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Binding of Neurohypophyseal Peptides to Neurophysin Dimer Promotes Formation of Compact and Spherical Complexes†

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ABSTRACT: Previous hydrodynamic studies [Rholam, M., & Nicolas, P. (1981) *Biochemistry 20*, 5837-5843] have demonstrated that the dimerization of a neurophysin monomer (prolate ellipsoid with an axial ratio, due to asymmetry, of 5.2) results in a decreased asymmetry (axial ratio, due to asymmetry, of 3.6) as the consequence of a side-by-side association process. By a combination of hydrodynamic measurements, including the use of sedimentation velocity, viscometry, and fluorescence polarization spectroscopy, the influence of hormone binding on the shape and asymmetry properties of the neurophysin dimer was evaluated. The binding of ocytocin, vasopressin, and the tripeptide analogue of the N-terminal sequence of ocytocin, Cys(*S*-Me)-Tyr-Ile-NH_2, results in an increase of $s^0_{20,w}$ and a decrease in both the reduced viscosity and rotational relaxation time of the bis-liganded dimeric species vs. the nonliganded form. The axial ratio (a/b) due to asymmetry of the ligand-bound dimers was found in each case to be equal to, or slightly greater than, 1.0, indicating a compact spherical shape (Stokes radius 21 Å). The profound alteration on molecular dimensions observed upon ligand binding is shown to be the consequence of a ligand-induced conformational change and might explain the intradimeric binding sites positive cooperativity. It is tentatively proposed that the pseudospherical shape of the neurophysin–hormone complexes may enhance the stability of neurophysin and contribute to the prevention of leakage of neuropeptides through the membrane of neurosecretory granules. The data provide a remarkable example of a small protein with a high content in disulfide links and that undergoes conspicuous changes in conformation under the influence of nonapeptide, or tripeptide, ligands.

Physicochemical studies of the neurophysins, hypothalamo-neurohypophyseal proteins associated with the transport and biosynthesis of the neuropeptide hormones ocytocin and vasopressin [for recent reviews see Pickering & Jones (1978), Breslow (1979), and Cohen et al. (1979)], have recently focused on the mechanism of assembly of neurophysin monomers into dimer (Nicolas et al., 1976, 1978b, 1980; Pearlmutter, 1979). Both the shape and asymmetry properties of these species in solution (Rholam & Nicolas, 1981) were studied. Data obtained from a combination of various hydrodynamic techniques (Rholam & Nicolas, 1981) indicated mainly that the single polypeptide chain monomer (M_r 10000) is rigid and highly asymmetrical with an axial ratio close to 5.2. The

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formation of the dimer involves a hydrophobically driven side-by-side association process, resulting therefore in a reduced asymmetry. The above, and previous, studies (Nicolas et al., 1978a, 1980) additionally suggested that the formation of the neurophysin dimer does not result in measurable conformational rearrangements of the monomer structure.

The complexes formed between neurophysins and neurohypophyseal hormones are particularly well suited for a similar analysis since the important influence of hormones binding on the in vitro dimerization has now been clarified. Ligand-facilitated dimerization is observed under conditions of preferential binding to the dimeric sites of the nonapeptide hormones and analogues (Nicolas et al., 1976, 1978a, 1980; Pearlmutter & McMains, 1977; Pearlmutter & Dalton, 1980), and a positive binding cooperativity has been detected between the two high-affinity dimeric sites for both ocytocin (Hope et al., 1975; Nicolas et al., 1978b; Pearlmutter & Dalton, 1980; Tellman & Winzor, 1980) and vasopressin (Nicolas et al., 1976, 1978a).

Although a wealth of information is now available on the thermodynamics and kinetics of both the dimer formation and the hormone binding process, much less is known about the shape and conformation of both the monomeric and dimeric neurophysin–hormone complexes. This knowledge is particularly desirable (i) in view of the suggested intragranular function of neurophysins in preventing the nonapeptide hormones transmembrane leakage (Cohen et al., 1979) by providing an appropriate size (Stokes radius ≥20 Å) for the complexes (Jamieson & Palade, 1977) and (ii) for a better understanding of the conformational changes that could arise upon binding of small ligands to a highly disulfide-linked protein (seven disulfide bridges per polypeptide chain; Capra et al., 1972). Previous studies have provided some evidence that complex formation produces local rearrangements in the neurophysin tertiary structure. UV absorption spectra arising from the single tyrosine side chain in position 49 indicate that this moiety is displaced from a restricted hydrophobic environment to a more polar one (Griffin et al., 1973; Wolff et al., 1975). Although these data did not argue in favor of important structural changes upon binding, CD spectroscopy additionally indicated perturbations above 290 nm that are not observed with tripeptide analogues and that arise from neurophysin disulfide perturbations (Breslow & Weis, 1972; Griffin et al., 1973). Also Raman studies suggested a ligand-induced increase in the α-helical content (Liu, 1975). These last findings indicate that the neurophysin tertiary structure undergoes more profound and extended changes upon binding than those detected through the tyrosine-49 probe.

We report herein results of sedimentation velocity, viscometric, and fluorescence polarization measurements that show drastic changes of the shape and asymmetry properties of the liganded neurophysin dimeric species when compared to those of the unliganded forms. These data, and their possible biological significance, are discussed in terms of various structural models and in connection with the possible intragranular function of neurophysin.

Materials and Methods

Preparation of Dansylated Neurophysin II. Highly purified neurophysins I and II were prepared by isoelectric focusing as previously described (Camier et al., 1973; Nicolas et al., 1976). The dansyl (Dns) fluorescent group, 5-(dimethylamino)-1-naphthalenesulfonate, was attached to the protein by reacting 39 mg of neurophysin II dissolved in 2 mL of 0.1 M Na_2CO_3 buffer, pH 8.1, with 0.1 mL of acetone solution containing 0.6 mg of the dye added in small aliquots. The reaction mixture was kept at 4 °C for 36 h under moderate stirring. After being filtered through 0.45-μm Millipore filters, the solution was eluted through a Sephadex G-25 superfine column (100 × 1.2 cm) equilibrated with 0.1 N HCOOH, in order to remove excess dye. The pooled protein fractions were dialyzed extensively against 0.1 N acetate buffer, pH 6.2, to ensure complete removal of any free label. The incorporation of covalent Dns labels into neurophysin II was determined from the absorption spectra of the labeled and unlabeled protein (Rholam & Nicolas, 1981).

Hormones and Tripeptide. Synthetic hormones (ocytocin and vasopressin) were a generous gift from Ferring (Uppsala, Sweden), and the tripeptide, Cys(*S*-Me)-Tyr-Ile-NH_2, was purchased from Bachem. The purity of these hormones and the tripeptide was asessed by thin-layer chromatography on cellulose plates run in two different solvent systems (Griffin et al., 1977) and by amino acid composition analysis, which gave the expected theoretical values.

Analytical Ultracentrifugation. Sedimentation velocity analyses were conducted in Beckman-Spinco Model E analytical ultracentrifuge equipped with a temperature-control unit and an electronic speed-control system. The runs were conducted at 60 000 rpm in a double-sector cell with sapphire windows. Sedimentation profiles were recorded on Kodak metallographic plates and analyzed on a Nikon 6C microcomparator. For dilute protein samples (less than 2 mg/mL) the sedimentation profiles were monitored with the Beckman split-beam photoelectric scanner as described in Nicolas et al. (1980). The weight-average sedimentation coefficients were evaluated from the rate of movement of the second moment of schlieren curves (Goldberg, 1953). Radial dilutions were corrected as described by Chervenka (1970), and the weight-average sedimentation coefficient was corrected to water at 20 °C. All these studies were conducted on samples previously dialyzed for 24 h against the appropriate buffer at 5 °C. When the experiments were run in the presence of hormone ligands, the protein solutions were dialyzed against the same hormone solutions for 24 h at 5 °C. After each experiment, both the neurophysins and the hormones were tested for homogeneity by using 0.1% sodium dodecyl sulfate ($NaDodSO_4$)–15% acrylamide slab gel electrophoresis and thin-layer chromatography on cellulose plates, respectively. The frictional ratio was calculated from the sedimentation coefficient by

$$(f/f_0)_{min} = \frac{M_r(1 - \bar{v}\rho)}{Ns^0_{20,w}6\pi\eta[3M_r\bar{v}/(4\pi N)]^{1/3}} \quad (1)$$

where $s^0_{20,w}$ is the sedimentation coefficient at infinite dilution, M_r is the molecular weight, $\bar{v}$ is the partial specific volume of protein, ρ is the density of the solution, N is Avogadro's number, and η is the viscosity of the medium.

The observed frictional coefficient is related to both hydration and shape by

$$(f/f_0)_{min} = (f/f_0)_{shape}[1 + \delta/(\bar{v}\rho)]^{1/3} \quad (2)$$

where δ is the water of hydration in grams per gram of protein.

Viscometry. Viscosity measurements were carried out in an Ostwald viscometer with a flow time of 1250 s in 0.1 M acetate buffer. The flow times were measured with an electronic stopwatch coupled to photoelectric cell detectors. All measurements were conducted at 23 °C, under controlled temperatures with an external Haake circulator unit. Protein stock solutions, in the presence of hormones, were first dialyzed against the same hormones and then filtered.

The reduced viscosity was evaluated from these measurements by

$$\eta_{sp}/C = (\rho t - \rho_0 t_0)/(C\rho_0 t_0) \quad (3)$$

where t and t_0 and ρ and ρ_0 are respectively the outflow times and densities of solution and hormone alone. The corrections for the kinetic energy and surface tension effects were found negligible down to a concentration of 1 mg/mL (Rholam & Nicolas, 1981).

Nanosecond Spectroscopy. The time dependence of fluorescence intensity was determined by the single-photon technique [for reviews see Yguerabide (1972) and Wahl (1975)]. The exciting band was selected at 350 nm. The exciting ray is vertically polarized, and the decay curves of the parallel and perpendicular polarized fluorescence are measured many times and averaged. The data were analyzed as described by Wahl & Timasheff (1969). The recorded decay curves $i_{\parallel}(t)$ and $i_{\perp}(t)$ are used to calculate the $s(t)$ decay curve defined as

$$s(t) = i_{\parallel}(t) + 2i_{\perp}(t) \quad (4)$$

The early portion of a measured fluorescence decay $s(t)$ is distorted by the finite duration of the exciting lamp pulse $g(t)S(t)$, and the undistorted time course of emission is related to the above curve by the convolution integral (Wahl, 1969)

$$S(t) = \int_0^T g(T)s(t - T)\, dT \quad (5)$$

To determine fluorescence lifetimes, we used the modulation function method (Valeur, 1978) to obtain $S(t)$ from measured decays $s(t)$ and $g(t)$. If $S(t)$ is represented by the expression in eq 6, the deconvolution procedure yields a value of a_i and

$$S(t) = \sum_{i=1}^{n} a_i e^{-t/\tau_i} \quad (6)$$

τ_i. $S(t)$ was then convoluted with $g(t)$ (eq 5) to calculate a new function $C(t)$. Goodness of fit was determined by calculating χ^2 defined in eq 7a. The deviation, J, is defined in

$$\chi^2 = \sum[S(t) - C(t)]^2/C(t) \quad (7a)$$

eq 7b. Mean lifetimes were calculated by using the following

$$J^2 = \sum[S(t) - C(t)]/C(t)^{1/2} \quad (7b)$$

expression

$$\langle\tau\rangle = \sum_{i=1}^{n} a_i\tau_i^2/\sum_{i=1}^{n} a_i\tau_i \quad (8)$$

Steady-State Fluorescence Polarization. Steady-state polarization measurements were made with a double-photomultiplier, Series-400 polarization spectrofluorometer manufactured by SLM Instruments, Inc. (Urbana, IL). Each experimental point was the average of a least 100 sets of data. The fluorescence excitation was 350 nm; the emission wavelength was selected with Corning glass 3-72 filters. The temperature was maintained at 23 °C unless otherwise stated.

The steady-state anisotropy, $\bar{A}$, a measure of the average angle through which the probes rotate during their fluorescence lifetime τ, was calculated from eq 9 and 10. In these equations, R is the corrected steady-state polarization ratio, and I_{vv} and I_{vh} are the vertical and horizontal components of the emission, viewed at 90° to the vertically polarized excitation light. T is equal to I_{hv}/I_{hh}.

$$\bar{A} = (R - 1)/(R + 2) \quad (9)$$

$$R = (I_{vv}/I_{vh})T \quad (10)$$

Other Analyses. Solution densities, either in the presence or in the absence of hormonal ligands, were measured at 23 °C in a Mettler-Paar mechanical oscillator as described previously (Nicolas et al., 1976). The partial specific volumes of hormones, analogue, and unliganded or liganded bovine neurophysin II were calculated from $\bar{v} = \sum_{i=1}^{n} M_{r_i}\bar{v}_i/\sum M_{r_i}$ by using their known aminoacid composition.

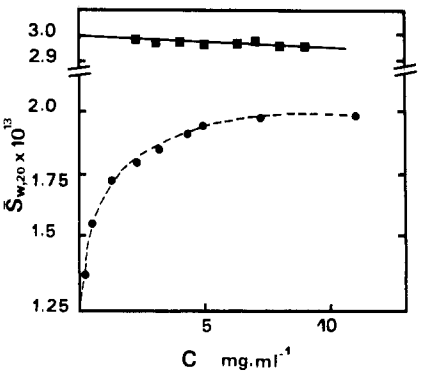

FIGURE 1: Sedimentation velocity of neurophysin. Concentration dependence of the weight-average sedimentation coefficient, corrected to water at 20 °C, of bovine neurophysin II in 0.1 M acetate buffer, pH 6.2, either in the absence (●) or in the presence (■) of 6 mM ocytocin. The dashed line represents the best fit of the experimental data, assuming a monomer–dimer equilibrium constant $K = 6000$ M^{-1} and $(s^0_{20,w})_D = 2.20$ S. The solid line drawn through the data points was fitted by linear-regression analysis.

Results

Sedimentation Velocity Studies. The weight-average sedimentation coefficient ($\bar{s}_{20,w}$) of neurophysin was determined in the absence of ligands by using the second-moment method (Goldberg, 1953) as a function of the corrected protein concentration ranging from 0.1 to 7 mg/mL (Figure 1). The sedimentation coefficient of the neurophysin II monomer $[(s^0_{20,w})_M]$ was found to be equal to 1.25 S by extrapolation of experimental data to infinite dilution. Analysis of the data in terms of a simple monomer–dimer equilibrium by using the method of nonlinear least squares showed (Figure 1) that this model fits the experimental points, assuming an equilibrium dimerization constant $K = 6 \times 10^3$ M^{-1} and a dimer sedimentation coefficient $[(s^0_{20,w})_D]$ equal to 2.20 S. Calculated Stokes radius and frictional and axial ratios of both the monomeric and the dimeric species are reported in Table I. Similar data have been previously reported (Rholam & Nicolas, 1981) and analyzed in terms of a side-by-side dimerization process of the highly asymmetrical monomers, resulting therefore in a reduced asymmetry.

A plot of the apparent sedimentation coefficient of neurophysin II in the presence of saturating amounts of ocytocin (6 mM) is shown in Figure 1. Under these conditions (ocytocin/neurophysin molar ratios varying from 10 to 20), the bis-liganded dimeric complex DL_2 is the only detectable species in solution even at the highest protein concentration tested (Nicolas et al., 1980). Linear extrapolation of the experimental data to infinite dilution yielded a sedimentation coefficient value of 3.00 ± 0.03 S for the DL_2 complex. However, on the assumption of only changes on the partial specific volume and molecular weight of neurophysin dimer with the binding of 2 mol of ocytocin (see Materials and Methods), an $s^0_{20,w}$ of 2.34 S is calculated for the bis-liganded dimer from

$$(s^0_{20,w})_{DL_2}{}^{calcd} = (s^0_{20,w})_D \frac{[M_r(1 - \bar{v}\rho)]_{DL_2}}{[M_r(1 - \bar{v}\rho)]_D} \quad (11)$$

where subscripts DL_2 and D are related respectively to the bis-liganded dimeric species and to the free dimer.

Table I: Hydrodynamic Parameters and Derived Axial Ratios of Neurophysins and Hormone Complexes

	monomer (M)	dimer (D)	liganded dimer (DL_2)
Sedimentation Velocity			
$s^0_{20,w}$ (S)	1.25	2.20	3.00
$(f/f_0)_{min}$	1.46	1.32	1.04
a/b[a]	5.25	3.55	≃1.0
$2a$[a] (Å)	98.4	95.5	42.4
$2b$[a] (Å)	18.7	26.9	42.4
Viscosity			
η (dL/g)	0.065	0.046	0.028
υ	5.97	4.22	2.57
a/b[a]	5.20	3.65	1.22
$2a$[a] (Å)	97.7	97.3	45.2
$2b$[a] (Å)	18.8	26.6	41.1
Fluorescence Polarization			
τ_1 (ns)	19.5	20.2	13.5
τ_2 (ns)	6.5	7.3	3.4
ρ_h (ns)	26.2	43.4	30.0
ρ_0 (ns)	13.2	26.4	29.5
ρ_h/ρ_0	1.99	1.62	1.02
a/b[a]	5.20	3.50	1.05
$2a$[a] (Å)	98.3	94.5	43.8
$2b$[a] (Å)	18.7	27.0	41.7

[a] A hydration of 0.38 g of H_2O/g of protein and a prolate ellipsoid model were assumed for these calculations.

The significant difference between this calculated value and the above observed values for the complex DL_2 indicates a decrease in the frictional ratio of the dimer upon ocytocin binding. From known values of the molecular weight and partial specific volume of ocytocin and neurophysin (see Materials and Methods), a Stokes radius of 21.2 Å and a frictional ratio $(f/f_0)_{min}$ = 1.04 were calculated (Table I), indicating that the effective hydrodynamic shape of the bis-liganded dimer cannot be far from a slightly hydrated compact sphere. Analysis of these data on the assumption of a hydration of 0.38 g of H_2O/g of protein (Nicolas et al., 1980) gave an axial ratio due to asymmetry of the dimeric complex $(a/b)_{DL_2} \simeq 1.0$. This value suggests that the binding of the nonapeptide ligand promotes a structural rearrangement within the dimer, resulting therefore in an increased symmetry.

Viscosity Studies. In order to provide further support for this hypothesis, we carried out viscosity studies of the neurophysin dimer in the presence of saturating amounts of an N-terminal tripeptide analogue of ocytocin, Cys(*S*-Me)-Tyr-Ile-NH_2. The small size of this ligand, when compared to that of the nonapeptide ocytocin, can allow us to detect any possible influence of the proper shape of the ligand tested on the effective hydrodynamic shape of the ligand-bound dimeric species.

A plot of the reduced viscosity η_{sp}/C vs. C of the bis tripeptide bound dimeric species is shown in Figure 2. For a nonassociating system, the concentration dependence of η_{sp}/C is described by

$$\eta_{sp}/C = (\eta) + k'(\eta)^2C + \ldots \quad (12)$$

where (η) is the intrinsic viscosity of the molecule and k' is the dimensionless Huggin constant. k' is a measurement of the concentration dependence between solute molecules (Bradbury, 1970) and is usually on the order of 10 or higher for associated rigid rods and 2 for spheres.

The linear representation obtained on Figure 2 is compatible with a single species in solution. Extrapolation of these data to infinite dilution, according to eq 12, gave a value of 0.028 dL/g for the intrinsic viscosity of the dimeric complex. From the deduced value of the intrinsic viscosity, it is possible to obtain an estimate of the axial ratio of the complex. The calculated Simha viscosity increment (Yang, 1963) and axial ratio of DL_2 are summarized in Table I. Hydrodynamic parameters of the complex, determined both from sedimentation velocity and viscosity studies suggest that the pseudospherical symmetry of the bis-liganded dimer is not the result of effects resulting from the shape of hormonal ligands.

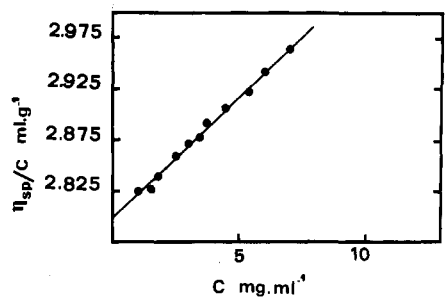

FIGURE 2: Concentration dependence of reduced viscosity of neurophysin II in 0.1 M acetate buffer, pH 6.2, at 23 °C in the presence of 6 mM Cys(*S*-Me)-Tyr-Ile-NH_2. The solid line represents the best fit of the experimental data, assuming a Huggin constant $k' = 3$.

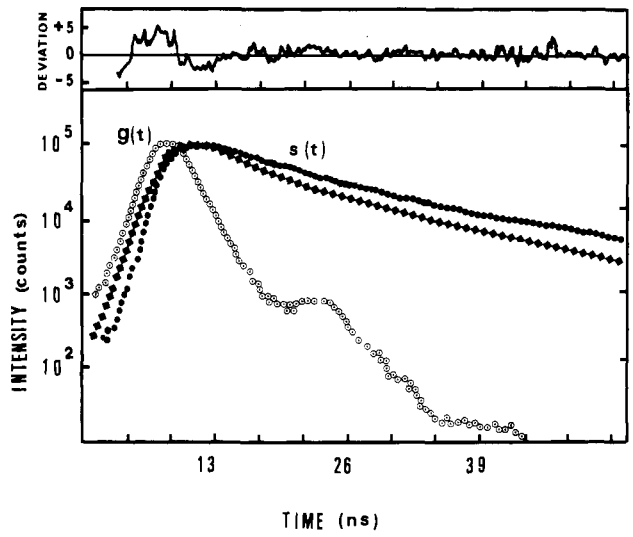

FIGURE 3: Decay curves $s(t)$ for Dns-labeled neurophysin II in the absence (●) and presence (♦) of 6 mM either ocytocin, vasopressin, or tripeptide analogue. The curve $g(t)$ is the flash response.

Nanosecond Spectroscopy Studies. Fluorescence lifetimes of dansylated neurophysin II, in the absence or in the presence of hormonal ligands, were measured under the same experimental conditions used for sedimentation and viscosity studies. The results, presented in Figure 3, showed that the deconvoluted fluorescence lifetime graphs were fit to the sum of two exponentials as described by the experimental procedure. These fitted deconvoluted curves were preferred because they gave lower χ^2 values. Data for dansylated neurophysin II alone (Table I) were similar to those previously reported at a different protein concentration (Rholam & Nicolas, 1981). The observation of two distinct lifetimes might be indicative of different microenvironments of the dansyl fluorophores attached to the protein.

Binding of ligands results in a faster decay of the deconvoluted curve $S(t)$ (Figure 3). Since the lifetime of DNS is very sensitive to its microenvironment (Stryer, 1968), the smaller lifetime values (Table I) can be interpreted, therefore, as resulting from a better exposure of residues to solvent upon ligands binding. Identical lifetime values (τ_1 = 3.5 ns and τ_2

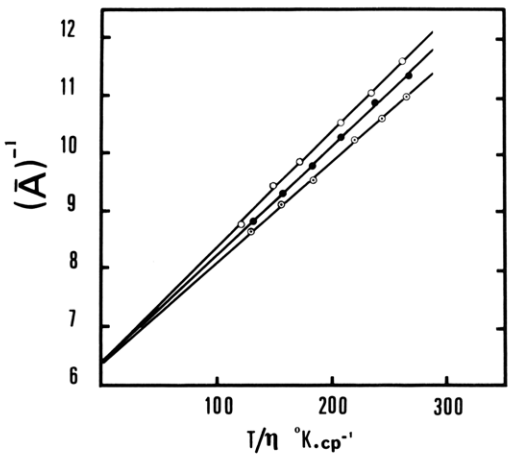

FIGURE 4: Perrin's plots of Dns-labeled neurophysin II in the presence of excess amounts of ocytocin (●), tripeptide (○), or vasopressin (⊕). Viscosity η was varied by adding sucrose to solutions at 23 °C. The solid lines represent the best fit of the data points.

= 13.4 ns) were found for dansyl groups of the complexes when the ligand is either ocytocin, vasopressin, or the tripeptide.

Steady-State Polarization Studies. The determination of the harmonic mean relaxation time (ρ_h) was based on Perrin's equation (Perrin, 1926):

$$\bar{A}^{-1} = A_0^{-1}(1 + 3\langle\tau\rangle/\rho_h) \quad (13)$$

where $\bar{A}$ is the observed average anisotropy, A_0 is the limiting anisotropy in the absence of Brownian rotation, and $\langle\tau\rangle$ is the fluorescence mean lifetime. The average anisotropy values were evaluated (eq 9) by varying the viscosity of the solution by addition of increasing amounts of sucrose. The dependence of $\bar{A}$ vs. T/η is plotted in Figure 4 according to Perrin's equation for the different complexes. Since the fluorescence mean lifetimes were found independent of viscosity, the anisotropy values reflected changes in the hydrodynamic volume of the complexes. Relaxation times of 32, 30, and 28.5 ns at 23 °C were calculated from the data in Figure 4 for neurophysin dimer upon vasopressin, ocytocin, and tripeptide bindings, respectively (Table I). With the assumption of a random orientation of chromophores on the protein (Weber, 1952, 1953), it is possible to calculate the axial ratio from

$$\rho_h = \rho_0 f(a/b) \quad (14)$$

where ρ_0 is the relaxation time of an equivalent sphere (Jablonski, 1961) and $f(a/b)$ is a function of the axial ratio, which may be calculated from Perrin's equations (Perrin, 1936). The (ρ_h/ρ_0) values for the three complexes (Table I), slightly greater than 1, are in accord with the hydrodynamic data deduced from sedimentation velocity and viscosity studies.

Discussion

The data obtained by various hydrodynamic methods clearly show changes in the neurophysin molecules upon ligand binding that can be analyzed (assuming a model for a rigid and impervious solid) in terms of a modification in the molecular dimensions and/or hydration of the dimeric species. The concomitant increase in $s^0_{20,w}$ and decrease in both reduced viscosity and rotational relaxation time would suggest that the bis-liganded dimer becomes more symmetric than the unliganded species. However, the interpretation in terms of shape requires the evaluation of a possible influence of both proper hydrodynamic shape of the ligands and change in hydration upon binding. Ocytocin and vasopressin are both disulfide-linked nonapeptide ligands of small size, but they are believed to possess different shapes and conformational properties in

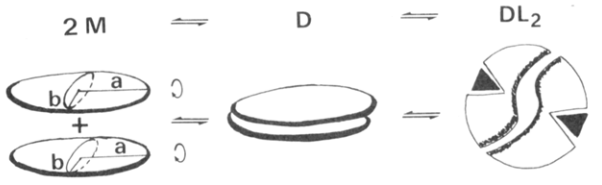

FIGURE 5: A diagrammatic representation of relative shape and asymmetry of neurophysin monomer (M), dimer (D), and bis-liganded dimer (DL_2).

aqueous solutions [for a discussion see Cohen et al. (1979)]. On the other hand, binding of the N-terminal tripeptide analogue of ocytocin, Cys(*S*-Me)-Tyr-Ile-NH_2, is accompanied by physicochemical changes that are also observed upon nonapeptide binding, indicating that their binding sites are identical (Breslow, 1979; Cohen et al., 1979). Similar values of axial ratio were obtained with either of the nonapeptide ligands or the tripeptide analogue. Hence, there is no evidence for any detectable effect of the proper shape of ligands on the asymmetry property of the bis-liganded neurophysin dimer. Therefore the profound alteration on hydrodynamic properties observed upon ligand binding should reflect structural modification and/or hydration changes of the dimer as the result of ligand-induced conformational changes rather than being a consequence of some particular geometrical arrangement of ligands within the dimeric complexes. Since frictional and axial ratio values are in each case only slightly greater than, or close to, 1.0, the effective hydrodynamic shape of the bis-liganded complexes cannot be far from a slightly hydrated compact sphere. Although a maximal hydration of 0.38 g of H_2O/g of protein was calculated from the amino acid composition of bovine neurophysin II (Nicolas et al., 1976), binding of hormonal ligands might produce some change in the hydration as a result of a modified degree of exposure of some residues. This could diminish the accuracy of a direct comparison between the shape of the bis-liganded vs. the unliganded dimeric species. The use of the recently described swelling-independent Λ function (Harding, 1980), which combines intrinsic viscosity and harmonic mean relaxation time, leads to a value of $\Lambda_{DL_2} = 2.54$ ($a/b = 1.0$) for the bis-liganded species compared to $\Lambda_{D_2} = 2.69$ ($a/b = 3.0$) for the unliganded one. These swelling-independent values indicate that, regardless of any minor undetectable hydration changes, the asymmetrical dimer acquires a pseudospherical symmetry upon ligand binding. Such a profound variation on the effective hydrodynamic shape of the dimeric complex upon ligand binding necessarily implies a variation of environment of some residues within the neurophysin structure. Previous spectroscopical experiments, in agreement with hydrodynamic data, suggest that the single tyrosine residue of neurophysin undergoes a reorientation upon binding (Griffin et al., 1973; Wolff et al., 1975) and that the disulfide links, as measured by CD, are perturbed (Griffin et al., 1973). On the other hand, Raman spectroscopy suggested an increase in the α-helix content of neurophysin upon binding (Liu, 1975). In this study, the modification in molecular dimensions is accompanied by a decrease in the two lifetimes, resulting in a variation in the degree of exposure of given residues to solvent (Dns-Ala and Dns-Lys). This variation ($\Delta\tau_1 = 3.5$ ns and $\Delta\tau_2 = 7$ ns) can be attributed, at least in part, to a structural reorganization of the protein rather than to a direct effect of ligands on the dansylated amino acids.

A representation model for the association pathway of neurophysin monomers (M) into a liganded dimer (DL_2) is shown in Figure 5. In this tentative scheme, the asymmetric dimer, formed by a side-by-side association process of highly

asymmetrical monomers, undergoes a conformational rearrangement upon ligand binding that leads to a slightly hydrated, compact, and spherical dimeric complex. Such a liganded-induced conformational change may also provide a basis for the structural interpretation of the mechanism by which occurs the intradimeric positive cooperativity between binding sites (Hope et al., 1975; Nicolas et al., 1978b; Tellman & Winzor, 1980). According to the above-proposed model, binding of the first molecule of the ligand would induce a conformational rearrangement (induced isomerization) leading to a pseudospherical monoliganded dimer. This, consequently, would enhance the affinity of the ligand for the second available binding site.

This model provides a clear example of a small protein containing a high proportion of disulfide links and that undergoes conspicuous changes in conformation under the influence of well-characterized ligands such as the nonapeptide hormones or a simple tripeptide.

These informations on the shape of bis-liganded neurophysin dimers may shed some light on the putative intragranular function of the complexes formed with the nonapeptide hormones. The biological relevance of these observations is not yet clear, but their significance can be tentatively envisioned in terms of the following main features: (i) dimerization decreases the osmolarity of the protein component; (ii) stabilization of the polypeptide chains of both the protein and the hormonal ligands results from a compact structure; (iii) reduction of transmembrane leakage of both neurosecretory compounds occurs as a result of an adequate Stokes radius (~21 Å) of the spherical complexes; (iv) the high concentration of the neurosecretory compounds, in the granule core, contributes to the presumed low solubility of the complexes [see Discussion in Gainer et al. (1977)].

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