SPECIFIC TRITIATION ONTO C-2 AND C-5 POSITIONS OF HISTIDINE - CONTAINING PEPTIDE. APPLICATION TO THYROLIBERIN.

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SUMMARY

Specific methods of tritium labelling onto C-2 and (or) C-5 positions of the imidazole ring were successfully applied to thyroliberin (TRF, L-[Glu-L-His-L-Pro-NH_2).

Diazocoupling by diazotized sulphanilic acid and iodination by iodine monochloride of the histidyl residue were chosen to prepare precursors and to quantify the distribution of tritium atoms which were introduced.

Catalytic hydrogenolysis in the presence of tritium gas of 5-iodohistidine[2] - and 2(4-sulfophenylazo)histidine[2] - derivatives allowed to label these positions with specific radioactivities of 30 Ci and 25 Ci/mmole respectively. For comparison thyroliberin tritiated by direct catalytic exchange is labelled essentially onto C_2 with a specific radioactivity of 30 Ci/mmole.

KEY WORDS : histidine-containing peptides - tritiation - specific positions

1 - INTRODUCTION

In order to study the behaviour of various polypeptidic hormones, our laboratory has evolved several specific tritiation methods. Their main characteristics rely on the labelling of aminoacid residue belonging to the completed cold hormone / 1 - 4 /, with the aim to respect both the primary structure and biological potencies.

To enlarge the field of general tritiation methods of biological histidine-containing peptides, we have investigated in more details the labelling of the smallest known peptide hormone : thyroliberin (or thyrotropin-releasing factor : TRF) / 5 /. This tripeptide (L-[Glu-L-His-L-Pro-NH_2) is mainly produced by the hypothalamus and is involved in the release of thyroid-stimulating hormone and prolactin.

In a previous short report / 6 / we have described the complete tritium labelling of TRF imidazole group in order to reach 60 Ci per mmole.

Aims other than the mere detection of TRF / 7, 8 / can be visualized and in particular those associated with the role of the imidazole side chain of the histidine residue, as active center for the biological potencies or as probe for molecular alteration of the hormone.

The rate of exchange of the C-2 proton of imidazole has been shown to be a function of the protonation of the vicinal nitrogen N_π / 9 /. In addition hydrogen bonding to N_π might mimic a protonation in this respect. For these reasons it was convenient to label TRF specifically on either the C-2 or the C-5 carbon and to know the amount of tritium bound onto the unspecific position.

The present work describes the specific tritium labelling of TRF by substitution of monoazoderivative at the C-2 and monoiododerivative at the C-5 position. Direct catalytic exchange on palladium catalyst leads also to an asymetric tritiation, the radioisotope being localized mainly on the C-2 carbon.

2 - MATERIALS AND METHODS

2.1 - Materials

TRF was kindly provided by Dr. R.O. Studer (Hoffman - La Roche, Basel). Iodine monochloride (ICl) came from Merck (Germany). The catalyst 10 % palladium on alumina was supplied by Engelhardt (Italy). Precoated cellulose and silicagel plates (20 x 20 cm) for thin-layer chromatography (TLC) and electrophoresis (TLE) were obtained from Schleicher and Schüll (Germany), 3 MM chromatographic paper from Whatman and silicagel 60 pre-packed columns from Merck. Ion exchange resins came from Biorad (U.S.A.). All other chemical products and solvents were of analytical grade from Prolabo (France) or Merck.

^{125}ICl (50 mCi/mmole, 2.5 mCi/ml) and ^{35}S sulphanilic acid (31.6 mCi/mmole) were purchased from New England Nuclear Corporation. Pure tritium gas was made by the Commissariat à l'Energie Atomique (Saclay, France). Absorption spectra were obtained with the use of a Beckman model DK-2A spectrophotometer.

Nuclear magnetic resonance spectra of TRF and derivatives in deuterium oxide (D_2O) solutions were performed with a Cameca TSN spectrometer operating at 250 MHz. NMR spectra of tritiated TRF were recorded on a Bruker WT spectrometer operating at 60 MHz.

Tritium and ^{125}I determinations were made with a SL 30 Intertechnique liquid scintillation and CG 4000 Intertechnique solid scintillation counters respectively.

Radioscans of electrophoresis and thin-layer chromatographic plates were performed with a Berthold scanner II.

Autoradiography was achieved on R-P Royal X-O mat medical X-ray films (Kodak).

The automatic tritium gas transfer unit used for catalytic hydrogenolysis built by Morgat, Desmares and Cornu was described elsewhere / 10 /.

Peptide weight determinations were carried out by amino-acid measurements with the Technicon autoanalyzer TSM.

Liquid chromatography under pressure (3 bars) was performed with a dosing unit Duramat (CFG).

The following solvent systems were used : n-butanol-acetic acid-water (75 : 10 : 25, v/v), chloroform-methanol-38 % acetic acid (60 : 40 : 20, v/v) or chloroform-methanol-ammonia (125 : 75 : 25, v/v) for TLC ; pyridine acetate buffer 0.17 M, pH 4.5 for TLE ; and chloroform-methanol-40 % acetic acid (50 : 40 : 20, v/v) for liquid chromatography.

2.2 - Preparation of iododerivatives

TRF in phosphate buffer (pH 7) and ICl in methanolic solution were mixed for 1 minute. The reaction was stopped by adding sodium thiosulfate solution (0.1 N). The iodinated products were separated by TLC on cellulose plate or by descending chromatography on 3 MM paper (Table I).

Mono and diiodo-TRF were identified by aminoacid analysis and measurement of the iodine content afforded by the use of ^{125}ICl. The stoechiometric ratio of 1/1.5 (TRF/ICl) was required to obtain the largest amount of monoiodo-TRF (iodination yield was close to approximately 90 %). Complete conversion into diiodo-TRF occured with a fourfold excess of ICl.

2.3 - Preparation of azoderivatives

Diazocoupling of TRF was achieved according to Pauly's method / 11 /. An hydrochloric solution of sulphanilic acid 0.03 M (1 ml) was diazotized with sodium nitrite 0.725 M (0.5 ml) and then was neutralized by sodium carbonate 1 N (1 ml). In order to identify and quantify the reaction products tracer ^{35}S sulphanilic acid was added to the cold sulphanilic acid. Then, TRF 0.1 M (10 µl) in aqueous solution was added to 100 µl of reagent. The diazotization mixture was immediately applied to a TLE on silicagel plate. After electrophoresis (Table II) two azocompounds were isolated. The dark orange band was identified as the 2,5-biazo-TRF by aminoacid analysis (the histidine of azoderivatives was destroyed by acidic hydrolysis), counting of incorporated ^{35}S and by NMR spectra. The yellow band was identified as monoazo-TRF.

TABLE I

Compounds	Rf (1)	Rf (2)
Iodide	0.33	0.13
TRF	0.37	0.11
5-iodohistidine[2] -TRF	0.53	0.32
2,5-diiodohistidine[2] -TRF	0.85	0.59

Chromatographic data of TRF iodination products.

(1) TLC on cellulose plates, and (2) descending paper chromatography on Whatmann 3 MM paper, in n-butanol-acetic acid-water (75 : 10 : 25).

TABLE II

Compounds	Electrophoretic mobility (mm)	Chromatographic Rf
TRF	- 33	
Sulphanilic acid	+ 92	
2-monoazo-TRF	+ 23	0.16
5-monoazo-TRF	+ 23	0.08
2,5-bisazo-TRF	+ 40	
Diazotized sulphanilic acid	+ 05	

Analytical data of TRF and diazotization products in electrophoresis and chromatography.

Electrophoresis on silicagel plate was carried out in pyridine acetate buffer 0.17 M, pH 4.5 for 2 hours (10 volts/cm) at 8°C.

TLC on silicagel plate was performed in the solvent system n-butanol-acetic acid-water (75 : 10 : 25).

The compounds were localized by Pauly reagent or by scanning.

From this last band, two yellow monosubstituted derivatives were separated by TLC on silicagel plate (Table II). The main compound was characterized as the 2(4-sulfophenylazo)histidine[2] -TRF or 2-monoazo-TRF and the minor compound as the 5(4-sulfophenylazo)histidine[2] -TRF or 5-monoazo-TRF. It is worthy to note that λ_{max} for 2-monoazo derivative (385 nm) was higher than that of its 5-monoazo isomer (352 nm) at pH $\leq$ 7 ; λ_{max} of 2,5-bis (4-sulfophenylazo)histidine[2] -TRF or 2,5-bisazo-TRF was measured at 425 nm.

In order to purify greater quantities of 2-monoazo intermediate, low pressure liquid chromatography on a silica gel column was performed with chloroform-methanol-40 % acetic acid (50 : 40 : 20). This compound was then separated from the native peptide by chromatography through a sulfonic resin column (AG 50 W - X2).

2.4 - Catalytic tritiation

TRF (1.5 mg), 5-iodohistidine[2] -TRF (0.8 mg) and 2,5-diiodohistidine[2] -TRF (0.8 mg) in aqueous solutions (1 ml) and 2-monoazo-TRF (3 mg) in anhydrous methanol (1 ml) were tritiated using an automatic gas transfer unit / 10 / in the presence of 10 % Pd/Al_2O_3 during 25 min at 0.7 bars, at room temperature. The labile tritium was removed by successive flash evaporations in water or in 1 % acetic acid. The tritiated products were purified by several TLC in different solvent systems (as above).

The reductive cleavage of the 2-monoazo-TRF resulted in a concomitant formation of TRF (10-15 %) and 2-aminohistidine[2] -TRF.

These compounds were both isolated by TLE and then separated by successive TLC in different solvent systems as described above.

2.5 - Biological assays

Weight peptide determinations of tritiated TRF was carried out using radioimmunological assays, according to the method described by Pradelles / 12 / and correlated to the biological effect (release of prolactine in GH_3 cells / 6 /).

3 - RESULTS AND DISCUSSION

3.1 - Tritiation in C-5 position by iodine substitution

It was established / 13, 14, 15 / that iodine, reacting with imidazole or histidine derivatives becomes attached to the C-5 position. It was not obvious that TRF imidazole chain would behave the same way in the presence of ICl, as a consequence of sterical hindrance and intramolecular hydrogen bonding. More precisely, it has been observed / 16 / that histidine and histidine containing peptides catalyses the hydrolysis of ICl to iodine, whereas TRF is devoided of this property. This behaviour which reflects conformational contraints as well as the lack of accessibility of imidazole nitrogen N_π / 17 / might alter the course of I^+ reaction.

Monoiodo TRF was therefore analyzed by ^{1}H-NMR in D_2O and as shown in Table III, the C-5 proton signal was missing, indicating that iodine was bound to C-5 carbon, like in the reference compounds. Thus, deshalogenation of monoiodo-TRF carried out in the presence of tritium gas might lead to a specific labelling at C-5 position.

The amount of label localized on the imidazole group was established, directly by ^{3}H-NMR, and indirectly by measuring the tritium displacement associated with re-synthesis of monoiodo-TRF or synthesis of 2-monoazo-TRF. Both approaches led to the same conclusion. The 30 Ci carried by a millimole of ^{3}H-TRF are for 27 Ci associated with the C-5 and for 3 Ci with the C-2 ; the label in the latter position originated from an exchange with the C-2 proton. The rate of this exchange is pressure dependent. For instance, increasing the tritium from 0.7 to 0.95 bars, other parameters remaining equal, increased the amount of tritium on C-2 to 15 Ci/mmole, whereas no changes occurred, nor where expected at the C-5 level.

3.2 - Tritiation in C-2 position by azo substitution

Among the reagents reacting with imidazole, diazonium salts are popular for a very long time / 18, 19 / and lead to mono and

TABLE III

Iodination reagent	Compounds	NMR characterization δC-2-H	δC-5-H	Reference
Iodine/hexane / 15 /	Imidazole	7.8	7.2	Bensusan et al. /14/
	4(5)-iodoimidazole	7.7	7.1	
Iodine/hexane / 15 /	Histidine	7.8	7.0	Bensusan et al. /14/
	5-iodohistidine	7.8	-	
Iodine monochloride	TRF	7.8	7.0	
	5-iodohistidine[2] -TRF	7.8	-	

Proton assignments spectra of the C-2 and C-5 protons of imidazole derivatives in D_2O.

δ = chemical shifts (ppm)

The 250 MHz. NMR spectra of TRF and monoiodo TRF were related to the internal standard TSP-d_4 (tetradeutero-trimethylsilyl-propionate, sodium salt) at pD 6.4. Trimethylsilyl propane-sulfonate was the internal standard for NMR spectra / 14 / at pD 8.0-8.5.

diazo derivatives. By contrast with monoiodo TRF which is unique, the monoazo-TRF are made of two separable entities corresponding to the substitution of C-2 and C-5 respectively, the former being predominant.

For a stoechiometric ratio of 1 : 1 between sulphanilic acid and TRF, optimal yields of the C-2 azoderivative were obtained, 55 % of the TRF having reacted. In that case, C-2 azo, C-5 azo and C-2 - C-5 bis azo are in the ratio 2 : 1 : 1.

These results showed the same trend as those reported by Nagai et al. / 20 / after reaction of p. bromo phenyl diazonium salt with N-acetyl histidine methylester, but to a lesser extent. In the latter case, the substitution of the imidazole C-2 position was 6.4 times more effective than that of the C-5.

The replacement of N-acetyl histidine methyl ester by N-benzoyl histidine methyl ester altered completely the pattern of substitution by an aryldiazonium salt and resulted in an almost exclusive bis-substitution / 20 /, all other conditions being equal.

The substitution of an azoderivative by a tritium atom was attempted after noticing that aryldiazomethanes and ethyldiazo acetate lose nitrogen when hydrogenated over palladium in neutral medium, as mentionned by Augustine / 21 /. We wondered whether a similar process would occur as a side reaction with ordinary aryl azoderivatives. This is the case indeed as can be readily observed when working under tritium gas. As a matter of fact the rupture between the carbon and the nitrogen occurs on both sides of the azo group. 2-monoazo-TRF when submitted to the tritium-palladium hydrogenation process is transformed to a small extent into ^{3}H-TRF of 25 Ci/mmole. Under the conditions used, the yield is low : 500 µg for 5 mg input material. Nearly all the label is localized on the imidazole C-2 with 2.5 Ci/mmole bound to C-5. In addition, as expected 2-amino-His -TRF is formed.

Thus, in the case of TRF, introduction of a tritium label mainly on one of the imidazole carbons of histidine has been realized with virtually no dilution of the tritium atom.

A small fraction of the label, of the order of 10 %,was localized on the imidazole carbon devoided of a leaving group. This labelling is due to a direct exchange reaction and not to

the presence of the corresponding derivative as contaminant. This point has been checked carefully, using labelled leaving groups.

It was therefore interesting to control the labelling of TRF when submitted only to a direct exchange, under the same conditions as above. To our surprise the TRF recovered (30 Ci/mmole) carried nearly one tritium atom on C-2 and 1/6 atom on C-5.

This result has to be compared to those obtained after deshalogenation of monoiodo-TRF, where the extend of the exchange at the C-2 is very much lower. It seems thus unlikely that the catalyst can act at the same time on the C-5 to replace the halogen by tritium and on the C-2 to exchange the proton.

Moreover, under the conditions used and for the peptide investigated, the rates of both reactions appear similar.

The asymetry of the exchange kinetics related to the C2-H and C5-H protons is founded very likely on the acidic character of the latter, with, in addition,other yet undefined parameters, like the positioning of the imidazole ring in the vicinity of the catalytic centers.

The present work affords an example of this possibility.

Why did the deshalogenation of the monoiodo-TRF not afford either a concomitant or sequential significative labelling of C-2 ?

Other examples were found in the behaviour of tryptophane containing peptides. It has been reported that palladium catalyst in the presence of tritium promoted the exchange of aromatic hydrogen of N-acetyl tryptophanamide leading to a specific radioactivity of 60 Ci/mmole, of which 3 Ci were localized on indole C-2. Under identical experimental conditions,the scission of the O-nitrophenyl thioether derivative introduced tritium (16 Ci/mmole) on indole C-2 ; no detectable amount being attached to the benzene moiety of the indole ring / 22 /.

This very impressive tritium labelling by direct exchange is not observed when tryptophan alone or BOC-Gly-Trp-Met-Asp-Phe-NH_2 (pentagastrine) are handled. Only 4 and 2 Ci/mmole are introduced respectively, essentially on the benzene ring.

Therefore whereas direct exchange might provide an intensive and specific labelling like in the TRF case, it is fairly exceptional and unpredictable.

Various studies on the rate of isotopic exchange with ^{3}H-C2 and ^{3}H-C5 TRF (unpublished results) showed a prefential lability of the C-2 -T (C-2 -T : 17 % ; C-5 -T : 4 %, after one day at 37°C, pH 7). The compared data with those obtained from N-acetyl-histidine suggested a slower rate (2.5 times). This observation could be in close connection with the privileged conformation of TRF molecule / 12, 17 /.

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