Side-by-Side Dimerization of Neurophysin: Sedimentation Velocity, Viscometry, and Fluorescence Polarization Studies†

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ABSTRACT: The shape and asymmetry properties of both neurophysin monomer and dimer were assessed by a combination of sedimentation velocity, viscometry, and steady-state or time-dependent fluorescence polarization techniques in 0.1 M acetate buffer, pH 6.2, solutions at 23 °C. Data from hydrodynamic studies were analyzed with respect to possible models of self-association and in order to characterize the particular geometry of both monomer and dimer. With the assumption that the monomer is a prolate ellipsoid of revolution with a hydration coefficient of 0.38 g of H_2O/g of protein, average axial ratios (a/b) due to asymmetry were found to be equal to 5.2 and 3.6 for the monomer and dimer, respectively. The overall length $2a$ and the minor axis $2b$ were respectively equal to 98 and 19 Å for the monomeric species. The same calculations conducted about the dimeric species gave an overall length $2a$ = 96 Å and a minor axis $2b$ = 27 Å. These data strongly suggest that the association of the highly asymmetrical monomers results in a reduced asymmetry possibly as a consequence of a side-by-side process of dimerization. Neurophysin II was dansylated to the extent of 0.35 mol of dansyl group per mol of protein. This chemical modification was not accompanied by any significant change in either tertiary or quaternary structures as assessed by sedimentation equilibrium, sedimentation velocity, and radioimmunoassay. Fluorescence polarization studies on dansylated neurophysin II are not indicative of more than one rotational relaxation time either for the monomer or for the dimer. However, two different fluorescence lifetimes were detected with values, 19.9 and 6.9 ns, respectively, that were found to be independent of the protein concentration (from 0.1 to 11 mg mL^{-1}). This might be indicative of different environmental conditions of the fluorescent reporter group attached to the protein. The best-fit model representing fluorescence polarization results was found to be an elongated ellipsoid (axial ratio a/b = 5.0) undergoing a side-by-side process of dimerization, in good agreement with viscometry and sedimentation velocity analyses. Results are discussed mainly with respect to possible relationships between the hydrophobic protomer association areas and the particular geometry of both monomer and dimer.

Neurophysins have been the subject of numerous investigations, and efforts were made to define their physical properties in solution and to relate them to their capacities to form noncovalent specific complexes with ocytocin and vasopressin [for recent reviews, see Breslow (1979) and Cohen et al. (1979)]. The basic subunit of neurophysin consists of a single polypeptide chain (M_r = 10000) cross-linked by seven disulfide bonds (Capra et al., 1972). This single-chain subunit shows a marked tendency to self-associate in aqueous solution in the form of a dimer (Nicolas et al., 1976, 1978a, 1980a; Pearlmutter, 1979). Ligand-facilitated dimerization (Nicolas et al., 1976, 1978a,b, 1980a) together with half-of-the-sites reactivity properties (Nicolas et al., 1976, 1978a; Cohen et al., 1978, 1979; Lord & Breslow, 1978) are observed under conditions of preferential binding to the dimeric sites of neurophysins of the nonapeptide hormones or peptide analogues. These half-of-the-sites reactivity properties are believed to be related to the duplicated domains of internal sequence homology lying on both sides of the unique central tyrosine in position 49 of the protein sequence (Camier et al., 1973; Nicolas et al., 1976).

The knowledge of the shape and asymmetry properties of neurophysins under various states of aggregation and experimental conditions might provide information about the relationships between tertiary and quaternary structure. In this context, it is interesting to note that the formation of the neurophysin dimer does not result in profound conformational rearrangements of the monomer structure as judged solely by near-ultraviolet absorption spectroscopy (Nicolas et al., 1978a), tyrosyl fluorescence emission (Sur et al., 1979), and near-ultraviolet circular dichroism (Nicolas et al., 1980a). Moreover, only minor conformational changes are associated with the binding of the hormonal ligands as assessed by the same methodologies (Breslow, 1979; Cohen et al., 1979). In this investigation, we have used sedimentation velocity, viscometry, and steady-state or decay-time fluorescence polarization measurements to estimate the shape and asymmetry of neurophysin under various states of aggregation in 0.1 M acetate buffer, pH 6.2, at 23 °C. Since these techniques are extremely sensitive to changes in the axial ratio of the macromolecules (Wahl & Timasheff, 1969; Bradbury, 1970), results were interpreted mainly with respect to possible models of self-association and to a characterization of the geometry of dissociated and associated neurophysin monomers.

Materials and Methods

Neurophysins. Highly purified neurophysins I and II were prepared as previously described (Camier et al., 1973), by isoelectric focusing from acetone powder of freshly collected bovine pituitaries. The samples, routinely tested for lipid content, were found to contain no more than 0.5% by weight of glyceride derivatives as judged by gas chromatography analysis of the fatty acid methyl esters produced after alkaline hydrolysis of the neurophysins (Camier et al., 1976). Neurophysins were routinely tested for their homogeneity by using slab gel electrophoresis in the absence or presence of 0.1% sodium dodecyl sulfate, gel isoelectric focusing, and amino acid composition determination (Nicolas et al., 1976).

Preparation of Dansylated Neurophysin II. The dansyl (Dns) fluorescent group, 5-dimethylamino-1-naphthalene-

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sulfonate, 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 an 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 then dialyzed extensively against 0.1 M 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 absorption spectra of the labeled and unlabeled protein. The amount of label bound per neurophysin monomer was assessed from the protein concentration and from the Dns absorbance at 330 nm (Weber, 1953). It was found to be equal to 0.35 mol of Dns bound per neurophysin monomer. Thin-layer chromatography of the amino acids from dansylated neurophysin II hydrolysates confirmed the existence of ϵ-dansyllysine and dansylalanine as the chemically modified residues.

Ultracentrifugal Studies. Sedimentation equilibrium experiments were conducted with a Beckman Model E analytical ultracentrifuge and two compartment cells as previously described (Nicolas et al., 1976). Ultracentrifugations were performed at 23 °C and 30000 rpm for 5 days. The protein distribution was obtained by using Rayleigh interference optics. Data from these studies were analyzed by the meniscus-depletion method of Yphantis (1964) by plots of ln of protein concentration against r^2 with a Wang 720 calculator. The experiments were run on samples dialyzed for 24 h against 0.1 M acetate buffer, pH 6.2, at 5 °C. Sedimentation velocity experiments were carried out at 60000 rpm with a double-sector cell with sapphire windows. The weight-average sedimentation coefficient was evaluated from the second-moment method (Golberg, 1953) as previously described (Nicolas et al., 1980a,b). Frictional coefficient ratios, $(f/f_0)_{min}$ (Schachman, 1959; Tanford, 1961), were calculated from

$$(f/f_0)_{min} = \frac{M_r(1 - \bar{v}\rho_1)}{Ns^0_{20,w}6\pi\eta[3M_r\bar{v}/(4\pi N)]^{1/3}} \quad (1)$$

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 the protein, and ρ_1 is the density of the solution.

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

$$(f/f_0)_{min} = (f/f_0)_{shape}[1 + \delta_1/(\bar{v}\rho_1)]^{1/3} \quad (2)$$

where δ_1 is the water of hydration in grams of H_2O per grams of protein.

Viscosity Measurements. An Ostwald-type viscometer with a long capillary (200 cm) was used for viscosity measurements at 23 ± 0.01 °C. The viscometer stand and photoelectric cells holder were constructed in such a fashion that the viscometer was always situated in the same position in the bath. Viscosity measurements were made on 2.5-mL samples. The flow time for the solvent was 1250 s, measured with an electronic stopwatch coupled to the photoelectric cell detectors. Six to ten replicate readings, reproducible to within 0.1 s, were made for each protein solution obtained by automatic serial dilution of the most concentrated sample. Protein stock solutions were dialyzed extensively against acetate buffer, pH 6.2, and centrifuged at 30000g for 60 min to remove particulate material. The supernatant was then carefully withdrawn and filtered through 0.22-μm Millipore filters. The reduced viscosity was obtained from these measurements with

$$\eta_{sp}/C = \frac{t - t_0}{Ct_0} + \left(\frac{1}{\rho_0}\right)\left(\frac{\delta\rho}{\delta C}\right)^0_\mu\left(\frac{t}{t_0}\right) \quad (3)$$

where t, t_0 and ρ, ρ_0 are respectively the outflow times and densities of solution and solvent.

The corrections for the kinetic energy and surface tension effects were found to be negligible down to 2 mg/mL protein. The reduced viscosity vs. concentration profile was analyzed by a least-squares computer program in order to evaluate the intrinsic viscosity of each species.

Fluorescence Polarization Studies. The fluorescence decay-time experiments were carried out in 0.1 M acetate buffer, pH 6.2, at 23 °C on a fluorescence polarization spectrophotometer, using an exciting flash of short duration. The principle of this instrument is based on the single photoelectron method (Pfeffer, 1965; Lami et al., 1966). The exciting band was selected at 350 nm. The exciting ray is vertically polarized, and the decay curves of the parallel and perpendicular polarized fluorescences are measured alternately many times and averaged. The data were analyzed as described by Wahl & Timasheff (1969). Briefly, the recorded decay curves $i_\parallel(t)$ and $i_\perp(t)$ are used to calculate the sum $s(t)$ and difference $d(t)$ decay curves defined as

$$s(t) = i_\parallel(t) + 2i_\perp(t)$$
$$d(t) = i_\parallel(t) - i_\perp(t) \quad (4)$$

The shapes of the $s(t)$ and $d(t)$ curves are related to the decay curves $S(t)$ and $D(t)$ which would be obtained with an ideal infinitely short flash by (Wahl, 1969)

$$s(t) = \int_0^t g(t')S(t - t')\,dt'$$
$$d(t) = \int_0^t g(t')D(t - t')\,dt' \quad (5)$$

where $g(t')$ is the apparatus response to the exciting flash.

The decay curve of the emission anisotropy, $R(t)$, is obtained from the ratio of the two decay curves

$$R(t) = D(t)/S(t) \quad (6)$$

The shape of the $R(t)$ curve is a function of size, shape, and structure of the fluorescent molecule. For the general case of symmetrical ellipsoids of revolution (Perrin, 1936)

$$R(t) = R_0\sum_i \alpha_i e^{-3t/\rho_i} \quad (7)$$

where ρ_i is the relaxation time of the molecule.

With the assumption of random orientation of the fluorescent groups on the protein molecule (Weber, 1952), it is possible to calculate the harmonic mean of the relaxation times, ρ_h, directly from the initial slope of the $R(t)$ curve:

$$\rho_h = \rho_0 f(p) \quad (8)$$

where ρ_0 is the relaxation time of an equivalent sphere and $f(p)$ is a function of the axial ratio p, $p = a/b$, which may be calculated from Perrin's equations (Perrin, 1936). Fluorescent excited lifetimes, τ, were measured from $S(t)$ decay curves as described by Wahl (1969). Steady-state fluorescence polarization measurements were made with an SLM 400 double photomultiplier polarization spectrofluorometer. Dns-labeled neurophysin II was excited at 350 nm in 0.1 M acetate buffer, pH 6.2, at 23 °C. The determination of rotational relaxation times, ρ_h, was based on Perrin's equation (Perrin, 1926):

$$1/p - 1/3 = (1/p_0 - 1/3)(1 + 3\tau/\rho_h) \quad (9)$$

where p is the observed polarization, p_0 is the limiting polarization in the absence of Brownian rotation, τ is the fluorescence lifetime, and ρ_h is the rotational relaxation time.

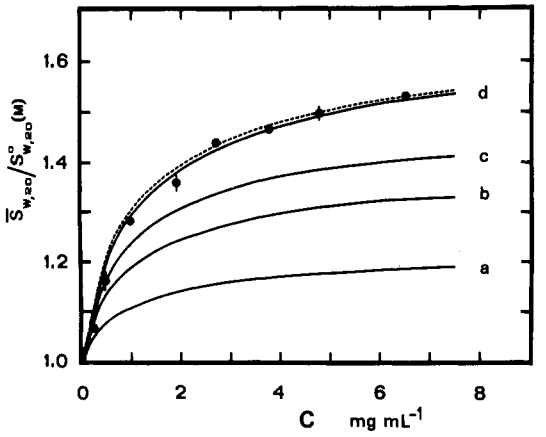

FIGURE 1: Sedimentation velocity of bovine neurophysin II. Concentration dependence of the reduced weight-average sedimentation coefficient corrected to water at 20 °C, $\bar{s}_{20,w}/s^0_{20,w}$(M), of bovine neurophysin II in 0.1 M acetate buffer, pH 6.2. Each point was made in triplicate, and the protein concentrations were corrected for radial dilution. The lines represent the theoretical variation of $\bar{s}_{20,w}/s^0_{20,w}$(M) vs. C for a monomer–dimer equilibrium with an equilibrium association constant K = 6000 M^{-1}, $(s^0_{20,w})$, = 1.25 S, and $(f/f_0)_{min,M}$ = 1.46, for which association of elongated monomers into a dimer occurs by means of (a) an end-to-end process (fraction of overlap E = 0), (b) a side-by-side process (fraction of overlap F = 0), (b) a side-by-side process (F = 0.50), (c) a side-by-side process (F = 0.75), and (d) a side-by-side process with F = 1.0. The axial ratio of dimer was calculated for each of the proposed models from the relationships described in the text. They were equal to (a) 10.5, (b) 6.8, (c) 5.2, and (d) 3.7. 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 limiting polarization p_0 was determined from Perrin's plot of $(1/p - 1/3)$ vs. η/T.

Other Analyses. The solvent density was measured at 23 °C in a Mettler-Paar mechanical oscillator as described previously (Nicolas et al., 1976). The partial specific volume of both native and dansylated neurophysin II was 0.709 as calculated from the amino acid composition. Radioimmunoassays were carried out as previously reported (Camier et al., 1979; Nicolas et al., 1980b).

Results

Sedimentation Velocity Studies. Figure 1 shows the concentration dependence of the reduced weight-average sedimentation coefficient, $\bar{s}_{20,w}/s^0_{20,w}$(M), of bovine neurophysin II in 0.1 M acetate buffer, pH 6.2, at 23 °C. The sedimentation coefficient of the monomeric species at $C \rightarrow 0$, $s^0_{20,w}$(M), was found to be 1.25 S. Direct analysis of the experimental data in terms of a simple monomer–dimer equilibrium by a polynomial and by using the method of least squares showed that this model fit the data, assuming a monomer–dimer equilibrium constant $K = 6 \times 10^3$ M^{-1} and $s^0_{20,w}(D)$ = 2.20 S (Figure 1). From known values of the molecular weight and partial specific volume, a Stokes radius of 20.6 Å and a frictional ratio $(f/f_0)_{min}$ = 1.46 were calculated for the neurophysin II monomer, indicating a highly asymmetrical shape and/or hydration. Analysis of these data, assuming that the monomer is a prolate ellipsoid of revolution with a hydration of 0.38 g of H_2O/g of protein (Nicolas et al., 1980a,b), gave an axial ratio due to asymmetry of the monomer, $(a/b)_M$, of 5.25. The same calculations conducted about the dimeric species gave a Stokes radius of 23.3 Å, a frictional ratio $(f/f_0)_{min}$ = 1.32, and an axial ratio $(a/b)_D$ = 3.55. By using the known relationships for the volume of an ellipsoid, we calculated values of $2a$ (the major axis of revolution) and $2b$ (the minor axis) both for monomer and for dimer (Table I). These values suggest that the association of asymmetrical monomeric units results in a reduced asymmetry as a consequence of a side-by-side process of dimerization.

Table I: Hydrodynamic and Asymmetry Properties of Bovine Neurophysin II. Sedimentation Velocity and Viscosity Measurements

	monomer	dimer
Sedimentation Velocity Data		
$s_{20,w}$ (S)	1.25	2.20
$(f/f_0)_{min}$	1.46	1.32
a/b[a]	5.25	3.55
$2a$ (A)	98.4	95.5
$2b$ (A)	18.7	26.9
Viscosity Data		
(η) (dL g^{-1})	0.065	0.046
ν	5.97	4.22
a/b[a]	5.20	3.65
$2a$ (A)	97.7	97.3
$2b$ (A)	18.8	26.6

[a] A hydration of 0.38 g of H_2O/g of protein and a prolate ellipsoid model were assumed. The dimensionless Huggin's constant (k') was assumed to equal 0.4.

In order to provide further support for this hypothesis, theoretical curves of $\bar{s}_{20,w}/s^0_{20,w}$(M) vs. C were generated from some representative models for the association of neurophysin monomers by end-to-end or side-by-side processes, differing in the fraction of overlap, F, of the monomers in the dimer structure (Figure 1). By assuming that no significant changes of shape accompany association of neurophysin monomers (see the introduction) and by minimizing molecular overlap, we calculated the expected $\bar{s}_{20,w}/s^0_{20,w}$(M) vs. C curves for each case according to

$$\frac{\bar{s}_{20,w}}{s^0_{20,w}} = f_M + 2^{2/3}\frac{(f/f_0)_{min,M}}{(f/f_0)_{min,D}}(1 - f_M) \qquad (10)$$

where f_M is the molar fraction of monomer.

The frictional ratio of the dimer, $(f/f_0)_{min,D}$, may be related to the axial ratio $(a/b)_D$ and to the overlap fraction of the monomer inside the dimer by the following equation (Burke & Harrington, 1972):

$$(a/b)_D = (a/b)_M(2 - F)\left(\frac{2 - F}{2}\right)^{1/2} \qquad (11)$$

where F is the overlap fraction, ranging from 0 (in the case of an end-to-end process of dimerization) to 1 (when a side-by-side process of dimerization occurs).

The semimajor axis of revolution, a_D, and the semiminor axis, b_D, of the dimer may be related to the fraction of overlap F by

$$a_D = a_M(2 - F)$$
$$b_D = b_M\left(\frac{2}{2 - F}\right)^{1/2} \qquad (12)$$

where a_M and b_M are respectively the semimajor and semiminor axes of the monomer.

The experimental data were consistent with a side-by-side dimerization process of the asymmetrical monomers with a fraction of overlap close to 1.0 (Figure 1, curve d). Furthermore, the predicted values of $2a_D$ = 98.4 Å and $2b_D$ = 26.4 Å for a side-by-side model with F = 1 were in close agreement with those deduced from a direct analysis of the experimental data (Table I).

Viscosity Studies. For a nonassociating system, the concentration dependence of η_{sp}/C is generally well fitted by a virial expansion such as

$$\eta_{sp}/C = (\eta) + k'(\eta)^2C + \ldots \quad (13)$$

where C is the protein concentration (in grams per deciliters) (η) is the intrinsic viscosity of the protein, and k' is the dimensionless Huggins' constant, which is a measure of the concentration dependence. It is usually on the order of 2 for spheres, 0.5 for rigid rods, and about 10 for associated rigid rods (Bradbury, 1970).

In the particular case of a monomer–dimer equilibrium, the reduced viscosity can be expressed as the sum of the specific viscosities of each species weighted by their respective concentration:

$$\eta_{sp}/C = [(\eta)_M + k'(\eta)_M^2C_M]f_M + [(\eta)_D + k'(\eta)_D^2C_D](1 - f_M) \quad (14)$$

where C_M, C_D and f_M, $(1 - f_M)$ represent the concentration (in grams per deciliter) and the weight fraction of monomer and dimer, respectively.

The weight fraction of monomer is defined as

$$f_M = \frac{[(1 + 8KC/M_1)^{1/2} - 1]M_1}{4KC} \quad (15)$$

where K and M_1 represent the dimerization equilibrium constant on a molar basis and the molecular weight of the monomer, respectively.

The equations show that if the asymmetry of the dimer is greater than that of the monomer, the η_{sp}/C plot will increase with increasing concentration with a slope greater than $k'(\eta)_M^2$ as $C \rightarrow 0$ and with a slope of $k'(\eta)_D^2$ as $C \rightarrow \infty$. On the other hand, if the asymmetry of the dimer is reduced compared to that of the monomer, the η_{sp}/C vs. C curve will decrease with increasing concentrations with an initial slope smaller than $k'(\eta)_M^2$ and then increase with a limiting slope of $k'(\eta)_D^2$ as $C \rightarrow \infty$.

Plots of the reduced viscosity η_{sp}/C vs. C of bovine neurophysin II in 0.1 M acetate buffer, pH 6.2, at 23 °C are presented in Figure 2. The reduced viscosity slowly decreases with increasing initial protein concentration, as expected for a rapid reversible dimerizing system in which the association of monomer results in a reduced asymmetry. The intrinsic viscosity of neurophysin monomer and dimer could not be obtained by extrapolation of the η_{sp}/C data to infinite dilution since the low values of both the dimerization constant (K = 6000 M^{-1}) and the apparent intrinsic viscosities would lead to unreliable results. The experimental data were then fitted by a least-squares-treatment computer program (see Material and Methods) in which K, η_{sp}/C, and C were used over the entire range of concentrations studied to estimate $(\eta)_M$ and $(\eta)_D$. Values of $(\eta)_M$ and $(\eta)_D$ which best represent the experimental data are in Table I. The simulated curve of η_{sp}/C vs. C generated from this set of calculated data is shown in Figure 2. Furthermore, a linear extrapolation of the variation of η_{sp}/C vs. f_M, the weight fraction of monomer, to $f_M = 0$ and $f_M = 1$ (insert of Figure 2) yielded values of $(\eta)_M$ = 0.067 dL g^{-1} and $(\eta)_D$ = 0.046 dL g^{-1}, respectively. The linear representation obtained was compatible with a monomer–dimer equilibrium with an equilibrium association constant of 6000 M^{-1}.

From the deduced values of the intrinsic viscosities of the dimer and monomer and their molecular weights, it was possible to obtain an estimate of the axial ratio and length of each species by assuming that their hydrodynamic properties can be mimicked by an equivalent prolate ellipsoid of revolution. In that case

$$(\eta) = \nu(\bar{v}_2 + \delta_1\bar{v}_1) \quad (16)$$

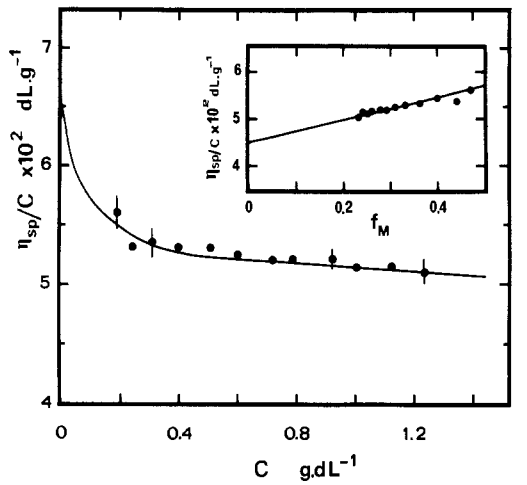

FIGURE 2: Concentration dependence of the reduced viscosity of bovine neurophysin II solutions in 0.1 M acetate buffer, pH 6.2, at 23 °C. Bears represent experimental variations obtained from six to ten replicate readings. The smooth line represents a computer simulation of η_{sp}/C vs. concentration plots for a monomer–dimer equilibrium with parameters given in Table I. The Huggin's constant, k', is assumed to be 0.4 both for monomer and for dimer. The inset shows the variation of the reduced viscosity vs. f_M, the weight fraction of monomer, assuming an equilibrium association constant of K = 6000 M^{-1}.

where ν, $\bar{v}_2$, and $\bar{v}_1$ are the Simha viscosity increment, the partial specific volume of the protein, and the partial specific volume of the solvent, respectively.

The axial ratio (a/b) of a prolate ellipsoid is then related to ν by the Simha relation (Doty et al., 1956);

$$\nu = (a/b)^2\left\{\left[15\left(\ln\frac{2a}{b} - 3/2\right)\right]^{-1} + \left[5\left(\ln\frac{2a}{b} - 1/2\right)\right]^{-1}\right\} + 14/15 \quad (17)$$

The calculated a/b ratios and viscosity increments ν of neurophysin II monomer and dimer are given in Table I, assigning a value of 0.38 g of H_2O/g of protein for the hydration coefficient δ_1 (Nicolas et al., 1980a,b). These values of the axial ratio for the monomer and the dimer deduced from viscosity measurements were in excellent agreement with those determined from sedimentation velocity studies. Furthermore, values of a (the semimajor axis of revolution) and b (the semiminor axis) were calculated for each species by using the known relationships for the volume of an ellipsoid. These values led to an average overall length $2a$ equal to 97 Å both for the monomer and for the dimer (Table I). Thus, the particular geometry of the dimer can be established by assuming no significant changes of shape of neurophysin upon dimerization and by assigning a mean diameter (d) of $2^{1/2}x$ (monomer) to neurophysin dimer (see Sedimentation Velocity Studies). The values obtained for both the overall length $2a$ and the minor axis $2b$ of the dimer compared to those of the monomer (Table I) are consistent with a side-by-side process of dimerization with a fraction of overlap F close to, or equal to, 1.

Fluorescence Polarization Studies. Physicochemical Properties of Dansylated Neurophysin II. Conformational changes induced in neurophysin II by dansylation proved to be minor as assessed by the following: (i) Sedimentation equilibrium conducted with Dns-neurophysin II in 0.1 M acetate buffer, pH 6.2, at 23 °C led to the same profile of $\bar{M}_w^{app}$ vs. C as that of unmodified neurophysin II (Figure 3). Analysis of the experimental data by the means of a least-squares computer program (Nicolas et al., 1980a,b) was in-

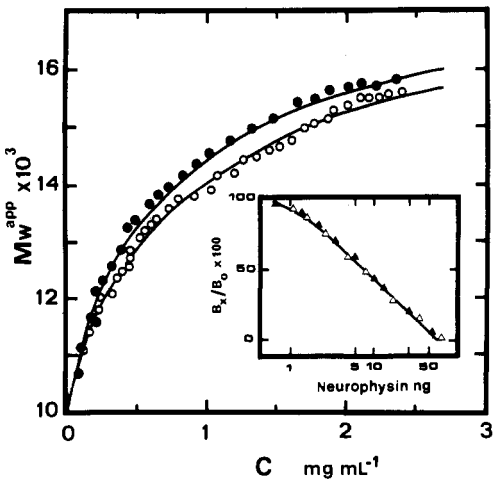

FIGURE 3: Variation of the apparent weight-average molecular weight ($\bar{M}_w^{app}$) of bovine neurophysin II (○) and dansylated neurophysin II (●) as a function of protein concentration from meniscus-depletion sedimentation equilibrium at 30 000 rpm in acetate buffer, pH 6.2, $\mu = 0.1$ (initial loading concentration 0.7 mg/mL). The smooth curves were drawn from the equilibrium constants calculated from a least-squares computer program (see text) for the ideal monomer–dimer model; (○) $K = 5.8 \times 10^3$ M^{-1}; (●) $K = 7.3 \times 10^3$ M^{-1}. The inset shows the comparison of the cross-reactivity of reference native neurophysin II (△) and of the dansylated neurophysin II (▲) as studied by radioimmunoassays using neurophysin antiserum S II04 and ^{125}I-labeled Tyr-49-neurophysin II as tracer (Camier et al., 1979).

dicative of an ideal monomer–dimer equilibrium with an equilibrium association constant of $K = 7.3 \times 10^3$ M^{-1}. This value is of the same order of magnitude as that of native neurophysin II [$K = (5.8–6 \times 10^3$ M^{-1}]. (ii) Sedimentation velocity experiments conducted with native or dansylated neurophysin II showed no significant difference of the sedimentation coefficient of both species at any concentrations tested. (iii) Parallel binding to neurophysin II antibodies is observed (Figure 3) for both native and dansylated neurophysin II by means of radioimmunoassay.

Steady-State Fluorescence Polarization Measurements. Since neurophysin shows a marked tendency to self-associate in aqueous solution, the principle of additivity of anisotropies (Weber, 1952) was used to relate the polarization measurements to the state of association. The average anisotropy of a mixture of components, $\bar{A}$, equals the sum of the anisotropy of each component weighted by their fractional contribution to the total fluorescence intensity:

$$\bar{A} = \sum_i \left\{ f_i \left[A_{0,i} \Big/ \left(1 + \frac{3\tau_i}{\rho_i} \right) \right] \right\} \qquad (18)$$

where ρ_i is the rotational relaxation time, τ_i is the excited state lifetime, and $A_{0,i}$ is the anisotropy corresponding to the limiting polarization, P_0, of each species i. $\bar{A}$ is linked to the observed polarization p by

$$\bar{A} = (2/3)(1/p - 1/3)^{-1} \qquad (19)$$

In the case of a monomer–dimer system:

$$\bar{A} = A_M f_M + A_D f_D = (A_M - A_D) f_M + A_D \qquad (20)$$

where f_M and f_D are the weight fractions of monomer and dimer, respectively. A plot of $\bar{A}$ vs. f_M should be linear with the intercept equal to A_D and the slope equal to $A_M - A_D$.

Figure 4 shows the increase of the average anisotropy $\bar{A}$ with increasing protein concentration from 0.1 to 6 mg/mL. The resulting curve is consistent with a reversible association of neurophysin monomers. When the data were plotted in keeping with eq 20, a linear dependence was found (inset of Figure 4) which confirmed a monomer–dimer equilibrium with an equilibrium association constant of $K = 7.3 \times 10^3$ M^{-1}. The values of monomer and dimer anisotropy are given in Table II. Since the fluorescence lifetimes (19.9 and 6.9 ns) were found to be independent of the protein concentration (see Fluorescence Polarization Decay-Time Measurements), the polarization values were a function of the rotational relaxation time and the limiting anisotropy of each species. Limiting anisotropies of $A_0(M) = 0.207$ for the monomer and $A_0(D) = 0.279$ for the dimer were determined from the intercept of a plot of $\bar{A}$ vs. η/T determined from eq 7 and are reported in Table II. Table II also gives the values for the minimum rotational relaxation time, ρ_0, calculated for equivalent spheres of the monomer and the dimer. From these data, axial ratios (a/b) for equivalent prolate ellipsoids were found to be equal to 5.2 and 3.5, respectively, for neurophysin monomer and dimer (Weber, 1953). These values are in good agreement with those obtained from sedimentation velocity and viscosity measurements. They also led to the same calculations and conclusions about the shape and geometry of the monomeric

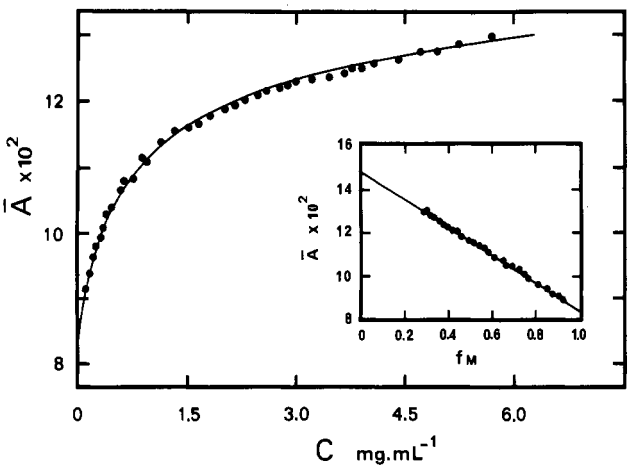

FIGURE 4: Concentration dependence of the average anisotropy $\bar{A}$ for Dns-labeled neurophysin II in 0.1 M acetate buffer, pH 6.2, at 23 °C. The inset shows data plotted in accord with eq 2. Line drawn through the data points was fitted by a linear least-squares computer program, assuming an equilibrium association constant of $K = 7.3 \times 10^3$ M^{-1}.

Table II: Fluorescence Lifetimes, Rotational Relaxation Times, and Axial Ratios of Dns-Labeled Neurophysin II Monomer and Dimer in 0.1 M Acetate, pH 6.2, at 23 °C

	Dns-labeled neurophysin II	
	monomer	dimer
A	0.084	0.147
A_0	0.207	0.279
τ_1[a] (ns)	19.5	20.2
τ_1	0.079	0.074
τ_2 (ns)	6.5	7.3
γ_2	0.272	0.244
$\langle\tau\rangle$[b] (ns)	12.5	12.8
ρ_h (ns)	26.2 (25.0)[c]	43.4 (42.0)[c]
ρ_0 (ns)	13.2	26.4
ρ_h/ρ_0 (ns)	1.99 (1.90)	1.62 (1.59)
a/b[d]	5.20 (4.75)	3.50 (3.13)
$2a$ (Å)	98.3 (91.9)	94.5 (87.7)
$2b$ (Å)	18.7 (19.3)	27.0 (28.0)

[a] τ_1, τ_2, and γ_1, and γ_2 are the time constants and the coefficients, respectively, that best fit the experimental data of the $S(t)$ curves. [b] Mean decay time constant. This value was calculated from $\langle\tau\rangle = (\Sigma x_i\tau_i^2)/(\Sigma x_i\tau_i)$ where $x_i = \gamma_i/\Sigma\gamma_i$. [c] Values in parentheses are obtained from decay-time fluorescence polarization measurements. [d] A hydration of 0.38 g of H_2O/g of protein and a prolate ellipsoid model were assumed.

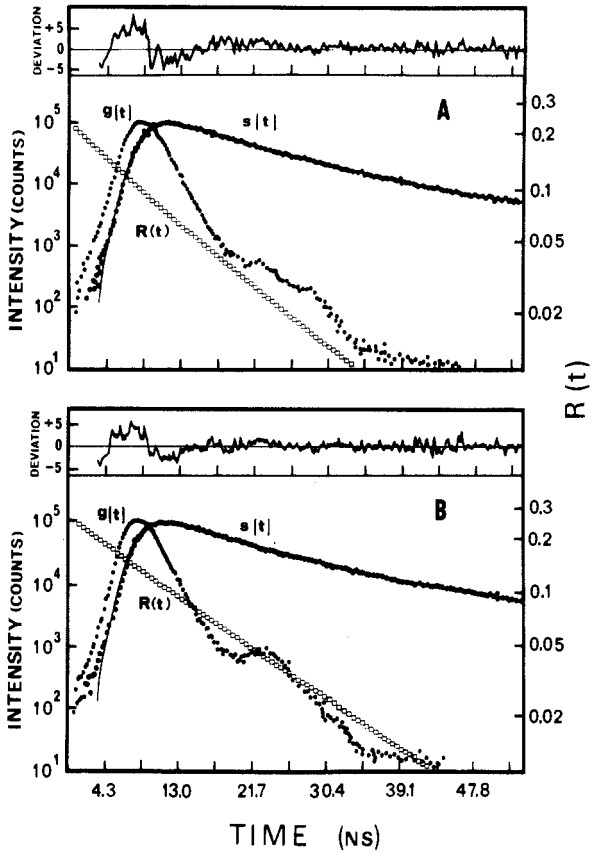

FIGURE 5: Decay curves $s(t)$ (●) and $R(t)$ (□) for (A) the monomer and (B) the dimer. The solid lines were calculated as described under Materials and Methods. Anisotropy of the monomer and the dimer is shown in a semilogarithmic scale by the $R(t)$ curves. The dotted lines are the flash response, $g(t)$.

and dimeric species (Table II).

Fluorescence Polarization Decay-Time Measurements. Fluorescence lifetimes of dansylated neurophysin II were measured at various initial concentrations in 0.1 M acetate buffer, pH 6.2, at 23 °C. The results of typical experiments carried out at conditions where either monomer or dimer predominate are presented in Figure 5. Analysis of the $S(t)$ curves showed two lifetimes (Table II), the values of which were found to be independent of protein concentration (from 0.1 to 11 mg/mL). This might be indicative of different microenvironments of the reporter groups attached to the protein. The average anisotropy, $R(t)$, of dansylated neurophysin II was calculated, giving the curves shown on a semilogarithmic scale in Figure 5, under conditions where the monomer or the dimer predominate (i.e., 0.4 and 8 mg/mL). Except at very short times, these curves are linear with slopes corresponding to apparent relaxation times of 33.3 and 39.9 ns, respectively, for 0.4 and 8 mg/mL protein. At both concentrations studied, the intercepts of the curves are different. This is indicative of different limiting anisotropy values for the monomeric and the dimeric species, as detected previously. The values of monomer and dimer molecular asymmetry (Table II) obtained from extrapolation to $f_M = 1$ and $f_M = 0$ of the experimental harmonic mean relaxation times and limiting anisotropies are for the rotation of the whole kinetic units and within the model of an ellipsoid of revolution. Thus, the side-by-side process of dimerization was independently confirmed by the dynamic fluorescence polarization measurements.

Discussion

The present data, interpreted in terms of an equivalent prolate ellipsoid model, strongly suggest that the monomer is highly asymmetrical with an axial ratio, due to asymmetry, close to 5. The association of these asymmetrical monomers to a dimer results in reduced asymmetry, possibly as a consequence of a side-by-side process of dimerization. From the numerous geometric arrangements considered for the association process, that which most likely describes the structure of neurophysin involves the formation of dimer through side-by-side association of highly asymmetric monomers.

It must be kept in mind that if the inferences about changes in molecular asymmetry when comparing monomer to dimer are correct, the information about the particular shape of both the neurophysin monomer and the dimer is essentially qualitative. Interpretation of results in terms of the molecular structure must be accepted with some caution since they involve a number of assumptions. The most important assumptions are a hydration value and an arbitrary choice of a model (prolate or oblate ellipsoids). In our case, the choice of a prolate model has been considerably facilitated since the Scheraga–Mandelkern coefficient (Scheraga & Mandelkern, 1953) was equal to 2.24×10^6 and 2.20×10^6 for the monomer and the dimer, respectively.[1] These values excluded the choice of an oblate model in both cases. On the other hand, a hydration value of 0.38 g of H_2O/g of protein was calculated (Nicolas et al., 1980a) from the amino acid composition of both neurophysins I and II. This value represents a maximal hydration since unknown buried amino acid side chains could not be taken into account in such a calculation. Although calculated values of hydration are generally in good agreement with experimental determinations, the deduced axial ratios might be underestimated for both the monomeric and dimeric species by the use of a possibly overestimated factor of hydration.

Neurophysin dimerization constants are small (Nicolas et al., 1976, 1980a) and cannot account for a strongly stabilized dimeric species in solution. Moreover, recent studies have shown that dimerization may involve mainly hydrophobic interactions (Camier et al., 1976; Cohen et al., 1979; Pearlmutter, 1979; Nicolas et al., 1980b), and it was proposed that the protomer association areas may involve the conservative common sequence of neurophysins (Nicolas et al., 1980a,b). Thus, it is tentative to think that a side-by-side model of the dimerization process must be satisfied by numerous weak interactions lying on a large area of each associated protomer. In contrast, an end-to-end process of dimerization would need strong interactive forces bringing together a limited area of the dimer.

The observation of two distinct lifetimes for the fluorescent probe appeared independent of the state of polymerization of the protein. This should be taken as indicative of the heterogeneity of the microenvironment of the dansyl moieties. The relative contributions of τ_1 and τ_2 to the emission were respectively 23 and 77%. According to Yguerabide et al. (1970) and Zagyansky et al. (1969), it can be hypothesized that the shorter constant ($\tau_2 = 7$ ns) may correspond to the emission of the solvent-accessible residues while the longer ($\tau_2 = 20$ ns) reveals groups excluded from the solvent, influencing, for example, those located in hydrophobic regions of the molecule.

The results obtained at various concentrations of protein

[1] The use of the recently described swelling-independent Λ function (Harding, 1980) which combines intrinsic viscosity and harmonic mean relaxation data leads to values of $\Lambda_M = 3.16$ ($a/b = 4.3$) and $\Lambda_D = 2.69$ ($a/b = 3.0$). These swelling-independent values strictly exclude the choice of an oblate ellipsoid model in both cases and independently reinforce the hypothesis of a side-by-side process of association.

show that the parameters, X_i and τ_i, remain constant; this suggests that the Dns environment is not located at the dimerization domains.

More sophisticated methods such as small-angle X-ray scattering and neutron diffusion are clearly needed in order to gain more information about the shape and geometry of each species in solution. Such studies are under way in this laboratory. However, the above reported results provide a basic working model which would prove to be useful to examine the conformational changes which occur upon ligand binding and environmental perturbations (Breslow, 1979; Cohen et al., 1979).

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References

Bradbury, J. H. (1970) in *Principles and Techniques of Protein Chemistry* (Leach, S. J., Ed.) pp 100–145, Academic Press, New York.

Breslow, E. (1979) *Annu. Rev. Biochem. 48*, 251.

Burke, M., & Harrington, W. F. (1972) *Biochemistry 11*, 1456.

Camier, M., Alazard, R., Cohen, P., Pradelles, P., Morgat, J. L., & Fromageot, P. (1973) *Eur. J. Biochem. 32*, 207.

Camier, M., Nicolas, P., & Cohen, P. (1976) *FEBS Lett. 67*, 137.

Camier, M., Lauber, M., Möhring, J., & Cohen, P. (1979) *FEBS Lett. 108*, 369.

Capra, J. D., Kehoe, J. M., Kotelchuck, O., Walter, R., & Breslow, E. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 431.

Cohen, P., Nicolas, P., Wolff, J., & Camier, M. (1978) in *The Cell Biology of Hypothalamic Neurosecretory Processes* (Kordon, C., & Vincent, J. D., Eds.) pp 543–560, CNRS, Paris.

Cohen, P., Nicolas, P., & Camier, M. (1979) *Curr. Top. Cell. Regul. 15*, 263.

Doty, P., Bradbury, J. H., & Holtzer, A. M. (1956) *J. Am. Chem. Soc. 78*, 947.

Goldberg, R. J. (1953) *J. Chem. Phys. 57*, 194.

Harding, S. E. (1980) *Biochem. J. 189*, 359.

Lami, H., Pfeffer, G., & Laustriat, G. (1966) *J. Phys. (Orsay, Fr.) 27*, 398.

Lord, S. T., & Breslow, E. (1978) *Biochem. Biophys. Res. Commun. 80*, 63.

Nicolas, P., Camier, M., Dessen, P., & Cohen, P. (1976) *J. Biol. Chem. 251*, 3965.

Nicolas, P., Wolff, J., Di Bello, C., Camier, M., & Cohen, P. (1978a) *J. Biol. Chem. 253*, 2633.

Nicolas, P., Dessen, P., Camier, M., & Cohen, P. (1978b) *FEBS Lett. 86*, 188.

Nicolas, P., Batelier, G., Rholam, M., & Cohen, P. (1980a) *Biochemistry 19*, 3565.

Nicolas, P., Camier, M., Lauber, M., Masse, M. J., Möhring, J., & Cohen, P. (1980b) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2587.

Pearlmutter, A. F. (1979) *Biochemistry 18*, 1677.

Perrin, F. (1926) *J. Phys. Radium 7*, 390.

Perrin, F. (1936) *J. Phys. Radium 7*, 1.

Pfeffer, G. (1965) Ph.D. Thesis, Strasbourg.

Schachman, H. (1959) in *Ultracentrifugation in Biochemistry*, Academic Press, New York.

Scheraga, H. A., & Mandelkern, L. (1953) *J. Am. Chem. Soc. 75*, 179.

Sur, S., Rabbani, L. P., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026.

Tanford, C. (1961) in *Physical Chemistry of Macromolecules*, Wiley, New York.

Wahl, P. (1969) *Biochim. Biophys. Acta 175*, 2945.

Wahl, P., & Timasheff, S. N. (1969) *Biochemistry 7*, 2945.

Weber, G. (1952) *Biochem. J. 51*, 155.

Weber, G. (1953) *Adv. Protein Chem. 8*, 415.

Yguerabide, J., Epstein, H. F., & Stryer, L. (1970) *J. Mol. Biol. 51*, 573.

Yphantis, D. A. (1964) *Biochemistry 3*, 297.

Zagyansky, Y. A., Nezlin, R. S., & Tumerman, L. A. (1969) *Immunochemistry 6*, 787.