SPECIFIC TRITIUM LABELLING OF LYSINE-VASOPRESSIN IN TWO DIFFERENT AMINOACID RESIDUES*

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SUMMARY

Lysine^{8}-vasopressin was specifically labelled with tritium in the tyrosine^{2} and phenylalanine^{3} residues. The 3,5-dibromo--tyrosine^{2} and 4-chloro-phenylalanine^{3} derivatives of the hormone were synthesized by solid phase peptide synthesis. The halogen containing peptides were catalytically reduced by tritium gas yielding preparations with high specific radioactivity and full biological activity as compared with reference material.

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The neurohypophyseal hormone vasopressin is a nonapeptide and exerts an important antidiuretic effect. Other endocrine effects, namely: pressor, oxytocic and corticotrophin-releasing activities have been attributed to vasopressin, as well. Lysine^{8}-vasopressin (LVP) has been isolated from porcine, while Arg^{8}-vasopressin (AVP)

* The nomenclature used in the text is in accordance with the IUPAC-IUB Rules on Biochemical Nomenclature: Biochem.J. 126:773 (1972) and J.Biol.Chem. 242: 555 (1967). Abbreviations used: Dbt: 3,5--dibromo-L-tyrosine, Cpa:4-chloro-L-phenylalanine, DMF:dimethylformamide, BOC:t-butyloxycarbonyl, DCC:dicyclohexylcarbodiimide, TFA:trifluoroacetic acid, TEA:triethylamin, Bzl:benzyl.

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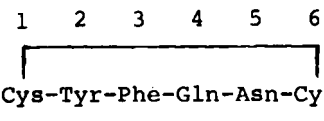
has been isolated from bovine pituitaries (1,2).

Structure of lysine-vasopressin:



Besides its endocrine effects, lysine-vasopressin has been found to possess a behavioural effect: it maintains a conditioned avoidance response in trained rats (3). Vasopressin has been found to stimulate protein metabolism and neurosecretion in the hypothalamus (4) and block the puromycin-induced inhibition of protein synthesis (5). There is further evidence that vasopressin stimulates protein synthesis (6) and it may have a synaptic function as a neurotransmitter (7). Vasopressin has also been found to increase ionic conductivity in lipid bilayers (8). However, these data have not unfolded the mechanism of action of vasopressin on memory processes and further studies are required to investigate the biochemistry of this central activity.

The investigation of these effects on the molecular level is greatly enhanced by the availability of radiolabelled peptides with high specific radioactivity, in view of the extremely low physiological concentration of vasopressin.

Earlier attempts on the labelling of vasopressin have started with the application of the Wilzbach method to lysine-vasopressin (9). A more advanced approach was applied by Sjöholm and Carlsson (10, 11) for the labelling of lysine-vasopressin. Tritium-labelled tyrosine was incorporated into LVP by classical techniques of peptide synthesis. A relatively high specific radioactivity (1.9 Ci/mmol) was achieved associated with full biological activity. Both arginine--vasopressin and lysine-vasopressin were synthesized incorporating ^{14}C-glycine by solid-phase peptide synthesis by Walter and Havran (12), and the application of these preparations has demonstrated the advantages of radiolabelling in studying biochemical mechanisms of vasopressin action (13). Lysine-vasopressin with very high specific radioactivity (10 Ci/mmole) was obtained by the application of the technique developed by Morgat and Fromageot et al. (14) The diiodo-tyrosine derivative of LVP was first prepared by direct iodination with iodine monochloride. This was catalytically tritiated to give

the labelled nonapeptide.Another attempt involving solid phase peptide synthesis was applied for the labelling of arginine-vasopressin with tritium in the phenylalanine residue by Holton et al (15), however with fairly low specific radioactivity.

In our laboratory, we have developed a method for the labelling of peptides based on the incorporation of 3,5-dibromo-L-tyrosine and 4-chloro-L-phenylalanine into peptides followed by their catalytic reduction with tritium gas. This method has been applied in the case of angiotensin II (16,17), α-melanotropin (18,19), ACTH fragments (20) and bradykinin (21) . Based on the experience gained from this earlier work we have applied the same technique in the case of lysine-vasopressin, however using the solid phase methodology of peptide synthesis.

The two peptides: 3,5-dibromo-tyrosine^2-lysine vasopressin (Dbt^2-LVP) and 4-chloro-phenylalanine^3-lysine vasopressin (Cpa^3-LVP) were sytnhesized on conventional, 1 % cross-linked styrene-divinyl-benzene copolymer resins. The sequence of the synthesis is outlined in the experimental section. The two protected peptides were cleaved from the resin by ammonolysis followed by the removal of protecting groups by liquid hydrogen fluoride. The formation of the disulfide bridges was carried out by hydrogen peroxide and aeration, respectively. Finally the peptides were purified in two consecutive steps by column chromatography.

The halogen-containing LVP derivatives were catalytically reduced using carrier-free tritium gas. After tritiation the peptides were purified by column chromatography. Specific activity of the tyrosine-labelled LVP batches ranged between 8 and 12 Ci/mmole, while the phenylalanine-labelled LVP preparations had specific activities between 4.3 and 5.0 Ci/mmole. Biological potencies of the labelled peptides were tested in alcohol-anaesthetized female Wistar rats using DeWied's method (3) . Both phenylalanine- and tyrosine-labelled lysine-vasopressin preparations had full (270 U/mg) antidiuretic activities as compared with synthetic standard material (Sandoz).

Experimental Section

All amino acids were of the L-configuration. BOC-derivatives and activated esters were prepared according to known procedures.

The purity of amino acid derivatives as well as that of peptides were tested by thin layer chromatography. TLC-s were developed on Merck DC-Fertigplatten Kieselgel 60 .R_f values refer to the following solvent systems: R_f^1 , n-butanol-acetic acid-water 8:5:4; R_f^2, n-butanol-acetic acid-water 4:1:1.

All reagents used for solid phase peptide synthesis were of analytical grade, while solvents were freshly distilled before use.

Column chromatographic elutions were followed by continuous monitoring of UV absorption of the eluent at 254 and 280 nms in LKB Uvicord 8300 and ISCO UA-5 photometers. Tritiations were carried out in a special vacuum manifold described elsewhere (17). Radioactivity determinations were carried out by liquid scintillation counting in Beckman LS-355 and Packard Tri-Carb 2425 equipment. Radiochromatograms were evaluated in a Nuclear Chicago Actigraph III. scanner.

Amino acid analyses were determined in samples hydrolyzed by 6N HCL at 105° for 24 hrs or (48 hrs for resin-peptides) on JEOL JLC-5 and Chinoin Lyz 75 amino acid analyzers. UV spectra were scanned in Beckman Model 25 spectrophotometers.

t-Butyloxycarbonyl-glycine-Resin

10 g chloromethylated BIO-BEADS SX-1 (1% crosslinked) styrene--divinylbenzene copolymer resin (capacity: 1.1 mmol/g) was suspended in 70 ml dimethylformamide. 1.25 g (7.15 mmol) t-butyloxycarbonyl-glycine dissolved in 20 ml dimethylformamide was added followed by 1.0 ml (7.15 mmol) triethylamine. The mixture was stired for two days at room temperature. Then the resin was filtered and washed thoroughly with dimethylformamide, ethanol, acetic acid, ethanol and methylene chloride, and dried in the desiccator. Yield: 10.35 g.

The degree of substitution was determined to be 0.25 mmol/g by amino acid analysis.

Z-Cys(Bzl) -Dbt-Phe-Gln-Asn-Cys (Bzl) -Pro-Lys (Z) -Gly-Resin

3.5 g BOC-Gly-Resin (0,25 mmol/g substituted, total amino acid content 0.875 mmol) was used as starting material for the synthesis. With the exception of coupling Gln and Asn, the synthesis involved the following cycles:

(Unless otherwise indicated all volumes are 40 ml.)

1. Deprotection
   CH_2Cl_2 wash: 3x3 min
   20 % TFA/CH_2Cl_2 reagent: 5 min, and 25 min, resp.
   CH_2Cl_2 wash: 3x3 min
   1M Isopropanol/CH_2Cl_2 wash:3 min
   CH_2Cl_2 wash: 6x3 min

2. Neutralization
   0.5 M TEA/CH_2Cl_2 reagent: 3x5 min
   CH_2Cl_2 wash:6x3 min

3. Coupling
   3 eq. BOC-amino acid in 20 ml CH_2Cl_2:5 min
   3 eq. DCC in 10 ml CH_2Cl_2:12 hrs
   CH_2Cl_2 wash: 6x3 min
   0.5 M TEA/CH_2Cl_2 reagent: 3x5 min
   CH_2Cl_2 wash: 6x3 min

4. Repeat coupling
   3 eq.BOC-amino acid in 20 ml CH_2Cl_2:5 min
   3 eq. DCC in 10 ml CH_2Cl_2: 12 hrs
   CH_2Cl_2 wash:3x3 min

5. Dorman titration and amino acid analysis
   0.3 M Pyridine hydrobromide / CH_2Cl_2 (Dorman) reagent: 3 min
   CH_2Cl_2 wash: 3x3 min
   DMF wash: 3x3 min
   4 ml TEA+36 ml DMF: 7 min
   DMF wash: 3x3 min

The last two solutions were collected and titrated according to the method of Dorman (22). If the result was less than 98 % coupling was repeated.

   CH_2Cl_2 wash: 3x3 min
   A sample was taken for amino acid analysis at this stage.
   CH_2Cl_2 wash 3x3 min
   0.5 M TEA/CH_2Cl_2 reagent: 3x5 min

CH_2Cl_2 wash 6x3 min

6. Blocking

10 eq. acetic anhydride + 10 eq TEA in CH_2Cl_2 reagent
30 ml: 3 hrs
CH_2Cl_2 wash:6x3 min

Gln and Asn were coupled as their p-nitrophenyl esters.
In these cases the coupling cycles were as follows:
DMF wash 3x3 min
6 eq. BOC-amino acid p-nitrophenyl ester in 30 ml DMF:48 hrs
DMF wash: 6x3 min
CH_2Cl_2 wash: 3x3 min
1 M isopropanol/CH_2Cl_2:3 min
CH_2Cl_2 wash: 6x3 min
0.5 M TEA/CH_2Cl_2 reagent: 3x5 min
CH_2Cl_2 wash: 3x3 min

After the last coupling the peptide-resin was washed 6x with DMF, 6x with CH_2Cl_2 and dried in the dessicator.

Z-Cys(Bzl)-Dbt-Phe-Gln-Asn-Cys(Bzl)-Pro-Lys(Z)-Gly-NH_2

3.5 g of the obtained peptide-resin was suspended in 70 ml methanol. At - 60°, 400 ml ammonia was distilled from sodium onto the suspension, which was further stirred for 8 hrs. After the evaporation of ammonia the procedure was repeated. The ammonia-saturated suspension was stirred for three days at room temperature in the closed reaction vessel. Then the mixture was evaporated to dryness. The peptide was dissolved in methanol and the resin removed by filtration. It was washed with 4x35 ml DMF and 2x35 ml methanol. The filtrates were evaporated to dryness, the oily residue dissolved in 30 ml methanol and left standing overnight in the refrigerator. The precipitated white crystalline material was filtered and washed with cold methanol and ether and dried. Yield: 193 mg.

H-Cys-Dbt-Phe-Gln-Asn-Cys-Pro-Lys-Gly-NH_2

The protected nonapeptide amide was suspended in 0,6 ml anisol.15 ml anhydrous hydrogen fluoride was distilled onto the material and the

mixture was stirred for 30 minutes at 0°. Then HF was distilled off and the peptide was powdered with peroxide-free ether. The material was filtered and dried. It was dissolved in 500 ml 0.2% acetic acid and the solution was adjusted to pH 6.8 with ammonium hydroxide. Air was bubbled through the solution for 5 hrs, then the solution was evaporated and lyophylized.

The crude material was first purified by gel filtration on Sephadex G-15 column (2x90 cm) eluting with 50% acetic acid. The main component was subjected to a second gel filtration on Sephadex G-15 column 1.1x110 cm eluting with 0.2 M acetic acid. After evaporation and lyophylization the main fraction yielded 58 mg homogeneous nonapeptide amide: R_f^1:0.58, R_f^2:0.31.

Amino acid analysis: Asp 0.96, Gln 0.99, Pro 0.97, Gly 1.0, Phe 1.06, Lys 1.06, Dbt 0.94.

[Tyr-3,5-^{3}H]^2, Lys^8-vasopressin

5 mg Dbt^2, Lys^8-vasopressin was dissolved in 0.5 ml 1 % acetic acid. The solution was frozen with liquid nitrogen and 50 mg 10 % Pd/Al_2O_3 catalyst was placed onto it. The reaction vessel was connected to the tritiation manifold. The catalyst was first saturated with carrier-free tritium-gas and then the frozen solution was melted and stirred for 30 minutes at room temperature. Gas pressure was 600 torr. The catalyst was removed by Millipore filtration and washed with 20 ml 1 % acetic acid. The solution was evaporated to dryness. 3x80 ml water was further distilled from the material to remove labile tritium.

The material was dissolved in 1.5 ml 0.2 M acetic acid and purified by gel filtration on Sephadex G-15 column (1.1x18 cm) eluting with the same solvent. The fractions containing the labelled nonapeptide amide were evaporated and dissolved in 10 ml water. According to UV absorption at 274 nm peptide content was 197 µg/ml. Radioactive concentration was found to be 1.54 m Ci/ml. This corresponds to a specific radioactivity of 8.3 Ci/mmol.

The material was found to be chemically and radiochemically homogeneous: R_f^1:0.51, R_f^2:0.20

Amino acid analysis: Asp:1.03, Glu 1.04, Pro 0.96, Gly 1.0, Tyr 0.98, Phe 0.96, Lys 1.06

Biological testing of the material in anaesthetized female Wistar rats showed it to have an activity of 270 U/mg as compared to Sandoz synthetic standard material.

H-Cys-Tyr-Cpa-Gln-Asn-Cys-Pro-Lys-Gly-NH_2

2.2 g of BOC-Gly-Resin (0.5 mmol/g) was the starting material of this synthesis. The synthetic sequence followed the one described above. The dry peptide-resin after the synthesis weighed 2.64 g.

2.5 g of this peptide-resin was ammonolyzed in the same fashion as described for the dibromo-tyrosine derivative to give 107 mg protected nonapeptide amide. This was deblocked in liquid hydrogen fluoride followed by cyclization in 500 ml pH 6.8 ammonium acetate solution. The crude material was purified by gel filtration on Sephadex G-15 columns successively eluting with 50 % and 0.2 M acetic acid solutions. Yield: 53 mg homogeneous nonapeptide amide; R_f^1:0.55, R_f^2:0,27

Amino acid analysis: Asp 0.99, Glu 0.98, Pro 1.0, Gly 1.0, Tyr 0.96 Lys 0.98, Cpa 0.97

[Phe-4-^{3}H]^3, Lys^8-vasopressin

5 mg Cpa^3-LVP was dissolved in 0.5 ml 1 % acetic acid and frozen by liquid nitrogen. 50 mg 10 % Pd/Al_2O_3 catalyst was placed onto the frozen solution and the reaction vessel connected to the tritiation apparatus. Carrier-free tritium gas was led into the system, the solution melted and the reaction mixture was stirred for 30 minutes at room temperature; tritium gas pressure was 500 torr. The catalyst was removed by Millipore filtration. The filtrate was evaporated to dryness and further 3x80 ml water was distilled from the material. The residue was dissolved in 1 ml 0.2M acetic acid and purified on Sephadex G-15 column (1.1x20 cm) eluting with the same solvent. The fractions containing the nonapeptide were collected and evaporated. The residue was dissolved in 10 ml water.

The peptide content was found to be 204 μg/ml photometrically

at 274 nm.

Radioactive concentration was determined as 0.83 m Ci/ml, which corresponds to a specific radioactivity of 4.3 Ci/mmol.
Amino acid analysis: Asp 1.02, Gln 0.98, Pro 0.95, Gly 1.0, Tyr 0.99, Phe 0.97, Lys 1.03.

Biological evaluation showed the material to have a potency of 260 U/mg as compared to Sandoz synthetic standard material.

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