SPECIFIC TRITIUM LABELLING OF THYROLIBERIN ON HISTIDYL AND PROLYL RESIDUES

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SUMMARY

Tritiation of thyroliberin (L-pGlu-L-His-L-Pro-NH_2) starting from the L-[Δ^3-Pro^3]-TRF, on prolyl and histidyl residues is being dealt with below.

By catalytic tritiation of the double bond, followed by iodination of histidyl residue and catalytic deshalogenation in presence of tritium gas, TRF was labelled with a specific radioactivity close to the theoretical value. ^{3}H-[2,5-His^2-3,4-Pro^3]-TRF retained all biological potencies.

Key words : histidyl - prolyl - thyroliberin - tritiation - tritium.

0362-4803/82/020171-12$01.20

Received December 29, 1980

1 - INTRODUCTION

The tritium labelling of TRF (L-pyroglutamyl-L-histidyl-L-proline-amide) was carried out either by total synthesis, starting from tritiated L-proline (1), or by catalytic deshalogenation of diiodo TRF (2). Both approaches led to labelled TRF with respective specific radioactivities of 40 Ci/mmole and 60 Ci/mmole.

Using the ^{3}H-[2,5-His^{2}]-TRF prepared in our laboratory (60 Ci/mmole) (2), the characteristics of binding sites on prolactin-secreting GH_3 cell lines have been examined (3) (cf. for review 4, 5). The penetration of unmodified ^{3}H-TRF in intact cells was shown by autoradiographic studies, radioactivity distribution in subcellular fractions (3) (6) and binding on isolated nuclei (7). The data concerning the analysis of tritiated material associated to intact cells (8) indicated that early biological response was induced by intact TRF. The existence of a continuous flow of TRF molecules on both sides of plasma membrane was suggested. The availability of ^{3}H-TRF rendered possible the performance of a radioreceptor assay applicable to biological samples (9) and of a radioimmunoassay (10). The stability of the labelling in the experimental conditions was determined with TRF, specifically tritiated on C_2 or C_5 position of the imidazole ring (11).

It appears that all the investigations involving the interaction of TRF with cellular receptors require still higher specific radioactivities. Particularly, the autoradiography which constitutes a prime approach to localize the peptide at subcellular level is predominantly dependent on the availability of highly labelled TRF.

Such a compound has been obtained by labelling two residues of the tripeptide, instead of one, namely proline and histidine. In the present work, the tritiation procedure recently developed by Felix et al. (12) and based upon the catalytic reduction of a double bond in the prolyl residue has been used in a first step. The ^{3}H-[Pro^{3}]-TRF has further been iodinated and the following catalytic deshalogenation led to TRF labelled on the two residues. ^{3}H-[2,5-His^{2}]-[3,4-Pro^{3}]-TRF (110 Ci/mmole) has been found to retain all its biological characteristics.

2 - EXPERIMENTAL

2.1 - Materials

TRF was obtained by Sigma Chemical Co (U.S.A.). L-[Δ^{3}-Pro^{3}]-TRF was purchased by Peninsula Laboratories Inc (U.S.A.). Halogenation was carried out with ICl from Merck (Germany). Palladium oxyde was obtained from Fluka (Switzerland) and 10% palladium/alumina from Engelhardt (Italie). Pure tritium gas was purchased by Commissariat à l'Energie Atomique (France). Silicagel plates came from Schleicher-Schüll and cellulose plates from Merck (Germany). The X-O mat R films (Kodak) were used for autoradiography of thin-layer plates. The ion exchange resin AG 11 A8 (50-100 mesh) came from Bio Rad Laboratories.

The following solvent systems were used : n-butanol-acetic acid-water (75 : 10 : 25) , chloroform-methanol-ammonia (5 : 3 : 1), chloroform-methanol - 38% acetic acid (3 : 2 : 1), for thin layer-chromatography and pyridine acetate buffer 0.17 M, pH 4.5 for thin-layer electrophoresis. Peptide weight determinations were carried out by amino acid measurement with the Technicon Autoanalyzer TSM.

The automatic gas transfer unit used for catalytic hydrogenolysis was described elsewhere (13). Tritium determinations were made with SL 30 Intertechnique liquid scintillation counter. Tritium NMR spectra were recorded on a Bruker WT spectrometer operating at 60 MHz.

2.2 - Tritiation of [Δ^3-Pro^3]-TRF : reduction of the double bond

7 µmoles [Δ^3-Pro^3]-TRF were dissolved in 500 µl methanol and frozen. 20 mg PdO catalyst were added and the tritiation flask connected to the vacuum line. Tritium gas was introduced under pressure of 1.1 bars. After one hour at ambiant temperature, the catalyst was removed by filtration and labile tritium exchanged by successive evaporations in 1% acetic acid. ^{3}H-TRF was separated from dehydro-derivative and tritiation products by TLC in ethanol-water (7 : 3) or by electrophoresis on silicagel plates in pyridine acetate pH 4.5, 15 volts/cm, for 2.5 hours. After hydrolysis of an aliquot of the labelled compound (6 N HCl, 110°C, 18 h), amino-acid weight and specific radioactivity were determinated.

The tritium atom distribution and the biological activity of ^{3}H-[Pro^3]-TRF were checked respectively by ^{3}H NMR, by radioimmunological assay (14) and on GH_3 cells (2).

2.3 - Halogenation of ^{3}H-[Pro^3]-TRF : iodination of the imidazole ring

1 µmole ^{3}H-[Pro^3]-TRF (60 mCi) was redissolved in 200 µl phosphate buffer (Titrisol-Merck) pH 7. 4 µmoles ICl were added by stirring. After one minute the mixture was neutralized by excess of sodium thiosulfate 0.1 N. Diiodo-His^2-^{3}H-[Pro^3]-TRF was purified by TLC on cellulose plate (solvent system : n-butanol-acetic acid-water, 75 : 10 : 25) and identified by co-chromatography of control substances.

2.4 - Tritiation of diiodo-His^2-^{3}H-[Pro^3]-TRF : catalytic deshalogenation

The diiodo intermediate (15 mCi : 0.25 µmoles) was dissolved in 1 ml water. 10% Pd/Al_2O_3 (10 mg) was added. The tritiation under pressure of 0.72 bars was carried out during 25 minutes. The mixture was then treated as above (2.2).

2.5 - Purification

The tritiated product was eluted on an AG 11 A8 column with distilled water. Identification of ^{3}H-[His^2-Pro^3]-TRF was achieved by TLC in different solvent systems and by amino acid analysis.

Physical and biological studies were performed by the same manner described in paragraph 2.2.

3 - RESULTS

3.1 - L-^{3}H[3,4-Pro^3]-TRF (55 Ci/mmole) was obtained by tritiation of L-[Δ^3-Pro^3]-TRF and this in experimental conditions almost similar to those chosen by Felix et al. (12).

Amino acids analysis of the purified ^{3}H-TRF showed the expected aminoacids ratio to be correct and to be free of unreduced Δ^3-Pro precursor.

The NMR spectrum revealed that radioactivity was mainly appearing in the prolyl residue : 85% were found on prolyl C-3 and C-4 carbons, and 15% on the histidyl residue (5% on C-5 and 10% on C-2, this last being the most exchangeable). Besides, it was observed (12) that in methanol and with PdO, the tritiation of substrates containing Δ^3-Pro was obtained with a theoretical value and a minimal amounts of randomization.

Radioimmunological and biological characteristics showed that tritiated ^{3}H-[Pro^{3}]-TRF was identical to the native molecule.

3.2 - After iodination and further catalytic deshalogenation ^{3}H-[Pro^{3}]-TRF conduced to ^{3}H-[His^{2}-Pro^{3}]-TRF with a specific radioactivity of 110 Ci/mmole.

NMR study showed that 55 Ci were associated to histidyl residue (on C2 and C5) and 55 Ci to prolyl residue (on C-3 and C-4).

Radioimmunological and biological characteristics correlated to the analytical properties proved that this tritiated TRF was identical to the native TRF.

Figure 1 shows the specific binding of ^{3}H-TRF to the target cells GH_3/B_6. The Scatchard plot was curvilinear. The affinity constant was identical to that previously defined with ^{3}H-TRF labelled on histidyl residue (17) (18) : $K_A = 1.8 \pm 0.7 \times 10^{8}\ M^{-1}$. Kinetics study performed at 37°C at two concentrations (2 nM, 20 nM) showed that the equilibrium of binding was reached within 30 min as described before (3).

The autoradiography (fig. 2) confirms the presence of tritiated material (3) in the cell at both cytoplasmic and nuclear levels, without specific concentration on the plasma membrane. The tritiated material was identified as ^{3}H-TRF (8) in the same experimental conditions. Contrary to the previous experiments reported (3) the relatively short time of exposure allowed here a very low background and an excellent quality of the autoradiographs.

Figure 1 - ^{3}H-[2,5-His^{2}-3,4-Pro^{3}]-TRF as a function of concentration⁂.

GH_3/B_6 cells were grown 4 days in serum free medium. Cells suspensions were incubated with ^{3}H-TRF (110 Ci/mmole) for 30 min at 37°C. Unspecific binding was determined by adding a large excess of TRF (200 times) and did not exceed 5%. Each value is the mean of quadriplicate ± SEM. Proteins Concentration was measured by Peterson's method (15) and DNA concentration by Hill and Whatler's method (16).

⁂(By courtesy of Dr N. Brunet).

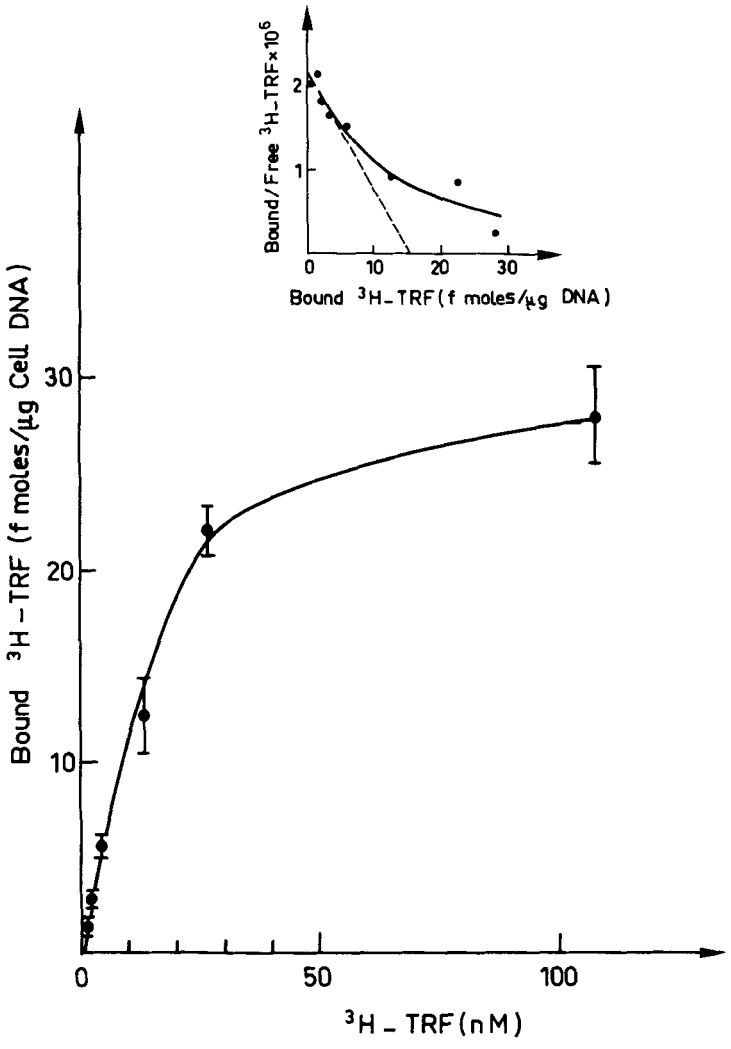
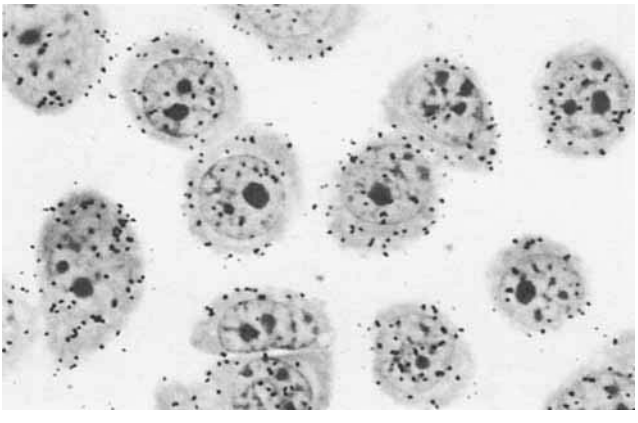


Figure 2 - Autoradiograph of GH_3/B_6 rat prolactin cells incubated with ^3H-TRF (110 Ci/mmole, 30 nM).

After incubation for 30 min at 37°C the monolayers were rinsed and fixed in 1% glutaraldehyde in cacodylate buffer and embedded in Epon. 1 µm thick sections were processed for light - microscopy using NTB_2 Kodak emulsion. Silvergranules appear located at both cytoplasmic and nuclear levels. A hundred fold excess of unlabelled TRF during the incubation decreased the average number of cell associated silver granules by ca 95%. Exposure time : six weeks. Magnification : X 950.

(By courtesy of Dr A. Tixier-Vidal and R. Picart).



4 - DISCUSSION

The obvious interest of biological peptides highly labelled prompted us to associate two tritiation methods : on prolyl and histidyl residues of thyroliberin.

Hence it was possible to choose between two labelling ways : either by direct iodination of Δ^3-[Pro^3]-TRF and subsequent deshalogenation, or by preliminar reduction of Δ^3-[Pro^3]-TRF, iodination of ^{3}H-[Pro^3]-TRF and deshalogenation of the diiododerivative.

With the first method, the theoretical specific radioactivity should not exceed 90 Ci/mmole. Iodination by iodine monochloride allowed to obtain disubstitution on imidazole ring and addition of I Cl on the double bond. Then, deshalogenation was effected, but with a very low yield (unpublished results) and led to TRF tritiated with 80 Ci per mmole.

In spite of the multiple steps needed, the second method was prefered since it allowed a theoretical specific radioactivity of 120 Ci/mmole. This direct tritiation seemed to us easier than total synthesis (NEN, ^{3}H-TRF) using Boc-2,3,4,5-dehydro-Pro as precursor, leading to ^{3}H-TRF labelled on Pro with 110 Ci/mmole. Direct labelling of L-[Δ^3-Pro^3]-TRF avoids problems of racemization due to the reduction of 2,3,4,5-dehydro-Pro which must be undoubled before its incorporation to histidine and pyroglutamic acid. Also the total synthesis requires great amounts of product and consequently by implies a considerable amount of radioactivity (1 Ci/mg).

Our procedure gave a full active ^{3}H-TRF (110 Ci/mmole). Two tritium atoms were positioned on prolyl residue (C-3 and C-4)

by reduction of the double bond and two tritium atoms on imidazole ring of histidyl residue (C-2 and C-5) by deshalogenation of the diiodo ^{3}H-Pro-TRF. We observed during this chemical treatment that labelling of prolyl residue was entirely preserved. Storing conditions of the tritiated peptide (50 % aqueous methanolic solution in cryotubes, -195°C in liquid nitrogen) were completely satisfactory to prevent autoradiolytic decomposition.

Thus, the highly enriched tritium peptides permit an increased sensitivity of detection estimated for studies at cellular level (autohistoradiography) such as for analysis of the role of the different labelled residues (metabolism , interaction, ...).

Other substrates having double bond (like ΔPro, ΔPhe, ΔTrp) and residues able to be directly halogenated will lead us to promote tritiation of active peptides upon different residues. Stereo selective synthesis of dipeptides by asymmetric reduction with chiral rhodium complexes (19) gave us promizing results.

ACKNOWLEDGEMENTS

We are indebted to Drs D. Gourdji (Collège de France), O. Bepoldin and P. Pradelles (Institut Pasteur) for performing biological and radioimmunological assays. We are grateful to J.P. Beaucourt (Département de Biologie, Service des Molécules Marquées, Saclay) for tritium NMR analysis.

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