Small Peptides as Analogs of Oxytocin and Vasopressin in Their Interactions with Bovine Neurophysin-II†

Esther Breslow,* Jane Weis, and Celia J. Menendez-Botet‡

ABSTRACT: Circular dichroism and proton titration studies of mixtures of native or nitrated bovine neurophysin-II and lysine vasopressin confirm that there is one principal site for lysine vasopressin of very similar properties to the single oxytocin site. A second, but markedly weaker lysine vasopressin site is allowed by the data, but two thermodynamically equivalent sites for lysine vasopressin are precluded. Binding constants to nitrated bovine neurophysin-II of oxytocin, lysine vasopressin, and a series of peptide analogs of the first two to three residues of the hormones were obtained by circular dichroism using a single-site model. The data indicate that peptides containing only the first three residues of the hormones contribute almost two-thirds of the binding free energy of the hormones and that half of the binding free energy is contributed by the cooperative binding of residues 1 and 2. Binding interactions at the side chain of residue 1 appear to be hydrophobic and an aromatic hydrophobic side chain at residue 2 is necessary for binding. The contribution of residue 3 to binding appears to be less in tripeptides than in the hormones suggesting that conformational differences between the hormones and peptides contribute to their differences in affinity for neurophysin-II. Oxytocin, lysine vasopressin, and appropriate tripeptides all increased the sedimentation velocity of bovine neurophysin-II by approximately 20%, indicating a change in protein conformation and/or a small shift in monomer $\rightleftharpoons$ dimer equilibrium on binding. Circular dichroism studies indicate small differences in optical properties among the complexes of oxytocin, lysine vasopressin, and tripeptides; differences between the hormone and tripeptide complexes suggest that the hormone disulfides are perturbed by binding.

Bovine neurophysins bind their natural ligands, oxytocin and vasopressin, and also bind di- and tripeptide analogs of the α-amino-terminal sequence of the hormones (Breslow, 1970; Breslow *et al.*, 1971). Circular dichroism and H^+ ion equilibria changes attending binding of the hormones and of the smaller peptides have been shown to be very similar (Breslow, 1970; Breslow *et al.*, 1971) and studies of a neurophysin derivative in which the single neurophysin tyrosine has been nitrated have indicated that both the smaller peptides and the hormones similarly perturb the neurophysin nitrotyrosine residue when equally bound (Breslow and Weis, 1972). The tyrosine of bovine neurophysin-II has been shown to be near or at the peptide binding site (Balaram *et al.*, 1972).

The interaction of neurophysin with the hormones and smaller peptides provides an ideal model system for the study of protein–protein interaction because neurophysin is of low molecular weight (monomer = 10,000) and the hormones themselves are only nonapeptides. We ultimately plan to define, on a quantitative basis where possible, the thermodynamic contribution of different segments of the hormones to their interaction with NP.[1] Qualitative estimates of the contribution of a few residues of the hormones and peptides have already been arrived at in other studies (Stouffer *et al.*, 1963; Breslow and Abrash, 1966; Breslow *et al.*, 1971; Hope and Walti, 1972). Semiquantitatively, it is known that substitution of the α-NH_2 of the hormones by H or removal of the aromatic ring in position 2 of the hormones diminishes binding by a factor of at least 100 while loss of the side chain in position 3 of the hormones diminishes binding by a factor of 30 (Breslow and Abrash, 1966). In tripeptide analogs of the first three residues of the hormones, the nature of the side chain in position 1 has been shown qualitatively to affect the strength of interaction (Breslow *et al.*, 1971). From the data compiled to date, the hormone–protein interaction appears in a general sense to involve an electrostatic bond between the protonated α-NH_2 of the hormones and an unprotonated side-chain carboxyl of the protein (Stouffer *et al.*, 1963; Ginsburg and Ireland, 1964; Breslow and Abrash, 1966; Breslow *et al.*, 1971; Hope and Walti, 1972) in the midst of a hydrophobic environment provided in part by the side chains of residues 1–3 (Breslow and Abrash, 1966; Furth and Hope, 1970; Breslow *et al.*, 1971). Modifications of the hormones at residues 4, 5, 8, and 9 have thus far had only small effects on binding (Breslow and Abrash, 1966; Breslow and Walter, 1972).

Because structural analogs of tripeptides are easier to obtain than are nonapeptide analogs of the hormones, a principal object of the present study was to utilize the binding ability of the smaller peptides to assess more completely the contributions of their individual segments to binding. In addition, we wanted to know whether tripeptide analogs of residues 1–3 of the hormones contained all, or only a part of, the binding features of the hormones. Our approach to the problem was to determine the binding constants to nitrated NP-II of a series of di- and tripeptide structural analogs of residues 1–3 of the hormones, and of the hormones themselves, utilizing the perturbation of the 350-nm nitrotyrosine ellipticity band by binding to determine binding constants.

† From the Department of Biochemistry, Cornell University Medica College, New York, New York 10021. *Received June 18, 1973.* Supported by Grant GM-17528 from the National Institutes of Health.

‡ Present address: Department of Clinical Chemistry and Physiology, Sloan-Kettering Institute, New York, N. Y. 10021.

[1] Abbreviations used are: NP, bovine neurophysin; NP-II, bovine neurophysin-II; θ, ellipticity in (deg cm^2)/dmol (defined per mole of NP-II); $\bar{\nu}$, moles of peptide bound per mole (10,000 g) of neurophysin. Abbreviations of peptides are standard, but the following should be specifically noted: (Cys-Tyr-NH_2)$_2$, L-cystinylbis(L-tyrosinamide); *S*-methyl-Cys-Tyr-Phe-NH_2, *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalaninamide; *S*-methyl-Cys-Phe-Ile-NH_2, *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucinamide; N-Ac-Tyr-Phe-NH_2, *N*-acetyl-L-tyrosyl-L-phenylalaninamide.

Secondarily, we also compared the effects of hormones and tripeptides on the structure of NP-II by looking, for the first time, at changes in sedimentation velocity which accompanied binding, and by looking more rigorously at the circular dichroic consequences of binding hormones and peptides to NP-II. Some of the peptides studied have been specially synthesized for binding studies to neurophysin. However, commercial availability influenced the choice of peptides in other instances.

Prior to the use of a circular dichroic method for determining binding constants, the number of peptide binding sites per mole of protein must be known. Previous studies (Breslow and Abrash, 1966; Breslow and Walter, 1972) indicated one binding site per neurophysin monomer for either oxytocin or vasopressin; binding of the two hormones was competitive. Preliminary tripeptide binding constants to neurophysin, derived from gel filtration studies (Breslow *et al.*, 1971), were also based on the observation of a single binding site for the peptide glycyl-L-tyrosyl-L-phenylalaninamide. Recently, Camier *et al.* (1972) have reported finding one binding site for oxytocin but two binding sites for lysine vasopressin, each of equal affinity to the single oxytocin binding site. Therefore, prior to an analysis of peptide binding, further data will be presented supporting the existence of only a single principal binding site for either oxytocin or vasopressin on bovine neurophysin-II.

Materials and Methods

Proteins, Peptides, and Hormones. Bovine neurophysin-II and nitrated bovine neurophysin-II were prepared as described previously (Breslow *et al.*, 1971; Breslow and Weis, 1972). Data obtained on several different samples of oxytocin and vasopressin contributed to the results presented in this paper. Oxytocin samples used were a former gift from Professor V. du Vigneaud, prepared by Dr. D. Yamashiro, a gift from Professor M. Manning and newly synthesized material prepared by H. L. Aanning. Lysine vasopressin samples were gifts from Dr. W. Cash, Dr. R. Walter, and Dr. P. Cohen. With the exception of one old sample of oxytocin which contained an insoluble contaminant, data obtained on the different oxytocin samples were consistent; moreover, removal of the insoluble contaminant from the old oxytocin sample by centrifugation, and recalculation of the solution oxytocin concentration by optical density measurements gave results with this oxytocin sample which agreed with the other oxytocin data. Lysine vasopressin samples ranged in pressor activity from 130 to 270 units per mg and no significant difference among the preparations in their behavior toward neurophysin was seen. For the most critical studies, such as titration of the number of binding sites, samples of approximately 230 units/mg were used.

The peptides glycyl-L-tyrosyl-L-phenylalaninamide, L-alanyl-L-tyrosyl-L-phenylalaninamide, L-methionyl-L-tyrosyl-L-phenylalaninamide, *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucinamide, L-methionyl-L-tyrosinamide, L-methionyl-L-tyrosine, L-cystinylbis(L-tyrosinamide), and L-methionyl-L-phenylalanylglycine were those previously described (Breslow *et al.*, 1971; Breslow and Weis, 1972). *S*-Methyl-L-cysteinyl-L-tyrosyl-L-phenylalaninamide was also that reported previously (Breslow *et al.*, 1971) but was further purified by removal of a water-insoluble contaminant and relyophilized. All other peptides were standard peptides obtained from Cyclo Chemical Corp. and were reported by Cyclo to give the correct nitrogen analysis and to be homogeneous in two or three solvent systems. With the exception of L-leucyl-L-tryptophanamide, we routinely checked the peptides by amino acid analysis according to the method of Spackman *et al.* (1958), H^+ ion titration and determination of the uv absorption spectrum. (L-Leucyl-L-tryptophanamide was studied only by uv spectrophotometry.) All peptides used here gave the correct amino acid analysis, and tyrosine (or tryptophan) extinction coefficients were within 10% of the expected values (Schellman and Schellman, 1964). H^+ ions titration studies gave the expected pK values except that some samples behaved as if they were contaminated with small amounts of NH_3 and one sample contained traces of a group titrating with a pK of 4.6 (probably acetate). In peptides in which the α-COOH was amidinated, no titrimetric evidence was found for a free α-COOH group (expected pK_a = 3–3.5). One peptide, purchased for this study as L-norvalyl-L-tyrosinamide, was clearly not the correct material as evidenced by its uv spectrum, amino acid analysis and nmr, and was not used.

Methodology. Sedimentation velocity studies were performed as previously described using the photoelectric scanner (265 or 280 nm) at protein concentrations up to 2 mg/ml and schlieren optics at protein concentrations of 5 mg/ml (Breslow *et al.*, 1971). The use of the peptide *S*-methyl-L-cysteinyl-L-phenylalanine-L-isoleucinamide, in studies of the effect of peptide binding, minimized complications (when using the photoelectric scanner) arising from absorbance contributions of the peptide itself. For binding studies in the presence of hormone, these complications were minimized by using the scanner only at 265 nm, a wavelength at which the absorbance of the hormones is at its lowest compared with that of the protein.

pH-Stat binding studies to measure the number of protons consumed or liberated on addition of increasing amounts of hormone were performed in 0.16 or 0.06 M KCl as previously described (Breslow *et al.*, 1971). The initial concentration of NP-II was 7 mg/ml and successive aliquots of 2×10^{-3} M hormone (at the identical pH as the protein) were added until no further proton uptake or liberation was observed. After each aliquot, the solution was back-titrated to the initial pH with a measured volume of 0.01 N HCl or 0.01 N NaOH. pH-Stat binding studies were performed only with native NP-II.

Circular dichroism studies of native NP-II and its complexes with hormone were performed at pH 6.2 in 0.16 M KCl as previously described (Breslow, 1970). Circular dichroism binding studies using nitrated NP-II were performed both at pH 6.2 and 8.0, in 0.16 M KCl and 0.16 M KCl plus 0.01 M Tris buffer, respectively. Binding studies were principally carried out at 2×10^{-4} M nitrated NP-II in a 1-cm cell, but several studies were also performed at concentrations of 4×10^{-5} M nitrated NP-II using a 5-cm path length. At each pH, for each different nitrated NP-II preparation, the ellipticity at 350 nm of the uncomplexed protein and the protein saturated with hormone was determined. For several peptides, such as Ala-Tyr-Phe-NH_2 (see Results), it was possible to independently check the 350-nm ellipticity representative of complete saturation and this was found to be within experimental error of the values obtained on saturation with hormone. Thus, the ellipticity at 350 nm representative of complete saturation was routinely assumed to be equal to that found by saturating the nitrated protein with hormone. However, the actual ellipticity values obtained with each peptide at concentrations approaching total binding were also checked against this value for internal consistency; in only one instance, Leu-Trp-NH_2, did the ellipticity values found for the peptide appear tentatively inconsistent with the assumption that the

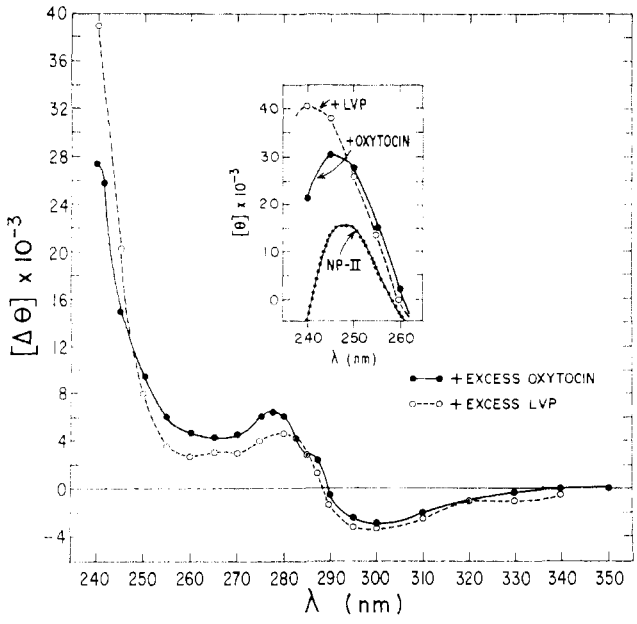

FIGURE 1: Near-uv difference CD spectra generated by mixing 2×10^{-4} M NP-II with 2 equiv of oxytocin or lysine vasopressin. The results were calculated by subtracting the individual ellipticities of the hormones and NP-II from the spectrum obtained when NP-II and the hormones were mixed. The inset represents the direct CD spectrum of NP-II alone and in the presence of 2 equiv of hormone. Conditions: pH 6.2, 0.16 M KCl.

complex with peptide had the same ellipticity at 350 nm as that with hormone. Using these assumptions, binding isotherms could be simply determined by adding known amounts of peptide or hormone to a solution of nitrated NP-II at constant pH and determining the ellipticity at 350 nm. (In practice, the entire CD spectrum from 500 to 290 nm was determined, so that auxiliary wavelengths could be used to calculate binding from the data if necessary.) Concentrations of peptide used ranged upward to 10^{-1} M if solubility permitted. The number of moles of peptide bound per mole of protein ($\bar{\nu}$) was calculated from the relationship

$$\bar{\nu} = \frac{[\theta]_{350\text{ nm obsd}} - [\theta]_{350\text{ nm protein}}}{[\theta]_{350\text{ nm complex}} - [\theta]_{350\text{ nm protein}}} \quad (1)$$

The unbound peptide concentration was determined by correcting the total peptide concentration for that removed by binding according to the relation

$$[\text{peptide}]_{\text{unbound}} = [\text{peptide}]_{\text{total}} - \bar{\nu}[\text{protein}]_{\text{total}} \quad (2)$$

The validity of these relationships demands that there is only one binding site for each peptide; evidence for this is given in Results. Once several valves of $\bar{\nu}$ were determined, the data were plotted according to the method of Scatchard and Black (1949). All peptides gave plots which approximated straight lines (see Results) and binding constants were accordingly calculated from the slope using the method of least squares to obtain the best fit. At pH 6.2, the least-squares straight lines generated by the data gave intercepts on the abscissa ($\bar{\nu}$) which averaged 1.03 ± 0.06; intercepts on the ordinate were used directly as the calculated apparent binding constant, K'. Data at pH 8 showed significantly more scatter than that at pH 6.2 because ellipticity differences generated by binding are 50% lower at pH 8 than at pH 6.2 due to ionization of the nitrotyrosine (Breslow and Weis, 1972). At pH 8, therefore, least squares straight lines were calculated using an intercept on the abscissa of 1.0 as a fixed point.

Duplicate isotherms using a single peptide with the same or different protein preparations were in satisfactory agreement. Where no evidence of binding was observed by CD, the lack of active-site binding was apparently confirmed by demonstrating that the peptide in question, at a concentration of 10^{-2} M or higher, did not diminish the ellipticity at 350 nm produced by binding peptides; in no instance was any competition observed, but it must be recognized that the instrumental "noise" in these studies is sufficiently great to prevent the observation of low levels of competition.

Results

Evidence for a Single Strong Binding Site for either Oxytocin or Lysine Vasopressin. In an interacting system, the change in physical properties of the system as a function of ligand/protein mole ratio gives important information bearing on the number of binding sites. Accordingly, we have studied changes in circular dichroism and proton equilibria of neurophysin-hormone mixtures as a function of hormone/NP ratio.

The circular dichroism spectra of oxytocin-NP-II complexes in the near- and far-uv regions have been reported previously (Breslow, 1970; Breslow and Weis, 1972). Comparative CD studies in the far-uv region of the complexes of NP-II with LVP and oxytocin show no differences which are clearly outside the range of experimental error, although this may be because experimental error in this region is large for NP because of the low signal to noise ratio. In the near-uv region, complexes with the two hormones are also very similar (Breslow, 1970) but differences are apparent, particularly when the difference CD spectra rather than the direct spectra are examined. Figure 1 shows the near-uv difference CD spectra observed on mixing NP-II with 2 equiv of oxytocin and lysine vasopressin. The origins of the CD changes have been previously ascribed (Breslow, 1970; Breslow and Weis, 1972). It is sufficient to note here that changes near 240 nm (probably disulfide) are consistently greater on binding lysine vasopressin than on binding oxytocin, while those in the 260- to 290-nm region (due principally to the hormone tyrosine) are somewhat greater on binding oxytocin than on binding lysine vasopressin. In addition, a shoulder near 287 nm, is consistently more distinct for oxytocin than for lysine vasopressin difference spectra and is also manifest as a greater degree of fine structure in the direct CD spectrum of the oxytocin NP-II complex. (Differences between the difference CD spectra generated by binding the two hormones principally reflect differences in the *bound* state. This is evidenced by the large differences in direct CD spectra of the two complexes, shown in the 240-nm region in the insert in Figure 1. The free hormones differ only slightly in this region.)

Despite the above differences in the CD spectra of oxytocin and lysine vasopressin complexes, and of greatest importance to the present argument, is the fact that CD changes accompanying addition of the first molar equivalent lysine vasopressin represent almost the same percentage of the total CD change seen on addition of excess lysine vasopressin as do CD changes accompanying addition of the first mole of oxytocin. This is seen in Figure 2 where changes in ellipticity accompanying addition of lysine vasopressin and oxytocin under identical conditions are plotted as a function of the number of equivalents added. In both cases, the first equivalent produces 80–90% of the total ellipticity change. Addition of oxytocin

and lysine vasopressin also lead to comparable changes in the CD of the nitrotyrosine residue of nitrated NP-II (Figure 3) (Breslow and Weis, 1972). Also plotted in Figure 2 are the CD changes at 350 nm accompanying addition of increasing aliquots of lysine vasopressin and oxytocin to nitrated NP-II at pH 6.2 under identical conditions. Clearly, perturbation of the nitrotyrosine residue is nearly complete upon addition of 1 equiv of either hormone.

Taken individually or collectively, the above data are not reconcilable with the presence of two sites for lysine vasopressin, each of equal affinity to the oxytocin site. Whether only one or both of the hypothetical two lysine vasopressin binding sites were responsible for the changes in ellipticity observed, the percentage of the total change accompanying addition of the first equivalent of lysine vasopressin should be only 50% of the total change (see "theoretical" line in Figure 2); clearly this is not the case. It should be stressed, however, that the present data do not preclude a second lysine vasopressin site which is considerably weaker than the first—but only indicate that the first lysine vasopressin (and oxytocin) sit predominantly on a single site and that this site is the source of the NP-II nitrotyrosine perturbation. If two sites for lysine vasopressin are allowed, it can be calculated that the weaker site must have less than $^1/_{15}$th the affinity of the stronger.[2]

Marked changes in proton equilibria accompany binding of hormones and peptides to native NP-II (Breslow *et al.*, 1971). In particular, at pH 7, an uptake of 0.6–0.7 proton/mol is observed on binding both lysine vasopressin and oxytocin; below pH 5, protons are released on binding. We have also studied the relative number of equivalent sites available to oxytocin and lysine vasopressin by assessing (under identical conditions) the completeness of changes in proton equilibria on addition of 1–3 equiv of either hormone (see Experimental Section). Under the conditions chosen at pH 7, addition of the first equivalent of oxytocin produced 85% of the total proton uptake observed on addition of 3 equiv and binding appeared complete upon addition of the second equivalent. For lysine vasopressin at pH 7 the first equivalent also produced 85 ± 5% of the total proton uptake observed on addition of 3 equiv, and binding was again complete on addition of the second equivalent. At pH 3.5, in 0.06 M KCl, both hormones produced identical changes in proton equilibria at identical concentrations. Interestingly, at pH 4 in 0.16 M KCl the lysine vasopressin complex is soluble while the oxytocin complex is insoluble. Nonetheless, the first mole of lysine vasopressin added produced 87% of the total H^+ ion release

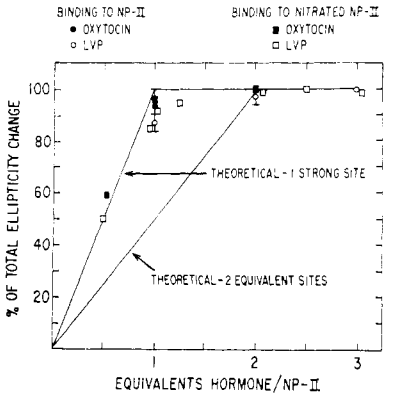

FIGURE 2: Relative ellipticity changes generated on addition of increasing molar ratios of hormone to NP-II and nitrated NP-II. Conditions: 2×10^{-4} M protein, pH 6.2, 0.16 M KCl. Ellipticity changes attending binding to nitrated NP-II are specifically shown for 350-nm data but relative ellipticity changes appeared the same at all wavelengths. For binding to native NP-II, ellipticity changes were calculated at 245, 280, and 295 nm from the difference CD spectra calculated as in Figure 1. Individual points represent the average of the data at the three wavelengths with the average deviation shown as an error bar. Theoretical lines represent those expected for infinitely strong binding to one site or to 2 equiv sites respectively and are independent of any assumptions as to the nature of the sites.

(0.5 equiv/mol of NP); no further proton release was observed after the second equivalent of LVP was added. Again these data are incompatible with the presence of two binding sites of lysine vasopressin of equal or near-equal affinity. It is relevant to note also that P. Cohen (personal communication)

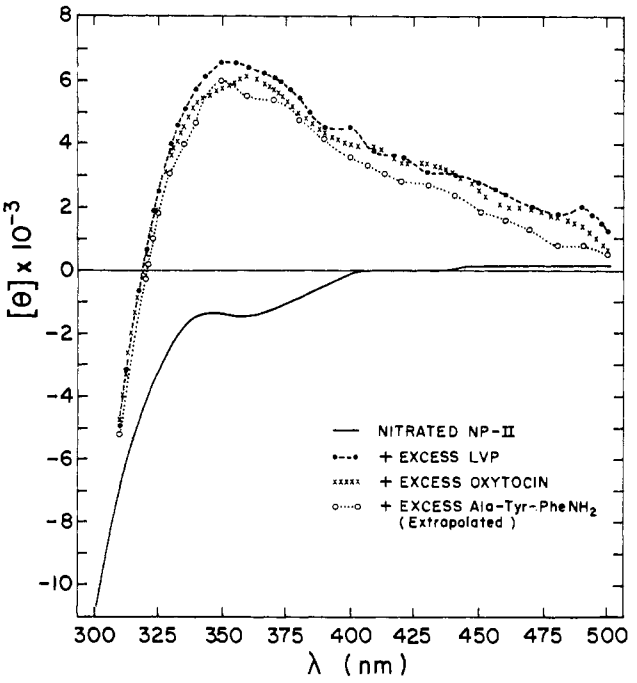

FIGURE 3: CD spectra of nitrated NP-II saturated with lysine vasopressin, oxytocin, and Ala-Tyr-Phe-NH_2. Conditions: pH 6.2, 0.16 M KCl. Data for the hormones represent single experiments in the presence of 3 equiv of hormone/mol of NP-II. Peptide data were obtained in the presence of 0.01 M Ala-Tyr-Phe-NH_2 and corrected for the fact (see reported binding constant) that this concentration represents only 93% saturation. (More recent experiments using a new photocell confirm that small differences among the spectra in the presence of hormones and peptide are due solely to instrumental noise.)

[2] The extent to which the ellipticity changes in Figure 2 are less than 100% complete upon addition of 1 equiv hormone is a reflection either of partial dissociation from a single binding site or of competition for the first hormone equivalent by a second site. There is general agreement on the presence of a single oxytocin binding site, and the oxytocin data in Figure 2 actually suggest a slightly stronger binding constant than reported by Camier *et al.* (1973) for these conditions. If the incompleteness of lysine vasopressin binding to either native or nitrated NP at 1 equiv is attributed *solely* to competition by a second lysine vasopressin site, the data at 1 equiv indicate on the average that approximately 15% of the first equivalent is bound to the second site, *i.e.*, that the second site has $^1/_{30}$th the affinity of the first. When maximum uncertainty in the actual ratio of hormone to NP is allowed for, the value of $^1/_{30}$th is raised to $^1/_{15}$th. Significantly, it has just been reported to us (P. Cohen, personal communication) that only one lysine vasopressin binding site is found on *nitrated* NP by equilibrium dialysis. While these new data do not explain differences between the data in Figure 2 and the equilibrium dialysis data of Camier *et al.* (1973) for lysine vasopressin binding to *native* bovine NP-II, they apparently confirm that the assumption of only a single lysine vasopressin site on the nitrated protein is valid.

TABLE I: Binding of Hormones and *S*-Methyl-Cys-Tyr-Phe-NH_2 to Nitrated Bovine NP-II.

pH	Peptide or Hormone	Protein Concn (M)	K' (±SD)[a]
8.0	Oxytocin	4×10^{-5}	$5.3 \times 10^4 \pm 0.49 \times 10^4$
8.0	Lysine vasopressin	4×10^{-5}	$5.7 \times 10^4 \pm 0.74 \times 10^4$
8.0	*S*-Methyl-Cys-Tyr-Phe-NH_2	4×10^{-5}	$1.2 \times 10^3 \pm 0.05 \times 10^3$
8.0	*S*-Methyl-Cys-Tyr-Phe-NH_2	2×10^{-4}	$1.0 \times 10^3 \pm 0.07 \times 10^3$
6.2	*S*-Methyl-Cys-Tyr-Phe-NH_2	4×10^{-5}	$3.7 \times 10^3 \pm 0.6 \times 10^3$
6.2	*S*-Methyl-Cys-Tyr-Phe-NH_2	2×10^{-4}	$5.5 \times 10^3 \pm 0.06 \times 10^3$

[a] Units are M^{-1}.

has found only 1 binding site for the peptide *S*-methyl-Cys-Tyr-Phe-NH_2. The results support the assumption that the data to be presented here can be interpreted in terms of a single binding site.

Perturbation of NP-II Nitrotyrosine by Hormones and Peptides; Determination of Binding Constants. Figure 3 shows representative CD spectra of nitrated NP-II in the vicinity of nitrotyrosine absorption in the absence and presence of oxytocin, lysine vasopressin and the peptide Ala-Tyr-Phe-NH_2. Under conditions of saturation, the spectrum near 350 nm is essentially independent of the ligand. Small differences in the effects of the different ligands may exist near 350 nm but they are within the range of instrumental noise; similarly, possible larger relative differences in spectra above 375 nm are not clearly outside the range of experimental error, particularly when the marked effects of small changes in pH in this wavelength region are noted (Breslow and Weis, 1972).

All other peptides studied, with the possible exception of Leu-Trp-NH_2, appear to give the same CD spectra at saturation within experimental error. Thus, it was possible to determine the degree of saturation, $\bar{r}$, of nitrated NP-II at any total peptide concentration, and the unbound peptide concentration, by determining the magnitude of the 350-nm nitrotyrosine CD band relative to its value at saturation with hormone (see Materials and Methods). From these data, Scatchard plots were constructed, as shown in Figure 4, for two typical peptides at pH 6.2. No curvature is noted which is clearly outside experimental error nor were any *systematic* effects of varying the protein concentration over a 5-fold range noted, signifying that any interactions between binding sites in the dimer form of NP-II or change in monomer $\rightleftharpoons$ dimer equilibrium (Breslow *et al.*, 1971) accompanying binding are not detectable by the techniques used. Linear Scatchard plots were previously obtained by Breslow and Walter (1972) for the binding of lysine vasopressin to nonnitrated NP-II at pH 7.4 and by Camier *et al.* (1973) for the binding of oxytocin and lysine vasopressin to nonnitrated NP-I and NP-II. However, it is important to note that potential changes in monomer $\rightleftharpoons$ dimer equilibrium accompanying binding are not precluded, as will be evident when changes in sedimentation velocity which accompany binding are considered.

Relative Binding of Peptides and Hormones to Nitrated NP-II. Binding of oxytocin and lysine vasopressin to nitrated NP-II is too strong at pH 6.2 to enable binding constants to be accurately determined by circular dichroism. However, binding is diminished enough at pH 8 such that binding constants can be determined if the protein concentration is 4×10^{-5} M. Table I shows the binding constants for both hormones and for the peptide *S*-methyl-Cys-Tyr-Phe-NH_2 determined at pH 8.0 at a protein concentration of 4×10^{-5} M, and for the peptide additionally at 2×10^{-4} M. No systematic effect of protein concentration is seen with the peptide (and it can probably be assumed that no effect on hormone binding would be detectable either). The essentially equivalent affinity of both hormones for nitrated NP-II is in keeping with observations on the binding of hormones to native NP-II (Breslow and Walter, 1972). The peptide binds with an *apparent* affinity (K') which is $^1/_{30}$th that of the hormones.

Also shown in Table I is the effect of pH on the binding of the peptide. The increase in binding constant between pH 8.0 and 6.2 is almost exactly in accord with theory if it is assumed that only the protonated form of the α-amino ($pK_a = 6.8$) binds to NP-II, taking into account the change in pK_a of the NP-II nitrotyrosine which accompanies binding (Breslow and Weis, 1972). Specifically, the pK_a of the nitrotyrosine decreases from 7.45 in the uncomplexed state to 6.85 in the complexed state, from which it can be demonstrated by the usual relationships for linked functions (Edsall and Wyman, 1958) that the species of NP-II in which the nitrotyrosine is ionized has four times the affinity for hormone as does the un-ionized species. Thus, for the peptide, the theroetical relationship between binding at the two pH values, using the assumption given above is

$$\frac{K'_{\text{pH } 6.2}}{K'_{\text{pH } 8}} = \frac{4[(1-\alpha)_{NH_3^+}][(\alpha)_{PhOH}]_{\text{pH } 6.2} + [(1-\alpha)_{NH_3^+}][(1-\alpha)_{PhOH}]_{\text{pH } 6.2}}{4[(1-\alpha)_{NH_3^+}][(\alpha)_{PhOH}]_{\text{pH } 8} + [(1-\alpha)_{NH_3^+}][(1-\alpha)_{PhOH}]_{\text{pH } 8}} \quad (3)$$

where K' is the apparent binding constant observed at each pH, $(1-\alpha)_{NH_3^+}$ is the fraction of the α-NH_3^+ normally protonated at each pH in the unbound state and $(\alpha)_{PhOH}$ and $(1-\alpha)_{PhOH}$ are defined in footnote 3. The observed change in binding is slightly greater than fourfold; the theoretical change is 3.4. The same assumptions can be used to calculate the expected binding constant of the hormones to nitrated NP-II at pH 6.2 using a pK_a of 6.3 (Breslow, 1961) for the α-amino. The expected binding constant at pH 6.2 for either of the hormones is approximately 5×10^5 M^{-1}.

The differences in K' between the peptides and the hormones can be used to calculate the relative *apparent* free energies of the binding of nitrated NP-II to the hormones and to the peptide. At pH 8 the *apparent* binding free energies are 4.2 and 6.5 kcal for the peptide and hormone, respectively. However, the hormones and peptide differ in their relative degrees of protonation at pH 8. Correcting both the peptide and hormone apparent constants at pH 8 to obtain the *intrinsic* affinities of the protonated species,[3] K^0, the *intrinsic* free

[3] The intrinsic binding constant, K^0, representing reaction of the protonated hormone or peptide with that species of protein in which the nitrotyrosine is completely protonated, can be calculated from the apparent constant at any pH, K', according to the relation: $K^0 = (K' + K'K_h[H^+])/K_h[H^+][(1-\alpha)_{PhOH} + 4(\alpha)_{PhOH}]$, where K_h is the affinity of the α-NH_2 for protons, $[H^+]$ is the H^+ ion activity, and $(1-\alpha)_{PhOH}$ and $(\alpha)_{PhOH}$ are the fractions of nitrotyrosine residues normally protonated and unprotonated, respectively, in the uncomplexed protein at the pH of the study.

TABLE II: Binding of Peptides to Nitrated NP-II at pH 6.2.[a]

Dipeptide	K' (±SD), M^{-1}	Tripeptide or Tetrapeptide	K' (±SD), M^{-1}
Gly-Tyr-NH_2	$5.3 \times 10^1 \pm 0.18 \times 10^1$	Gly-Tyr-Phe-NH_2	$2.5 \times 10^2 \pm 0.2 \times 10^2$
Ala-Tyr-NH_2	$2.4 \times 10^2 \pm 0.3 \times 10^2$	Ala-Tyr-Phe-NH_2	$1.2 \times 10^3 \pm 0.03 \times 10^3$
Ser-Tyr-NH_2	$5.7 \times 10^2 \pm 0.2 \times 10^2$		
α-NH_2-butyryl-Tyr-NH_2	$1.3 \times 10^3 \pm 0.07 \times 10^3$		
Nle-Tyr-NH_2	$2.4 \times 10^3 \pm 0.07 \times 10^3$		
Met-Tyr-NH_2	$1.7 \times 10^3 \pm 0.02 \times 10^3$	Met-Tyr-Phe-NH_2	$7.0 \times 10^3 \pm 0.3 \times 10^3$
Phe-Tyr-NH_2	$1.4 \times 10^4 \pm 0.13 \times 10^4$	Phe-His-Gly-Lys	$<10^1$
[Cys-Tyr-NH_2]$_2$	$3.9 \times 10^3 \pm 0.4 \times 10^3$	*S*-Methyl-Cys-Tyr-Phe-NH_2	$5.5 \times 10^3 \pm 0.07 \times 10^3$
		S-Methyl-Cys-Phe-Ile-NH_2	$4.1 \times 10^3 \pm 0.2 \times 10^3$
Pro-Tyr-NH_2	$<10^1$		
Met-Tyr	$<10^1$	Met-Phe-Gly	$\sim 10^2$
Leu-Gly-NH_2	$<10^1$	Gly-Gly-Tyr-NH_2	$<10^1$
Leu-Ala-NH_2	$<10^1$	N-Ac-Tyr-Phe-NH_2	$<10^1$
Leu-Tyr-NH_2	$1.2 \times 10^3 \pm 0.05 \times 10^3$	Leu-Tyr-Ile-NH_2	$3.6 \times 10^3 \pm 0.8 \times 10^3$
Leu-Trp-NH_2	$\sim 10^2$		

[a] Conditions: 0.16 M KCl, 2×10^{-4} M protein concentration.

binding energies of peptide and hormone are 5.2 and 8.3 kcal, respectively. Intrinsically, *S*-methyl-Cys-Tyr-Phe-NH_2 has 63% of the binding free energy of oxytocin or vasopressin.

Binding Constants of Small Peptides at pH 6.2. Table II shows the binding constants to bovine NP-II obtained with a series of small peptides at pH 6.2. The data should be viewed from the perspective that the peptide *S*-methyl-Cys-Tyr-Phe-NH_2 is almost an exact analog of the first three residues of lysine vasopressin and that Met-Tyr-Phe-NH_2 is apparently an equally appropriate analog since it has been shown (Breslow and Abrash, 1966) that changing residue 1 of oxytocin by adding an additional methylene between the sulfur and the α-NH_2 is without effect (or slightly increases) the binding constant. It also should be noted, as shown above, that lysine vasopressin (position 3 = Phe) and oxytocin (position 3 = Ile) differ only slightly in their affinity for NP-II and it is assumed here that effects (or lack of them) produced by structural changes at a particular position among residues 1–3 in one hormone will be duplicated in the other. Finally, it should be stressed that the binding constants reflect (among other properties) differences in the state of protonation of the α-amino of each peptide at pH 6.2. Fortunately, only the α-amino groups of (Cys-Tyr-NH_2)$_2$, the *S*-methyl-Cys-peptides, and Met-peptides are significantly deprotonated at pH 6.2; in the latter two cases the degree of deprotonation is only 20 and 10%, respectively. Thus, differences in the pK_a's of the different α-amino groups can, for the most part, be neglected.

Several facts are immediately apparent from Table II. First, with respect to the side chain of residue 1, sulfur does not play a unique role; substitution of sulfur in Met-Tyr-NH_2 by CH_2, as in Nle-Tyr-NH_2, leads to no decrease in binding. However, in both dipeptides and tripeptides, loss of the side chain in position 1 (substitution of methionine by glycine) leads to a 30-fold decrease in binding; compare Met-Tyr-NH_2 with Gly-Tyr-NH_2 and Met-Tyr-Phe-NH_2 with Gly-Tyr-Phe-NH_2. This decrease is due principally to loss of the β- and γ-methylene groups since α-aminobutyryl-Tyr-NH_2 has 2/3 the binding constant of Met-Tyr-NH_2. Within experimental error, the contribution of the β-methylene group is the same in both di- and tripeptides as evidenced by the similar increases in binding affinity on changing residue 1 from glycine to alanine in the two series. Other pertinent facts about substituents in the side chain of position 1 is that addition of an OH to the β-carbon does not increase binding as much as addition of a CH_3 group (compare Ser-Tyr-NH_2 and α-aminobutyryl-Tyr-NH_2) and that, as in the hormones, increasing the number of carbon atoms between the α-NH_2 and the sulfur is without effect or slightly increases binding (compare Met-Tyr-Phe-NH_2 and *S*-methyl-Cys-Tyr-Phe-NH_2 taking small differences in their respective degrees of protonation at pH 6.2 into account). A striking feature of changes in position 1 are the high binding constants shown by (Cys-Tyr-NH_2)$_2$ and particularly by Phe-Tyr-NH_2. It is possible that the strong binding shown by (Cys-Tyr-NH_2)$_2$ is related to its potential to form a bidentate complex, but the same is not true of Phe-Tyr-NH_2. The data are best interpreted in terms of hydrophobic interactions at the side chain of residue 1.

Among the other modifications of note at position 1 was the substitution by proline. The lack of binding of Pro-Tyr-NH_2 can probably be attributed to restricted access to the α-NH group in the prolyl residue. In addition, the lack of detectable binding by Gly-Gly-Tyr-NH_2 indicates that, as in the hormones (Kluh *et al.*, 1973), the distance between the

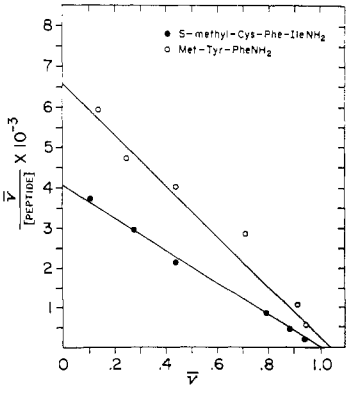

FIGURE 4: Representative binding isotherms of two peptides to nitrated NP-II calculated from single experiments as explained in the text. The lines through the data points represents the least-squares fit of the data. Conditions: 2×10^{-4} M protein, pH 6.2, 0.16 M KCl.

α-NH_2 and the tyrosine cannot be varied without a loss of binding affinity.

In position 2, it is clear that only peptides having an aromatic hydrophobic residue in position 2 bind with any affinity. A similar situation was previously demonstrated in oxytocin where it was shown that analogs having a glycine or isoleucine in position 2 bind with less than $^1/_{100}$th the affinity of oxytocin (Breslow and Abrash, 1966). We have not yet done a detailed study of the effect of a Tyr→Phe substitution in position 2, but, as in oxytocin, comparison of *S*-methyl-Cys-Phe-Ile-NH_2 and *S*-methyl-Cys-Tyr-Phe-NH_2 suggests that Phe is as effective, or almost as effective, as Tyr in position 2. Trp in position 2 also seems to allow binding (see Leu-Trp-NH_2) but the shape of the binding isotherm generated by this peptide was nonlinear, suggesting that quantitatively it may perturb the nitrotyrosine differently than the other peptides. Most interestingly, no binding was observed of the peptide Phe-His-Gly-Lys. This cannot be due to the positively charged side chain in position 4, since [4-ornithine]oxytocin binds more strongly to NP-II than does oxytocin (Breslow and Walter, 1972). It is also unlikely that the failure of this peptide to bind resides in the free α-carboxyl in position 4. Although binding is affected (see below) by free α-carboxyl groups, the effect is dependent upon the distance between the carboxyl and α-NH_2 and would not be expected to be major when the carboxyl is at position 4. Therefore, the failure to bind is most readily attributed to the Tyr → His substitution. That His does not bind with an affinity approaching that of Phe, Trp, or Tyr, despite its aromaticity and structural similarities to both Trp and Phe, is most readily explained by the assumption of a water-free pocket for the bound tyrosine in position 2. Insertion of a histidine side chain into this pocket would necessitate both complete deprotonation (which in itself probably is not too energetically expensive at pH 6.2) and more importantly, loss of hydrogen binding between the imidazole nitrogens and water.

The influence of a negatively charged α-carboxyl group on the peptide near to residues 1 and 2 region can be seen by the extremely weak binding of Met-Tyr and the weak binding of Met-Phe-Gly relative to Met-Tyr-NH_2. This may reflect either the hydrophobic nature of the binding site or repulsion of the negatively charged protein carboxyl group presumed to participate in the binding process.

Last, the effect of position 3 can be ascertained by comparison of tripeptides with Phe or Ile in position 3 with the corresponding dipeptides. When position 3 is Phe-NH_2, the tripeptides almost uniformly show a four- to fivefold increase in binding relative to the dipeptides. Within experimental error, this increase is independent of whether position 1 is Gly, Ala, or Met. In the one case examined in which Ile-NH_2 was added as position 3 (compare Leu-Tyr-NH_2 with Leu-Tyr-Ile-NH_2), the increase in binding was only threefold. Further studies on the effect of Ile-NH_2 in position 3 are clearly needed to determine whether Ile-NH_2 binds more weakly than Phe-NH_2 or whether the smaller effect of adding an Ile-NH_2 in this instance is due to the nature of residue 1. We tend to favor the latter explanation, since oxytocin (position 3 = Ile) binds at least as strongly to NP-II as does lysine vasopressin (position 3 = Phe).

Effects of Binding on the Sedimentation Velocity of NP-II. Although binding plots were apparently linear at pH 6.2 and no effects of protein concentration on binding were noted which were clearly outside the range of experimental error, sedimentation velocity studies indicate that an increase in $s_{20,w}$ accompanies binding of both peptides and hormones to both native and nitrated NP-II. Neurophysin-II (Breslow *et al.*, 1971) and nitrated NP-II behave in the ultracentrifuge as if they were in monomer $\rightleftharpoons$ dimer equilibrium. At pH 6.2, addition of saturating concentrations of *S*-methyl-Cys-Phe-Ile-NH_2 (3×10^{-3} M) increased the $s_{20,w}$ of nitrated NP-II at 0.25 mg/ml from 1.37 ± 0.04 to 1.78 ± 0.03 and at 1 mg/ml from 1.76 ± 0.03 to 2.10 ± 0.04. Lower concentrations of peptide (3×10^{-4} M) led to only half the increase in $s_{20,w}$ at a given protein concentration. This effect was studied further using native NP-II over a wider range of protein concentrations (Table III). The results indicate that both hormones and binding tripeptides cause comparable increases in $s_{20,w}$ of NP-II (20–30%); nonbinding peptides such as Leu-Gly-NH_2 did not produce any change in sedimentation velocity. In addition the data indicate that the $s_{20,w}$ values of the peptide- or hormone-saturated protein are dependent on the concentration of protein in much the same manner as are those of the free protein. Such data rule out the possibility that either monomer or dimer is the sole binding species, since in these hypothetical instances the $s_{20,w}$ of the saturated complex would be independent of protein concentration. It remains then to assign the origin of the increase in $s_{20,w}$ of which only a small portion can be attributed to the weight of the bound peptide. In brief the data indicate either a shift to a slightly higher value of the monomer $\rightleftharpoons$ dimer equilibrium constant when peptide is bound (a slightly greater preference of the peptide for dimer than for monomer) and/or a conformational change. Although firm proof of a conformational change is not found in these data, it should be noted that changes in monomer $\rightleftharpoons$ dimer equilibrium are often a reflection of changes in conformation (with attendant changes in intersubunit contacts). Interestingly, the fact that differences in $s_{20,w}$ between NP-II and its complexes remain constant or slightly increase with a 20-fold increase in protein concentration also suggests that the sedimentation velocity of the dimeric forms of free NP-II and its complexes are different; *i.e.*, that extrapolation to 100% dimer in each case would yield a higher $s_{20,w}$ for the complex. Thus, it is probable that a conformational change attends binding. In either event, the data indicate that both hormones and tripeptides have very similar effects on the sedimentation velocity of NP-II.

Comparison of the Effects on Circular Dichroism of Binding Hormones and Small Peptide Analogs. The overall similarity of circular dichroism changes attending binding of hormones and small peptides has already been noted and the origin of many of these changes ascribed (Breslow, 1970; Breslow and Weis, 1972). We have now noted however, after a very large number of studies of peptide and hormone binding, that there are certain small but reproducible near-uv differences between the peptide and hormone binding situations. (Again, potential small differences in the far-uv region are difficult to assess because of the poor signal to noise ratio in this region.) Figure 5 shows the near-uv ellipticity difference spectra arising from binding oxytocin, Phe-Tyr-NH_2 and *S*-methyl-Cys-Tyr-Phe-NH_2. Qualitatively, as noted previously, ellipticity changes attending binding are the same in all wavelength regions–an increased negative ellipticity above 290 nm (attributed to disulfides), an increased positive ellipticity in the 265- to 290-nm region (shown to be due principally to the peptide or hormone tyrosine with small contributions from the protein tyrosine) and a markedly increased positive ellipticity near 240 nm (attributed in large part to disulfides). Figure 5 shows that tyrosine changes in the 265–290 nm are quantitatively greater for the small peptides shown here than for oxytocin, but that oxytocin (and as can be seen from comparison

TABLE III: Effect of Hormone and Peptide on the Sedimentation Velocity of NP-II.[a]

Peptide or Hormone Concn	$s_{20,w}$ (S) at NP-II Concentration (mg/ml)						
	0.25	0.5	0.8	1	1.55	2	5
	1.30 ± 0.06	1.49 ± 0.05	1.50	1.54 ± 0.04	1.66 ± 0.03	1.68 ± 0.03	1.87
S-Methyl-Cys-Phe-Ile-NH_2 (5×10^{-4} M)	1.52	1.64		1.83		1.93	
S-Methyl-Cys-Phe-Ile-NH_2 (1×10^{-3} M)	1.61	1.81 ± 0.05		2.01		1.97 ± 0.04	
S-Methyl-Cys-Phe-Ile-NH_2 ($3–5 \times 10^{-3}$ M)	1.64	1.70		1.93		1.99	2.27
Oxytocin (0.15 mg/ml)				1.92			
Oxytocin (0.5 mg/ml)	1.25	1.60		1.86			
Oxytocin (1 mg/ml)							2.32
Lysine vasopressin (0.5 mg/ml)				1.86			
Lysine vasopressin (1 mg/ml)							2.39

[a] Conditions: pH 6.2 in 0.16 M KCl, 0.02 M in phosphate buffer; temperature = 24.5°. Where average deviations from the mean are reported, results represent the average of from two to eight determinations at each concentration. Where no average deviations are reported, only a single sedimentation run was performed. Standard deviations on each single run were computed by the method of least squares and ranged from ±0.03 to ±0.07 S unit. In experiments performed with schlieren optics (See Materials and Methods) only a single symmetrical peak was observed in all studies. In experiments in the presence of hormone and peptide, NP-II is saturated with hormone at all concentrations used and is essentially saturated with peptide at the highest peptide concentrations used. At the lower peptide concentrations, NP-II is 50–60% saturated at 5×10^{-4} M total peptide and 70–80% saturated at 1×10^{-3} M total peptide at the protein concentrations used.

with Figure 1, vasopressin as well) shows quantitatively greater negative ellipticity changes near 300 nm and greater positive ellipticity changes near 240 nm. We do not have sufficient data to ascertain whether all peptides differ from oxytocin in the 265- to 290-nm region, or to what extent different peptides differ among themselves in this region. However, the data shown here indicate that the presence of Phe in position 3 of lysine vasopressin in itself does not account for the lower difference curve generated by lysine vasopressin than by oxytocin (see Figure 1) since one of the peptides has Phe in position 3. In the 240- and 300-nm regions, moreover, *all* peptides differ from the hormones as do these shown here; these differences are well outside the limits of experimental error and are consistently reproduced. Of the changes near 300 nm, the data suggest that a single difference ellipticity band found with the hormones and centered at 300 nm is absent in the peptide complexes. Previously, ellipticity changes above 290 nm and near 240 nm were attributed solely to the disulfides of the protein, because qualitatively they were observed on binding both hormone and non-disulfide-containing peptides (Breslow, 1970). The new results indicate most probably that the disulfides of the hormone also contribute significantly to CD changes near 300 and 240 nm; it is alternatively possible, however, that binding of hormone perturbs the disulfides of the protein differently than binding of the smaller peptides.

Discussion

A potential pitfall in using analogs to define quantitatively the binding interactions of the intact hormone is that binding of analogs may occur through different interactions than do the hormones. In the present system, with the possible exception of Phe-Tyr-NH_2, this seems unlikely. The analogs strongly resemble positions 1–3 of the hormones, appear to produce the same changes in H^+ ion equilibria (Breslow *et al.*, 1971) and sedimentation velocity as do the hormones, and qualitatively produce very similar changes in circular dichroism as do the hormones; moreover, since CD spectra are markedly sensitive to small differences in orientation, no fundamental differences in the binding of analogs and that portion of hormone which they represent need be postulated to explain the CD differences which are observed. Finally, the data presented in Table II indicate that in those instances where specific modifications of the intact hormone have been studied, comparison

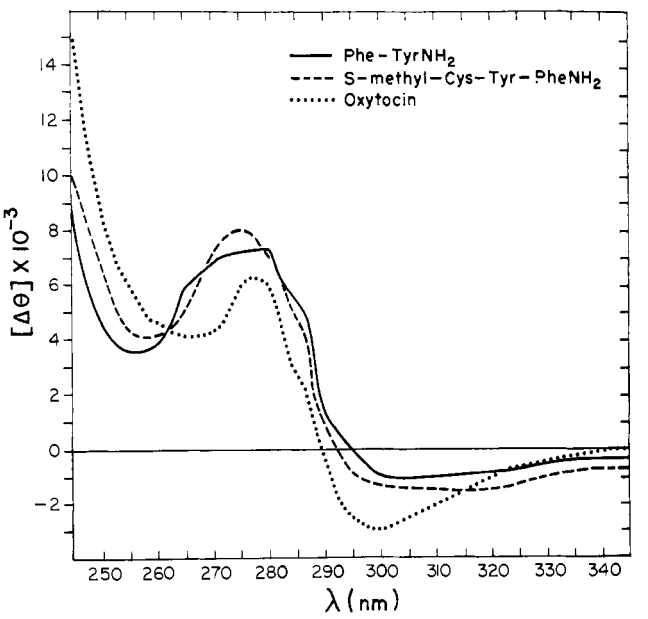

FIGURE 5: Near-uv difference CD spectra generated by mixing 2×10^{-4} M NP-II with 2 equiv of oxytocin (100% saturation), 2×10^{-3} M *S*-methyl-Cys-Tyr-Phe-NH_2 (90% saturation) or 1×10^{-3} M Phe-Tyr-NH_2 (90% saturation). Conditions: pH 6.2, 0.16 M KCl.

with the same modifications in the peptides produces, at least qualitatively, the same effect on binding affinity to neurophysin.

The binding data therefore indicate that two-thirds of the binding free energy of the hormones is to be found in the isolated tripeptide from the amino terminus. Two possible sources of the remaining one-third of the binding free energy of the hormones can be postulated: either positions 1–3 of the hormones are preconstrained by the ring conformation of the hormones into a configuration necessary for binding such that they suffer less of an entropy decrease on binding than the same residues in the small peptide, or there are binding positions on the hormone located somewhere in residues 4–9. It is probable that both situations are present. Evidence for the role of conformation in particular comes from an apparent difference in the quantitative contribution of position 3 in tripeptides and in the hormones. In the latter, substitution of position 3 by glycine leads to a decrease in binding affinity to $^1/_{30}$th of its original value (Breslow and Abrash, 1966). In the small peptides studied here, loss of position 3 leads to a decrease in binding to about one-fourth of the original value. Such data must mean either that position 3 is more preconstrained in the unbound hormones into a conformation favorable for binding than it is in the unbound tripeptides or that position 3 in the hormones influences the conformation of other hormone residues involved in binding. It should be added that the effect of position 3 in the tripeptides cannot be due solely to its potential effect on tripeptide conformation; nmr studies (Balaram *et al.*, submitted for publication) indicate that the side-chain protons of position 3 in model tripeptides (particularly protons associated with the β-carbon) are broadened by binding to NP-II.

A role of residues other than 1–3 in binding to NP-II is suggested by previous studies (Breslow and Abrash, 1966; Breslow and Walter, 1972) which indicate that although modifications of residues 4, 5, and 9 do not cause major decreases in binding affinity, small changes do occur. Specifically, substition of position 4 by glycine leads to a 40% decrease in binding while substitution by ornithine leads to an apparent increase in binding to NP-II of somewhere between 4- and 30-fold (although no major effect on binding to NP-I was seen in the latter case). Substitution of Gly-NH_2 in position 9 of oxytocin by Gly leads to an apparent decrease in binding to about one-half the original value. In the present studies, differences in the CD spectra of complexes of oxytocin and vasopressin may also indicate some participation of the tail of one or both of the hormones in binding; such differences do not seem to be due to differences in position 3 as judged by our peptide studies (Figure 5). It is possible that different interactions of the tails of the two hormones with NP change the preferred orientation of residues 1–3 sufficiently to perturb the CD spectra. However, with any of the above effects, it is again not certain whether they reflect direct participation of other residues than 1–3 in binding or an effect of these residues on hormone conformation which in turn modifies interactions at positions 1–3.

The peptide binding data indicate not only that the principal free energy of hormone binding is derived from residues 1–3, but also suggest that slightly more than half of the binding free energy lies in residues 1–2 alone. Thus, the calculated intrinsic binding constant, K^0, of Met-Tyr-NH_2 (corrected from the binding data at pH 6.2 using a determined pK_a of 7.25 for the peptide α-amino) is 2.0×10^3; the intrinsic free energy of binding of the protonated species is 4.4 kcal compared with 8.3 kcal for the hormones themselves. Within the dipeptide, it is possible to sort out the contributions of some of the segments of the side chain in position 1 (see below) but is not possible rigorously to distinguish between the contributions of residue 1 and 2. This is because the data provide clear evidence for cooperativity between residues 1 and 2 on binding to NP-II as can be seen from several comparisons. First, substitution of Tyr in Leu-Tyr-NH_2 by Gly reduces binding by a factor of greater than 100; if all of this effect were attributed to the loss of tyrosine alone, the free energy of binding of tyrosine in position 2 would be calculated as at least 2.9 kcal. Substitution of Gly in Gly-Tyr-NH_2 by Pro leads to a greater than 5.3-fold decrease in binding and substitution of Gly in Gly-Tyr-Phe-NH_2 by an acetyl group leads to a decrease in binding of greater than 25-fold; if these effects were attributed solely to loss of the α-NH_2 the free energy of binding of the α-NH_2 would be calculated as at least 0.99 kcal in the first case and at least 1.9 kcal in the second. When it is recognized that these are all minimum values, it becomes clear that the estimated individual contributions of residues 1 and residues 2 exceed their summed contributions in the peptides. For example, the total free energy of binding of Gly-Tyr-NH_2, in which both the α-NH_2 and tyrosine are present together is only 2.4 kcal, a value which is less than the above estimated contribution of the tyrosine alone. Such results indicate that loss of tyrosine in position 2 leads to weakening of binding at residue 1 and that loss of the α-NH_2 reduces binding by the tyrosine in position 2; *i.e.*, positive cooperativity in binding exists between residues 1 and 2. In view of this, and in view of the fact that the dipeptide Met-Tyr-NH_2 provides more than half of the binding free energy of the hormones, we postulate that the cooperative binding of residues 1 and 2 provides the principal driving force for the binding of the hormones. This is supported by, but not as explicitly evident from, the marked effects of substituting the α-NH_2 and tyrosine in position 2 of the hormone. It is also of interest that nmr evidence for a linkage between binding at positions 1 and 2 exists. The relative broadening of ortho *vs.* meta protons on the tyrosine in position 2 of small peptides, on binding to NP, is influenced by the nature of the side chain in position 1 (Balaram *et al.*, submitted for publication).

If there are cooperative interactions between the binding of residues 1 and 2, there is little apparent cooperativity between the side chain of residue 1 and residue 3. The fact that the same free-energy increases (the same relative increase in binding constant) accompany the addition of Phe-NH_2 in position 3, irrespective of whether residue 1 is Gly, Ala, or Met, suggests a large degree of independence in the binding of these two side chains, at least in the case of small peptides. This is inevitably paralleled by the similar free-energy increases observed on increasing the length of the side chain in position 1 irrespective of whether the binding peptide has two or three residues.

Analysis of the rest of the binding data further supports the presence of a hydrophobic region at the binding site. The free-energy increase observed on increasing the length of the carbon chain in residue 1 is 0.9 kcal on going from Gly to Ala, 1.0 kcal on going from Ala to α-aminobutyryl and 0.4 kcal on changing from α-aminobutyryl to norleucine (the norvalyl compound was omitted from this study, as explained in Materials and Methods). Clearly, the β- and γ-carbon atoms contribute more in free energy than do the more distant carbons; interestingly, nmr studies suggest that protons attached to the β-carbon are broadened by binding slightly more than those at the γ-carbon (Balaram *et al.*, submitted for publication). The magnitude of approximately 1 kcal/CH_2 residue

for the first two CH_2 groups is in good agreement with theoretical and observed values for the transfer of a CH_2 from water to a nonaqueous environment (Kauzmann, 1959; Holler *et al.*, 1973). While this value must be viewed with caution since it may well include a possible free energy of interaction between the α-amino and the side chain in position 1, the data are consistent with a hydrophobic environment for the bound side chain in position 1. The unexpectedly tight binding of Phe-Tyr-NH_2 supports this interpretation. No specific role for the sulfur in position 1 is evident from our data (*i.e.*, its electronegativity does not appear to be important in binding) but it is possible that, in the hormones, the disulfide *bridge* constrains the hormones into a favorable conformation for binding, and it may also provide a more sterically acceptable binding site for neurophysin than an extended carbon chain.

In position 2, data for both the hormones and peptides indicate that only residues which are both aromatic and hydrophobic can bind. While such a situation might suggest that the aromatic residue in position 2 of the peptide "stacks" with an aromatic residue in the protein, this is not in accord with nmr data (Balaram *et al.*, 1972) which show no significant upfield shift in the position of the aromatic protons of position 2 in bound peptides as would be expected in a stacking situation (Deslauriers and Smith, 1970). Cohen *et al.* (1972) have shown that the ring protons of the tyrosine of oxytocin are not shifted upfield on binding and have similarly argued against stacking for the hormone tyrosine. Moreover, even if proximity between the protein tyrosine and the peptide tyrosine is allowed (Balaram *et al.*, 1972), the protein tyrosine "ortho" protons clearly move downfield on binding (Balaram *et al.*, 1972) a situation not compatible with stacking by the protein tyrosine. Thus, it is possible that the apparent requirement for aromaticity and hydrophobicity is, in reality, a steric requirement for a hydrophobic residue which is planar, rather than for one which is necessarily aromatic.

Finally the data presented here illustrate the usefulness of small peptide analogs of the first three residues of the hormones in extending our insights into the mode of hormone binding. In addition to demonstrating cooperativity between residues 1 and 2 on binding, and further defining the role of residues 2 and 3, peptide binding studies have allowed the importance of the side chain in position 1 to first be observed (Breslow *et al.*, 1971) and now quantitated. It is therefore of particular interest that evidence for participation in binding of the side chain in position 1 of the hormones themselves is now emerging. Thus, the probable contribution of the peptide disulfide to the difference CD spectra generated by binding hormone supports involvement of the side chain of residue 1, and recent studies of the binding of deuterated hormones (Glasel *et al.*, 1973) indicate that nmr signals from deuterium atoms on the side chain of residue 1 are markedly broadened by binding. Additional peptide binding studies are currently in progress to resolve the free-energy contributions to binding of the different segments of residues 1–3 into their enthalpic and entropic components.

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