Some Biological and Chemical Properties of a Lysine-Vasopressin Dimer

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In the course of fractionation of neurohypophysial extracts in search for corticotropin-releasing factor (1) we sometimes observed materials with low pressor activity, consistent corticotropin-releasing activity, and with the same molar ratios of amino acids as lysine-vasopressin. In view of the report by Ressler on a possible dimerization of oxytocin (2), it was decided to investigate these materials with the hypothesis that their peculiar biological properties may be due to polymerization of vasopressin.

EXPERIMENTAL PROCEDURE

All the bioassays (for vasopressor, CRF, and ACTH activities) were performed as described previously (3, 4). All the details of chromatography on carboxymethylcellulose have been reported (4). The techniques of gel filtration of posterior pituitary hormones on beds of dextran were described by Porath and Lindner (5) and Porath and Schally (6). For the determination of approximate molecular size, Sephadex columns G-25 and G-50 were carefully calibrated with lysine-vasopressin, oxytocin, α-MSH, β-MSH, corticotropin A, and bovine serum albumin. Fraction V. Paper chromatography was carried out by the descending method on Whatman No. 1 or No. 3 filter paper with a system consisting of 1-butanol-acetic acid-water (4:1:5). High voltage paper electrophoresis was done in a water-cooled E-C 451 apparatus on Whatman No. 3MM paper, in pyridine-acetic acid-water (100:4:900) at pH 6.5. The peptide spots were routinely located by ultraviolet scanning and ninhydrin spray, followed by cupric nitrate and Amido black stain. The amino acids were determined after hydrolysis in a vacuum in redistilled 6 N hydrochloric acid in a Beckman/Spinco model 120 amino acid analyzer.

The polymerization of lysine-vasopressin which was found to occur under a variety of conditions (see below), among them repeated lyophilizations from solutions slightly alkaline with ammonia, was accomplished as follows. Solid material (1.5 g) resulting from a single lyophilization of the lysine-vasopressin peak from preparation of α-CRF (7) by chromatography on carboxymethylcellulose were placed in a beaker in an evacuated vacuum desiccator over sodium hydroxide pellets. The mixture contained about 1 g of purified lysine-vasopressin and 0.5 g of ammonium acetate. Phosphorus pentoxide or phosphoric acid, which are normally used to absorb traces of ammonia, that remain even after two lyophilizations from ammonium acetate solutions were omitted. These materials were allowed to stand at room temperature for a period of 3 months. When the desiccator was opened, it was found to contain ammonia fumes. The dry weight of this material had decreased to about 1 g.

RESULTS AND DISCUSSION

The partially polymerized lysine-vasopressin was subjected to gel filtration on Sephadex G-25. The results are shown in Fig. 1.

The material in the first five peaks, but not the lysine-vasopressin monomer peak, exhibited CRF activity in the rat blocked with morphine and Nembutal (3) when tested at an equivalent of 12 to 20 pressor milliunits. All the peaks yielded material that was completely inactive in the hypophysectomized rat, i.e. exhibited no direct adrenocorticotropic activity. The specific pressor activity of the material in these first peaks ranged from 10 to 26 units per mg; the monomer peak, which accounted for about 60% of the total material recovered from the column, assayed 250 pressor units per mg in bracketed assays. The heterogeneity of materials in each of the first four peaks was established by paper chromatography. The fifth peak (tubes 115 to 138) gave 118 mg of solids on lyophilization and one major spot on paper chromatography, corresponding in RF to lysine (RF = 0.2). This material was tentatively identified as lysine-vasopressin dimer on the basis of its behavior on Sephadex G-25. The material also contained a small contaminant, which gave a spot corresponding to α-MSH (RF = 0.6). By bioassay it was confirmed that the area contained about 3 × 10⁴ MSH units per mg or a 20% contamination. This α-MSH contamination and minute traces of lysine-vasopressin monomer were removed by chromatography on carboxymethylcellulose (see below). Although we have shown before that LVP monomer and α-MSH can be separated on Sephadex G-25 (6), it appeared that the dimer (mol. wt. 2108) could not be easily separated from α-MSH (mol. wt. 1729) on Sephadex G-25. The material recovered from tubes 115 to 138 was thus applied to a carboxymethylcellulose column, 1 × 63 cm, equilibrated with 0.035 M ammonium acetate, pH 5.7 (9). Although traces of α-MSH and traces of lysine-vasopressin monomer were eluted by a gradient to 0.1 M buffer, pH 7, as shown in our previous publications (1, 4, 9), the lysine-vasopressin dimer was not, because of its affinity for carboxymethylcellulose. It could be eluted, however, in a fairly high yield from carboxymethylcellulose by the application, in a stepwise fashion, of 400 ml of 2 N...
acetic acid and it was recovered as a solid by lyophilization. This material, which had an $R_f = 0.15$ on paper chromatography and the molar amino acid ratios of lysine-vasopressin, was rechromatographed on a Sephadex G-25 column. The pattern obtained is shown in Fig. 2.

Lyophilization of tubes 102 to 126 yielded 50 mg of material with a pressor activity of 8 to 12 pressor units per mg computed on the basis of the maximal blood pressure peak obtained. The profiles of the blood pressure responses obtained were different from those of vasopressin monomer; the dimer gave a response that developed considerably more slowly than the response caused by the monomer and lasted much longer. The natural dimer had CRF activity at a dose of 1.5 µg as evidenced by 15- and 30-minute blood compound B levels in the rat blocked with morphine and Nembutal.

The molar ratios of amino acids and ammonia obtained after acid hydrolysis were exactly the same as those of lysine-vasopressin. Paper chromatography (Fig. 3) showed that the dimer was homogeneous and that it had a much smaller $R_f$ than lysine-vasopressin monomer.

After oxidation with performic acid (10), both the monomer and the dimer had the same mobility (Fig. 4). A synthetic dimer made by Dr. S. T. Guttman, Sandoz Ltd., Basle (Lot 23323-111), behaved chemically and biologically in the same fashion as the "natural" dimer. Both substances had similar pressor activity and gave similar shapes of pressor responses and
cyclic disulfides. A linear polymer should have a number of free —SH groups. No such groups could be demonstrated in the dimer in the present work by the nitroprusside reaction.

Preliminary studies to establish the parallel or antiparallel character of these dimers by digestion with chymotrypsin, in which case parallel and antiparallel dimers would yield different digestion products, gave inconclusive results. Chymotryptic digestion of the dimer followed by electrophoresis gave five products instead of the expected one from an antiparallel dimer and two from a parallel dimer plus an anticipated slow liberation of lysine and vasopressin.

Both were active in the CRF assay at doses corresponding to less than 30 pressor milliunits. The electrophoretic behavior of the "natural" and synthetic dimer is shown in Fig. 5.

Both "natural" and synthetic dimers had an electrophoretic mobility somewhat greater than that of the monomer.

The molecular weight of the dimer, estimated from the gel filtration experiments, was confirmed by Dr. L. C. Craig by his technique of thin film dialysis (11). The behavior of the dimer on dialysis was consistent with that of a molecule with twice the molecular weight of the monomer. The results are shown in Tables I and II.

By a similar gel filtration technique, but on Sephadex G-50, not G-25, it was possible to isolate from Fractions 79 to 90 (see Fig. 1) 2.2 mg of a substance tentatively identified as a trimer from its rate of filtration on calibrated gel columns. This material had 1.3 to 2.5 pressor units per mg and CRF activity at 3μg level.

**FIG. 4.** Paper chromatography of the lysine-vasopressin (LVP) monomer and dimer after performic acid oxidation.

**FIG. 5.** Electrophoresis of "natural" and synthetic dimer and lysine-vasopressin monomer in pyridine-acetic acid-water (100:1:900) at 1200 volts, 51 μA, for 12 hours at 16°. A and B, lysine-vasopressin, ninhydrin stain; C, synthetic dimer, ninhydrin stain; D and F, "natural" dimer, ninhydrin, and Amido black stain.

**DISCUSSION**

The process of formation of polymers seems to involve the cleavage of the intramolecular disulfide bond of vasopressin and the formation of an intermolecular disulfide between 2 or more molecules of this hormone. Rydon and Schofield with Heaton (12), Large (13), and Jarvis (14) have shown that in such reactions with model compounds the possible products can include the parallel and antiparallel cyclic dimers, analogous higher cyclic polymers, and acyclic polymers. Moreover, they have demonstrated that such reactions yield predominantly the...
TABLE I

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Half-escape time</th>
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<tr>
<td>$\beta$-Aspartic acid amide-angiotensin</td>
<td>3.2</td>
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<tr>
<td>Lysine-vasopressin</td>
<td>6.1</td>
</tr>
<tr>
<td>&quot;Natural&quot; dimer lysine-vasopressin</td>
<td>40*</td>
</tr>
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* In 4 hours 6.5% escaped.

TABLE II

<table>
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<tr>
<th>Peptide</th>
<th>Half-escape time</th>
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</thead>
<tbody>
<tr>
<td>Bacitracin, mol. wt. 1422</td>
<td>1.3</td>
</tr>
<tr>
<td>Lysine-vasopressin</td>
<td>1.0</td>
</tr>
<tr>
<td>&quot;Natural&quot; dimer lysine-vasopressin</td>
<td>3.4</td>
</tr>
<tr>
<td>Synthetic dimer lysine-vasopressin</td>
<td>4.2</td>
</tr>
</tbody>
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of free phenylalanine from each dimer. These results were probably caused by disulfide interchange at the pH of the digestion.

It is interesting that the polymerization of vasopressin, which involves a great reduction in pressor activity gives, nevertheless, a product with a definite and long lasting pressor activity and a significant CRF activity. The ratio of CRF activity to pressor activity of these dimers is higher than that of the monomer.

SUMMARY

The isolation of a lysine-vasopressin dimer was achieved by means of gel filtration on Sephadex and chromatography on carboxymethylcellulose from partially polymerized lysine-vasopressin.

The dimer derived from natural lysine-vasopressin was characterized by paper chromatography and electrophoresis, amino acid analysis, performic acid oxidation, and by molecular sieving and thin film dialysis.

The biological and chemical properties of the dimer derived from natural lysine-vasopressin and the synthetic dimer made by Dr. S. T. Gutman were compared and found to be similar.

The lysine-vasopressin dimer has a low but definite pressor activity (6 to 11 units per mg) and gives a curve of pressor response different from that of the monomer.

The dimer also has corticotropin-releasing factor activity at a dose equivalent to less than 30 pressor milliunits as evidenced by the release of adrenocorticotropic hormone in the rat blocked with morphine and Nembutal.

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REFERENCES

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