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# Binding Properties of Bovine Neurophysins I and II: An Equilibrium Dialysis Study

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SUMMARY

Native bovine neurophysin II has one binding site per monomer for lysine-vasopressin at physiological pH (pH 7.38), as revealed by equilibrium dialysis. Oxytocin is bound to neurophysin II with the same or slightly greater affinity than vasopressin, but binding of the two hormones is competitive. The affinities of vasopressin and oxytocin for neurophysins I and II are essentially the same. Substitution of the asparagine (position 5) and glycinamide (position 9) residues in oxytocin by valine and glycine, respectively, has no substantial effect on binding of the resulting analogues to neurophysin; substitution of the glutamine residue by ornithine in position 4 of oxytocin slightly increases the affinity of the analogue for neurophysin II. The results are in agreement with the contention that the specific association of neurophysin I with oxytocin and of neurophysin II with vasopressin in neurosecretory granules is a result of compartmentalization during the biosynthesis of the neurohypophysial hormones and neurophysin proteins. The relatively low binding constants of neurohypophysial hormones to neurophysin proteins at physiological pH assure complete dissociation of the hormone-protein complexes after their release into the blood. Our observations also show that the specificity of the hormone-binding site of neurophysin proteins for the neurohypophysial hormones differs from that of the hormone receptors.

## INTRODUCTION

The neurophysins are a family of related proteins which function physiologically as "carriers" (1) of the hormones oxytocin and vasopressin within the secretory neurons of the hypothalamo-neurohypophysial system. Of the two principal neurophysins found in the ox, neurophysin I appears to be associated principally with oxytocin in fractionated neurosecretory granules, while neurophysin II is found in association with vasopressin (2, 3). It is desirable to know the number of hormone-binding sites on neurophysin, to determine the strength of hormone-neurophysin interaction, and to elucidate the sites of both hormone and protein involved in binding as well as the nature of this interaction. In addition, possible specificity differences between neurophysins I and II for different neurohypophysial hormones are clearly relevant to the physiology of these proteins.

Interpretation of previous neurophysin

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studies is complicated by the fact that preparations were used which are now known either to have been impure (4) and/or to have undergone partial proteolysis (4, 5). Nevertheless the following conclusions appear secure at this time: bovine neurophysin C (6) and partially degraded neurophysins (4, 7) bind approximately 1 molecule of hormone per monomer of protein (10,000 mol wt). Both native and partially degraded bovine neurophysins interact principally with positions 1–3 of the hormones (4, 7), but the participation of other residues of the hormones is not excluded. Different fractions of partially degraded neurophysins, which are now known to have been nonhomogeneous, show no significant differences in their relative affinities for oxytocin and vasopressin (4, 7).

The purpose of the present study was to determine the strength of the interaction between hormones and native bovine neurophysins, particularly at physiological pH, and to determine whether neurophysins I and II differ in their specificity and/or affinity for the two hormones. We also explored the ancillary problem of the role of positions 4, 5, and 9 of the hormones in binding to neurophysins by examining hormonal analogues with limited structural modifications in these positions. The technique chosen for these studies was thin-film equilibrium dialysis using $^{14}$C-labeled lysine-vasopressin or $^{14}$C-labeled oxytocin. Binding of labeled hormones was measured directly; binding of unlabeled oxytocin and oxytocin analogues was measured by competition with labeled lysine-vasopressin.

## MATERIALS AND METHODS

*Hormones and hormone analogues.* The following peptides were used: [9-*glycinamide*-1-$^{14}$C]lysine-vasopressin and [9-glycinamide-1-$^{14}$C]oxytocin (8, 9) with specific activities of 25 and 30 Ci/mole, respectively; synthetic unlabeled [8-*lysine*]vasopressin (10), [4-*ornithine*]oxytocin (11), [5-*valine*]oxytocin (12), oxytocin (13), and oxytocinoic acid (14). The two latter compounds were resynthesized by Dr. R. T. Havran, Department of Physiology, Mount Sinai School of Medicine, according to the general solid-phase procedure of Merrifield (15). Unlabeled peptides were stored as lyophilized powders, while the labeled hormone was kept in ampoules at a concentration of 0.01 mg/ml in either 0.9% NaCl or 0.25% acetic acid containing 0.5% chlorobutanol. Prior to use the compounds were tested for their avian vasodepressor or rat pressor activities (16); their potencies were found to correspond to the values reported in the references cited above.

*Preparation of Neurophysins.* Neurophysins I and II were isolated from acetone-dried bovine posterior pituitary powder (generously supplied by Parke, Davis and Company). First the crude hormone–neurophysin complex was prepared by the method of Hollenberg and Hope (17). The hormones were chromatographically separated from the protein, and the resultant crude neurophysin was resolved by ion-exchange chromatography into the individual species, I and II, as described elsewhere (7, 18). Some samples were subjected to an additional purification step by preparative gel electrophoresis.[1] The isolated neurophysins were homogeneous on analytical polyacrylamide gel electrophoresis at pH 9.5 and 7.5 (7) and gave amino acid analyses in agreement with those reported elsewhere (7, 18).

*Equilibrium dialysis.* Thin-film equilibrium dialysis was performed as follows. A piece of 18/32 Visking tubing was soaked in water, equilibrated with buffer, and blotted to remove excess liquid. The tubing was tied at the bottom around a 13-mm (outer diameter) hollow glass rod, and 0.5 ml of the protein-containing solution ("inner" solution) was inserted through the top, which was then tied off. The rod and its surrounding membrane were attached to a motor-driven plunger and immersed in a test tube [16 mm (inner diameter) × 150 mm] containing 4.5 ml of buffer and hormone. Stirring was accomplished by driving the plunger with 25 vertical strokes/min of 1-inch amplitude. The temperature was maintained at 24 ± 1° with a water bath surrounding the test tube.

The time necessary to attain equilibrium concentrations of hormone on both sides of

[1] Unpublished observations and procedures.

the membrane was first determined in the absence of protein. Labeled lysine-vasopressin was placed inside the membrane (in the absence of protein), and the outer solution was sampled until the "inner" and "outer" hormone concentrations were equivalent or remained unchanged. By these criteria, an approximate equilibrium of 95% was attained within 90 min. Therefore, for experiments involving a combination of both protein and hormone, a dialysis time of 2 hr was chosen in order to ensure equilibration and minimize protein leakage through the membrane. For direct binding studies with labeled oxytocin, 4-hr equilibration times were used with no significant protein leakage. A single 2-hr experiment with labeled oxytocin gave results within the range of experimental error of the 4-hr data.

For direct hormone-neurophysin binding studies, the inner solution contained 2–4 mg of protein in 0.5 ml of buffer. The outer solution contained various initial concentrations of labeled hormone diluted with unlabeled hormone to a specific activity of $3 \times 10^5$–$6 \times 10^5$ dpm/mg. After equilibration, aliquots of the "inner" and "outer" solutions were assayed for radioactivity and for total protein content according to Lowry *et al.* (19); a standard neurophysin-Folin curve was always obtained along with the unknown solutions. Protein determinations of the outer solution were used as a check on protein leakage, which was always negligible relative to free hormone concentrations. Protein concentrations in the inner solution were corrected for the contribution of "inner" hormone; calculations of the amount of bound hormone were made as previously described (4).

Competitive binding studies with other neurohypophysial analogues were performed using 0.5 mg of labeled lysine-vasopressin ($3 \times 10^5$ dpm/mg) in the presence of 0.5 and/or 1 mg of unlabeled analogue. In this series an *apparent* lysine-vasopressin binding constant, $K'_{LVP}$, was first calculated as if no unlabeled competitor had been added, except that the "inner" total protein concentration was corrected for unlabeled competitor assuming free distribution of this peptide on both sides of the membrane. $K'_{LVP}$ is related to the intrinsic lysine-vasopressin binding constant measured in the absence of unlabeled peptide ($K^0_{LVP}$) and to the intrinsic binding constant ($K^0$) of peptides other than lysine-vasopressin by the relationship

$$K'_{LVP} = \frac{K^0_{LVP}}{1 + K^0_{peptide}\,[\text{free peptide}]} \qquad (1)$$

From the "first approximation" value of $K'_{LVP}$, an initial estimate of the number of moles of peptide bound per mole (10,000 g) of protein ($\bar{\nu}_{peptide}$) was obtained from

$$\bar{\nu}_{peptide} = \frac{\bar{\nu}_{LVP}\,[K^0_{peptide}]\,[\text{free peptide}]}{K^0_{LVP}\,[\text{free LVP}]} \qquad (2)$$

where $\bar{\nu}_{LVP}$ is the initial estimate of moles of lysine-vasopressin bound per mole of protein and the term $[K^0_{peptide}$ (free peptide concentration)] is calculated from Eq. 1. From values of $\bar{\nu}_{peptide}$ so calculated, the free peptide concentration was then corrected for the amount of peptide removed by binding, and all calculations were repeated using the revised free peptide concentration. Under the experimental conditions used, values of $K'_{LVP}$, $K^0_{peptide}$, $\bar{\nu}_{LVP}$, and $\bar{\nu}_{peptide}$ were not changed by more than 7% by this first correction, and further refinement was therefore held unwarranted. [Intrinsic to the above calculations is the assumption that no significant amount of peptide is lost by binding to the membrane. This assumption appears valid; we have observed no significant binding of labeled lysine-vasopressin or labeled oxytocin (4) to the membrane.]

*Reagents and other methods.* Radioactivity was measured with a Unilux II liquid scintillation system (Nuclear-Chicago), using a stock scintillation mixture containing 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene and 4 g of 2,5-diphenyloxazole dissolved in 1 liter of toluene and methyl cellosol (2:1). Efficiencies on all samples were determined adding 10,000 dpm of [$^{14}$C]toluene and recounting; they averaged 80 ± 2%. All chemicals were reagent grade or its equivalent except as noted, and deionized water was used throughout.

## RESULTS

The results of direct binding studies of labeled hormone to neurophysin II at pH

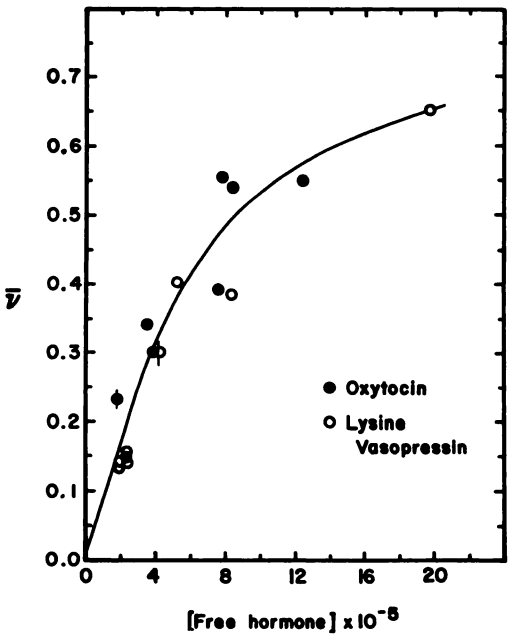


FIG. 1. *Binding of oxytocin and lysine-vasopressin to neurophysin II at pH 7.38 (in 0.06 M Tris-HCl buffer containing 0.10 M KCl)*

$\bar{\nu}$ is defined as the number of moles of hormone bound per 10,000 g of neurophysin. Lines through data points, where evident, represent spread of values in $\bar{\nu}$ due to poor agreement between different estimates of protein concentration or different estimates of counting efficiency.

7.38 are shown in Fig. 1. The data for both oxytocin and vasopressin fall on almost the same binding curve, but allow a slightly greater binding constant for oxytocin than for lysine-vasopressin. Assuming one binding site per monomer, the calculated lysine-vasopressin binding constant is $8.4 \times 10^3 \pm 1.1 \times 10^3$ (standard deviation), while that for oxytocin is $1.21 \times 10^4 \pm 0.35 \times 10^4$. The presence of one binding site for the hormone per monomer of neurophysin is demonstrated in Fig. 2; here the lysine-vasopressin binding data are plotted according to the method of Scatchard and Black (20) and extrapolate linearly to $\bar{\nu} = 1$. It is relevant that the data shown encompassed a 2.5-fold range of total protein and of final protein concentration; the linearity of the plot in Fig. 2 indicates that potential effects of protein concentration were either absent or undetectable under the conditions used.

Lysine-vasopressin binding studies were also conducted at pH 6.2, but at this pH the data showed a marked scatter in three different buffer systems. The scatter is tentatively attributed to the insolubility of the neurophysin II–hormone complex below pH 6.2 (7).

The apparently simple lysine-vasopressin binding pattern observed at pH 7.38 permitted exploration of the relative binding affinities of different neurophysin preparations for lysine-vasopressin, using only one concentration of the latter. The simple binding pattern also permitted an evaluation of the binding of unlabeled neurohypophysial hormones and analogues by competition with labeled lysine-vasopressin, using the standard concentration of the latter. This standard concentration (see MATERIALS AND METHODS) was selected to give $\bar{\nu}_{LVP}$ values of about 0.6 in the absence of competing peptide. The results of these studies, summarized in Table 1, indicate the following: first, three different preparations of neurophysin II, one of which had been sub-

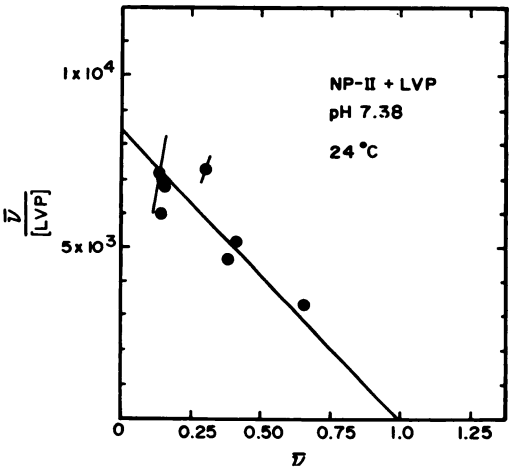


FIG. 2. *Binding of lysine-vasopressin (LVP) to neurophysin II (NP-II) at pH 7.38, plotted according to Scatchard and Black (20)*

Data, symbols, and lines through data points are same as in Fig. 1. The line through the points is drawn to intercept the abscissa at $\bar{\nu} = 1.0$. A least-squares analysis of the data gives a value for the intercept of $\bar{\nu} = 0.95$, with a standard deviation of $\pm 0.15$.

TABLE 1

*Binding of neurohypophysial hormones and analogues to neurophysin proteins*

Equilibrium dialysis studies were performed in 0.06 M Tris-HCl buffer containing 0.10 M KCl, pH 7.18, for a total of 2 hr. Results are averages of two determinations, except where the standard error of the mean is not reported.

| Protein | Preparation[a] | Peptide | $K^0$ |
|---|---|---|---|
| | | | M (± SE) |
| Neurophysin II | A | Lysine-vasopressin | $2.5 \times 10^4 \pm 0.9 \times 10^4$ |
| Neurophysin II | B | Lysine-vasopressin | $1.9 \times 10^4$ |
| Neurophysin II | C | Lysine-vasopressin | $2.0 \times 10^4 \pm 0.5 \times 10^4$ |
| Neurophysin II | A | Oxytocin | $3.0 \times 10^4 \pm 0.5 \times 10^4$ |
| Neurophysin I | A | Lysine-vasopressin | $2.6 \times 10^4 \pm 0.2 \times 10^4$ |
| Neurophysin I | A | Oxytocin | $3.7 \times 10^4 \pm 0.4 \times 10^4$ |
| Neurophysin I | A | 5-Valine-oxytocin | $3.6 \times 10^4 \pm 0.7 \times 10^4$ |
| | | 4-Ornithine-oxytocin | $4.9 \times 10^4$ |
| Neurophysin II | B | 4-Ornithine-oxytocin[b] | $1.8 \times 10^5$ |
| Neurophysin II | B | Oxytocinoic acid[c] | $1.2 \times 10^4$ |

[a] A and B refer to neurophysin samples prepared independently according to Breslow *et al.* (7). Sample C was prepared according to Walter *et al.* (18) followed by preparative gel electrophoresis[1].

[b] The affinity of neurophysin II for 4-ornithine-oxytocin was also determined at pH 7.38 by competition with labeled oxytocin. A constant of $4.4 \times 10^4$ was obtained, which is to be compared with a value of $1.2 \times 10^4$ for oxytocin at this pH.

[c] Only a single binding experiment was performed by equilibrium dialysis. The approximate binding constant so determined was corroborated by optical rotatory dispersion studies, as in ref. 7.

jected to preparative gel electrophoresis, give indistinguishable lysine-vasopressin binding constants. These data indicate that neurophysin II is stable enough to resist random differences in handling, and suggest that the observed binding constant is characteristic for the pure protein. However, the magnitude of binding constants (for both lysine-vasopressin and oxytocin) reported in Table 1 are 2–3 times those obtained from the data in Fig. 1. This difference appears to be due largely to the difference of 0.2 pH unit in the two studies (see DISCUSSION). Second, Table 1 confirms that the affinity of neurophysin II for oxytocin is essentially indistinguishable from or just slightly greater than its affinity for lysine-vasopressin. Moreover, neurophysin I binds oxytocin and lysine-vasopressin with affinities of the same order of magnitude as does neurophysin II. It is of interest in this context that previous studies of the competition between *unlabeled lysine-vasopressin* and *labeled oxytocin* for crude preparations of neurophysins also suggested a slight preference by neurophysins for oxytocin relative to lysine-vasopressin (4).

Finally, the data show that replacement of the asparagine residue in position 5 of oxytocin by valine is without effect on binding of the analogue to neurophysin, while substitution at position 4 by ornithine actually increases binding to neurophysin II. Replacement of the carboxyl-terminal carboxamide moiety by a free carboxyl group in oxytocin (oxytocinoic acid) does not prevent binding, but may slightly reduce the binding constant.

## DISCUSSION

The lysine-vasopressin binding data at pH 7.38 clearly indicate the presence of one binding site per neurophysin II monomer. That this same neurophysin site is involved in the binding of both hormones is supported by the similarity in relative oxytocin and lysine-vasopressin binding constants as measured by two methods: direct binding studies and competition studies. The agreement between these two types of studies indicates that no site on neurophysin can bind one hormone and not the other with any significant affinity.

The calculated oxytocin binding constant

at pH 7.38 ($1.21 \times 10^4$) can be compared with the value of $1.8 \times 10^5$ previously found for the binding of oxytocin to partially degraded neurophysin preparations at pH 5.8 (4). The latter value, which should be revised downward to approximately $1.4 \times 10^5$ to accommodate the revised molecular weight for neurophysin (6, 7, 18), is in good agreement with the pH 7.38 data when a decline in binding proportional to the extent of deprotonation of the hormone $\alpha$-amino group ($pK_a = 6.3$) (7, 21) is allowed for. A change in the state of protonation of the hormone $\alpha$-amino group probably also accounts largely for the differences between binding constants observed at pH 7.18 and those at pH 7.38 in the present study. The report by Ginsburg and Ireland (21) and our own circular dichroism studies[1] support the thesis that interaction of the hormones with neurophysin rapidly declines above pH 7.

A surprising feature of neurophysin-hormone interaction at pH 7.38, however, is that the lysine-vasopressin data (Fig. 2) are characterized by a single binding constant over a substantial range of saturation. The neurophysins are self-aggregating; bovine neurophysin II has recently been shown to consist of an approximately 2:1 molar mixture of monomer and dimer at pH 8.1 at the protein concentrations used here (7), and there is some indication that the equilibrium of porcine neurophysin is shifted toward oligomers at lower pH values (22). In such self-aggregating systems, single binding constants should not be found unless the different protein species present (e.g., monomer and dimer) do not differ significantly in their affinities for the hormone, or unless the equilibrium in the absence of hormone is highly in favor of the species with the stronger hormone-binding affinity. Clearly, more detailed studies of monomer-aggregate equilibria in neurophysin are necessary to distinguish between these possibilities. However, if it is tentatively assumed that the monomer does not bind more strongly than the dimer—an assumption supported by chromatographic studies (23) and by earlier molecular weight studies of the neurophysin-hormone complex (24)—then the binding constants observed here at pH 7.38 indicate that, at the low neurophysin and hormone levels found in plasma (well below $10^{-8}$ M in hormone), the "carrier" and hormone should be totally dissociated.[2] This conclusion is of interest in the light of recent studies which suggest that neurophysin and hormone are released simultaneously from the pituitary (26–28).

The lack of a major difference in specificity or binding affinity between neurophysins I and II with respect to oxytocin and vasopressin parallels results obtained with different fractions of partially degraded neurophysin (4). These data strongly suggest that the reported association of oxytocin with neurophysin I and of vasopressin with neurophysin II in neurosecretory granules (2, 3) is a direct consequence of the difference in the biosynthesis of these hormones [i.e., involvement of different neurosecretory cells at different loci of the hypothalamus, probably via different precursor molecules (29) rather than a selective association of neurophysin from a mixed protein-hormone pool following release of the hormonal octapeptides]. In addition, the finding that modification of positions 4, 5, and 9 of the hormones does not prevent binding indicates that the carboxamides play no major role in binding at these positions. These results conform with other data. For example, substitution of glycine at position 4 of oxytocin has only minimal effects on binding to partially degraded neurophysins (4), and recent studies have shown that the major *qualitative* features of neurophysin-hormone interaction are reproduced by model peptides containing only the first 3 residues of the hormones (7). In this respect, the increase in binding to neurophysin II caused by sub-

[2] This conclusion should not be altered by the fact that the present studies were conducted at 24° whereas the physiological temperature is 37°. For most documented intermolecular protein-protein interactions (26), binding diminishes with increase in temperature; i.e., $\Delta H$ of binding is negative. In those instances when $\Delta H$ of binding is positive, the magnitude of $\Delta H$ would not permit an increase in binding over the temperature interval 24–37° that would be significant in the present context (i.e., an increase in binding constant from $10^4$ at 24° to $10^8$ at 37° is highly improbable).

stitution of ornithine at position 4 is unexpected and unexplained at present.

It becomes increasingly clear that no parallelism exists between the binding of neurohypophysial hormones and analogues to neurophysin proteins, on the one hand, and to receptor proteins of target tissues, on the other. Illustrative examples are (a) the highly similar binding properties of oxytocin and vasopressin to neurophysins compared with their markedly different hormonal activities and (b) the critical importance of the $\alpha$-amino group for binding to neurophysins relative to its nonessentiality for biological activity (30, 31). The present study furnishes additional examples. [5-*valine*] Oxytocin (12) and, in most systems, oxytocinoic acid (14) have little biological activity but associate with neurophysin with a binding constant comparable to natural hormones. These results clearly indicate that the active site of the physiological receptor of the hormones bears little topological or chemical resemblance to the binding site of neurophysin.

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