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PRESENCE OF DITYROSINE BRIDGES IN THYROGLOBULIN AND THEIR RELATIONSHIP WITH IODINATION

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The presence of dimeric thyroglobulin (19S), after reduction and alkylation, suggests that within thyroglobulin there may be intermolecular cross-links, other than disulphide bridges. However, the nature of these intermolecular cross-links is still unknown. In this study, we show the presence of 3-3' dityrosine bridges in the molecule of bovine thyroglobulin by NMR and fluorescence studies. Also, we evaluated the role of iodination in dityrosine formation in vivo and in vitro.

Thyroglobulin (Tg) is the major protein synthesised in the thyroid gland. It is constituted of two subunits of 330,000 Da and represents the molecular site for the synthesis of thyroid hormones. Tg is heterogeneous with respect to the degree of iodination (1), glycosylation (2), phosphorilation (3), and to the different way by which the two subunits are bound to each other. In fact, it is well known that some Tg dimers may be dissociated into the two subunits by various denaturants (4), alkaline conditions (5), and low temperature (6), or only upon reduction. Some dimers persist even after reduction and alkylation (7), suggesting the presence of intermolecular cross-links other than

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disulphide bridges. Until now no firm evidence has been reported for their presence in Tg. In this study we show the presence of 3-3' dityrosine bridges in bovine Tg and their relationship with in vivo, and in vitro iodination.

MATERIALS AND METHODS

Purification and hydrolysis of thyroglobulin from bovine thyroid : Bovine Tg, purified as previously reported (8) was hydrolysed in 6M HCl at 120°C for 24 hours under nitrogen. The resulting mixture was evaporated in vacuo, and applied to a Dowex 50W-X8 column. The column was washed with water, and eluted with 2M ammonium hydroxide. Fluorescent fractions were pooled and concentrated by lyophilization. The preparation was adjusted to pH 9.5 and then loaded at 1 ml/min onto a Mono Q HR 5/5 column (Pharmacia). The column was equilibrated with buffer A (carbonate buffer pH 9.5) using a fast protein liquid chromatography (Pharmacia). After washing with 50 mM NaCl in buffer A, the column was developed at 1 ml/min with a linear 50-200 mM NaCl gradient in buffer A. Fluorescent fractions were pooled, concentrated by lyophilization and further purified by HPLC using a Delta Pack C_{18} column (300 X 7.8 mm). The column was developed with a linear gradient of CH_3CN in 10mM trifluoroacetic acid (0-10 % CH_3CN in 45 min). Flow rate was 1.5 ml/min , detection at 280 nm.

Purification and iodination of thyroglobulin from FRTL-5 cells medium : Tg was purified from FRTL-5 cells medium by fractional precipitation in 1.4-1.8 M $(NH_4)_2SO_4$ and by chromatography with Sephacryl S-300 HR (Pharmacia) equilibrated and eluted with Tris 10 mM, NaCl 100 mM, pH 7.5. Fractions containing Tg were applied onto a DEAE-Sepharose column (Pharmacia) equilibrated with the same buffer and eluted with a linear gradient 100 - 300 mM NaCl. TG was iodinated as earlier described (12).

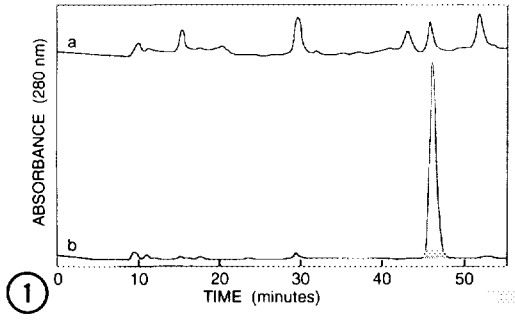
Preparation of dityrosine standard : Dityrosine was synthesised by oxidation of L-tyrosine (Sigma) with lactoperoxidase (Sigma) according to Andersen (9). L- tyrosine (181 mg; 1 mmol) was dissolved in 200 ml of 0.02 M sodium borate buffer pH 9.5; 1.1 ml (1.05 mmol) of a 3% v/v hydrogen peroxide solution, and 2 mg of lactoperoxidase was added. The solution was oxidised at a constant temperature of 37°C for 24 hr, concentrated in rotatory evaporator under vacuum, and acidified by adding concentrated HCl. The mixture was applied to a cellulose-phosphate column that was equilibrated with 0.2 M acetic acid. Elution of the column was performed with 0.2 M acetic acid containing 0.5 M NaCl. Fractions containing dityrosine were pooled, acidified to pH 2.0 and applied on a Dowex 50W-X8 column. The column was extensively washed with water, and the dityrosine was eluted with 2 M ammonium hydroxide. Dityrosine was monitored either

spectrophotometrically (E_{315} = 5 mM^{-1} at pH 7.5) (10) or by fluorescence using excitation and emission wavelengths of 330 and 410 nm, respectively (9 ,11).

NMR methods : Nuclear Magnetic Resonance spectra were collected at ^1H 500 Mhz on a Bruker AM500 spectrometer equipped with an Aspect 3000 computer. Data size was 8K on a spectral width of 4500 Hz. All the spectra were recorded at 298 K, in D_2O with presaturation of the residual HDO signal and at pH 4.0. pH was measured directly in the NMR tube, the values reported are the meter readings not corrected for the isotopic effect. Data were processed with FELIX software (Biosym Technologies) on a Silicon Graphics machine (Indigo). DC offset was estimated by the tail of the FID and corrected. A 60 degree shifted square sine window function was applied before zero filling to 16K. Chemical shifts are referenced to internal HDO at 4.8 ppm.

RESULTS AND DISCUSSION

In order to evaluate the presence of dityrosine in Tg, bovine Tg was subjected to acid hydrolysis and to HPLC with a C_{18} column. The hydrolysate yelded a fluorescent peak exactly coincident with standard dityrosine (Fig.1). Both at alkaline and acid pH, the fluorescent emission



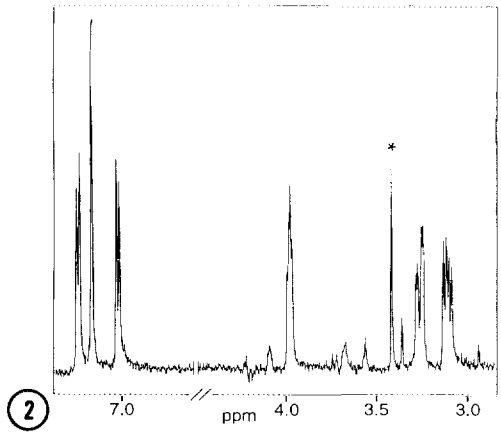


Fig. 1. HPLC profiles of acid hydrolysate bovine Tg (a) and standard dityrosine (b).
Bovine Tg was hydrolysed and the aminoacid mixture was subjected to HPLC, using a C_{18} column. The shaded peaks display fluorescence (maximum emission at 410 nm).

Fig. 2. ^1H NMR spectra in D_2O.
Aromatic and aliphatic region of fluorescent material isolated from bovine Tg. The largest impurity is indicated by an asterisk. Chemical shift (in ppm) is referenced to internal HDO (4.8 ppm).

spectra of the compound isolated from bovine Tg and dityrosine were essentially identical (not shown). To firmly establish the presence of dityrosine in Tg, we used NMR spectra. The spectra of the substance isolated from bovine Tg (Fig.2) were identical with that reported in literature (13) and with standard dityrosine (not shown).

In order to investigate if dityrosine formation is related to Tg iodination, we isolated Tg from rats treated with propylthiouracil (PTU) (an inhibitor of iodide organification) (14) and from controls. SDS-PAGE analysis under reducing and non reducing conditions showed that only iodinated Tg presented high molecular weight species containing intermolecular disulphide and non disulphide cross-links (Fig.3). In fact, only Tg from control rats presented a band of 660 KD and a band of 1200 KD corresponding to dimers and tetramers , which largely persisted under reducing conditions. These results were confirmed in an

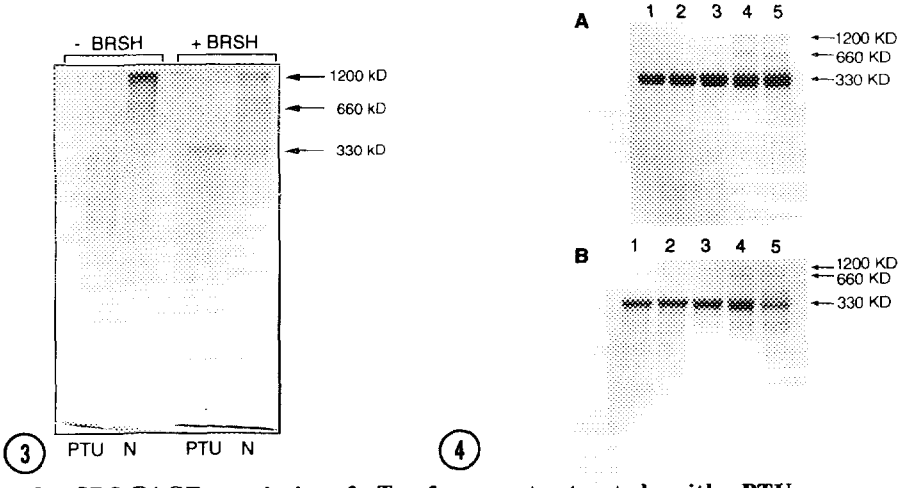


Fig. 3. SDS-PAGE analysis of Tg from rats treated with PTU and from control rats under non-reducing and reducing conditions.
Tg from rats treated with propylthiouracil (PTU) and from control rats (N) was subjected to SDS-PAGE analysis under non-reducing and reducing conditions.

Fig. 4. SDS-PAGE analysis of in vitro iodinated Tg labelled with ^{35}S methionine under non-reducing (A) and reducing (B) conditions.
Tg purified from the medium of FRTL-5 cells was iodinated as reported previously (12). Lane 1: Tg purified from FRTL-5; Lane 2: complete Tg / lactoperoxidase / H_2O_2 system; Lane 3: complete system plus $1{\cdot}1\ 0^{-5}$ M NaI; Lane 4: complete system plus $5{\cdot}1\ 0^{-5}$ M NaI; Lane 5: complete system plus $1{\cdot}1\ 0^{-4}$ M NaI.

in vitro system. Tg secreted by FRTL-5 cells, which lacks iodine (15), was purified from the culture medium and subjected to in vitro iodination as described in materials and methods. The reaction mixture was analysed by SDS-PAGE under both reducing and non reducing conditions. Fig. 4 shows that a band of 660 KD and a band of 1200 KD formed after iodination and persisted under reducing conditions. These high molecular weight bands were not formed when the reaction mixture lacked iodide or the lactoperoxidase / H_2O_2 generating system (not shown). Therefore, intermolecular cross-links in addition to disulphide bridges were formed during Tg iodination. In order to have some insights on the nature of the intermolecular cross-links occurring during Tg iodination, we have performed a fluorescence study on Tg iodinated in vitro. The protein mixture, when excited with light of 330 nm, exhibited a spectrum with a fluorescence emission maximum at about 410 nm which was identical to the spectrum of pure dityrosine. Treatment of Tg with increasing amount of iodide caused an increase in fluorescence at 410nm (not shown). The presence of a specific emission at 410nm suggests that oligomerization of Tg may involves dityrosine formation.

These results demonstrate that 3-3' dityrosine bridges are involved in the intermolecular cross-links of Tg. A correlation between Tg iodination and dityrosine formation exists. In fact, Tg purified from the medium of FRTL-5 cells and from PTU treated rats does not show intermolecular cross-links. Moreover, iodinated Tg purified from bovine thyroid contains 3-3' dityrosine bridges, Tg from control rats and in vitro iodinated Tg from FRTL-5 contains non reducible cross-links. Our observations are consistent with the presence in bovine thyroid of Tg highly iodinated and extremely resistant to various solubilization procedure (16). It is generally known that more iodinated Tg, degrades more rapidly than less iodinated Tg (17). Recently, dityrosine has been identified as an endogenous marker for the selective proteolysis of oxidatively modified haemoglobin (18). Therefore, it is possible that dityrosine may have a similar role within the thyroid, tagging the Tg molecules highly iodinated, subjected to preferential proteolysis.

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