TRITIUM LABELLING OF 8-LYSINE VASOPRESSIN AND ITS PURIFICATION BY AFFINITY CHROMATOGRAPHY ON SEPHAROSE BOUND NEUROPHYSINS

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1. Introduction

The antidiuretic hormone vasopressin is a nonapeptide containing one disulfide bridge and tyrosyl and phenylalanyl residues in position -2 and -3, respectively. 8-Lysine vasopressin (LVP) is found in pig and hippopotamus while 8-arginine vasopressin (AVP) occurs in several other mammals (man, ox, rat...). These pituitary polypeptides exert selective effects on the permeability of the distal part of the mammalian nephron and on the active sodium transport and passive permeability to water and other low molecular weight molecules of the amphibian skin and bladder epithelial cells. These effects have been shown to result from a specific activation of an adenyl cyclase located within the membrane of the target cells (for review, see for instance [1]).

Either LVP or AVP is associated with oxytocin, a related peptide, and carried in the post-hypophysis by a family of relatively small proteins, the neurophysins [2], with which they form non covalent complexes [3].

The isotopic labelling of polypeptidie hormones has been successfully applied, in this laboratory, to oxytocin [4], angiotensine II [5] and other compounds [6]. The principle is an iodination of the tyrosyl (or histidyl) residue(s) followed by catalytic substitution of the peptide bound iodine with tritium.

In this report we describe a similar procedure which produces, after purification by affinity chromatography on a column of bovine neurophysins immobilized on Agarose, a highly radioactive compound (10 Ci/mmole) which exhibits all the biological and biochemical properties of the native hormone. This, obviously, provides a very useful tool for the examination of the molecular mechanisms by which this important biological compound interacts with neurophysins [7] and biological membranes.

2. Results and discussion

2.1. *Iodination*

4 μmoles of 8-lysine-vasopressin (as a hydrochloride, 285 ± 20 IU/mg; a generous gift from Dr. Guttmann, Sandoz) dissolved in 2 ml 0.1 M phosphate buffer pH 6.0 were allowed to react with 15 μmoles ICl containing traces of ^{125}ICl in anhydrous methanol at 5° under continuous stirring. After 1 min a slight excess of $Na_2S_2O_3$ was rapidly added to the mixture in order

to stop the reaction and destroy unreacted iodine.

Quantitative evaluation of iodine incorporation into the peptide molecule was checked by paper electrophoresis separation of free mineral from bound iodine [4]; 70% of the total iodine was found associated with the peptide. The reaction product was then passed through a Biogel P2 column (Biorad Laboratories) to remove salts and elution was performed with 1 mM acetic acid. After elution of a first peak of UV absorbing material, 1% acetic acid was then used and a second peak of peptide was recovered. This represented roughly 80% of the total product and was used for further tritiation. UV absorption of the reaction product indicated the characteristic bathochromic shift of the tyrosyl ring after iodination [4, 5]. The behavior of this compound on thin-layer chromatography (cellulose plate MN 300; solvent system: n-butanol–acetic acid–water (75:10:25, v/v) indicated the absence of any significant amount of impurity.

Aliquots samples were counted for ^{125}I radioactivity on a Saip Gammatic solid scintillation counter. About 2 iodine atoms were found incorporated per peptide molecule.

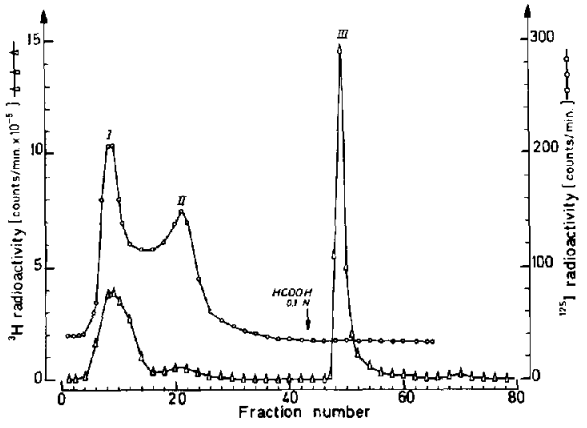

Fig. 1. Affinity chromatography on a neurophysins–Sepharose conjugate. Elution diagram obtained after the product of the tritiation reaction was applied at the top of a column containing 5 ml of Sepharose-bound neurophysins in 0.1 M acetate buffer, pH 5.7. Elution was performed with the same buffer and peaks I and II were recovered. Then 0.1 N HCOOH pH 2.5 was used and peak III eluted. ^{3}H radioactivity was measured on a 5 µl aliquot of each fraction. ^{125}I radioactivity was evaluated on the total fraction volume (1 ml).

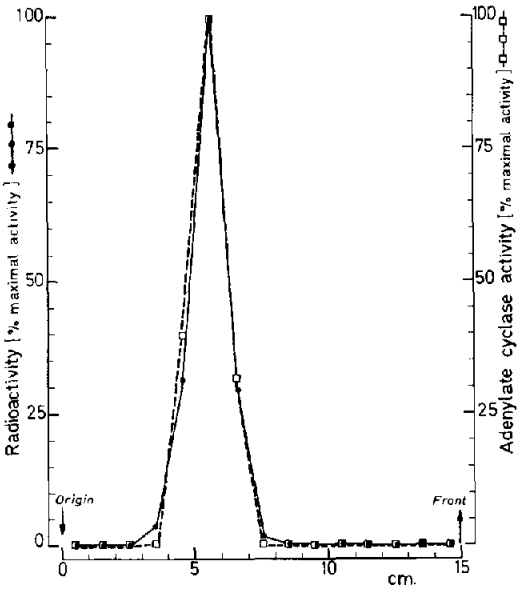

Fig. 2. Radiochromatogram of [^{3}H]LVP. A tracer amount of the neurophysins–Sepharose column eluate was spotted together with 10 IU unlabelled LVP on a cellulose plate. Elution profile of radioactivity (•–•–•) and biological activity of the unlabelled LVP measured by the activation of pig kidney medulla adenylate cyclase (□- -□- -□).

2.2. *Tritiation*

The iodo derivative was lyophylized and then redissolved in a minimum volume (about 0.5 ml) of 1% acetic acid. The solution placed in the special flask described elsewhere [4] was then frozen and 10 mg of 10% palladium on alumina catalyst (Engelhardt and Co.) added. 10 Ci of pure tritium (Commissariat à l'Energie Atomique) gas was then introduced at a pressure of 500 mm Hg. After the mixture was brought to room temp. the reaction was performed under stirring at 25° during 20 min. Tritium gas was removed and the labile hydrogens eliminated by successive washes and evaporations with large volumes of distilled water. Salts were then removed from the reaction product by passage through a Biogel P2 column in acetic acid using a similar stepwise elution as mentioned before (see sect. 2.1.). In the second peak eluted with 1% acetic acid a partial separation of compounds containing either ^{3}H or ^{3}H and ^{125}I radioactivities was apparent. Since the separation could not be achieved by this method with a sufficient efficiency,

purification was attempted using affinity chromatography. Tritium determination was made using a SL 30 Intertechnique scintillation spectrometer.

2.3. *Purification and biological assays*

In this reaction mixture, the tritiated peptide could be separated from other by-products, including halogenated derivatives, dimeric forms and others, by selective adsorption on a conjugate of bovine neurophysins covalently bound to Agarose gel. This was prepared according to the general coupling technique of Axen and Ernback [8] and Cuatrecasas [9]. 10 ml decanted Sepharose 4 B (Pharmacia, Uppsala) were activated by reaction with BrCN (2 g) at pH 11 in the ice bath during 8 min, then rapidly washed with cold 0.1 M $NaHCO_3$ and the coupling buffer (0.1 M phosphate, pH 7.2). 10 μmoles of bovine neurophysins [7] were then rapidly added to the activated gel and allowed to react at pH 7.2 at 4° during 24 hr. The product of the reaction was washed with 120 ml of the coupling phosphate buffer in order to remove the remaining uncoupled proteins. The latter fraction was evaluated to 5 μmoles by UV absorbance at 260 nm assuming $\epsilon = 3400\ M^{-1}\ cm^{-1}$.

The affinity absorbent was placed on a column and washed thoroughly by large volumes of the following solvents: 1000 ml each: phosphate 0.1 M buffer containing 0.5 M glycine at pH 7.1; 0.1 M acetate, pH 5.7; 0.1 N HCOOH; and again 0.1 M acetate pH 5.7.

The adsorption of the tritiation reaction product on the Sepharose conjugate was performed in 0.1 M acetate at pH 5.7 optimal for binding of the vasopressin to neurophysins [7]. This product was first lyophylized then dissolved in a minimum volume of acetate buffer and applied at the top of the affinity absorbent column (5 ml).

Elution was performed with the same buffer. In the void volume (peak I; fig. 1), ^3H and ^{125}I radioactivities were recovered as a single peak. Partially excluded from the adsorbant was another compound (peak II; fig. 1) containing both ^3H and ^{125}I radioactivities. This product was not identified with certainty: its chromatographical behavior suggests that it might be a monohalogenated derivative of LVP. When no significant amount of radioactivity could be measured in the column effluent, the acetate buffer was replaced with 0.1 N HCOOH pH 2.5 to displace the tritiated LVP from its complex with Sepharose-bound neurophysins [7]. A single peak of ^3H radioactivity (peak III; fig. 1) was then eluted without any measurable contamination by ^{125}I radioactivity. This represented about 50% of the total ^3H radioactivity.

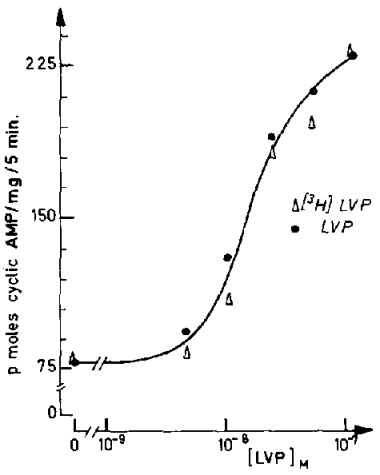

Fig. 3. Pig kidney adenylate cyclase activation by [^{3}H]LVP and unlabelled LVP. Pig kidney medulla membranes (0.2 mg) were incubated at 30° for 5 min in presence of: 100 mM Tris-HCl pH 8.0; 10 mM $MgCl_2$; 0.25 mM ATP; 0.65 μCi [^{32}P] αATP; 1 mM 3'-5'- AMP; 20 mM phosphocreatine; 100 μg creatine kinase, and LVP or [^{3}H]LVP. Labelled 3'-5'- AMP was separated by the aluminium oxide method of Ramachandran and Lee [11]. Values are the mean of two determinations.

The radiochemical purity of this product was controlled by thin-layer chromatography. 5 μl aliquots of the labelled product (containing about 420,000 cpm) were spotted on cellulose plates together with 10 IU of unlabelled peptide. The chromatogram was developed in a solvent system: n-butanol–pyridine–acetic acid–water (15:10:3:6, by vol.) for 8 hr. Then the chromatogram was separated in successive strips (1 cm width) and the peptide extracted from the cellulose powder with 0.5 ml 5% acetic acid. After centrifugation, the concentration, radioactivity and biological potencies of the supernatants were determined.

The radiochromatogram of the labelled peptide (fig. 2) reveals the absence of any labelled impurity and illustrates the identical behavior of the labelled and unlabelled molecules.

2.4. *Activation of the adenyl cyclase*

A low speed sediment of pig kidney medulla homogenate was used as a source of adenylate cyclase. The enzyme activity was assayed as previously described

[10]. Activation of the cyclase by the tritiated material was found perfectly comparable with the standard process obtained by action of the unlabelled peptide (fig. 3).

2.5. *Pressor activity*

The rat pressor activity of the tritiated material was measured according to Stürmer [12]. A value of 228 IU/mg, i.e. 80%, of the maximum biological activity of the starting material was found; the concentration of the peptide was measured by the Lowry's method [13] using unlabelled lysine-vasopressin as standard. The [^{3}H]LVP presented a specific radioactivity of 10 Ci/mmole.

Thus, this labelling technique leads to a rapid preparation of tritiated LVP of high specific activity. The affinity chromatography purification step proved to be very selective in allowing the recovery of peptide molecules retaining the ability both to bind neurophysins and to trigger the biological responses examined here. This method has been applied to the purification of tritiated oxytocin [7] and could be extrapolated to other neurophysin-binding peptides.

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