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Neurophysin Binding of Vasopressin Analogues Altered at the NH_2- and COOH-Terminal Positions

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SUMMARY

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Binding studies were carried out at pH 5.8 with bovine neurophysin II and two series of analogues of lysine-vasopressin. In one series the peptide chain of the parent hormone was extended at the NH_2 terminus by 1 leucine or 1 phenylalanine residue or by 1, 2, or 3 glycine residues, which represented a progressive steric separation of the N^{α}-amino group from the cyclic portion of the molecule. The second series of analogues consisted of shortened peptide chains at the COOH terminus, enzymatically prepared, and involved the removal of glycinamide from sequence position 9 and both terminal amino acids, lysine and glycine. Gel filtration was used to separate the neurophysin from the small peptide moieties, and spectrophotometry, dansylation analysis, and biological activity were used to investigate the fractional positions of the small peptides in the eluate. There was clear evidence of neurophysin binding of the parent hormone itself and the analogue shortened at the COOH termini. None of the analogues extended at the NH_2 termini showed any binding. This is taken as evidence that only the composition of the NH_2 terminus is critical for binding of the intact hormone molecule to neurophysin, provided that the N^{α}-amino group not only is present and free but also is not sterically separated from an adjacent aromatic side chain.

INTRODUCTION

Neurophysins are large carrier polypeptides which originate in the mammalian hypothalamus and are stored in the posterior pituitary. They have no known biological activity per se, but reversibly bind such neurohypophysial hormones as vasopressin and oxytocin and some of their structural analogues. They are released into the circulation, often under conditions that produce the release of hormones which they bind (1, 2), but their presence there has not yet been associated with a biological function. Those mammalian species so far investigated possess at least two different neurophysin molecules, all of molecular weight about 10,000. The complete amino acid structures of one bovine and one porcine molecule (3, 4) have recently been reported, and the amino acid contents of all the others show a great similarity regardless of species.

The chemical nature of the binding has been studied, and can be summarized as follows: (a) it takes place roughly in a pH range of 5–7, and the complex breaks down reversibly at higher and lower (e.g., 0.1 M formic acid) pH values; (b) binding of cyclic

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nonapeptide hormones requires the presence of a free N^{α}-amino group. It has been suggested that binding of these hormones involves a hydrophobic interaction in the region of sequence positions 2 and 3, evidently with the aromatic side chain of tyrosine or phenylalanine, combined with an electrostatic or hydrogen bond interaction between the N^{α}-amino grouo of the hormone and a carboxyl group of the neurophysin molecule (5–8).

The present work involves studies of binding to bovine neurophysin II of the following analogues of 8-lysine-vasopressin as compared with the parent hormone. Series I consisted of N^{α}-leucyl-vasopressin, N^{α}-phenylalanyl-vasopressin, N^{α}-glycyl-vasopressin, N^{α}-glycylglycyl-vasopressin, and N^{α}-glycylglycylglycyl-vasopressin. Series II contained des-9-glycinamide-vasopressin and des-8-lysine-des-9-glycinamide-vasopressin. The nomenclature of these analogues was taken from published suggestions (9). Series I represents various degrees of separation of the free N^{α}-amino group from the cyclic portion of the molecule (or from tyrosine in sequence position 2), the ring and positions 7–9 of the COOH terminus remaining constant. This group of analogues has been called "hormonogens" and is of pharmacological interest because, although they have very little activity per se, enzymatic removal of the added residues *in vivo* results in very prolonged duration of activity of the parent hormone by virtue of slow-release kinetics (10, 11). Series II represents a progressive shortening of the peptide chain from the COOH terminus and is of biological interest because the octa- and heptapeptides involved have lost practically all their pressor activity (12). In other words, is there any relation between biological activity and propensity for binding to neurophysin for this group of cyclic peptide hormones?

MATERIALS AND METHODS

LVP[2] was a commercial product of Spofa, Prague. The Leu-LVP, Phe-LVP, Gly-Gly-LVP, and Gly-Gly-Gly-LVP hormonogens were synthesized by E. Kasafírek of the Research Institute of Pharmacy and Biochemistry, Prague (13). The basic synthetic procedure was to attach the given NH_2-terminal sequence to a heptapeptide COOH terminus, which excludes the possible presence of the parent nonapeptide hormone, or any other biologically active fragment, as a contaminant. All these analogues gave satisfactory elemental and amino acid analyses, and migrated as a single spot on paper electrophoresis. Gly-LVP was synthesized by Dr. Lars Carlsson of Ferring, Ltd., Malmö, Sweden. Since this was a 1 + 9 synthesis, there was slight contamination with a LVP nonapeptide (less than 0.5%), which was removed by neurophysin binding and separation of unbound decapeptide on a Sephadex G-25 column (see below and RESULTS, Fig. 2). The resulting product gave an amino acid analysis of Lys, 0.95; Asp, 0.95; Glu, 1.02; Pro, 0.92; Gly, 2.20; Cys/2, 1.81; Tyr, 0.93; Phe, 1.02; so that it could be considered a pure substance. All other chemicals were analytical grade, and the Sephadex gels were commercial products of Pharmacia, Ltd., Sweden.

Enzymatic methods were used to prepare series II analogues. Glycinamide was cleaved from the COOH terminus of LVP by a modification of the method of Barth *et al.* (14): 10 mg of LVP were dissolved in 20 ml of 0.05 M $(NH_4)_2CO_3$ and the pH was adjusted to 7.4 by bubbling in gaseous CO_2. Then 0.4 mg of L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone-treated trypsin (Worthington Biochemical Corporation) was added in 100 µl of 1 mM, HCl and the mixture was incubated for 60 min at 36°. Following incubation the sample had only 0.4% of its initial pressor activity. The reaction mixture was freeze-dried, and the components were separated by preparative descending electrophoresis (15) at 1500 V in a pyridine-acetate buffer, pH 5.6, on Whatman No. 1 paper. The zone representing des-GlyNH_2^9-LVP had a mobility of 0.54 in comparison

[2] The abbreviations used are: LVP, 8-lysine-vasopressin; Leu-LVP, N^{α}-leucyl-vasopressin; Phe-LVP, N^{α}-phenylalanyl-vasopressin; Gly-LVP N^{α}-glycyl-vasopressin; Gly-Gly-LVP, N^{α}-glycylglycyl-vasopressin; Gly-Gly-Gly-LVP, N^{α}-glycylglycylglycyl-vasopressin; des-GlyNH_2^9-LVP, des-9-glycinamide-vasopressin; des-Lys^8, des-GlyNH_2^9-VP, des-8-lysine-des-9-glycinamide-vasopressin.

with LVP itself, and was eluted from the paper with distilled water. After 20 hr of hydrolysis in 5.7 M HCl, the amino acid analysis showed the following molar ratios: Asp, 1.00; Glu, 0.93; Pro, 1.00; Cys/2, 1.72; Phe, 1.00; Lys, 0.97; Tyr, 0.78. The yield was 70%.

For the preparation of the heptapeptide des-Lys^8,des-GlyNH$_2^9$-VP, 20 mg of LVP were treated with trypsin in the same reagent ratios as above. The reaction mixture, following freeze-drying, was dissolved in 3 ml of 0.02 M $(NH_4)_2CO_3$, pH 8.3, and the trypsin was removed by gel filtration on Sephadex G-25. The fractions containing the octapeptide were pooled (total volume, 12 ml), and to this were added 400 μl of a solution of diisopropyl fluorophosphate-treated carboxypeptidase B, 1.8 mg/ml (Worthington Biochemical Corporation). The mixture was incubated for 18 hr at 36°, then diluted with 10 ml of water and freeze-dried. Separation of the heptapeptide from lysine and glycinamide was achieved by descending electrophoresis under the same conditions as above, and the product (yield, 60%) was eluted from the paper with water. Amino acid analysis showed the following molar ratios: Asp, 0.94; Glu, 0.99; Pro, 0.92; Cys/2, 1.6; Tyr, 0.80; Phe, 1.00. Splitting of the Pro–Lys bond by carboxypeptidase B, which under these conditions left the disulfide bridge intact, would have been more rapid at a higher pH, but the latter condition might have resulted in a transulfidation reaction and dimer formation.

Neurophysins I and II were prepared from frozen beef posterior pituitary glands. The first extract was made according to the method of Hollenberg and Hope (16) by Dr. Hans Norén of Ferring, Ltd., Malmö, Sweden. Smaller peptides were separated from the protein carrier by gel filtration on Sephadex G-25 in 0.1 M formic acid. The crude neurophysin thus collected was further separated on a Sephadex G-75 column in 0.1 M formic acid. The low molecular weight fraction from the latter separation was further divided on sulfoethyl Sephadex C-25 in a sodium acetate concentration gradient (17) which yielded neurophysins I and II. Moiety II, used for the binding studies below, gave on amino acid analysis the same contents as those previously published for this molecule (Table 1), and dansylation analysis gave alanine as the only NH_2-terminal amino acid, in agreement with published results (4, 5).

TABLE 1

Comparison of amino acid molar ratios of bovine neurophysin II used in the present work (column C) with data reported by Rauch et al. (18) (column A) and Walter et al. (3) (column B)

Samples of neurophysin were hydrolyzed in parallel in 5.7 M HCl under vacuum at 105° for 20 and 70 hr. Serine values were extrapolated to zero time. Half-cystine was determined by performic acid oxidation. Proline values were calculated from the oxidized sample.

Amino acid	A	B	C
Lysine	2	2	2.1
Arginine	6	7	6.6
Aspartic acid	5	5	5.0
Threonine	2	2	2.0
Serine	6	6	5.6
Glutamic acid	14	14	13.5
Proline	8	8	7.9
Glycine	15	16	14.8
Alanine	6	6	6.1
Half-cystine	12	14	14.0
Valine	4	4	3.3
Methionine	1	1	0.8
Isoleucine	2	2	2.1
Leucine	6	6	5.9
Tyrosine	1	1	1.0
Phenylalanine	3	3	2.9

Binding studies involved solution of a 10:1 mixture by weight of neurophysin II and the LVP analogues (either 10 and 1 or 5 and 0.5 mg), respectively, in 1 ml of 0.05 M pyridine-acetate buffer, pH 5.8. This mixture was approximately equimolar in terms of large and small peptides. These samples were all filtered through the same Sephadex G-25 column (75 × 0.9 cm), which had been equilibrated and was eluted with the same buffer, all at room temperature. The course of elution was followed spectrophotometrically at 280 nm, and 1.7-ml fractions, containing peaks of neurophysin and small peptides, were separately pooled and freeze-dried. The NH_2-terminal amino acid in each peak was determined by dansylation, provided that the acid involved was not cysteine, which does not show up by this technique. The samples were also titrated biologically for

pressor activity (19) for those analogues which showed measurable levels (series I only) along with samples of the original analogue for comparison, using despinalized Wistar strain rats weighing 180–220 g. A Statham A23 strain gauge was attached to a carotid cannula, and the pressure records were made with a strip-chart recorder.

RESULTS

As will be presented in detail below, the binding data could be divided into two groups, qualitatively all-or-none, in which all the series I analogues (with pressor activity) did not bind and all the series II (without pressor activity) did bind. For this reason, Fig. 1 presents only sample spectrophotometric curves to illustrate the two categories of experimental result without repetition. The integrated area-under-the-curve data for all the analogues are presented numerically in Table 2. Figure 1, top curve, shows a plot of the fraction number vs. absorbance at 280 nm distribution for the parent LVP hormone alone; the second curve shows neurophysin II alone; the third curve shows a nonbinding mixture of neurophysin and Gly-LVP; and the fourth curve shows a binding mixture of neurophysin and des-GlyNH_2^9-LVP, all in equimolar quantities in the same buffer. Nonbinding curve 3 was

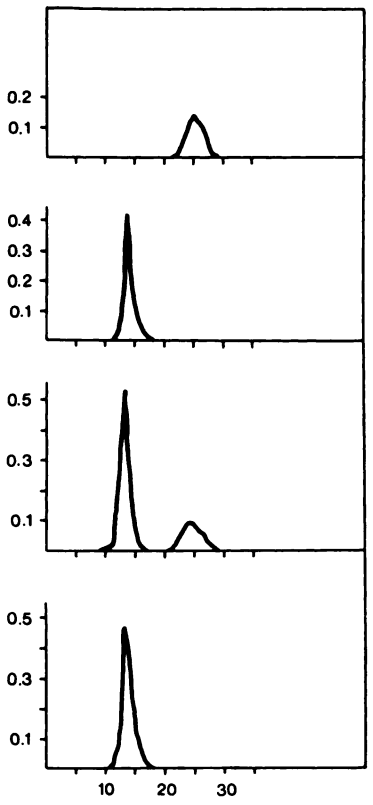

FIG. 1. *Optical density at 280 nm plotted with respect to fraction number (1.7-ml fractions) in eluate from Sephadex G-25 gel filtration of LVP alone (top curve), neurophysin II alone (second curve), neurophysin + Gly-LVP (third curve), and neurophysin + des-GlyNH_2^9-LVP (bottom curve)*

All reactions were carried out at pH 5.8 (see the text). In all cases 5.0 mg of neurophysin and 0.5 mg of small peptide were used.

TABLE 2

Integrated A_{280} data from first (I) and second (II) peaks of elution from Sephadex G-25 column for LVP analogues in combination with neurophysin II (0.5 mg of small peptide, 5.0 mg of neurophysin)

For details, see the text. In the case of the series II analogues, following gel filtration at pH 5.8, the single peak I recorded was pooled and freeze-dried, and the dry powder was dissolved in 0.1 M formic acid and gel-filtered again on Sephadex G-25 with the same column geometry, but in the formic acid medium. The eluate, as recorded in the last two lines of the table, shows dissociation of the neurophysin-peptide complex.

Substances	Integral A_{280} values	
	Peak I	Peak II
Pyridine-acetate buffer, pH 5.8		
Neurophysin II	1.510	
LVP		0.710
Neurophysin II + LVP	1.920	
Neurophysin II + Leu-LVP	1.430	0.590
Neurophysin II + Phe-LVP	1.320	0.655
Neurophysin II + Gly-LVP	1.540	0.660
Neurophysin II + Gly-Gly-LVP	1.370	0.680
Neurophysin II + Gly-Gly-Gly-LVP	1.275	0.620
Neurophysin II + desGly-NH_2^9-LVP	2.010	
Neurophysin II + desLys^8, desGlyNH_2^9-VP	1.930	
0.1 M formic acid		
Neurophysin II + desGly-NH_2^9-LVP	1.380	0.530
Neurophysin II + desLys^8, desGlyNH_2^9-VP	1.250	0.580

repeated for all the series of I analogues. Binding curve 4 was typical for series II analogues and the parent hormone.

Table 2 presents integrated A_{280} data for all the analogues in comparison with neurophysin II alone, LVP alone, and an equimolar mixture of neurophysin and LVP. All data here are for 5 mg of neurophysin and 0.5 mg of small peptide. The integration was simply calculated from a summation of fractions as follows:

$$(A_{280,I,1} + A_{280,I,2} \cdots + A_{280,I,n})(1.7 \text{ ml})$$

and

$$(A_{280,II,1} + A_{280,II,2} \cdots + A_{280,II,n})(1.7 \text{ ml})$$

where the subscripts I and II refer to the first and second elution peaks, representing neurophysin and the small peptide, respectively. The data are not quantitative in the sense that a binding constant can be calculated, but are sufficient to suggest qualitatively that LVP and series II analogues did bind in an all-or-none fashion to neurophysin II at pH 5.8, while none of the series I analogues were bound.

The spectrophotometric data were supplemented by dansylation analysis (except for cysteine) of the NH_2-terminal amino acid in the two peaks, and titration of pressor activity in the separate peaks for those analogues (LVP and series I only) with a measurable degree of activity. The nature of the pressor response to LVP is very different from that to the series I hormonogen analogues, since the latter display prolonged activity with a slow approach to peak values because of the slow-release mechanism of action. Were we to compare areas under the curves, the decrease in potency of a hormonogen vs. the parent hormone would only be about 1 order of magnitude. Since it is often difficult to measure this area accurately with very prolonged responses (e.g., with Gly-Gly-Gly-LVP), only peak response values have so far been used for such a comparison, and here the difference is 2–3 orders of magnitude. However, mixtures of LVP and a hormonogen can be detected in the pressor assay. Figure 2 shows control responses to LVP alone and to the same doses of three different preparations of Gly-Gly-Gly-LVP: a sample synthesized in Prague by a 6 + 7 procedure with no nonapeptide contamination, a sample synthesized in Malmö by a 3 + 9 procedure with a 0.19% LVP contamination, and the Malmö sample after mixing with neurophysin II at pH 5.8 and separation of the second peak in the eluate from Sephadex G-25 filtration, with removal of the LVP contamination. The neurophysin, in this purification procedure, can then be treated with formic acid and recycled again in pure form.[3] Table 3 contains the dansylation and pressor activity data. The latter are presented as a percentage of the activity of the small peptide added to the mixture, titrated separately with pure samples, and so represent a "yield" of how much of the known added activity can be biologically detected in the first or second peak of the gel filtration eluate after mixing with an equimolar amount of neurophysin.

Figure 1 and Tables 2 and 3 clearly show that LVP and series II analogues bind to neurophysin, whereas series I analogues do not bind. Pressor activity, however, was restricted to LVP and series I analogues.

DISCUSSION

Binding studies *in vitro* with neurophysin have been carried out by both gel filtration and equilibrium dialysis. The latter enables an estimate of binding constants but requires larger amounts of small peptides, and since such binding constants show a large degree of scatter, only large differences have a significant meaning. Gel filtration was used here because in most cases the amounts of small peptides available were limited, and in any case the qualitative all-or-none nature of the results seemed to justify the approach, particularly since there was such a clear difference between series I and II analogues.

As illustrated in Fig. 2, the results have a practical significance in that should a 1 + 9, 2 + 9, or 3 + 9 synthesis of hormonogens prove more convenient than a 3 + 7, 4 + 7, or 5 + 7 synthesis, neurophysin can be used to purify the product of nonapeptide contaminant at pH 5.8, and can itself be cleaned at a lower pH and recycled.

There are two biological points arising

[3] Patent applied for.

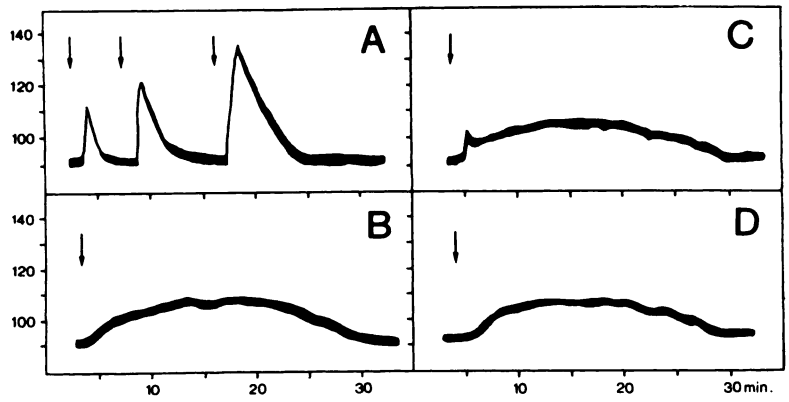

FIG. 2. *Pressor activity records*

Ordinates are in millimeters of mercury; abscissae, time in minutes. A. LVP assay. Arrows show injection times; responses, from left to right, are to 5, 10, and 20 ng of LVP. B. Response to 2 μg of Gly-Gly-Gly-LVP synthesized in Prague by a 6 + 7 procedure. C. Response to 2 μg of Gly-Gly-Gly-LVP synthesized in Malmö by a 3 + 9 procedure. D. Response to the same material as in C following neurophysin purification.

TABLE 3

Dansylation analysis and pressor activities of pooled peaks I and II from Sephadex G-25 filtration of mixtures of neurophysin II with LVP analogues (see Table 2)

The pooled peaks were freeze-dried and dissolved in water. Pressor activities were calculated as peak amplitude and expressed as a percentage of the activity added initially to the mixture, titrated separately in each case.

Substances	Peak I (neurophysin II)		Peak II (small peptides)	
	Pressor	NH_2 terminus	Pressor	NH_2 terminus
Neurophysin II	0	Ala only		
LVP			100	
Neurophysin II + LVP	100	Ala	0	
Neurophysin II + Leu-LVP	0	Ala only	98.6	Leu only
Neurophysin II + Phe-LVP	0	Ala only	97.4	Phe only
Neurophysin II + Gly-LVP	0	Ala only	98.7	Gly only
Neurophysin II + Gly-Gly-LVP	0	Ala only	100	Gly only
Neurophysin II + Gly-Gly-Gly-LVP	0	Ala only	96.4	Gly only

from these results. Older work (20) had suggested that the plurality of neurophysins had a biological meaning with reference to species and molecule specificities for oxytocin and vasopressin. LVP species specificity resides in the basic amino acid residue in sequence position 8 of lysine or arginine. It is clear from this and previously published data (6) that the structure of the NH_2 terminus of cyclic nonapeptide hormones is critical for binding—the remnant of the molecule still being capable of binding after removal of 2 COOH-terminal residues, including the one at position 8. Thus the molecular basis of species specificity of neurophysin binding of vasopressin has been discredited since bovine neurophysin binds the porcine hormone (6). The series II analogues have lost almost all their pressor activity, which means that the COOH terminus is important for at least this receptor interaction while playing no role in binding to neurophysin. It would appear difficult, therefore, to consider such binding as a "model of receptor interaction." As is also evident in the work of Breslow and Walter (7), there would appear to be no correlation between biological activity and neurophysin binding. At the present state of

our knowledge it is not clear whether the known plurality of neurophysin molecules in separate mammalian species has a biological meaning. We have so far been unable to detect any difference between neurophysin II binding of LVP an oxytocin.

In terms of molecular interactions, these results confirm published conclusions that neurophysin binding of this category of peptide hormone involves only the NH_2-terminal moiety (7, 8), i.e., an aromatic side chain in sequence position 2 and a free N^{α}-amino group. A further steric requirement has been added, however: the free N^{α}-amino group and the aromatic side chain must be present in adjacent residues. Any greater steric separation of the two groups resulted in loss of binding. Since, however, Phe-LVP did not bind, the two steric factors cannot be present in the same residue.

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