Radiolabelling by Tritium and [^{125}I]Iodine of an Angiotensin II related Peptide.

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Summary : We describe the ^{3}H- and ^{125}I- labelling of Glu-Gly-Val-Tyr-Val-His-Pro-Val, (hIIA), an octapeptide encoded by an RNA strand complementary to the human angiotensin II (AII) mRNA

The labelling of this peptide with ^{125}I was performed by two ways : 1/ Mono-iodination of the Tyr residue, using Na^{125}I in the presence of Chloramine T ; 2/ Coupling of the [^{125}I]Bolton-Hunter reagent to the terminal amine group of the octapeptide.

The synthesis of the tritiated (3,5-$^{3}H_2$-Tyr^4)octapeptide was achieved by a two step synthesis : di-iodination of the peptide followed by its catalytic dehalogenation in the presence of tritium gas.

The compounds obtained inhibited the binding of AII to its receptors or antibodies but showed no direct binding to these proteins, suggesting no competition between AII and hIIA for these protein binding sites.

Résumé : Nous avons effectué le marquage radioactif par le tritium et l'iode 125 d'un octapeptide codé par l'ARN complémentaire de l'ARN messager de l'angiotensine II humaine.

Deux types de marquage à l'iode 125 ont été utilisés : 1/ introduction de l'iode en α de l'hydroxyle phénolique du résidu Tyr par Na^{125}I en présence de Chloramine T ; 2/ couplage du réactif [^{125}I]Bolton Hunter au groupement amine terminal du peptide.

La synthèse du dérivé tritié au niveau du résidu tyrosyle est réalisée en deux étapes : formation d'un dérivé diiodé (3,5-I_2-Tyr^4)octapeptide, puis déshalogénation catalytique en présence de tritium gazeux.

Les produits obtenus inhibent les liaisons AII-recepteur ou -anticorps mais ne se lient pas spécifiquement à ces protéines, suggérant l'absence de compétition entre AII et hIIA pour ces sites protéiques.

Keywords : Radiolabelling, tritium, iodine ^{125}I, [^{125}I]Bolton Hunter reagent, angiotensin, mRNA complementary peptide.

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Introduction

The synthetic octapeptide E-G-V-Y-V-H-P-V (or hIIA) encoded by a RNA strand complementary to human angiotensin II (AII) mRNA has been shown to inhibit the binding of (^{125}I-Tyr^4)AII to hepatic or Ig receptors. It also antagonized the physiological AII induced - $(Ca2+)_i$ increase or -PGE_2 release in mesangial cells, and it reduced the hypertensive response to AII in the rat (1).

To investigate direct binding measurements of hIIA to antibodies or receptors of AII, we synthesized one tritium labelled and two mono-iodinated derivatives of hIIA.

Results and discussion

Radioactive [^{125}I]iodine labelling of hIIA was performed in one step using Na^{125}I or ^{125}I-Bolton Hunter reagent. The ^{3}H-peptide was prepared by tritiation of an iodinated precursor.

A- Radioactive ^{125}I labelling

Substitution of a hydrogen by an iodine atom modifies the physico chemical properties of a compound, due to the high polarity and steric hindrance of the iodine atom. However, labelling peptides with ^{125}I still remains popular due to high specific activities obtained (around 2000Ci, 74TBq/mmol). In order to check the binding properties of hIIA, two iodinated derivatives (see Abbreviations) were synthesized : I , having an ^{125}I-atom in the side chain of the Tyr residue, and II , having an hydroxy-[^{125}I]iodophenylpropionyl group (HIPP) on the terminal amine group of hIIA.

Glu-Gly-Val-[^{125}I]Tyr-Val-His-Pro-Val or (^{125}I-Tyr^4)hIIA, I

A preliminary study of iodination of hIIA using non-radioactive NaI and chloramine T (ChlT) indicated that increased yields of monoiodinated peptide were obtained with higher ratios of ChlT/NaI (15-40/1). A short reaction time (10-15 sec) and an excess of peptide compared to iodine were used, so that the ChlT/peptide ratio remained inferior to 4 in order to reduce the peptide degradation and the formation of di-iodinated derivatives, as already shown for other peptides (2).

Analytical HPLC optimal conditions consisted in the use of a Nucleosil C-18 or a TSK-ODS-120T column and mixtures of acetonitrile and triethylammonium-phosphate (TEAP) buffer (3,4).

The monoiodinated ^{125}I-peptide was obtained with good yields (about 70% based on Na^{125}I after purification) when using 40µg (30 nmol) of the octapeptide hIIA and ratios of peptide/Na^{125}I = 30 and ChlT/NaI = 20.

Identification of the peptide was carried out by analytical HPLC and comparisons to an authentic non radioactive monoiodinated derivative : (3-I-Tyr^4)AII. The specific activity of I was around 2000Ci, 74 TBq/mmol.

Degradation of I after 40 days storage at -80°C or -20°C was followed by HPLC. Fig.1 shows that the labelled peptide was best stored at -80°C.

3-(4-hydroxy 3-[^{125}I]iodophenyl) propionyl-hIIA, [^{125}I]HIPP-hIIA, II.

To avoid possible disturbancies of binding or biological properties of hIIA due to the presence of an iodine atom in a central position in I, we introduced an iodinated hydroxyphenyl-propionyl group (5) onto the free terminal amine of hIIA.

HPLC analysis showed a major radio-iodinated product when hIIA was allowed to react with [^{125}I]Bolton Hunter reagent under usual conditions (5,6). This derivative was identified as II, using non-radioactive HIPP-hIIA obtained in a preliminary synthesis.

After HPLC purification, pure [^{125}I]HIPP-HIIA or II was obtained with a specific activity of 1.8 kCi/mMol, 66.6 TBq/mmol.

B- Tritium labelling

The octapeptide hIIA inhibited binding of radio-iodinated angiotensin, (^{125}I-Tyr^{4})AII, to rat hepatocyte membranes or to various monoclonal antibodies (mAb) against AII. However, no direct binding of [^{125}I]AII to AII receptor or mAb could be observed (1). In order to confirm this absence of direct binding to the proteins concerned and also to avoid possible physico-chemical modifications due to the iodine atom, we synthesized a tritium labelled derivative (3,5-$^{3}H_2$-Tyr^{4})hIIA or III.

Tritiated tyrosyl peptides can be prepared by catalytic dehalogenation of iodinated precursors (2,7,8). Iodination of hIIA with an excess of NaI (NaI/peptide=2) and chloramine T (ChlT/NaI=10) provided (3,5-I_2-Tyr^{4})hIIA or IV. Analyzed by HPLC, the three peptides hIIA, monoiodinated (3-I-Tyr^{4})hIIA and diiodinated peptide IV showed distinct peaks at retention volumes of 5ml, 15ml and 33ml respectively (isocratic elution by 19% acetonitrile in TEAP 0.15M, pH 2.5 buffer, on a TSK ODS-120T column). After purification by HPLC, IV was desalted on a Sep-Pak (Waters) cartridge, concentrated to dryness and submitted to tritiation in the presence of tritium gas and PdO catalyst under conditions already described (7). Filtration of the catalyst, treatment on Sep-Pak C-18 cartridge and purification by HPLC on a semi-prep column (TSK-ODS-120T, 7.8 x 300mm) yielded the tritiated peptide III. This peptide was identical to the hIIA standard peptide, as examined by analytical HPLC in various solvent systems and by amino acid analysis. Its specific radioactivity, estimated by U.V.absorbancy and radioactivity measurements, was 43 Ci or 1.59 GBq/mmol.

We observed a competitive inhibition between Angiotensin II (or ^{125}I-AII) and hAII (or I or III) for the binding AII - receptors or - antibodies (1). In contrast, no direct binding of labelled ^{3}H- or ^{125}I-hIIA to AII receptors or antibodies could be detected (1). Probably, binding between the two octapeptides AII and hAII occured and was responsible for the competitive inhibition observed. Experiments to examine direct binding of the two "complementary" octapeptides are in progress in our laboratory.

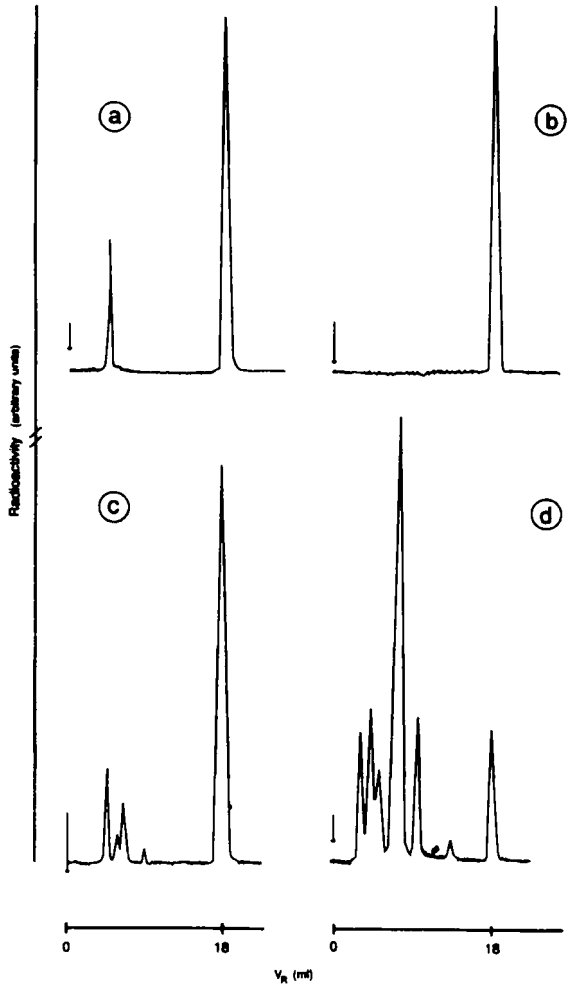

Figure 1 :
Analytical HPLC analysis (Nucleosil ODS 5µm, 250x4.6 mm column, isocratic elution 0.8ml/min of a mixture 16% acetonitrile in TEAP 0.15M, pH 2.5) :
a/ Mixture of hIIA, $Na^{125}I$, Chl T, $Na_2S_2O_5$
b/ **I** after purification
c/ **I** after 40 days storage at -80°C
d/ **I** after 40 days storage at -20°C

Experimental

Material and methods

The octapeptide hIIA was synthesized by solid phase peptide synthesis by Neosystem Laboratories (Strasbourg, France) with a purity of 98% (as controled by HPLC and aminoacid analysis). Other common chemicals were purchased from Aldrich, Sigma (USA), Fluka (CH) or Merck (D); solvents were from Merck or Prolabo (F). $Na^{125}I$ was from OrisI (F) and Bolton Hunter reagent from Amersham (GB).

Purity controls and reaction progress were checked by TLC and HPLC. Various HPLC systems were used, including Waters or Hitachi pumps, gradient controler and on-line U.V. absorbance (variable wavelength) detection. On-line radioactivity detection was monitored with Berthold instruments.

Specific radioactivity was calculated from radioactivity and U.V. absorbancy measurements. Total radioactivity of a solution was measured on a LKB (1211 Rack Beta) counter for tritium and a Berthold (LB2040) detector for ^{125}I-labelled derivatives. Peptide concentration was determined by their U.V. absorbancy on a Uvikon 860 (Kontron inst.,CH) spectrophotometer. Reference molar U.V. absorbance values in an acidic medium were ε_M = 1400 at 275nm for tyrosine and ε_M = 2750 at 283nm and at 287nm for monoiodo-tyrosine and diiodo-tyrosine respectively (9).

Aminoacid analysis was performed on a LKB 4000 auto analyzer. Distilled azeotropic 6N HCl was used for peptide total hydrolysis (20h at 105°C) and Milli Q deionized water for aqueous solutions.

(^{125}I-Tyr^{4}) hIIA (I)

hIIA (65µg, 72nmol) was dissolved in 30µl phosphate buffer (0.1M, pH 8) and added to $Na^{125}I$ (2mCi,74Mbq/10µl). This mixture was stirred using a reacti-vial and a conic magnetic stirrer, then chloramine T (0.5mg/ml, 10µl) was added. After 10 sec, the reaction was stopped by addition of $Na_2S_2O_5$ (1mg/ml, 10µl) . The solution was stirred for 1 min and then brought to 200µl with a NaI solution (2mg/ml). Aliquots of 2µl were taken for HPLC analysis and comparisons with non radioactive monoiodinated hIIA. Purification of I was carried out by two injections of 90-98µl of the reaction mixture on a HPLC column (Nucleosil C-18, 4.6x250mm) and isocratic elution (0.8ml/min) with a mixture of acetonitrile (16%) and triethylammonium-phosphate buffer (0.15M, pH2.5, 84%). The iodinated peptide was collected at V=16-18ml. Purity of the peptide was controlled by analytical HPLC. U.V. absorbancy and radioactivity measurements gave a specific radioactivity of 2000Ci, 74TBq/mmol.

[^{125}I]HIPP-hIIA (II)

Bolton Hunter reagent (0.5mCi, 185MBq, in 100µl benzene and 0.2% dimethylformamide) was concentrated to dryness by a gentle stream of argon. 67µg of hIIA in 20µl borate buffer (0.1M, pH 9) were then added.

After stirring at 0°C for 9 min, 20µl of borate buffer were added, and the mixture was stirred again for 9 min. The reaction was stopped by addition of 80µl of TEAP buffer (triethylammonium-phosphate buffer 0.15M, pH 2.5). The solution was then immediately injected on a HPLC column (Nucleosil C-18, 5µm, 4.6x250mm) and was eluted with an isocratic mixture of acetonitrile (28%) in TEAP buffer. The radioactivity of pure II was 60µCi, 2.2MBq/ml and its specific activity was 1.8kCi, 66.6TBq/mmol.

^{3}H-hIIA (III)

To hIIA (1.6mg, 2µMol) dissolved in 80µl of phosphate buffer (0.5M, pH 7.5) were added 40µl of 0.1M NaI and 40µl of ChlT solutions. The mixture was stirred for 30sec, then the reaction was stopped by addition of 60µl of 0.2M $Na_2S_2O_5$. After stirring for 1 min, the mixture was brought to 0.5ml with a 1M NaI solution. Purification on a HPLC column (TSK-ODS-120T, 4.6x250mm, 5 injections, isocratic elution with a mixture of 17% acetonitrile in TEAP 0.15M, pH2.5 buffer) gave a major fraction of diiodo-Tyr^{4}-hIIA and a minor fraction of the monoiodinated derivative. These derivatives were identified by uv absorption spectra (9) and by aminoacid analysis. The fraction corresponding to the diiodinated-hIIA, IV, was concentrated, desalted on a C-18 Sep-Pak cartridge, and brought to dryness in a small tritiation glass reactor (1ml).

Tritiation of IV was performed with tritium gas (P=0.8 bar) in the presence of PdO catalyst (2 mg) in 0.4ml of phosphate buffer (0.05M, pH7) with stirring for 1.5 hr at room temperature. After filtration of the catalyst (Millipore FHLP filter), the reaction mixture was concentrated to dryness, dissolved in acetic 1% solution and concentrated again to dryness. Purification of III was performed by HPLC on a semi-preparative column (TSK-ODS-120T, 10µl, 7.8x300mm, isocratic elution with a mixture of 12% of acetonitrile in TEAP buffer). The purity of III was checked by analytical HPLC. Its aminoacid content was identical to standard hIIA, as checked by aminoacid analysis. The specific radioactivity of III was 43Ci, 1.59GBq/mmol.

Abbreviations :

AII = Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
hIIA = Glu-Gly-Val-Tyr-Val-His-Pro-Val
I = (^{125}I-Tyr^{4})hIIA or Glu-Gly-Val-[^{125}I]Tyr-Val-His-Pro-Val
II = [^{125}I]HIPP-hIIA or 3-(4-hydroxy-3-[^{125}I]iodophenyl)propionyl-hIIA
III = (3,5-$^{3}H_2$-Tyr^{4})hIIA or Glu-Gly-Val-(3,5-$^{3}H_2$)Tyr-Val-His-Pro-Val
IV = (3,5-I_2-Tyr^{4})hIIA or Glu-Gly-Val-(3,5-I_2)Tyr-Val-His-Pro-Val

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