iodine appeared in the medium. The purity of dityrosine was verified both by its spectrophotometric (λ_{max} = 315 nm) and fluorometric ($Ex_{\lambda max}$ = 330 nm, $Em_{\lambda max}$ = 410 nm) properties and by nuclear magnetic resonance spectra.

2.8. Other procedures

Desialylation of the different forms of NTD non-retained on ConA-Sepharose column was done with 20 mU of sialidase from *Vibrio cholerae* (Boehringer Mannheim) for 24 h at 37°C in 100 mM acetate buffer pH 5.5 in the presence of 100 mM $CaCl_2$.

Amino acid analysis was done with a PicoTag system (Waters Millipore) after the sample had been hydrolyzed under vacuum in 6 N HCl at 110°C for 24 h.

SDS-PAGE was performed without reduction of the sample with a 10% acrylamide and a 1% SDS gel system. Immunoblotting was donen with a mouse monoclonal antibody directed against T4 residues; the second reagent was peroxidase-conjugated goat antimouse antibody diluted 1/1000.

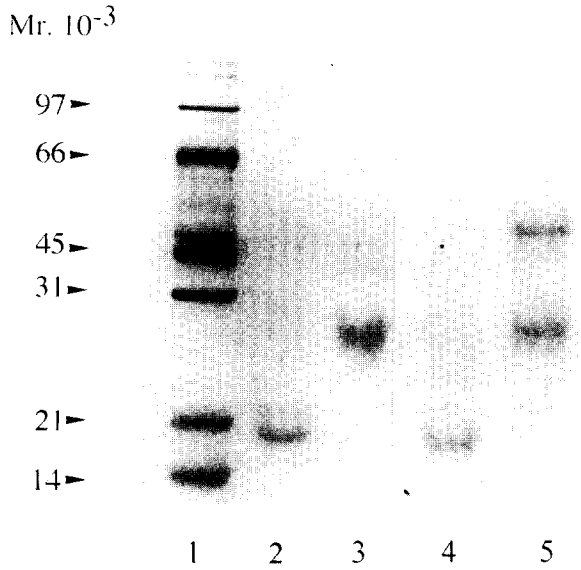

Fig. 1. SDS-PAGE (10% acrylamide) of the two isoforms of the NTD separated by affinity chromatographies as described in Section 2. Lane 1, low molecular standards (Bio-Rad); lane 2, native 19 kDa; lane 3, native 25 kDa; lane 4, 19 kDa after in vitro iodination and coupling; lane 5, 25 kDa after in vitro iodination and coupling. Proteins were detected by Coomassie brilliant blue staining.

3. Results

3.1. Iodoamino acid content of NTD in relationship with N-glycans

In this study, we investigated two characteristic forms of NTD: the unglycosylated isoform (19 kDa) and the isoform bearing at least one high mannose type structure (25 kDa). These peptides were prepared from a poorly iodinated human Tg treated with CNBr and purified by lectin affinity chromatography. Chromatography on ConA-Sepharose column yielded, first, a non-retained fraction containing both isoforms bearing triantennary complex type structures and unglycosylated NTD. This mixture was desialylated and put on an RCA_{120} Sepharose column. The NTD 19 kDa unglycosylated isoform, not retained on the column, was collected in the void volume. The NTD isoforms bearing at least one high mannose type structure were eluted from the Con A-Sepharose column using 300 mM α-methyl-D-mannopyranoside after previous washing with 10 mM α-methyl-D-glucopyranoside to eliminate the isoforms bearing biantennary complex type structures (see Section 2).

Table 1
Iodoamino acid composition of the NTD unglycosylated (19 kDa) and of the NTD bearing high mannose structures (25 kDa)

Iodoamino acid	19 kDa			25 kDa		
	Native	After coupling	After iodination and coupling	Native	After coupling	After iodination and coupling
MIT	80 ± 6	71 ± 8	602 ± 21	203 ± 23	192 ± 18	434 ± 14
DIT	18 ± 3	16 ± 5	102 ± 28	80 ± 10	19 ± 6	62 ± 16
T4	Und[a]	Und	Und	8 ± 2	16 ± 5	208 ± 15
Total iodine	116 ± 12	103 ± 18	806 ± 77	395 ± 51	294 ± 50	1390 ± 106

For the two types of NTD, obtained from poorly iodinated Tg, iodoamino acid analysis was performed in the native form, after in vitro coupling and after in vitro iodination and coupling.
Results are given as mmol of iodoamino acid residues/mol of NTD. Values are expressed as the mean ± S.D. of three experiments for 25 kDa and of two experiments for 19 kDa.
[a]Und = undetectable.

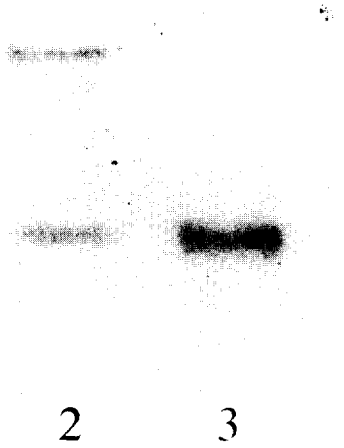

Fig. 2. After in vitro iodination and coupling of the two isoforms of the NTD, immunoblot was probed with anti-T4 monoclonal antibody. Lane 1, unglycosylated isoform (19 kDa); lane 2, 25 kDa; lane 3, sample of NTD obtained from a Tg containing a high level of thyroxine. Detection was performed with 4-chloro-1-naphtol as substrate.

For the native form of the unglycosylated NTD (19 kDa) and for the NTD firmly retained on ConA (25 kDa), the analysis of iodoamino acid residues (Table 1) showed that the 25 kDa contained a small amount of thyroxine and exhibited a higher degree of iodination than the 19 kDa, which did not contain T4. These two NTD were subjected to coupling of iodotyrosine residues in the presence of TPO or LPO and the H_2O_2 generating system but without addition of iodide in the medium. After coupling and in spite of the low level of iodination, we noted the formation of thyroxine but only for the 25 kDa. This result was comparable with TPO and LPO as enzyme (data not shown). To amplify this process, we iodinated and simultaneously coupled the two isoforms (see Section 2). The iodoamino acid composition of the two in vitro iodinated and coupled NTD showed that the 19 kDa contained more MIT and DIT residues than the 25 kDa but these iodotyrosines were unable to form hormone whereas the NTD bearing high mannose structures formed about 0.2 mol of T4 per mol of NTD.

3.2. *Process of dimerization of the NTD*

SDS-PAGE analysis of the two types of NTD identified the native forms of the 19 kDa and the 25 kDa (Fig. 1, lanes 2 and 3). After coupling, the 25 kDa, which was able to form hormone, exhibited the presence of a new band with a molecular weight about 50 kDa (Fig. 1, lane 5). This peptide could correspond to a dimer of the 25 kDa. This process of dimerization was observed only for the glycosylated NTD but not for the 19 kDa because no band was identified in the 35–40 kDa region. Taken together, our results show that the fraction able to form hormone was also able to form a peptide with a molecular weight corresponding to the double of NTD. After in vitro iodination and coupling, the two types of NTD were analyzed by immunoblotting with a monoclonal antibody to T4 (Fig. 2). The 19 kDa was not revealed because this isoform did not contain thyroxine, whereas the hormone-forming 25 kDa (lane 2) revealed, once again, the presence of a dimer. Also interesting was the presence of thyroxine in this dimer.

During the oxidative process of the coupling, it was possible to create some intermolecular disulfide bridges explaining the formation of a dimer of the NTD. On the other hand, Leonardi et al. [3] have shown the presence of intermolecular dityrosine bridges in the polymers of Tg, besides the disulfide bridges. Accordingly, we further separated the dimer of the NTD in order to determine whether dityrosine was present.

3.3. *Identification of the dityrosine bridges in the dimer of the NTD*

After in vitro iodination and coupling of the NTD, the mixture was filtered on HPLC with a Zorbax GF-250 size exclusion column. The elution was monitored at 280 nm and 315 nm (Fig. 3). The persistence of the signal at 315 nm (corresponding to the λ_{max} of pure dityrosine) in the dimer of the NTD suggested that dimerization of the peptide was, in part, caused by intermolecular dityrosine bridges. To gain insight into the number of intermolecular dityrosine bridges involved in the dimerization process, the NTD dimer was acid hydrolyzed and dityrosine was analyzed by spectrofluorometry. The dimer contained 0.18 mol of dityrosine residue per molecule of dimer. This result shows that some specific forms of NTD were able to form at least one dityrosine bridge.

4. Discussion

Among the numerous tyrosine residues of Tg that can be iodinated, only a few are involved in hormone formation. The strict specificity of the hormone-forming sites obviously requires not only consensus sequences [10] but also stringent spatial organization of the Tg molecule, which is modified by the degree of iodination [11] and by the type of *N*-glycans linked to Tg [12]. This specific organization of the molecule is essential because the formation of thyroid hormones from free diiodotyrosines has been unsuccessful so far whereas in the same conditions dityrosine formation is possible with free tyrosines, the presence of iodide enhancing the coupling [13]. On the other hand, it has been shown that the formation of dityrosine bridges in Tg was also associated with the presence of iodide [3] but until now this dimerization process has never been studied with respect to hormone synthesis and the nature of the carbohydrate moieties.

In this work, we used the N-terminal domain of human Tg because after separation from the core molecule, this peptide maintains most of its initial three-dimensional conformation, as demonstrated by surface epitope mapping [14]. Furthermore, it remains able to form thyroid hormone according to the type of *N*-glycan structures borne at Asn^{57} and Asn^{91} [5]. Notably, the NTD isoforms bearing at least one high mannose type structure are the richest in T4 residues while the unglycosylated isoform is devoid of T4. It has been previously shown that thyroid hormone formation can be obtained from a chemically iodinated Tg with few if any T4 residues, after incubation with TPO or LPO [15,16]. Similar results are reported here regarding the 25 kDa NTD which formed T4 without addition of iodide in the incubation medium. More interesting is the observation that, in the same conditions, the 25 kDa is also able to form intermolecular dityrosine bridges. By coupling of the two native forms of the NTD, only the 25 kDa containing 0.3 atom iodide per mol of NTD formed a dimer, whereas the 19 kDa containing 0.1 atom iodide per mol of NTD did not. However, the role of iodide could be minor in this process of dimerization because after iodination and coupling of the two types of NTD, the 19 kDa NTD

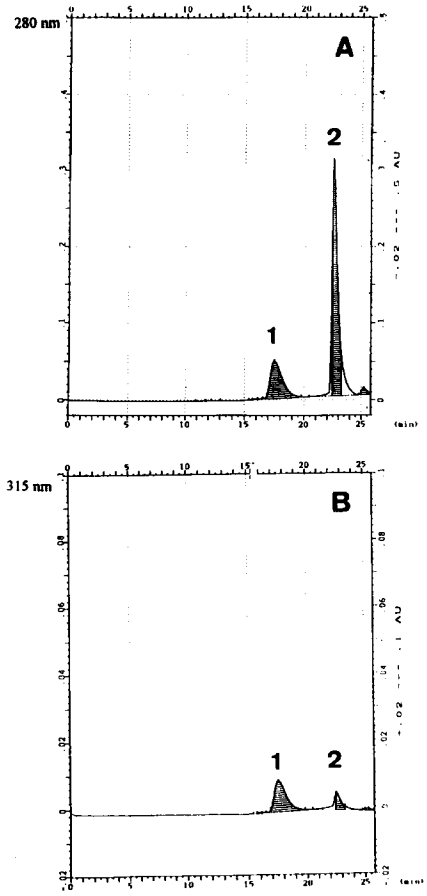

Fig. 3. HPLC analysis of the NTD in vitro iodinated and coupled. About 500 μg of T_4-NTD was chromatographed on a Zorbax GF-250 size exclusion column (250×8 mm) equilibrated in phosphate buffer pH 7.0. The elution was performed at room temperature and at a flow rate of 0.6 ml/min. The absorbance was monitored at 280 nm (A) and 315 nm (B). Peak 1 with a retention time of 17'52 corresponded to the dimer of the NTD, and peak 2 with a retention time of 22'51 represented the NTD.

contained 0.8 atom of iodide per mol of NTD and remained unable to create dityrosine bridges.

The oxidative reaction catalyzed by TPO or LPO results in coupling of iodotyrosines to form T4 and of tyrosines to create dityrosine bridges. These reactions are very similar. In both cases two molecules of the monophenolic substrates behave as single electron donors, producing free radicals of the substrate, which then undergo radical addition to form the 3,3'-biphenyl derivative in the case of dityrosine and 4,1'-biphenyl ether in the case of thyroid hormone: specific DIT residues would form T4 and specific tyrosine residues would form dityrosine. Indeed only tyrosine residues are able to form a 3,3'-dityrosine bridges while iodotyrosyl forms are poorly efficient (see Section 2). The most intriguing result of the present work is that iodotyrosine coupling and dityrosine formation appeared equally dependent on structural features of NTD isoforms: both are observed with the 25 kDa isoform and none with the unglycosylated 19 kDa isoform. Furthermore the dimer formation is not restricted to the NTD bearing high mannose type structures. Dimerization was also observed, but with a lower efficiency, with the other isoforms of the glycosylated NTD bearing complex type structures (data not shown).

Dimerization of a protein through formation of dityrosine bridges is not specific to the Tg molecule. Several proteins form dityrosine bridges, either to harden the extracellular coat of fertilized sea urchin eggs [17] and yeast spore walls [18] or as the result of oxidative stress in the course of degenerative and inflammatory diseases [19,20]. In the latter case, dityrosine formation is a marker of oxidative damage and aging of proteins which may be selectively degraded by intracellular proteases [21]. In the case of the N-terminal domain of Tg, formation of dityrosine bridges is also the result of a reaction of oxidation. However, several lines of evidence indicate that this reaction is part of a physiological process: dityrosine formation and thyroid hormone synthesis occur in the same conditions; both reactions are dependent on conformational structure of the prothyroid hormone peptide; TPO and the H_2O_2 generating system are normally present in the thyrocyte.

Our results extend the significance of the formation of intermolecular dityrosine bridges which could be a signal for routing and/or degradation of proteins not only in pathological but also in physiological processes.

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