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Thermodynamics and Kinetics of Bovine Neurophysins Binding to Small Peptide Analogues of Oxytocin and Vasopressin†

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ABSTRACT: Thermodynamic binding constants for the interactions of mononitrated neurophysins with oxytocin, vasopressin, and peptide analogues of the hormones were determined by using a spectrophotometric titration technique. The data were fit to a binding model which included all known interactions in these systems. From an examination of the free energies for the binding reaction, we concluded that residues 1–3 contribute 84% of the binding energy for formation of the neurophysin dimer mono complex and 79% for the formation of the bis complex. Rate constants for complex formation and dissociation with native bovine neurophysin were determined by using temperature-jump relaxation. The association rate constants for neurophysin dimer binding to oxytocin, vasopressin, and the peptide analogues were all in the range of 1.3 $\times 10^6$ M^{-1} s^{-1} for mono complexation and 1.5 $\times 10^6$ M^{-1} s^{-1} for bis complexation. Thus, formation rate constants are identical for both mono and bis complexation, and no significant differences exist between formation constants for hormones and peptides. On the other hand, a clear distinction in dissociation rate constants is apparent when one compares the hormones (k_r = 4 to 16 s^{-1}) with the peptide analogues (k_r = 54 to 182 s^{-1}). There is roughly a tenfold increase in overall dissociation rate constant when one compares the peptides to the hormones. From these data, we conclude that the rate-determining step in the association reaction involves the first two or three residues on the hormone. After the initial binding takes place, only with intact hormone, i.e., oxytocin or vasopressin, can additional bonding interactions in the complex take place. These additional interactions are reflected in the slower off-rate of the hormone complexes relative to the peptide complexes.

The neurohypophyseal hormones, oxytocin and vasopressin, are stored in granules in the posterior pituitary in a covalent association with a binding protein, neurophysin. In the cow, two neurophysins of closely related structure are found, bovine neurophysin I, which is associated with vasopressin, and bovine neurophysin II, associated with oxytocin. In vitro, each neurophysin is capable of association with either hormone with similar binding affinity. Indirect evidence (Brownstein & Gainer, 1977; Gainer & Brownstein, 1977; Lauber et al., 1979) indicates that the bovine neurophysins and the hormones are synthesized as part of a larger precursor, which is cleaved to form the neurophysin and its associated hormone, which are then stored in granules.

The neurophysin–hormone system is an ideal vehicle for studying protein–hormone interactions; neurophysins are readily purified and oxytocin, vasopressin, and peptide analogues of the hormones are available. The amino acid sequences of the bovine neurophysins are known (Walter et al., 1971; North et al., 1975; amended by Wuu & Crumm, 1976).

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Thermodynamic studies have indicated that neurophysin exists in a monomer–dimer equilibrium in solution (Breslow et al., 1971; Nicolas et al., 1976) and that the dimer binds hormone more strongly than the monomer (Nicolas et al., 1976, 1978a,b). Dipeptide and tripeptide analogues of the amino terminus of the hormones are known to bind to the hormone binding site of neurophysin and mononitrated neurophysin (Breslow & Weis, 1972; Breslow et al., 1973; Breslow, 1975). The first two residues bind with almost 60% of the free energy of the hormones, while the first three residues provide about 70% (Breslow, 1975). Kinetic studies of the binding reaction have shown that rate constants for dimer–hormone complexation are one order of magnitude faster than those for monomer complexation (Pearlmutter & McMains, 1977). Neurophysin dimerization occurs with rate constants comparable to those that have been found for other dimerizing proteins ($\sim 10^5$ M^{-1} s^{-1}) and is compatible with a pH-independent dimerization that involves hydrophobic interactions between the monomer subunits (Pearlmutter, 1979). Because identical kinetics are observed for native and mononitrated neurophysin dimerization, the single tyrosine residue (Tyr-49) does not participate directly in this process.

As part of a continuing investigation of the dynamics of neurophysin–hormone interactions, we have examined the binding kinetics of bovine neurophysins interacting with oxytocin, vasopressin, and small peptide analogues. We have extended the concentration range of hormone in order to decipher the characteristics of the bis complexation reaction. These results can be used to explain some of the discrepancies that have appeared in the literature with regard to the NMR measured lifetimes of neurophysin–hormone complexes which were determined under different stoichiometric conditions.

Materials and Methods

Bovine neurophysins were prepared from bovine posterior pituitaries (Pel-Freez) as described previously (Pearlmutter & McMains, 1977). Mononitrated neurophysins I and II were prepared according to the method of Furth & Hope (1970). Nitrated neurophysin II (NO_2NP)[1] preparations were routinely subjected to chromatography on DEAE-Sephadex A-50 in pH 5.9 pyridine acetate buffer, as described by Breslow & Gargiulo (1977), to remove any dinitrated material. All neurophysin preparations were stored as solids in a vacuum desiccator at 4 °C.

Oxytocin and [8-lysine]-vasopressin were a gift from Sandoz Ltd. and were purified on Sephadex G-15 according to the method of Manning et al. (1968). Purity was checked by thin-layer chromatography in a butanol–acetic acid–water (4:1:5) solvent system.

L-Phenylalanyl-L-tyrosinamide (Phe-Tyr-NH_2) and *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalaninamide (*S*-methyl-Cys-Tyr-Phe-NH_2) were gifts from Professor E. Breslow. L-Methionyl-L-tyrosyl-L-phenylalaninamide (Met-Tyr-Phe-NH_2), L-cystinylbis(L-tyrosin)amide (bis-Cys-Tyr-NH_2), and L-leucyl-L-tyrosinamide (Leu-Tyr-NH_2) were purchased from Vega Biochemicals. Peptides and hormones were stored as solids in a vacuum desiccator at 4 °C.

[1] Abbreviations used: DEAE, diethylaminoethyl; NP, bovine neurophysin; NO_2NP, mononitrated bovine neurophysin; Phe-Tyr-NH_2, L-phenylalanyl-L-tyrosinamide; *S*-Me-Cys-Tyr-Phe-NH_2, *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalaninamide; Met-Tyr-Phe-NH_2, L-methionyl-L-tyrosyl-L-phenylalaninamide; bis-Cys-Tyr-NH_2, L-cystinylbis(L-tyrosin)amide; Leu-Tyr-NH_2, L-leucyl-L-tyrosinamide; LVP, [8-lysine]-vasopressin; mol wt, molecular weight; HL, ligand with protonated α-amino group.

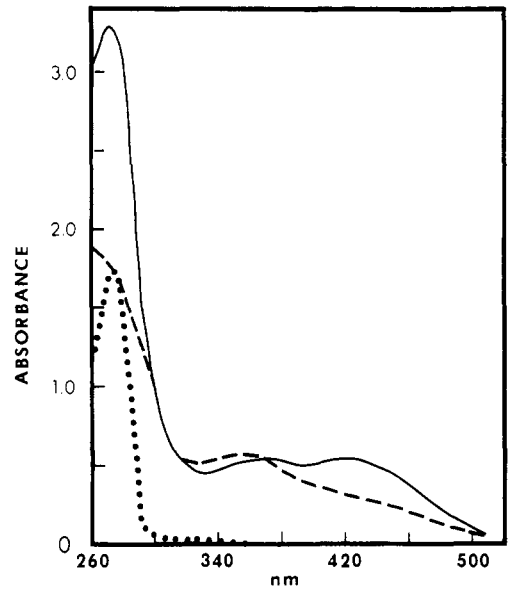


FIGURE 1: Absorption spectra at pH 7.00 of mononitrated neurophysin I, 2.7×10^{-4} M (---), of Phe-Tyr-NH_2, 1.4×10^{-3} (···), and of a mixture of mononitrated neurophysin I, 2.7×10^{-4} M, with Phe-Tyr-NH_2, 1.4×10^{-3} M (—). Spectra were obtained in a Gilford spectrophotometer at 25 °C in 0.1 M phosphate buffer, pH 7.

The pK_a values for the peptides obtained from Vega Biochemicals were determined by hydrogen ion titration at 25 °C in 0.1 M KNO_3. Values for Phe-Tyr-NH_2 and *S*-methyl-Cys-Tyr-Phe-NH_2 were communicated by Professor E. Breslow.

Association constants for binding of oxytocin, vasopressin, and the peptide analogues to bovine neurophysins were determined using a spectrophotometric titration technique. All solutions were maintained at 25 °C in 0.1 M phosphate buffer, pH 7. It has been shown that mononitration of the single tyrosine residue, Tyr-49, of neurophysin does not affect its ability to bind oxytocin or vasopressin (Furth & Hope, 1970). However, the environment of the nitrotyrosine does change with hormone binding, and thus the nitrotyrosine can be used as a reporter group to monitor binding (Breslow & Weis, 1972). At pH 7, the visible spectrum of either NO_2NPI or NO_2NPII exhibits an absorption maximum at 360 nm due to the protonated nitrotyrosine hydroxyl group and a broad absorption band in the region of 400–440 nm attributable to the ionized form of the nitrotyrosine (Figure 1). In the presence of saturating amounts of ligand (either peptide or hormone), the absorbance of NO_2NP decreases at 360 nm and increases in the region of 400–440 nm (Figure 1). These changes are due to an increased degree of ionization of the nitrotyrosine group, which is a reflection of the decrease in pK_a from 7.4 to 6.8 for the nitrotyrosine hydroxyl group upon binding of ligand (Breslow & Weis, 1972; Wolff et al., 1975). The NO_2NP–ligand mixture also shows increased absorption in the UV (maximum at 275 nm) due to the absorption by aromatic groups of the peptide or hormone. None of the peptides or hormones exhibit any absorption in the region of 400–440 nm; therefore, any change in absorbance in this region, if the solution is maintained at constant pH, is due solely to changes in the NO_2NP spectrum caused by interaction with ligand. Because the largest change occurs at 440 nm, ligand binding was monitored at this wavelength.

In a typical experiment (Figure 2), aliquots of a concentrated solution of peptide ($\sim 10^{-2}$ M) were added to a 2.5–3 $\times 10^{-4}$ M solution of NO_2NPI or NO_2NPII in 0.1 M phosphate buffer, pH 7. Final concentrations of peptide ranged

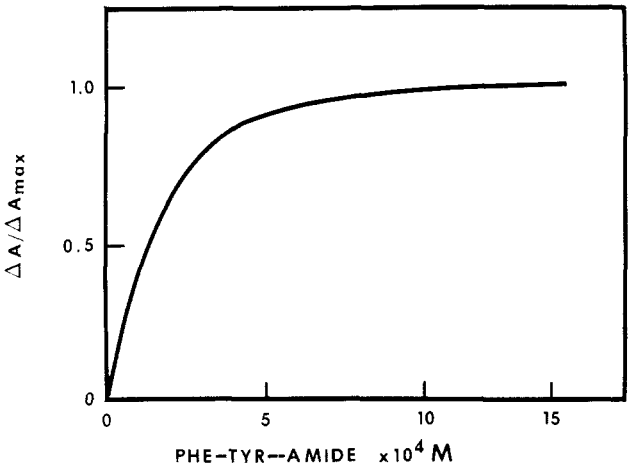


FIGURE 2: Titration of mononitrated neurophysin I, 2.9×10^{-4} M, with Phe-Tyr-NH_2 in 0.1 M phosphate buffer, pH 7.0. The results are expressed as the ratio of ΔA, the difference at 440 nm of the absorbance of the mononitrated neurophysin I–peptide solution and the mononitrated neurophysin I–buffer solution, to ΔA_{max}, the difference between the maximal change in absorbance at saturating peptide concentration and the change in absorbance due to dilution of the mononitrated neurophysin I.

from 1×10^{-5} to 2×10^{-3} M. Aliquots of ligand were added to a solution of NO_2NP; to an identical solution of NO_2NP were added equal aliquots of phosphate buffer. The pH of both solutions was maintained at 7 throughout the titration. The absorbance at 440 nm of both solutions was measured using a Gilford spectrophotometer. Titration was continued until there was no further change in absorbance with addition of ligand. In most cases, the titrations were done in duplicate.

Kinetic studies were carried out using a Gibson-Durrum stopped-flow temperature-jump spectrophotometer. The magnitude of the temperature jump was calibrated as previously described (Pearlmutter & McMains, 1977). Measurements were made at I = 0.1 M KNO_3, and the initial temperature was maintained at 9 °C so that after a 5-kV discharge through the cuvette the final temperature was 25 °C. The indicator phenol red (Eastman Kodak) was used to monitor the relaxation time, since binding of oxytocin, vasopressin, and the peptide analogues to neurophysin involves a redistribution of protonated and unprotonated species. Even though the neurophysin–hormone binding reaction does not involve a direct proton transfer (Pearlmutter & McMains, 1977), it is possible to monitor the overall binding reaction by following the change in distribution of protons on the unbound hormone upon perturbation of the system (Eigen & DeMaeyer, 1963). The nature of relaxational kinetic analysis predicts that the relaxation time is a function of all of the steps which occur, not only those which are directly pH dependent. Any conformational change in neurophysin or neurophysin–hormone complex that is equal to or faster in rate than the binding reaction itself would be mathematically coupled to the observed relaxation time. Therefore, with this technique we can monitor the overall reaction and not "one microscopic step in the oxytocin–neurophysin interaction" (Blumenstein et al., 1978).

For the temperature-jump experiments, known amounts of purified native NPI or NPII and ligand (oxytocin, LVP, or peptide) were dissolved in degassed 0.1 M KNO_3 containing 1×10^{-5} M phenol red. The pH was adjusted using measured amounts of KOH and HCl. For each system studied, we examined solutions where the ligand:NP ratio was <1, ~1, and >1. Relaxation spectra were monitored at 558 nm, which corresponds to the absorption maximum of phenol red.

Relaxation times were determined from at least three oscilloscope tracings by enlarging the Polaroid photograph and plotting log amplitude vs. time and obtaining the half-times, $t_{1/2}$. Half-times were converted to relaxation times using the relationship $\tau = t_{1/2}/\ln 2$ (Eigen & DeMaeyer, 1963).

Results

Values for α-amino pK_as for the peptide analogues were determined by hydrogen ion titration. Calculations were straightforward except in the case of bis-Cys-Tyr-NH_2, which is a symmetrical molecule with two identical α-amino groups whose pK_as are close together and influence each other. For bis-Cys-Tyr-NH_2, the two α-amino pK_as were calculated from the titration curve using the Noyes method for separating overlapping pK values as described by Albert & Serjeant (1962). Values for α-amino pK_as for ligands used in these studies are as follows: oxytocin and LVP, pK_a = 6.3 (Breslow, 1961); Phe-Tyr-NH_2, pK_a = 7.15; *S*-methyl-Cys-Tyr-Phe-NH_2, pK_a = 6.8; Met-Tyr-Phe-NH_2, pK_a = 6.9; Leu-Tyr-NH_2, pK_a = 7.6; bis-Cys-Tyr-NH_2, pK_{a1} = 5.82, pK_{a2} = 7.15.

Thermodynamic Binding Studies. From the titration data obtained with nitrotyrosine neurophysin and the appropriate ligand, binding isotherms for each system were constructed. For each point, the change in NO_2NP absorbance due to ligand binding is the difference between the absorbance at 440 nm of the NO_2NP–ligand solution and absorbance at 440 nm of the NO_2NP–buffer solution, ΔA. We assumed that NO_2NP was saturated with ligand when the absorbance no longer increased with additional ligand, and this plateau value was called ΔA_{max}. Plots of fractional saturation, $\Delta A/\Delta A_{max}$, vs. ligand concentration gave smooth curves (Figure 2). With oxytocin and vasopressin as ligands, we were able to do the titrations at lower NO_2NP concentrations (typically $\sim 1.5 \times 10^{-4}$ M) and lower final ligand concentrations (9×10^{-6} to 6×10^{-4} M) because the magnitude of the absorbance changes per mole of ligand added was much larger.

In order to plot the titration data in a form suitable for detailed analysis, we assumed that the change in NO_2NP absorbance at 440 nm reflects protonated ligand binding, that the extinction coefficient changes are the same for the first and second ligand bound, and that absorbance changes are due to ligand binding to NO_2NP with an ionized nitrotyrosine residue. This last assumption is supported by spectral experiments with a mixture of NO_2NPI and Phe-Tyr-NH_2, whose pH was maintained at either 6 or 8.5 in a buffered solution. At pH 6, 96% of NO_2NPI nitrotyrosine hydroxyl groups are protonated (using pK_a = 7.4); saturation at this pH with Phe-Tyr-NH_2 causes very little change in the NO_2NPI spectrum. This suggests that binding of ligand to NO_2NPI with un-ionized nitrotyrosine groups cannot be detected by absorbance changes. At pH 8.5, 93% of NO_2NPI nitrotyrosine hydroxyl groups are ionized; addition of a half-saturating amount of Phe-Tyr-NH_2 results in a decrease in absorbance at 360 nm and an increase in the region of 400–440 nm. This corresponds to the change in the NO_2NPI spectrum in the titration experiments. From these experiments we concluded that most, if not all, of the spectral change observed on titration of NO_2NP with ligand at pH 7 is due to binding to NO_2NP with an ionized nitrotyrosine group.

The experimental data were expressed according to Scatchard (1949). Fractional saturation, $\bar{\nu}$, moles of ligand bound per 10000 mol wt unit, was calculated from the relationship, ligand bound = $(\Delta A/\Delta A_{max}) \times$ [NO_2NP ionized]. [NO_2NP ionized] is calculated from the total concentration of 10000 mol wt units using pK = 7.4. Unbound ligand concentrations were corrected for the presence of ligand with

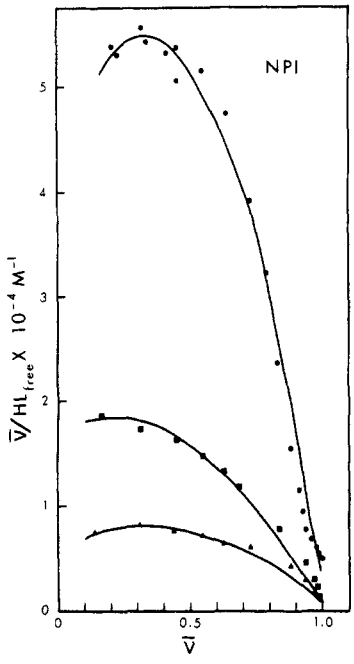


FIGURE 3: Scatchard plots of the binding of oxytocin (●), bis-Cys-Tyr-NH_2 (■), and Phe-Tyr-NH_2 (▲) to ionized mononitrated neurophysin I. Fractional saturation, $\bar{\nu}$, was calculated from spectrophotometric titration data at pH 7 as described in the text. The solid lines represent theoretical curves which were calculated with K_{1B} and K_{2B} shown in Table I. HL_{free} represents unbound, protonated ligand.

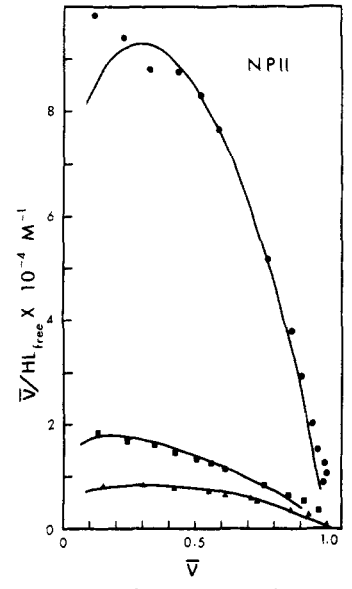


FIGURE 4: Scatchard plots of the binding of oxytocin (●), bis-Cys-Tyr-NH_2 (■), and Phe-Tyr-NH_2 (▲) to ionized mononitrated neurophysin II. Fractional saturation, $\bar{\nu}$, was calculated from spectrophotometric titration data at pH 7 as described in the text. The solid lines represent theoretical curves which were calculated with K_{1B} and K_{2B} shown in Table I. HL_{free} represents unbound, protonated ligand.

an unprotonated α-amino group, which is incapable of binding neurophysin with high affinity, and for the presence of ligand bound to un-ionized nitrotyrosine neurophysin. The correction factor for the latter, 1/3.17, was determined by pH titration of bound vs. unbound nitrotyrosine neurophysin. The inclusion of the latter correction factor had negligible (<5%) influence on the data. Plots of $\bar{\nu}/HL_{free}$ vs. $\bar{\nu}$ were nonlinear for all systems (Figures 3 and 4).

In order to determine binding constants from the nonlinear Scatchard plots, it was necessary to construct a theoretical model of the binding systems and to compare the predictions

Table I: Equilibrium Association Constants for Hormone and Peptide Binding to Ionized Mononitrated Neurophysins

ligand	K_{1B} $(M^{-1})^a$	K_{2B} $(M^{-1})^b$
NPI		
LVP	1.2×10^5	2.0×10^5
oxytocin	1.0×10^5	1.9×10^5
bis-Cys-Tyr-NH_2	4.8×10^4	3.5×10^4
Met-Tyr-Phe-NH_2	2.6×10^4	1.2×10^4
S-Me-Cys-Tyr-Phe-NH_2	2.3×10^4	1.8×10^4
Phe-Tyr-NH_2	1.4×10^4	2.8×10^4
Leu-Tyr-NH_2	8.5×10^3	4.5×10^3
NPII		
LVP	1.5×10^5	2.8×10^5
oxytocin	2.2×10^5	3.2×10^5
bis-Cys-Tyr-NH_2	5.2×10^4	3.0×10^4
Met-Tyr-Phe-NH_2	2.8×10^4	1.7×10^4
S-Me-Cys-Tyr-Phe-NH_2	1.3×10^4	2.5×10^4
Phe-Tyr-NH_2	1.6×10^4	2.8×10^4
Leu-Tyr-NH_2	2.8×10^3	4.2×10^3

[a] K_{1B} is the association constant for the reaction $(NP)_2 + HL = (NP)_2\text{-}HL$. [b] K_{2B} is the association constant for the reaction $(NP)_2\text{-}HL + HL = (NP)_2\text{-}(HL)_2$.

of our theoretical model with the actual experimental data. The correctness of our model could be verified by comparing our calculated binding constants for oxytocin and vasopressin interacting with NO_2NPI and NO_2NPII with those determined by other workers utilizing different techniques (Breslow & Walter, 1972; Nicolas et al., 1976).

In the theoretical model which was utilized to fit the experimental data, we made the following assumptions: (a) NP exists in a monomer–dimer equilibrium; (b) binding involves interaction of protonated ligand (hormone or peptide) with NP dimer, and binding to NP monomer is an order of magnitude weaker than to NP dimer; (c) each NP dimer has two ligand binding sites, one on each monomer subunit. A correction factor was included to account for the binding of ligand to un-ionized NO_2NP, which was not spectrally detectable at 440 nm.

In the calculation of the theoretical Scatchard plots, we tried trial sets of values for K_{1B} and K_{2B}, association binding constants of the NP dimer for the mono and bis complexes, respectively. We included the known values of the dimerization constants for NPI and NPII (Breslow & Weis, 1972; Nicolas et al., 1976) and a value for the association constant for NP monomer binding to ligand of 0.1 the value of K_{1B}. Equilibrium concentrations of all species involved were calculated by a successive approximation technique using a PDP/11 computer. In constructing Scatchard plots, we included only species with ionized NO_2NP, where $\bar{\nu}$ is defined as (moles of ligand in complexes with ionized NO_2NP)/(total moles of ionized NO_2NP), and NO_2NP concentrations are expressed in terms of 10000 mol wt units. We sought the best possible fit of the theoretical Scatchard plot of $\bar{\nu}/HL_{free}$ vs. $\bar{\nu}$ to the experimental plot, both by visual comparison of the plots and by minimizing the error function $\Sigma\sigma$, where

$$\Sigma\sigma = \Sigma\sigma_x + \Sigma\sigma_y = \Sigma\left|\frac{\bar{\nu}_{calcd} - \bar{\nu}_{exptl}}{\bar{\nu}_{exptl}}\right|^2 + \Sigma\left|\frac{\bar{\nu}/[HL]_{calcd} - \bar{\nu}/[HL]_{exptl}}{\bar{\nu}/[HL]_{exptl}}\right|^2$$

The subscripts calcd and exptl refer to calculated and experimental values, respectively. The best fit binding constants, which were determined using this procedure, are summarized in Table I. The lines in Figures 3 and 4 represent the best fit theoretical Scatchard plots for each system. The values

Table II: Initial Concentrations and Experimental and Calculated Relaxation Times for Representative Neurophysin Binding System[a]

[neurophysin] × 10^4 (M)	[ligand] × 10^4 (M)	pH	$1/\tau_1$ (s^{-1})	$1/\tau_2$ (s^{-1})
		NPII + Oxytocin		
4.84	5.25	7.61	(192)	100 (70.5)
4.05	3.49	7.67	(195)	81 (51.8)
2.02	2.62	7.51	(91.8)	70.9 (66.5)
2.88	2.88	7.45	(130)	60 (62.7)
0.98	2.81	7.47	(96.8)	53.6 (59.0)
0.96	1.23	7.49	(53.6)	53.0 (47.9)
0.96	1.23	7.49	(53.6)	45.0 (47.9)
2.23	1.61	7.67	(114)	43 (39.1)
0.33	0.42	7.60	39.0 (32.7)	(15.2)
0.31	0.40	7.41	36.0 (36.8)	(17.3)
0.95	0.95	7.52	(52.2)	35.0 (39.4)
0.49	1.40	7.43	(67.4)	33.7 (36.1)
0.53	0.80	7.43	(47.4)	33.0 (30.7)
0.24	0.70	7.43	(47.6)	27.2 (19.9)
0.44	0.32	7.45	27.0 (33.6)	(18.0)
0.06	0.18	7.42	27.0 (27.1)	(8.8)
0.50	0.80	7.43	(47.8)	26.0 (29.7)
0.12	0.35	7.44	(34.7)	20.4 (11.4)
		NPI + Bis-Cys-Tyr-NH_2		
1.94	6.90	7.34	730 (659)	(375)
0.97	3.45	7.34	410 (432)	(229)
2.00	2.30	7.32	316 (307)	(288)
0.48	1.70	7.32	308 (306)	(145)
0.24	0.85	7.36	265 (221)	(97)
1.00	1.15	7.34	(237)	194 (174)
2.00	0.97	7.32	(290)	186 (199)
1.00	0.48	7.33	176 (187)	(146)
0.50	0.58	7.33	159 (194)	(111)
0.12	0.42	7.39	158 (173)	(81)
0.44	0.31	7.30	(165)	102 (99)
0.22	0.16	7.36	(140)	93 (84)
0.11	0.08	7.35	(127)	85 (83)

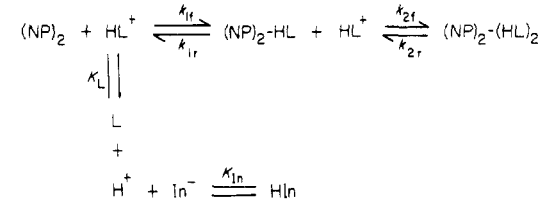
[a] The values calculated using the equation for $1/\tau_{1,2}$ in the text are shown in parentheses. The complete set of raw data for all of the systems is available upon request to the corresponding author.

for the oxytocin and vasopressin systems (with the exception of the NO_2NPI + oxytocin system) are in general agreement with previously published values (Nicolas et al., 1976; Breslow & Walter, 1972).

Kinetic Studies. In the temperature-jump experiments using native neurophysins, a single relaxation time in the millisecond time region was usually observed, as in previous studies with oxytocin and LVP (Pearlmutter & McMains, 1977). In a few cases, a second relaxation time could be extracted from the curve. The initial concentrations used and relaxation times obtained for two representative systems are given in Table II.

To construct a mechanism for these interactions, we considered the following interactions to be important: reversible dimerization of NP, with known dimerization constants of 7.7×10^3 M^{-1} for NPI (Nicolas et al., 1976) and $5\text{–}5.8 \times 10^3$ M^{-1} for NPII (Nicolas et al., 1976; Breslow et al., 1971); enhancement of NP dimerization induced by binding of ligand, which binds preferentially to the dimer (Nicolas et al., 1978a,b); binding by each dimer of 2 mol of ligand, with equilibrium association constants as shown in Table I. Because rate constants for neurophysin dimerization are at least a factor of 10 slower than those for the binding reactions, the ligand-binding system is kinetically uncoupled from the dimerization and it need not be included in the mechanism (Hammes, 1968). In previous kinetic studies with oxytocin and LVP (Pearlmutter & McMains, 1977), complexes of the form DL_2 were not significant because of the relatively low concentrations

Scheme I



of protonated hormone that were utilized. In this study, we extended the concentration range of the hormone. The binding of hormone to NP monomer was found to account for less than 5% of the relaxation time in the previous study, and therefore should not play a significant role in this analysis.

The mechanism which fits all of the data involves interaction between NP dimer and protonated ligand to form mono and bis complexes as outlined in Scheme I. HL^+ represents protonated ligand with a positively charged α-amino group; L represents the unprotonated, neutral ligand; $(NP)_2$ represents neurophysin dimer; $(NP)_2HL$ represents the ligand–neurophysin dimer mono complex; $(NP)_2$–$(HL)_2$ represents the ligand–neurophysin dimer bis complex; In^- and HIn represent the unprotonated and protonated forms of the indicator, respectively; and H^+ represents the proton. Rapid preequilibria are indicated by equal signs, and the rate-determining reactions are shown by the arrows. The two relaxation times for this mechanism are obtained by expressing the rate equations for restoration of equilibrium in the form of a determinant (Eigen & DeMaeyer, 1963) and by solving for the two roots:

$$\frac{1}{\tau_{1,2}} = (1/2)\{(a_{11} + a_{22}) \pm \sqrt{[(a_{11} + a_{22})^2 - 4(a_{11}a_{22} - a_{12}a_{21})]}\} \quad (1)$$

where τ_1 corresponds to the + sign and τ_2 corresponds to the − sign. The a_{ij} factors, calculated from mass balance and preequilibrium considerations, are $a_{11} = k_{1f}([\overline{(NP)}_2/(1+\beta)] + \overline{HL}) + k_{1r}$, $a_{12} = k_{1r} - k_{1f}[\overline{(NP)}_2/(1+\beta)]$, $a_{21} = k_{2f}(\overline{HL} - [\overline{(NP)}_2\overline{HL}/(1+\beta)])$, and $a_{22} = k_{2f}([\overline{(NP)}_2\overline{HL}/(1+\beta)] + \overline{HL}) + k_{2r}$. The factor $\beta = K_L/([\bar{L}]/(1+\alpha) + \bar{H})$, where $\alpha = \overline{In}/(K_{In} + \bar{H})$, and the bars denote equilibrium concentrations. Stability constants used to calculate equilibrium concentrations are $K_{In} = 2 \times 10^{-8}$ M (Yapel & Lumry, 1971), $K_D = 7.7 \times 10^3$ for NPI and 5.8×10^3 for NPII (Nicolas et al., 1976), and K_L = the acid association constant reported earlier in the text. Hydrogen ion concentrations were calculated by dividing the measured hydrogen ion activity by γ_H ($\simeq$0.80).

The rate constants which gave the best fit to the experimental data for each system were determined by choosing trial values for k_{1f} and k_{2f} and inserting these values in eq 1 to calculate $1/\tau_1$ and $1/\tau_2$. Calculated $1/\tau$ values were then compared with the experimental values. The best fit was defined by minimizing the factor σ:

$$\sigma = \frac{1}{N}\left[\Sigma\left(\frac{\frac{1}{\tau_{calcd}} - \frac{1}{\tau_{exptl}}}{\frac{1}{\tau_{exptl}}}\right)^2\right]$$

where N is the number of data points, calcd refers to calculated values, and exptl refers to experimental values. The reverse rate constants k_{1r} and k_{2r}, were calculated by dividing the forward rate constants by the appropriate stability constants determined for each system. The best fit rate constants

Table III: Best Fit Rate Constants[a] for Hormone and Peptide Binding to Bovine Neurophysins in 0.1 M KNO_3 at 25 °C

ligand	$10^{-6}k_{1f}$[b] (M^{-1} s^{-1})	k_{1r} (s^{-1})	$10^{-6}k_{2f}$ (M^{-1} s^{-1})	k_{2r} (s^{-1})
Neurophysin I				
LVP	1.3	11	0.8	4
oxytocin	0.9	9	1.1	6
bis-Cys-Tyr-NH_2	4.0	83	3.1	89
Met-Tyr-Phe-NH_2	1.4	54	1.9	158
S-Me-Cys-Tyr-Phe-NH_2	1.6	70	1.7	94
Phe-Tyr-NH_2	1.3	93	2.1	75
Neurophysin II				
LVP	1.7	11	1.6	6
oxytocin	3.5	16	3.1	10
bis-Cys-Tyr-NH_2	3.1	60	4.2	140
Met-Tyr-Phe-NH_2	2.8	100	3.1	182
S-Me-Cys-Tyr-Phe-NH_2	1.5	115	1.7	68
Phe-Tyr-NH_2	1.6	100	2.4	86

[a] The rate constants were determined from the relaxation times as described in the text. The rate constants are assigned as shown in Scheme I. [b] The error in the rate constants is estimated to be ±25%.

for all of the systems are summarized in Table III.

Discussion

This study is the first to determine equilibrium constants for both mono (K_{1B}) and bis (K_{2B}) complexation of neurophysin dimer with a series of peptide analogues of oxytocin and vasopressin. In the analysis we included all of the most significant known interactions: protein dimerization, ligand p*K*, and the weaker binding of neurophysin monomer. By avoiding the use of equilibrium dialysis techniques, no long incubations are necessary and radioactive ligands which may decompose during the course of the experiment are not required. Binding constants determined by others have either required the use of radioactive ligands (Nicolas et al., 1978a,b) or were reported before it was clear that the major neurophysin species which binds ligand is the dimer (Breslow et al., 1973; Breslow, 1975).

In general, the free-energy values (Table IV) for ionized nitrotyrosine–neurophysin binding are parallel with those determined by Breslow and co-workers for the un-ionized protein (Breslow et al., 1973; Breslow, 1975; Sur et al., 1979), with the exception of the two tripeptide analogues, where our values for the association constants are from 2 to 5 times greater, and bis-Cys-Tyr-NH_2. The latter peptide contains two protonated amino groups, and the binding free energy reported in the literature, which did not take this into account, is not accurate (Breslow, 1975). Because the association constants for hormone binding to the ionized NO_2NP are similar to those determined for the native protein (Breslow, 1975; Nicolas et al., 1976), we have utilized binding constants determined for ionized NO_2NP in the analysis of our kinetic data, which was obtained with native neurophysin. An examination of Table IV shows that, when both mono and bis complexations are incorporated into the binding model, residues 1–3 contribute 84% of the binding energy for formation of the mono complex and 79% for the formation of the bis complex. It has been suggested that the difference in binding affinity between the hormones and tripeptides may be due either to stronger interaction between residue 3 on the hormone than on the peptide because of the specific conformation of the hormone or to additional bonding interactions in the hormone complexes which are associated with binding (Breslow, 1979). Our kinetic findings can be used to distinguish between these two alternatives, as discussed below.

Cohen and his associates (Nicolas et al., 1978a,b) have used equilibrium dialysis techniques with tritiated oxytocin and bovine neurophysin II to generate Scatchard plots of oxytocin binding as a function of neurophysin concentration. From curve fitting these data to a binding model similar to ours, these workers have reported values for K_{1B} of 1.30×10^5 M^{-1} and K_{2B} of 5.35×10^5 M^{-1}; this analysis shows positive cooperativity between subunits of the neurophysin dimer by a factor of 4. The data presented in this paper show positive cooperativity for all four of the hormone–neurophysin binding systems, with an average cooperativity factor of 1.7 (± 0.2 SD). Our data are not compatible with an interdimeric cooperativity factor as high as 4. These differences in results are probably due to the different experimental techniques as well as the different conditions under which measurements were made.

The association rate constants for neurophysin I dimer binding to oxytocin, vasopressin, and the peptide analogues are all in the range of 1.3 (± 0.2 SD) $\times 10^6$ M^{-1} s^{-1} for the formation of the mono complex; the bis-Cys-Tyr-NH_2 peptide contains two protonated amino groups and the forward rate constant is faster, a reflection of the greater statistical probability of an effective collision with this ligand (Table III). For formation of the bis complex with neurophysin I dimer, the association rate constants are 1.5 (± 0.5) $\times 10^6$ M^{-1} s^{-1}, which is identical with that for mono complexation within the statistical accuracy of our data. Therefore, formation rate constants for binding of both the first and the second ligand to the dimer appear to be identical. No real differences between hormone and peptide binding formation rate constants are apparent.

On the other hand, the dissociation rate constants are clearly different when one compares the hormones with the peptide analogues. With neurophysin I and the hormones, the mean k_{1r} value is 10 s^{-1} for mono complex dissociation, and the corresponding value for k_{2r} if 5 s^{-1}, a factor of 2 different. The positive cooperativity observed in the equilibrium thermodynamic measurements is reflected in a faster dissociation rate

Table IV: Free Energies of Ligand Binding to Mononitrated Bovine Neurophysins I and II[a]

ligand	K_{1B} NPI	K_{1B} NPII	K_{2B} NPI	K_{2B} NPII	lit. NPII values[b]	% binding energy[c] K_{1B}	% binding energy[c] K_{2B}
LVP	6.9	7.0	7.2	7.4	7.4	99	99
oxytocin	6.8	7.3	7.2	7.5	7.8	100	100
bis-Cys-Tyr-NH_2	6.4	6.4	6.2	6.1	5.2	86	78
Met-Tyr-Phe-NH_2	6.0	6.1	5.6	5.8	5.2	86	78
S-Me-Cys-Tyr-Phe-NH_2	5.9	5.6	5.8	6.0	5.1	82	80
Phe-Tyr-NH_2	5.6	5.7	6.1	6.1	5.6	80	83
Leu-Tyr-NH_2	5.4	4.7	5.0	4.9	4.1	72	67

[a] Data were obtained at I = 0.1 and 25 °C and were analyzed as described in the text. These free energies refer to binding constants involving ionized nitrotyrosine neurophysin. [b] Values taken from Breslow (1975), which were determined for un-ionized nitrotyrosine neurophysin. [c] Computed by taking the means of NPI and NPII and comparing the values obtained with that for oxytocin.

constant for the mono complex when compared with the bis complex. This dissociation rate constant for the bis complex is in excellent agreement with the value obtained by NMR, 2 s^{-1}, at neutral pH with an excess of hormone (Blumenstein et al., 1978). For the peptide analogues, the mean k_{1r} value is 75 s^{-1} for bis complex dissociation. Thus, there is roughly a 10-fold increase in overall dissociation rate when one compares the peptide analogues with the parent hormone molecules. It is the increase in dissociation rate that reflects the decreased stability of the peptide complexes when compared with oxytocin and vasopressin.

The results with neurophysin II parallel those for neurophysin I. For both major bovine neurophysin species, cooperativity is reflected by a lower dissociation rate constant for the bis complex than for the mono complex, and the peptide analogues show an enhanced dissociation rate constant for both mono and bis complexation of about a factor of 10.

From these results, we can propose the following dynamic model of the binding interaction between neurophysin dimer and hormone and hormone analogues. The rate-determining step in the association reaction involves the first two or three residues on the hormone; this is reflected in the identity of the association rate constants for hormone and peptide analogues. After binding takes place, only with hormone can secondary stabilizing binding interactions in the complex take place. This is reflected in the 10-fold slower dissociation rate constants for the hormones when compared with the peptide analogues.

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