Scarlata, S. F. (1991) *Biophys. J. 59*, 319a.
Scarlata, S., Rholam, M., & Weber, G. (1984) *Biochemistry 23*, 6789-6792.
Shinitzky, M., & Barenholz, Y. (1978) *Biochim. Biophys. Acta 515*, 367-394.
Urry, D. W. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 672-676.
Wallace, B. A. (1990) *Annu. Rev. Biophys. Biophys. Chem. 19*, 127-188.
Wang, S., Martin, E., Cimino, J., Omann, G., & Glaser, M. (1988) *Biochemistry 27*, 2033-2039.
Weber, G. (1989) *J. Phys. Chem. 93*, 6069-6073.
Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 10*, 89-112.
Weber, G., Scarlata, S., & Rholam, M. (1984) *Biochemistry 23*, 6785-6789.
Varga, S., Muller, N., Pikula, S., Papp, S., Varga, K., & Martonosi, A. (1986) *J. Biol. Chem. 261*, 13943-13956.
Veatch, W. R., & Blout, E. R. (1976) *Biochemistry 15*, 3026-3030.
Veatch, W. R., Fossel, E. T., & Blout, E. R. (1974) *Biochemistry 13*, 5249-5256.
Veatch, W. R., Mathies, R., Eisenberg, M., & Stryer, L. (1975) *J. Mol. Biol. 99*, 75-92.

Binding and Fluorescence Studies of the Relationship between Neurophysin–Peptide Interaction and Neurophysin Self-Association: An Allosteric System Exhibiting Minimal Cooperativity†

Esther Breslow,*,‡ Thirleen LaBorde,‡ Shakti Bamezai,‡ and Suzanne Scarlata*,§
Departments of Biochemistry and Medicine, Cornell University Medical College, New York, New York 10021
Received February 5, 1991; Revised Manuscript Received April 17, 1991

ABSTRACT: The mechanism of peptide-enhanced neurophysin self-association was investigated to address questions raised by the crystal structure of a neurophysin–dipeptide complex. The dependence on protein concentration of the binding of a broad range of peptides to the principal hormone-binding site confirmed that occupancy of this site alone, and not a site that bridges the monomer–monomer interface, is the trigger for enhanced dimerization. For the binding of most peptides to the principal hormone-binding site on bovine neurophysin I, the affinity of each dimer site was at least 10 times that of monomer under the conditions used. No interactions between the two sites of the dimer were evident. Fluorescence polarization studies of pressure-induced dimer dissociation indicated that the volume change for this reaction was almost 4 times greater in the liganded than in the unliganded state, pointing to a significant alteration of the monomer–monomer interface upon peptide binding. Novel conformational changes in the vicinity of the single neurophysin tyrosine, Tyr-49, induced by pressures lower than required for subunit dissociation, were also observed. The bovine neurophysin I dimer therefore appears to represent an allosteric system in which there is thermodynamic and functional communication between each binding site and the monomer–monomer interface, but no communication across the interface to the binding site of the other subunit. A model for the peptide-enhanced dimerization is proposed in which intersubunit contacts between monomers reduce the large unfavorable free energy associated with binding-induced intrasubunit conformational change. Structural origins of the lack of communication across the interface are suggested on the basis of the low volume change associated with dimerization in the unliganded state and monomer–monomer contacts in the crystal structure. Potential roles for the peptide α-amino group and position 2 phenyl ring in triggering conformational change are discussed.

The binding to neurophysin (NP)[1] of oxytocin, vasopressin, and related small peptides is associated with an increase in the neurophysin dimerization constant, reflecting the stronger binding of peptides to dimer than to monomer (Cohen et al., 1979). This property contrasts with that of such classic allosteric systems as hemoglobin A, in which overall interactions among subunits are weaker in the O_2-saturated tetramer than in the deoxy state (Ackers, 1980), but is potentially related to the O_2-linked strengthening of intersubunit interactions in hemoglobin H (Valdes & Ackers, 1978) and in the final microscopic O_2-binding step of hemoglobin A [see Ackers and Johnson (1990) for a review]. Different mechanisms for the peptide-induced increase in neurophysin dimerization have been proposed (Breslow & Burman, 1990), including the possibility that peptide stabilizes the dimer by binding across the two monomer subunits (Peyton et al., 1986). The demonstration that several peptides produced an identical increase in neurophysin self-association as measured by affinity chromatography was used to argue against the latter mechanism (Fassina & Chaiken, 1988). However, the crystal structure

† Supported by NIH Grants GM-17528 to E.B. and GM-39924 to S.S.
* To whom correspondence should be addressed.
‡ Department of Biochemistry.
§ Department of Medicine. Present address: Department of Physiology and Biophysics, SUNY Stony Brook, Stony Brook, NY 11794-8661.

[1] Abbreviations: NP, neurophysin; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DMF, dimethylformamide; $\bar{p}$, moles of peptide bound per polypeptide chain.

0006-2960/91/0430-7990$02.50/0

of the complex of bovine neurophysin II with Phe-TyrNH_2 has recently been solved at 2.8-Å resolution (Chen et al., 1991), raising new questions about this and other potential allosteric mechanisms.

In the asymmetric unit of the unit cell of the crystal, neurophysin is present as two dimers arranged as a tetramer or pseudotetramer containing five bound peptides. Four of these peptides occupy the principal hormone-binding sites (one per subunit), located distant from the monomer–monomer interface, but adjacent to the dimer–dimer interface. A fifth peptide straddles the monomer–monomer interface and a tetramer–tetramer interface and therefore potentially stabilizes both the dimer and higher oligomers relative to monomer. The relationship of the fifth peptide site to sites present in solution is uncertain. Species higher than dimer have not yet been demonstrated in solution (Nicolas et al., 1980), and this site, if present, would represent a nonintegral contribution to the total number of sites per chain that might elude detection.[2] However, nonintegral values for the total number of sites have been reported in at least one study (Glasel et al., 1976), and a weak site for spin-labeled peptides, different from the principal site, was demonstrated in solution by NMR (Lord & Breslow, 1980). Additionally, a second more controversial site has been claimed for vasopressin and also for oxytocin under selected conditions [cf. Nicolas et al. (1978, 1980) and Breslow and Burman (1990)]. Because the affinity chromatography studies do not allow unambiguous distinction between occupancy of the principal hormone-binding site as opposed to alternative sites, a potential additional contribution of a site other than the principal hormone-binding site to the enhanced dimerization is not precluded.

In the present study, we specifically examined the relationship between occupancy of the principal hormone-binding site and enhanced neurophysin self-association, by looking at the dependence on protein concentration of the affinity of this site for a broad spectrum of ligand peptides. Occupancy of the principal hormone-binding site was measured by the perturbation of the nitrotyrosine CD signal of neurophysin mononitrated at its single tyrosine, Tyr-49, as described elsewhere (Breslow et al., 1973; Breslow & Gargiulo, 1977).[3] The peptides investigated represent a 200-fold range of binding constants to the strong site and include modification of essentially all the positions that can be modified or deleted without complete loss of binding, i.e., the side chains in positions 1 and 3, the hydroxyl group on the aromatic ring in position 2, and the backbone–CONH between residues 2 and 3. Since the principal peptide-binding sites and extra site in the crystal are nonidentical (Chen et al., 1991), these structural modifications can be expected to have different effects on their relative affinities, with potential alterations of allosteric effects if both sites are operative (see Discussion).

We further investigated mechanisms underlying the peptide-enhanced dimerization by monitoring the steady-state fluorescence anisotropy (A) of neurophysin covalently labeled with 5-(dimethylamino)-1-naphthalenesulfonate (dansyl). Labeling proteins with a long-lived probe such as dansyl permits determination of changes in anisotropy that are primarily due to the rotational volume (V_m), which in turn is a measure of the degree of self-association. The relative stability of the unliganded and liganded dimers was estimated by high-pressure techniques. Subjecting an oligomer to high hydrostatic pressure results in subunit dissociation due to more efficient packing of solvent between the separated subunits than in the intact dimer, replacement of dispersion forces with stronger dipole interactions, and electrostriction of water resulting from the breaking of salt bonds [see Weber and Drickamer (1983)]. Thus, the pressure needed for dissociation provides information on both the stability of the dimer at atmospheric pressure and the nature of its subunit interface. Relevant to all of these studies are the very strong structural homology between bovine neurophysins I and II and their common binding and self-association properties [e.g., see Breslow and Burman (1990)], the latter extending to their ability to form mixed dimers (Abercrombie et al., 1984).

The results of these studies confirm that occupancy of the principal hormone-binding site is the trigger for enhanced dimerization and indicate significant differences in the interfaces of unliganded and liganded dimers. On the basis of the data, a new mechanism for peptide-enhanced self-association is proposed.

Materials and Methods

Preparation and Modification of Neurophysin. Bovine neurophysins were purified and converted to their mononitrated derivatives as described elsewhere, using affinity chromatography and gel filtration steps to remove incompetent and high molecular weight protein, respectively (Virmana-Sardana & Breslow, 1983; Sardana et al., 1987). Native bovine neurophysin I was dansylated as follows. To a 2 mg/mL solution of protein in 0.1 M sodium phosphate buffer at pH 8 was added a 3-fold molar excess of dansyl chloride (from a 50 mM stock solution in dimethylformamide). The mixture was stirred at room temperature for 1 h, the pH was lowered to 3, and the solution was lyophilized to reduce the volume. To remove excess reagent, the sample was then chromatographed on Sephadex G-25 in 10% acetic acid and the protein peak lyophilized. The dansylated product was further purified by affinity chromatography [e.g., see Sardana et al. (1987)]. The extent of dansylation was determined by absorption spectroscopy using a molar extinction coefficient for the dansyl group of 4500/cm at 340 nm. Typical dansyl group incorporation was 0.1–0.2 mol per neurophysin subunit. Occasional samples of dansylated neurophysin that had been used for fluorescence studies were recovered by gel filtration in 0.2 M acetic acid followed by lyophilization. These samples were reanalyzed by affinity chromatography prior to use to ensure that they did not contain denatured protein, but are treated as different lots of protein.

Peptides. Peptides used in this study are largely those described earlier [e.g., see Whittaker et al. (1985)]. Oxytocin was purchased from Boehringer Manheim. A new lot of L-phenylalanylphenylethylamine was resynthesized for this study as follows. Boc-L-phenylanine (345 mg) was activated for 45 min at −20 °C with isobutyl chloroformate in 7 mL of dry DMF in the presence of N-methylmorpholine. To this solution was added 158 mg of phenylethylamine in 2 mL of

[2] Published neurophysin–peptide binding isotherms have been based on the quantitation of neurophysin by weight or amino acid analysis [e.g., see Nicolas et al. (1976, 1978) and Glasel et al. (1976)]. Because the protein was not purified by affinity chromatography and the relative content of competent protein unknown, these calculations would underestimate the number of peptides bound per chain to the extent that inactive protein was present; e.g., the presence of 20% incompetent protein would reduce a saturation value of 5 peptides bound per 4 chains (1.25/chain), as observed in the crystal, to an apparent value of 1/chain, or a value of 3 sites per dimer (1.5/chain) to an apparent number of sites of 1.2/chain.

[3] The single neurophysin tyrosine, Tyr-49, is adjacent to the principal hormone-binding site (Chen et al., 1991), and the CD signal of nitrated Tyr-49 is perturbed by peptide binding (Breslow & Weis, 1972). This perturbation reflects binding to the principal site and not to alternative sites as evidenced, for example, by the linear CD response under conditions of stoichiometric binding to the strong site (Breslow et al., 1973).

dry DMF containing *N*-methylmorpholine and the reaction stirred for 24 h at room temperature. The sample was diluted with water, the pH adjusted to 8.5 with $NaHCO_3$, and the solution extracted with ethyl acetate. The organic layer was washed with a solution of saturated NaCl, dried, and evaporated. The product was crystallized from the oily residue by using a mixture of hexane and ethyl acetate; yield = 218 mg; mp = 125–126 °C. Mass spectrum analysis gave a mass of 369 u ($M + H^+$) to be compared with the theoretical mass of 368. Concentrations of peptide in stock solutions were calculated by using a molar extinction coefficient of 400/cm at 256 nm.

Monitoring of Peptide-Enhanced Neurophysin Self-Association by Affinity Chromatography. Bovine neurophysin II (5 mg) was coupled overnight at 4 °C with 1 g (3.5 mL) of commercial CNBr-activated Sepharose 4B support (Pharmacia), and the remaining groups on the Sepharose were blocked with ethanolamine. The final concentration of protein bound to the gel was 1.25 mg/mL gel. For binding studies, the column was equilibrated with 0.1 M ammonium acetate buffer, pH 5.7, with or without added peptide as indicated, and protein was applied to the column in the same buffer. Elution was continued with the same buffer for ~17 mL. Protein not eluting at this stage was eluted with 0.2 M acetic acid at pH 2.5 without peptide. Elution was monitored by fluorescence for the dansylated protein (using 310–410-nm excitation and 480–520-nm emission) and by the absorbance at 350 nm for the nitrated protein.

Circular Dichroism Peptide-Binding Studies. Studies of the binding of peptides to nitrated neurophysin were conducted at pH 6.2, 25 ± 1 °C as described elsewhere (Breslow et al., 1973) using a Jobin-Yvon Mark 5 CD spectrometer. Concentrations of stock peptide solutions were determined by absorbance. Protein solutions of 2 and 0.2 mg/mL were prepared from a common stock solution by weight, with final calculations assuming a water and ash content of 10%. Random analysis of protein solutions by monitoring the nitrotyrosine absorption band [E = 2000/(M·cm) at 382 nm] gave good agreement with the protein concentration calculated by weight. (The subunit molecular weights of bovine neurophysins I and II are 9389 and 9869, respectively.) Path lengths used were 1 cm for studies at 2 mg/mL and 2–5 cm for studies at 0.2 mg/mL.

Studies of far-ultraviolet CD changes associated with oxytocin binding were conducted on the same spectrometer using unmodified affinity-purified neurophysin and a path length of 0.02 cm. Concentrations of neurophysin and hormone in stock solutions were determined by absorbance using molar extinction coefficients of 2800/cm at 280 nm and 1400/cm at 278 nm for protein and hormone, respectively.

Fluorescence Spectroscopy. Fluorescence measurements were carried out on a Greg-PC° spectrofluorometer (ISS, Inc., Champaign, IL). Atmospheric measurements were performed in a 3-mm cuvette at room temperature. Intrinsic fluorescence intensities were obtained by using an excitation wavelength of 278 nm and either monitoring the emission at 325 nm or integrating the emission spectrum from 300 to 400 nm. Polarization of dansyl-labeled neurophysin was measured by using an excitation wavelength of 360 nm; emission was typically monitored at 480 nm, with selected studies at other wavelengths. Single photon counting lifetime measurements were made by using the instrumentation at the Mt. Sinai School of Medicine (Hasselbacher et al., 1991), monitoring the emission at 480 nm. Unless otherwise reported, studies were carried out at pH 6.2 in 0.1 M ammonium acetate typically containing 2 mM Mes buffer; the latter was particularly introduced to reduce pH changes with increased pressure and was independently demonstrated to have no effect on fluorescence data at atmospheric pressure. For studies of the effects of pressure, samples were subjected to high pressure using an apparatus based on the design of Paladini and Weber (1981a). Anisotropy values were corrected for pressure-induced birefringence by using the method of Royer and Scarlata (unpublished results).

Average spherical rotational volumes (V_m) were calculated from the steady-state anisotropies (A) or polarization (P) and average lifetimes ($\langle\tau\rangle$) through the Perrin equation:

$$A_0/A = 1 + RT\langle\tau\rangle/\eta V_m = (1/P - 1/3)/(1/P_0 - 1/3) \quad (1)$$

where A_0 and P_0 are the limiting anisotropy and polarization, respectively, A_0 is equal to 0.31 for dansyl (Weber, 1951), and η is the viscosity of the solvent. Since the viscosity of water does not change significantly between 1 and 2000 bar (Bridgeman, 1970), it was treated as a constant.

For pressure studies, the degree of dissociation at pressure p (α_p) was calculated by using the relationship:

$$\alpha_p = [1 + Q(A_p - A_M)/(A_D - A_p)]^{-1} \quad (2)$$

where Q is the ratio of the monomer quantum yield to that of the dimer, A_p is the anisotropy at pressure p, and A_M and A_D are the anisotropies of the monomer and dimer, respectively. The value of the dimer dissociation constant, K_d, at atmospheric pressure and the change in the volume of the whole system associated with dissociation, ΔV, were calculated by plotting $\ln [\alpha^2/(1 - \alpha)]$ as a function of pressure, utilizing the relationships (Paladini & Weber, 1981b):

$$\ln [\alpha_p^2(1 - \alpha_p)] = \ln [\alpha_0^2/(1 - \alpha_0)] + p\Delta V/RT \quad (3)$$

and

$$K_d = 4D[\alpha^2/(1 - \alpha)] \quad (4)$$

where D is the total protein concentration expressed by using the molecular weight of the dimer; this plot yields the atmospheric value of K_d from the y intercept and ΔV from the slope of the line between α = 0.1 and 0.9.

Stabilization of a protein dimer by ligand can be estimated from the shift in $p_{1/2}$ (the pressure needed for 50% dissociation relative to the degree of dissociation at atmospheric pressure) from the equation:

$$\Delta p_{1/2}(\Delta V/RT) = \ln (K_2/K_1) \quad (5)$$

where K_2 and K_1 are the dissociation constants in the presence and absence of ligand, respectively.

Results

Effect of Protein Concentration on Peptide Binding to the Principal Hormone-Binding Site. The binding of different peptides to the principal hormone-binding sites of mononitrated bovine neurophysins I and II was compared at protein concentrations of 0.2 and 2 mg/mL (2×10^{-5} and 2×10^{-4} M, respectively, in protein subunits), a range of concentration over which the weight fraction dimer changes by a factor of ~3 (see below for dimerization constant). Figure 1 shows representative peptide-binding isotherms for nitrated neurophysins I and II at the two protein concentrations in ammonium acetate/Mes buffer. The approximate 100% increase in binding affinity for both neurophysins between 0.2 and 2 mg/mL is similar to that reported for the binding of Phe-TyrNH_2 to nitrated neurophysin II in 0.16 M KCl (Breslow

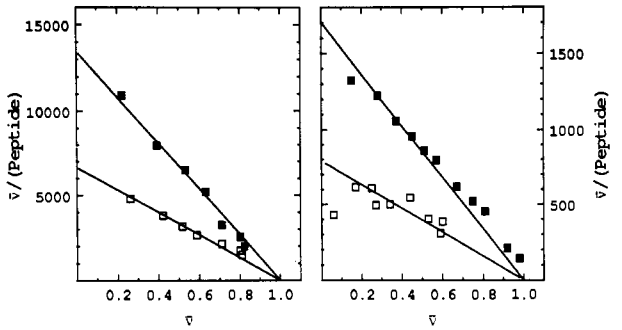

FIGURE 1: Representative Scatchard plots illustrating the dependence on protein concentration of the binding of peptides to nitrated neurophysin. Each plot represents a single binding experiment at pH 6.2 in ammonium acetate/Mes buffer. For each neurophysin, data for the different concentrations were obtained by using dilutions from a common stock solution of protein: (■) 2 mg/mL protein; (□) 0.2 mg/mL protein. Lines are derived from linear least-squares analysis constraining the x intercept to $\bar{\nu} = 1.0$ and are shown to depict the relationship of the data to linearity. (Left) Binding of Phe-PheNH$_2$ to bovine neurophysin I. (Right) Binding of Ala-Tyr-PheNH$_2$ to bovine neurophysin II.

& Gargiulo, 1977). Within the reliability of the data [e.g., see Breslow and Gargiulo (1977)], neurophysin I isotherms exhibited negligible cooperativity. Weak cooperativity in the neurophysin II isotherms was evidenced by a slight deviation from linearity in Figure 1, as reported in greater detail earlier (Breslow & Gargiulo, 1977). Table I reports the data obtained for a series of peptides, approximating the binding constant at each protein concentration from the slope at values of $\bar{\nu} = 0.5$ as described previously (Whittaker et al., 1985). More detailed analyses are given below. With the exception of Gly-Tyr-PheNH$_2$, only small nonsystematic differences among peptides in the effects of protein concentration, not clearly outside the range of experimental error, are seen in Table I. Reasons for the anomalous behavior of Gly-Tyr-PheNH$_2$ are unclear. This peptide induces the same increase in neurophysin self-association as Phe-PheNH$_2$ when analyzed by affinity chromatography (vide infra), and available evidence does not support the possibility that the lack of effect of protein concentration on its binding to the principal site arises from its potential interactions with the extra peptide site.[4]

The binding constants reported here for nitrated neurophysin II at 2 mg/mL are significantly higher than those reported earlier (Breslow et al., 1973; Whittaker et al., 1985) which were obtained in 0.16 M KCl. This was demonstrated to be an effect of buffer on neurophysin II binding constants, values being significantly higher in ammonium acetate than in KCl (Table II). Preliminary studies suggest that buffer effects on neurophysin I are nonidentical with those on neurophysin II, a difference also noted elsewhere (Whittaker et al., 1985).

[4] The possibility was considered that binding of this peptide to the extra site was stronger than to the principal site, such that this site was saturated at very low occupancy of the principal site. However, the concentration dependence of the binding of Phe-PheNH$_2$ was unaffected by the addition of sufficient Gly-Tyr-PheNH$_2$ to cause 10% occupancy of the principal site. Interestingly, the binding constant of nitrated neurophysin for Gly-Tyr-PheNH$_2$ was increased in the presence of native neurophysin, suggesting that, if this peptide does not increase the dimerization constant of nitrated neurophysin, it increases the formation of mixed dimers between native and nitrated neurophysin. This result is qualitatively consistent with the affinity chromatography results (text). Attempts to study the effects of binding this peptide by fluorescence polarization were complicated by a fluorescent impurity that was not removed by recrystallization. This impurity was indetectable by thin-layer chromatography or high-resolution NMR and is assumed to be present in too low a concentration to account for the unusual results.

Table I: Dependence on Protein Concentration of Binding Peptides to Mononitrated Bovine Neurophysins I and II[a]

peptide	app binding constant ratios: neurophysin I	neurophysin II
Phe-PheNH$_2$	14000/6500 = 2.2	17000/9800 = 1.7
Phe-TyrNH$_2$	14000/6000 = 2.3	19500/9000 = 2.2
Gly-TyrNH$_2$	130/70 = 1.9	80/40 = 2.0[b]
Ala-Tyr-PheNH$_2$	1100/530 = 2.1	1700/880 = 1.9
Met-Tyr-PheNH$_2$		7300/4400 = 1.7[b]
Gly-Tyr-PheNH$_2$	275/275 = 1.0	400/360 = 1.1
Phe-phenylethylamine	430/170 = 2.5	440/260 = 1.7

[a] Conditions: pH 6.2 in 0.1 M ammonium acetate/2 mM Mes buffer, 25 ± 1 °C. For each peptide, results are presented as the ratio of the apparent binding constant (in units of M^{-1}) obtained at a protein concentration of 2 mg/mL to that obtained at a concentration of 0.2 mg/mL. Apparent binding constants were calculated from the midpoints of the binding isotherms as previously described (Whittaker et al., 1985). Estimated errors in binding constants are ± 10%. [b] In ammonium acetate buffer alone, no Mes.

Table II: Effects of Buffer Composition on the Binding of Peptides to Nitrated Neurophysin II[a]

peptide	app binding constant (M^{-1}): ammonium acetate/Mes	KCl/citrate[b]
Phe-TyrNH$_2$	19500	11400
Gly-TyrNH$_2$	80	55
Ala-Tyr-PheNH$_2$	1700	940
Gly-Tyr-PheNH$_2$	400	190

[a] Apparent binding constants were obtained as in Table I and at a protein concentration of 2 mg/mL. Buffers compared are 0.1 M ammonium acetate/2 mM Mes and 0.1 M KCl/0.05 M sodium citrate, both at pH 6.2, 25 °C. [b] Binding constants in KCl/citrate are within experimental error of those reported earlier in 0.16 M KCl alone (Breslow et al., 1973).

Table III: Effects of Peptides on the Binding of Dansylated and Nitrated Neurophysin I to a Neurophysin II–Sepharose Support[a]

peptide additions	protein elution volume (mL): nitrated NP-I	dansylated NP-I	$\bar{\nu}$[b]
none	8.0	8.5	0
Phe-PheNH$_2$ (0.01 mM)	10.0	14.0	0.1
Phe-PheNH$_2$ (0.02 mM)	10.5		0.2
Phe-PheNH$_2$ (0.05 mM)	17.0		0.4
Phe-PheNH$_2$ (0.1 mM)		>16[c]	0.6
Gly-Tyr-PheNH$_2$ (0.4 mM)	9.5	10.0	0.1
Gly-Tyr-PheNH$_2$ (0.8 mM)	10.5	12.0	0.2
Gly-Tyr-PheNH$_2$ (4 mM)	>16[c]		0.6

[a] Buffer: 0.1 M ammonium acetate, pH 5.7. In each study, 100 μg of dansylated protein or 140 μg of nitrated protein was applied to the column in an initial volume of 50 μL in buffer containing peptide as indicated. Elution was continued as described under Materials and Methods. [b] These are approximate values and are obtained using the average of the binding constants for neurophysin-I and -II at 2 mg/mL in Table I. Binding constants for both neurophysins are relevant because they are interacting on the column. [c] pH 5.7 buffer containing peptide was discontinued at 16 mL to conserve peptide.

Affinity Chromatography. Ligand peptides increase the binding of neurophysin to neurophysin–Sepharose, reflecting the increased dimerization constant (Angal et al., 1982). We used this system to determine whether neurophysin dansylation, essential for fluorescence studies, interfered with peptide-enhanced self-association. We also investigated the effects of Gly-Tyr-PheNH$_2$ on neurophysin chromatographic behavior. Table III reports retention volumes for dansylated and nitrated neurophysins I in the absence and presence of Phe-Phe-NH$_2$ and Gly-Tyr-PheNH$_2$. The data demonstrate that the ligand-enhanced binding of dansylated neurophysin to the affinity column is at least as great as that of nitrated neuro-

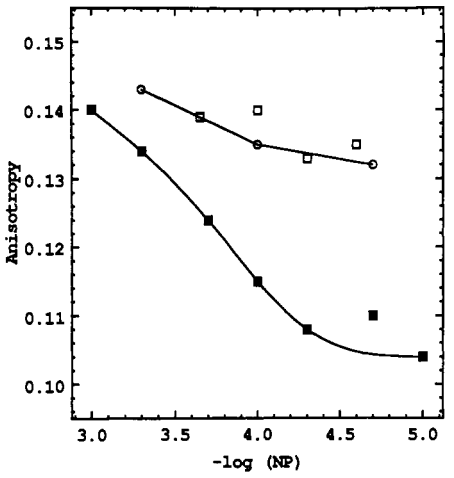

FIGURE 2: Fluorescence anisotropy of dansylated neurophysin I as a function of concentration at pH 6.2 in ammonium acetate/Mes buffer. (■) In the absence of peptide; (○) in the presence of 2 mM Phe-PheNH$_2$; (□) in the presence of 0.1 M Gly-TyrNH$_2$. The protein is calculated to be at least 90% saturated with peptide at all concentrations when peptide is present. Lines serve only to delineate the data.

physin. Gly-Tyr-PheNH$_2$ enhances binding of both the dansylated and nitrated proteins and, for the nitrated protein, to the same extent as Phe-PheNH$_2$ at equivalent levels of strong site saturation.

Fluorescence Polarization Studies. Dimerization of dansylated bovine neurophysin II has been demonstrated to be accompanied by increased anisotropy of dansyl fluorescence (Rholam & Nicolas, 1981). In the studies reported here of dansylated neurophysin I, we observed that dimerization-induced anisotropy changes are significantly more marked at 480 nm than at longer wavelengths, presumably because lower wavelengths sample more solvent-shielded dansyl groups that have less local motion. Accordingly, emission was monitored at 480 nm. We additionally observed that absolute anisotropies at 480 nm and the shape of the emission spectra showed small variations with different preparations of dansylated protein. These differences were interpreted to reflect differences in the dansyl site population in different preparations. Comparisons were therefore made only within single preparations of dansylated protein.

Figure 2 shows the effect of concentration on the fluorescence anisotropy of dansylated bovine neurophysin I at pH 6.2 in ammonium acetate buffer over the range 0.2–10 mg/mL at ~23 °C. The results indicate a single transition with an apparent midpoint, K_d, at 2×10^{-4} M, giving a dimerization constant of 5×10^3 M^{-1}. This value compares well with dimerization constants of $(7–11) \times 10^3$ M^{-1} calculated for native and nitrated neurophysin I at pH 5.6 (Nicolas et al., 1978, 1980; Whittaker & Allewell, 1984), particularly when the increase in dimerization constant of neurophysin I with decreasing pH (Peyton et al., 1986) is considered. [Fluorescence polarization studies here confirmed an increase in dimerization below pH 6.2 (data not shown).]

Heterogeneity lifetime analyses of solutions containing the same preparation of dansylated neurophysin I were carried out under the same conditions at 0.4 and 5 mg/mL. From these measurements, the average lifetimes for the monomer and dimer were extrapolated to be 7.2 ± 0.1 and 9.1 ± 0.1 ns, respectively. Increases in dansyl lifetime are consistent with increased shielding from solvent and are in agreement with the demonstration that neurophysin lysine residues are affected by dimerization (Sardana & Breslow, 1986). A small increase in dansyl lifetime upon neurophysin II dimerization was noted by Rholam and Nicolas (1981). When the additive property of anisotropies is used and the differences in lifetime are taken into account, the anisotropies of monomer and dimer are extrapolated to be 0.10 and 0.15, respectively. These results are in reasonable agreement with the 75% increase in anisotropy on dimerization calculated (Rholam & Nicolas, 1981) for bovine neurophysin II.

From the anisotropy and lifetime, the rotational volume, V_m, was calculated (see Materials and Methods). For a hydrated spherical protein, V_m is equal to approximately 1 mL/g; however, if local motions of the probe contribute to the depolarization, the calculated V_m represents a lower limit of the molecular weight. For the monomer, we obtained V_m = 8400 mL/mol, within range of the expected value for a protein of 9389 daltons. However, a value of 23 800 mL/mol was calculated for the dimer, which is 2.8 times that of the monomer. Differences between monomer and dimer can be at least partially explained by a decrease in the local dansyl group motion associated with dimerization, which would be consistent with the increased shielding from solvent (vide supra). However, the fact that the calculated volume for the dimer is greater than 19000 mL/mol additionally suggests that either asymmetry or the presence of higher order species contributes to the apparent volume. Earlier data, which were interpreted differently (Rholam & Nicolas, 1981), can be shown to lead to the same conclusion when the correct values for limiting anisotropies are used.[5] Although the structure of the unliganded state is not known, it is relevant that the crystal structure of liganded neurophysin indicates that the dimer is more asymmetric than the monomer (Chen et al., 1991). Additionally, no species higher than dimer have been detected by analytical ultracentrifugation over a very broad range of concentration (Nicolas et al., 1980). The most direct interpretation, therefore, is increased asymmetry of the dimer relative to the monomer.

Figure 2 also shows the anisotropy of the same preparation of dansylated neurophysin I as a function of concentration in the presence of essentially saturating concentrations of Phe-PheNH$_2$. The results indicate that the peptide induces a large increase in anisotropy at the lower protein concentrations and a decreased sensitivity of the anisotropy to changes in protein concentration over the range shown. A plot in the presence of Gly-TyrNH$_2$ shows a similar effect. The increase in anisotropy at this concentration is attributable to a peptide-induced increase in dimerization constant, as demonstrated for several other peptides (Nicolas et al., 1978), and consistent with the dependence of binding affinity on protein concentration (vide supra).

For the protein preparation in Figure 2, when monitored at 480 nm, we found no significant changes in dansyl lifetime upon ligation of peptide to the protein dimer, indicating that the environment of dansyl groups emitting at this wavelength was unperturbed. A decreased lifetime was observed at 580 nm, however, indicating a change in environment of the more exposed dansyl groups and possibly accounting for the decrease noted by Rholam et al. (1982). (Different dansylated protein preparations varied in the extent to which the 480-nm lifetime was sensitive to binding, again apparently reflecting differences in the distribution of dansylated amino groups.) Using the

[5] Rholam and Nicolas (1981) used a value for the limiting anisotropy obtained by linear extrapolation of the Perrin plot (Perrin, 1926) over a range of viscosities insufficient for complete dansyl group immobilization. This procedure gives too low a value for the limiting anisotropy (Weber, 1951; Cantor & Schimmel, 1980), leading to a calculated value for the rotational volume of the dimer that is smaller than the true value.

lifetime data, and assuming that the protein is essentially 100% dimer at 5 mg/mL in the presence of peptide, we calculated the apparent rotational volume of the dimer in the liganded state to be 19 500 mL/mol. However, the observed anisotropy at 5 mg/mL (0.143) is probably influenced by the presence of oligomers of higher order than dimer, as discussed further below. If the anisotropy of the bound dimer is instead calculated from smooth extrapolation of the data in Figure 2 at lower protein concentrations, a value of 0.138 is obtained, which yields a rotational volume of 18 100 mL/mol. In either event, the results are qualitatively consistent with an increase in dimer symmetry on binding peptide, as proposed elsewhere (Rholam et al., 1982).

Calculation of Relative Binding Constants of Monomer and Dimer. By use of the dimerization constant of unliganded neurophysin I calculated from the fluorescence data (5×10^3 M^{-1}), the dependence of binding on protein concentration can be analyzed in terms of a monomer–dimer equilibrium, with dimer binding ligand more strongly than monomer. As exemplified in Figure 1, the linearity of neurophysin I isotherms at protein concentrations of 2 mg/mL (50% dimer by weight in the unliganded state) suggests little cooperativity between the two dimer sites. Accordingly, different models were tested, each containing two identical and independent sites on the dimer, but differing in the intrinsic affinities allowed for monomer relative to dimer sites (Figure 3 legend). Theoretical curves for these models, plotted together with the collected binding data for Phe-PheNH$_2$, Ala-Tyr-PheNH$_2$, and Gly-TyrNH$_2$, indicate that the average data fall between the curves predicted for no binding to monomer and that in which the intrinsic affinity of a monomer site is one-tenth that of each dimer site. A model in which the monomer site has one-fifth the affinity of a dimer site appears to be precluded. The data therefore indicate that the intrinsic affinity of a dimer site is minimally 10 times greater than that of the monomer under these conditions, in turn indicating a minimum 100-fold increase in the dimerization constant for the fully liganded state relative to the unliganded state. This value can be compared with the peptide-induced 130-fold increased association of bovine neurophysin I with neurophysin II and the ~35-fold increased self-association of bovine neurophysin II measured by affinity chromatography (Abercrombie et al., 1984; Kanmera & Chaiken, 1985; Fassina & Chaiken, 1988). Sedimentation equilibrium patterns of native neurophysin II (Nicolas et al., 1975) and nitrated neurophysin I (Nicolas et al., 1978) also suggest at least a 100-fold increase in the dimerization constant in the presence of bound peptide hormone. However, reported binding constants for unmodified neurophysin I and II monomers and dimers in the same buffers (Cohen et al., 1979) predict only a 10–20-fold peptide-induced increase in dimerization.[6]

Circular Dichroism Analysis of Binding-Induced Conformational Change. The binding of peptides to the principal hormone-binding site is accompanied by major changes in far-ultraviolet CD spectra that presumably reflect, at least in part, the protein conformational change demonstrated by other techniques [e.g., see Rholam et al. (1982) and Virmani-Sardana and Breslow (1983)]. The noncooperativity of the binding isotherms for neurophysin I is paralleled by a linear correlation between binding-induced CD changes in the far-ultraviolet region and the amount of peptide bound, as exemplified by studies of oxytocin binding (Figure 4). [CD changes

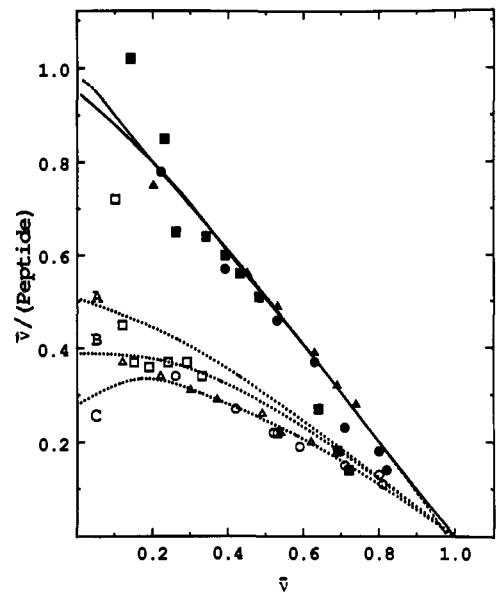

FIGURE 3: Comparison of theoretical and observed effects of protein concentration on the binding of peptides to bovine neurophysin I. For each peptide, closed symbols represent data at 2 mg/mL; open symbols represent data at 0.2 mg/mL. Binding data for Phe-PheNH$_2$ (●, ○), Ala-Tyr-PheNH$_2$ (▲, △), and Gly-TyrNH$_2$ (■, □) were normalized by dividing observed values of $\bar{\nu}$/[peptide] by the apparent binding constant at 2 mg/mL as determined from a linear analysis. Data at 2 mg/mL accordingly intercept the y axis at a value of $\bar{\nu}$/[peptide] = 1; the relationship between data at the two protein concentrations is unaffected by the normalization. Theoretical curves represent the relative binding isotherms that would be obtained for a monomer–dimer system in which the two dimer sites were equivalent and independent (same intrinsic binding constant) with the following assumptions: (A) the affinity of a dimer site is 5× that of a monomer site; (B) the affinity of a dimer site is 10× that of a monomer site; (C) only the dimer binds. Theoretical curves for 2 mg/mL, represented by solid and dashed lines, were adjusted to match the observed data at 2 mg/mL at values of $\bar{\nu}$ = ~0.5 and are not significantly distinguished from each other except at very low values of $\bar{\nu}$. Theoretical curves for relative binding at 0.2 mg/mL are shown by dotted lines for each of the three models. At each ligand concentration, the relative fractions of bound and unbound monomer were calculated from the assumed monomer binding constant. Relative proportions of unliganded, monoliganded, and diliganded dimer were calculated from the assumed intrinsic binding constant for the dimer by using standard statistical relationships such that the "apparent" binding constant for formation of all monoliganded species was 4 times that for formation of the diliganded species from monoliganded species. Total concentrations of bound species were then constrained by the dimerization constant of the unliganded protein, 5×10^3 M^{-1}.

associated with oxytocin binding are comparable to those for the smaller peptides (Breslow & Weis, 1972), but can be titrated more accurately as a function of the amount bound because of the tighter binding of hormone than of the smaller peptides.] The significance of the linear relationship between binding and conformational change is discussed further below.

Effects of Pressure on Neurophysin Self-Association in the Absence and Presence of Peptides. The pressure needed to induce the dissociation of protein oligomers is a function of the stability and nature of the interactions between subunits (Weber & Drickamer, 1983). Figure 5 shows representative studies of the effects of pressure on the polarization of 0.05 mM dansylated neurophysin I (same preparation as used above) in the absence and presence of an essentially saturating concentration ($\bar{\nu} \geq 0.91$) of Phe-PheNH$_2$. The unliganded protein at 0.05 mM shows a transition midpoint at ~1.2 kbar, reflecting dissociation of the relatively low fractional concentration of dimer. By use of the anisotropies of the unliganded monomer and dimer derived above for this preparation, and anisotropies independently determined for a second

[6] This discrepancy was first brought to our attention by Catherine Royer.

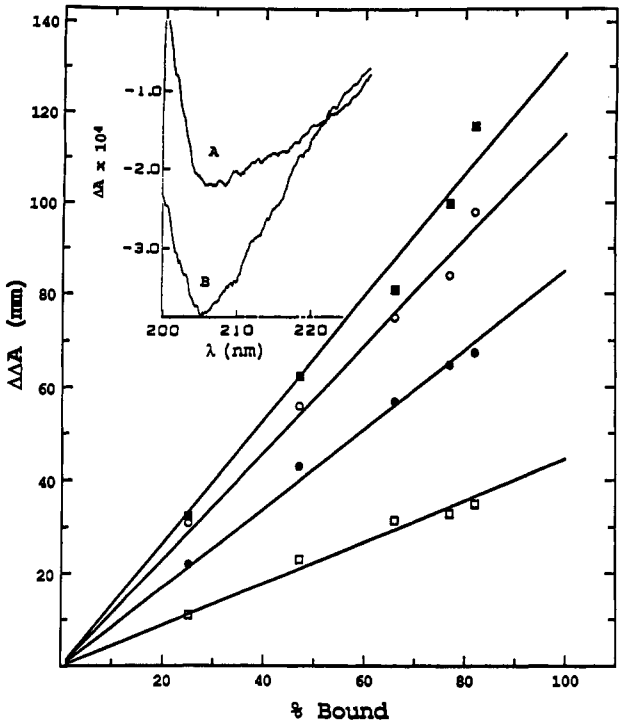

FIGURE 4: Far-UV circular dichroism changes associated with the binding of oxytocin to bovine neurophysin I at pH 6.2 in ammonium acetate buffer. The inset compares the observed spectrum (A) of a sample of neurophysin saturated with oxytocin with the calculated spectrum (B) for the same sample of protein and hormone in the absence of interaction. The main graph shows the change in ΔA (plotted in millimeters displacement on the chart) as a function of the percent bound protein at 202.5 (■), 205 (○), 210 (●), and 215 nm (□). For these studies, the spectra of samples of protein (1 mg/mL) with different concentrations of oxytocin were analyzed to give an apparent binding constant for oxytocin of 1×10^5 M^{-1}, in agreement with literature values. The percent bound protein in each sample is calculated by using this value.

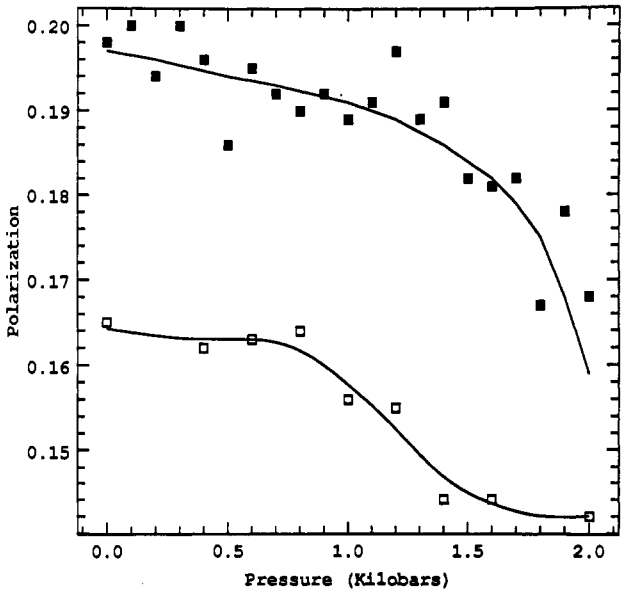

FIGURE 5: Effect of pressure on the polarization of fluorescence of dansylated bovine neurophysin I (0.5 mg/mL) at pH 6.2 in ammonium acetate/Mes buffer in the absence and presence of peptide. The results of two individual experiments, using the same preparation of protein, are shown. (□) No peptide; (■) in the presence of 1 mM Phe-PheNH$_2$. Lines serve only to delineate the data.

preparation, pressure data from several studies were analyzed (Materials and Methods) to calculate a dimerization constant at atmospheric pressure and the volume change of the entire

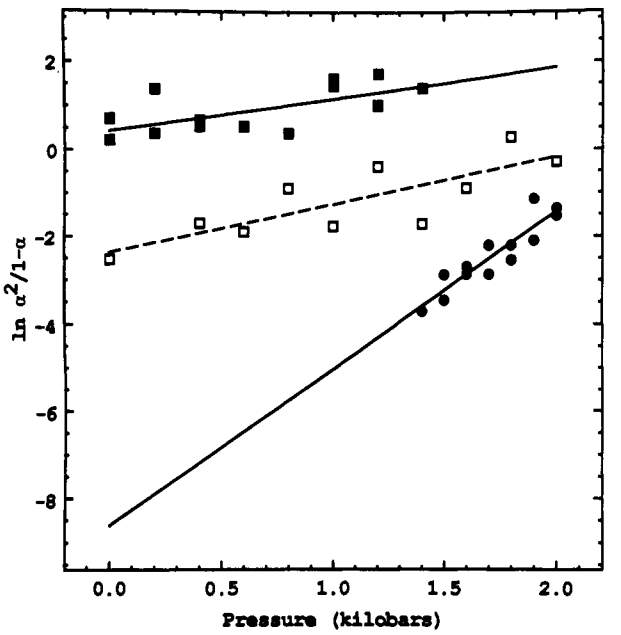

FIGURE 6: Analysis of the effects of pressure on subunit dissociation in the absence and presence of peptide. In the absence of peptide, results from two experiments at 0.5 mg/mL protein and one experiment at 5 mg/mL protein are shown: (■) 0.5 mg/mL protein; (□) 5 mg/mL protein. Data in the presence of Phe-PheNH$_2$ (●) represent runs at 0.5 mg/mL protein in the presence of 1 and 2 mM peptide. No significant difference in dissociation profile between the two peptide concentrations was seen. For each set of data, lines represent the linear least-squares fit. Dimerization constants at atmospheric pressure and volume changes associated with subunit dissociation are obtained from the intercepts and slope, respectively (see Materials and Methods).

system, ΔV, associated with dimer dissociation. Linear least-squares analysis of plots of ln $[\alpha^2/(1-\alpha)]$ as a function of pressure for the unliganded protein derived from data at 0.05 and 0.5 mM (Figure 6) gave values for the dimerization constant at atmospheric pressure of 6.5×10^3 and 10.8×10^3 M^{-1}, respectively, in reasonable agreement with the value obtained from dilution data. The average calculated value of ΔV for subunit dissociation, -22 ± 5 mL/mol, can be compared to a value of -39 mL/mol derived for neurophysin II from pressure studies (Royer, 1985).

In the presence of saturating Phe-PheNH$_2$, the apparent transition midpoint for the protein at 0.05 mM shifts to ~2 kbar, reflecting stabilization of the dimer by bound peptide (Figure 5). Similar data were obtained in the presence of saturating concentrations of Gly-TyrNH$_2$ (not shown). As above, the pressure dissociation data can be analyzed to give the apparent dimerization constant at atmospheric pressure in the presence of peptide (Figure 6). With an anisotropy of 0.138 for the liganded dimer (vide supra) and assuming the same anisotropy for the liganded and unliganded monomer (the calculations are not very sensitive to reasonable errors in this assumption because of the relatively low concentration of monomer), the results from two studies in the presence of Phe-PheNH$_2$ gave an apparent dimerization constant of 5 (± 3.5) $\times 10^7$ M^{-1} and a volume change of ~-84 mL/mol. In the presence of Gly-TyrNH$_2$ (not shown), the calculated dimerization constant and ΔV were 8 (± 6) $\times 10^6$ M^{-1} and -81 mL/mol, respectively, allowing the possibility of subtle differences among peptides. It is possible that the high calculated dimerization constants partially reflect the limited pressure range over which dissociation can be monitored in the presence of peptide. However, the data clearly indicate an increased volume change on dissociation of the liganded dimer relative to the unliganded dimer. If the value of ΔV was unchanged

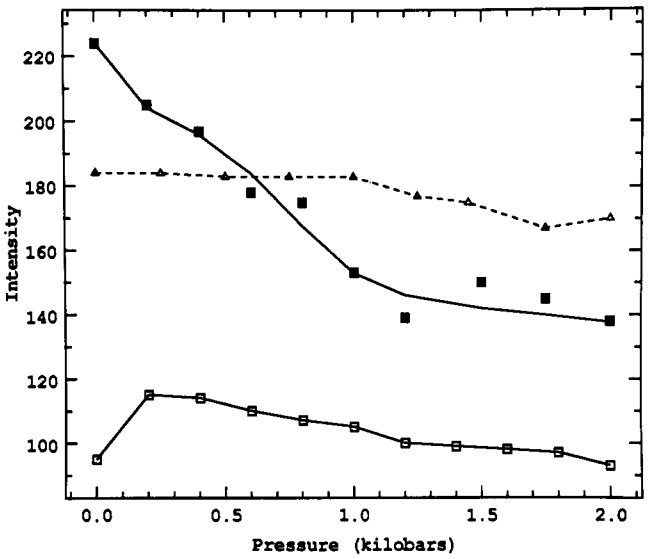

FIGURE 7: Effect of pressure on the fluorescence intensity of Tyr-49 of bovine neurophysin I in the absence and presence of peptide. Conditions: pH 6.2, ammonium acetate/Mes buffer, 0.5 mg/mL protein. (□) No peptide present; (■) in the presence of 2 mM Phe-PheNH$_2$. For comparison, the effect of pressure on the tyrosine fluorescence of the peptide Gly-TyrNH$_2$ in the same buffer is shown (△).

from that of the unliganded state (−22 mL/mol), the transition midpoint would be shifted to ~6 kbar for a dimerization constant of 5×10^5 M^{-1} and to higher pressures for higher dimerization constants (eq 5; see Materials and Methods), in marked contrast to the observed midpoint near 2 kbar. The increased value of ΔV in the presence of peptide suggests that peptide binding increases the surface involved in dimerization or diminishes the accessibility of the interface to water [e.g., see Weber and Drickamer (1983)].

A potential contribution to the results is dissociation of bound peptide with increased pressure. The higher anisotropy at all pressures in the presence of peptide (Figure 5) indicates that peptide remains bound, at least until pressure-induced subunit dissociation occurs. Nonetheless, partial dissociation of peptide upon subunit dissociation, arising from the weaker binding to monomer, might drive subunit dissociation. This was investigated by examining the effect of pressure on the fluorescence intensity of the single fluorescent chromophore of the undansylated protein, Tyr-49, in the absence and presence of peptide; Tyr-49 fluorescence intensity increases by a factor of ~2 upon protein ligation (Sur et al., 1979). In ammonium acetate/Mes buffer, pH 6, the fluorescence of the unliganded state was relatively insensitive to pressure, but the fluorescence of the liganded state exhibited marked quenching between 0 and 1 kbar (Figure 7), a range over which the anisotropy data (Figure 5) preclude ligand dissociation. In the 1.2–2-kbar region, where subunit dissociation is significant, the intensity in the presence of peptide stabilized at a value higher than in the absence of peptide, indicating that peptide dissociation was also negligible in the region over which the volume changes were calculated. (It is relevant that calculated values of ΔV can be shown to be relatively insensitive to 20% ligand dissociation at the highest pressure, a dissociation that probably represents the maximum that could have gone undetected.)

The pressure-induced quenching of Tyr-49 fluorescence was further investigated to determine its origin. A change in buffer to KCl/Mes at pH 6 doubled the magnitude of the quenching in the presence of peptide and induced a similar percentage quenching in the unliganded state. At pH 3 in KCl/Mes, the pressure-induced quenching of the unliganded protein was approximately half that seen at pH 6 (ligand dissociation precludes study of the liganded protein at pH 3). The results suggest that interactions between Tyr-49 and a proximal carboxyl group demonstrated earlier (Sur et al., 1979) are increased by a local pressure-induced conformational change. In ammonium acetate/Mes, this effect is significant only for the liganded state.

The possibility that the pressure-induced conformational change affects the stability of the subunit interface and contributes to the calculated value of ΔV in the liganded state is not precluded. However, in preliminary studies, we observed no significant differences in pressure-induced subunit dissociation between the ammonium acetate and KCl buffer systems, despite differences in Tyr-49 fluorescence behavior under the two conditions, suggesting that the conformational change does not affect the subunit interface. It is relevant that Tyr-49 is distant from the monomer–monomer interface in the crystal structure, although solution studies (Peyton et al., 1986) had suggested such a proximity in the unliganded state. Other evidence also suggests that differences in ΔV between liganded and unliganded states, which would be indicative of differences in the nature of their subunit interfaces, are likely to be real. For example, by analogy with the participation of residues 78–82 of neurophysin II in dimerization (Chen et al., 1991), His-80 of neurophysin I would be expected to participate in the subunit interface, and is known to be perturbed by peptide binding (Griffin et al., 1975; Virmani-Sardana & Breslow, 1983). Peptide-induced changes in other probable interface protons have been noted elsewhere (Peyton et al., 1986, 1987). Finally, in the absence of clear evidence of changes in 2° or 3° structure associated with dimerization in the unliganded state (Breslow & Burman, 1990), the difference in dimerization between liganded and unliganded states is best explained by a difference in the subunit interface.

Evidence for Oligomers Higher than Dimer in the Liganded State. Assuming that the dimerization constant of the liganded state is minimally 100-fold greater than that of the unliganded state, the weight fraction of liganded dimer should minimally be 87% and 90% at 0.5 and 1 mg/mL, respectively, consistent with the relative plateau in anisotropy of the complex over this concentration range (Figure 2). Sedimentation equilibrium studies of the liganded protein also indicate essentially complete dimerization at 1 mg/mL, with no increase in the apparent molecular weight over the range 1–10 mg/mL (Nicolas et al., 1980). Nonetheless, we find increases in anisotropy at concentrations of the liganded protein above 1 mg/mL that are too large to be explained by viscosity effects. With one lot of dansylated protein, the anisotropy increased by 24% between 1 and 10 mg/mL. The increased anisotropy at higher protein concentrations was not reduced by sonication and showed a reversible pressure-induced decrease, suggesting that it was not due to the formation of nonspecific aggregates. The data suggest that highly anisotropic oligomers, at concentrations too low to significantly affect sedimentation equilibrium behavior, may be in equilibrium with dimer in the liganded state and become manifest at protein concentrations above 1 mg/mL. Oligomers of higher order than dimer have also been proposed on the basis of NMR evidence (Blumenstein & Hruby, 1977) and are of potential importance in the packaging of neurophysin precursors into neurosecretory granules (Blumenstein & Hruby, 1977; Breslow & Burman, 1990).

Discussion

The results demonstrate that peptides of very different structure show similar, if not identical, discrimination between

neurophysin monomers and dimers when binding to the principal hormone-binding site. While this conclusion is analogous to that derived from affinity chromatography (Fassina & Chaiken, 1988), the earlier work did not address the potential problem of two classes of binding sites. Here, the earlier studies are extended in two ways. First, binding to the principal hormone-binding site, as opposed to total binding, has been specifically monitored, permitting a clearer distinction between effects from different sites. Second, the spectrum of peptides studied has been extended to include those with no side chain in position 1 (Gly-peptides) and with no carboxamide terminus (Phe-phenylethylamine), allowing investigation of a greater range of binding affinities.

With respect to the specific monitoring of the principal hormone-binding site, the question addressed is whether the observed concentration dependence is influenced by the extra site seen in the crystal that straddles the monomer–monomer interface; this additional site would be expected to exist principally in the dimer. If this site were sufficiently stronger than the principal site that it was saturated prior to significant occupancy of the principal site, little or no concentration dependence of binding to the principal site would be expected. With the possible exception of Gly-Tyr-PheNH_2, this situation is precluded for all peptides. If binding to the additional site and the principal site occurred with overlapping affinities, the apparent concentration dependence of binding to the principal site would be influenced by the exact relationship of the two binding constants. However, within experimental error, all peptides other than Gly-Tyr-PheNH_2 show the same concentration dependence of binding to the principal site, despite very large differences in overall affinities. Since the crystal structure indicates that the additional site is nonhomologous with the principal site (Chen et al., 1991), the possibility that all the other structural factors that so profoundly affect binding to the principal site would in each case similarly affect binding to the additional site is remote.

The results therefore validate earlier assumptions that stronger binding to the principal hormone-binding site in the dimer than in the monomer is, for most peptides, the essential basis of the peptide-enhanced dimerization. Together with the crystal structure, which indicates that the principal site is not near the monomer–monomer interface, this conclusion precludes any mechanism for the peptide-enhanced dimerization that depends on the binding of peptide across the monomer–monomer interface. Although the additional site straddles the monomer–monomer interface, the results here indicate that this site is too weak in solution to influence the thermodynamics of strong site occupancy.

Any allosteric model of the interaction of peptides with the principal binding site on neurophysin should be consistent with three properties of this system. First, binding to either site on the dimer is stronger than to the same site on the monomer. This is implicit in the data analysis here and is also evident in data elsewhere [e.g., see Cohen et al. (1979)]. Second, cooperative interactions between the two dimer sites are weak. Binding to nitrated neurophysin I in the present studies occurred with negligible cooperativity (weak negative interactions between the two dimer sites are not excluded), and for nitrated neurophysin II, binding to the second site on the dimer maximally occurs with only twice the intrinsic affinity of that to the first site, for peptides investigated so far [e.g., see Breslow and Gargiuolo (1977) and Pearlmutter and Dalton (1980)]. Third, significant conformational changes within the dimer occur upon binding (Rholam et al., 1982; Virmani-Sardana & Breslow, 1983), which, as discussed above, partially

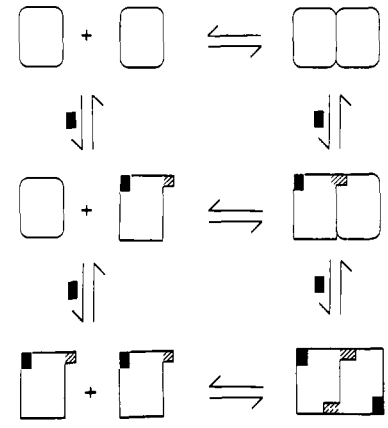

FIGURE 8: Proposed mechanism for stabilization of the dimer by binding to the principal hormone-binding site. The hatched area of the protein represents a region that interacts unfavorably with solvent.

reflect alteration of the monomer–monomer interface. The necessarily positive value of ΔG associated with this conformational change is likely to be a significant contributor to the large uphill energy shown to be a component of the peptide-binding reaction (Breslow & Burman, 1990).

A general model to explain the stronger binding by dimer than by monomer is suggested such that binding-induced conformational rearrangements in the monomer lead to unfavorable interactions of potential subunit interface residues with solvent. These interactions contribute a positive free energy term to the binding reaction and are relieved in the dimer via intersubunit interactions (Figure 8). This model can be compared to one previously described (Breslow & Burman, 1990) in which a dimerization-induced conformational change in the unliganded state increases the affinity of the binding pocket. In view of evidence arguing against a significant dimerization-induced conformational change (Breslow & Burman, 1990), the latter model can be generalized to stipulate only that the monomer–monomer interface of the unliganded dimer reduces the uphill free energy of conformational change associated with binding. To the extent that the monomer–monomer contacts which stabilize binding-induced conformational change are the same in liganded and unliganded states, both models are the same. However, the evidence here is that significant changes in the interface are associated with binding.

While the stronger binding to dimer can therefore be rationalized, it is difficult to develop a two-state model that accounts on one hand for the intradimer conformational changes associated with binding and their apparently large associated energy cost and, on the other, for the apparent weak cooperativity of binding. In a classic two-state system (Monod et al., 1965), a high energy cost of conformational change from T to R states is associated with weakened cooperativity only to the extent that ligand binds with measurable affinity to both T and R states. Even allowing for more cooperativity than we see here, available physical–chemical evidence, including temperature jump relaxation data (Pearlmutter & Dalton, 1980), has not so far pointed to two forms of ligated dimer. Moreover, for such a model, the relative fractions of T and R states should change nonlinearly with the amount of peptide bound, a nonlinearity not evident in the CD data (Figure 4). The data therefore suggest that the system does not follow two-state behavior. Interestingly, the behavior of Tyr-49 in fully liganded neurophysin has been interpreted to suggest differences between the two subunits of the dimer (Scarlata & Royer, 1986). However, these studies were conducted in 80% glycerol, where the state of oligomerization is uncertain.

Given the energetic linkage between the subunit interface and binding site, the lack of two-state behavior raises the question as to why conformational changes in one subunit are not significantly transmitted to the binding site of the other subunit. One reason (Figure 8) might be that the binding-exposed surface contacts the other subunit at a nonidentical locus which does not interact energetically with the binding site of its own chain. Contacts between Val-36 of one subunit and Ile-72 of the other in the crystal structure (unpublished observations) might be particularly significant in this context. A more general potential explanation is suggested by the relatively low volume change associated with pressure-induced dimer dissociation in the unliganded state. This would be consistent with weak van der Waals packing in the monomer–monomer contact region, allowing pressure-induced penetration of solvent without subunit dissociation. In the fully liganded state, ΔV increases, presumably reflecting stronger or more extensive packing interactions. If it is assumed that interface packing is still relatively loose in the semiliganded state, a change in orientation of a side chain on one subunit would not necessarily demand a complementary change in the other. We note that in the crystal (liganded state), the interface consists of hydrogen-bonded β-sheet regions that are further stabilized by side-chain and backbone van der Waals contacts. It is possible that the β-sheet hydrogen bonds, but not the side-chain contacts, are in place in the unliganded dimer, the absence of the latter allowing increased interactions with solvent. Such a difference between structural forms has also been postulated along the ribonuclease folding pathway (Udgaonkar & Baldwin, 1990).

Finally, the generally similar discrimination between monomer and dimer by most of the peptides studied, together with the crystal structure (Chen et al., 1991), suggests specific structural origins of the induction of interface changes when peptides are bound. The similarity in this property between peptides as different as Ala-Tyr-PheNH_2, Phe-phenylethylamine, and Gly-TyrNH_2 limits the principal structural elements responsible for this induction to the α-NH_3^+ group, the $-CH_2$-phenyl side chain at position 2, and the backbone between residues 1 and 2, all of which participate in binding. In the crystal structure, interactions of the α-NH_3^+ group involve the carboxyl terminus of a stretch of helix that connects the binding pocket to a segment of the subunit interface; this helix terminates in Tyr-49. In view of the demonstrated strength of these interactions [e.g., see Breslow and Burman (1990)] and the large binding-induced change in the environment of Tyr-49 (Furth & Hope, 1970; Breslow & Weis, 1972; Cohen et al., 1979; Sur et al., 1979), a reasonable trigger of the binding-induced change in dimerization is a change in either the organization or the tilt of this helix on binding. Additionally, the binding site of the peptide phenyl ring in position 2 communicates with the interface through a multilayer β-sheet (Chen et al., 1991), allowing the possibility that a restructuring of β-sheet hydrogen bonds on binding facilitates dimerization.

Acknowledgments

We express our appreciation for use of the fluorescence lifetime instrumentation at the Mt. Sinai School of Medicine which is supported by NSF Grant DMB-85-16318 to J. B. A. Ross and to the Mass Spectrometry Facility at Rockefeller University which is supported by NIH Grants RR00862 and GM-38724. We also gratefully acknowledge extensive consultation with Liqing Chen of the Departments of Crystallography and Biology, University of Pittsburgh, on details of the neurophysin crystal structure.

References

Abercrombie, D. M., Kanmera, T., Angal, S., Tamaoki, H., & Chaiken, I. M. (1984) *Int. J. Pept. Protein Res. 24*, 218–232.

Ackers, G. K. (1980) *Biophys. J. 32*, 331–346.

Ackers, G. K., & Johnson, M. L. (1990) *Biophys. Chem. 37*, 265–279.

Angal, S., & Chaiken, I. M. (1982) *Biochemistry 21*, 1574–1580.

Blumenstein, M., & Hruby, V. J. (1977) *Biochemistry 16*, 5169–5177.

Breslow, E., & Weis, J. (1972) *Biochemistry 11*, 3474–3482.

Breslow, E., & Gargiulo, P. (1977) *Biochemistry 16*, 3397–3406.

Breslow, E., & Burman, S. (1990) *Adv. Enzymol. Relat. Areas Mol. Biol. 63*, 1–67 (1990).

Breslow, E., Weis, J., & Menendez-Botet, C. J. (1973) *Biochemistry 12*, 4644–4653.

Bridgeman, P. W. (1970) *The Physics of High Pressure*, p 346, Dover Publishers, New York.

Cantor, C. R., & Schimmel, P. R. (1980) *Biophysical Chemistry*, Part II, p 465, W. H. Freeman, San Francisco, CA.

Chen, L., Rose, J., Breslow, E., Yang, D., Chang, W.-R., Yoo, C. S., Furey, W. F., Jr., Sax, M., & Wang, B.-C. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 4240–4244.

Cohen, P., Nicolas, P., & Camier, M. (1979) *Curr. Top. Cell. Regul. 15*, 263–318.

Fassina, G., & Chaiken, I. M. (1988) *J. Biol. Chem. 263*, 13539–13543.

Furth, A. J., & Hope, D. B. (1970) *Biochem. J. 116*, 545–553.

Glasel, J. A., McKelvy, J. F., Hruby, V., & Spatola, A. F. (1976) *J. Biol. Chem. 251*, 2929–2937.

Griffin, J. D., Cohen, J. S., Cohen, P., & Camier, M. (1975) *J. Pharm. Sci. 64*, 507–511.

Hasselbacher, C. A., Waxman, E., Galati, L. P., Contino, P., Ross, J. B. A., & Laws, W. (1991) *J. Phys. Chem.* (in press).

Kanmera, T., & Chaiken, I. M. (1985) *J. Biol. Chem. 260*, 8474–8482.

Lord, S. T., & Breslow, E. (1980) *Biochemistry 19*, 5593–5602.

Monod, J., Wyman, J., & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.

Nicolas, P., Wolff, J., Camier, M., Di Bello, C., & Cohen, P. (1978) *J. Biol. Chem. 253*, 2633–2639.

Nicolas, P., Camier, M., Dessen, P., & Cohen, P. (1976) *J. Biol. Chem. 251*, 3965–3971.

Nicolas, P., Batelier, G., Rholam, M., & Cohen, P. (1980) *Biochemistry 19*, 3565–3573.

Paladini, A., & Weber, G. (1981a) *Rev. Sci. Instrum. 53*, 419–427.

Paladini, A., & Weber, G. (1981b) *Biochemistry 20*, 2587–2591.

Pearlmutter, A. F., & Dalton, E. J. (1980) *Biochemistry 19*, 3550–3556.

Perrin, F. (1926) *J. Phys. Radium 1*, 390–401.

Peyton, D., Sardana, V., & Breslow, E. (1986) *Biochemistry 25*, 6579–6586.

Peyton, D., Sardana, V., & Breslow, E. (1987) *Biochemistry 26*, 1518–1525.

Rholam, M., & Nicolas, P. (1981) *Biochemistry 20*, 5837–5843.

Rholam, M., Nicolas, P., & Cohen, P. (1982) *Biochemistry 21*, 4968–4973.

Royer, C. (1985) Doctoral Thesis, University of Illinois at Urbana—Champaign, Urbana, IL.
Sardana, V., & Breslow, E. (1986) *Fed. Proc. 45*, 1538 (Abstr. 335).
Sardana, V., Carlson, J. D., Breslow, E., & Peyton, D. (1987) *Biochemistry 26*, 995–1003.
Scarlata, S., & Royer, C. A. (1986) *Biochemistry 25*, 4925–4929.
Sur, S. S., Rabbani, L. D., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026–1035.
Udgaonkar, J. B., & Baldwin, R. L. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8197–8201.
Valdes, R., Jr., & Ackers, G. K. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 311–314.
Virmani-Sardana, V., & Breslow, E. (1983) *Int. J. Pept. Protein Res. 21*, 182–189.
Weber, G. (1951) *Biochem. J. 51*, 155–164.
Weber, G., & Drickamer, H. G. (1983) *Q. Rev. Biophys. 16*, 89–112.
Whittaker, B. A., & Allewell, N. M. (1984) *Arch. Biochem. Biophys. 234*, 585–590.
Whittaker, B. A., Allewell, N. M., Carlson, J., & Breslow, E. (1985) *Biochemistry 24*, 2782–2790.

Neutron and X-ray Scattering Studies on the Human Complement Protein Properdin Provide an Analysis of the Thrombospondin Repeat

Kathryn F. Smith,[‡] Kathleen F. Nolan,[§] Kenneth B. M. Reid,[§] and Stephen J. Perkins*[,‡]

Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, Rowland Hill Street, London NW3 2PF, U.K., and MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.

Received February 15, 1991; Revised Manuscript Received May 22, 1991

ABSTRACT: Properdin is a regulatory glycoprotein of the alternative pathway of the complement system of immune defense. It is responsible for the stabilization of the C3 convertase complex formed between C3b and the Bb fragment of factor B. Neutron and X-ray solution scattering experiments were performed on the dimeric and trimeric forms of properdin. These have R_G values of 9.1 and 10.7 nm, respectively. The scattering curves were compared with Debye sphere modeling simulations for properdin. Good agreements were obtained for models similar to published electron micrographs showing that the properdin trimer has a triangular structure with sides of 26 nm. Such a structure also accounted for sedimentation coefficient data on properdin. Primary structure analyses for mouse and human properdin have shown that this contains six homologous motifs known as the thrombospondin repeat (TSR), which is the second most abundant domain type found in the complement proteins. Sequences for these 12 TSRs were aligned with 19 others found in thrombospondin and the late complement components. Three distinct groups of TSRs were identified, namely, the TSRs found in thrombospondin and properdin, the TSRs mostly found at the N-terminus of the late complement components, and the TSRs found at the C-terminus of the late components. Averaged secondary structure predictions suggested that all three groups contain similar backbone structures with two amphipathic turn regions and one hydrophilic β-strand region. The mean dimensions of the TSRs of properdin in solution were determined to be approximately 4 nm × 1.7 nm × 1.7 nm, showing that these are elongated in structure.

The components of the complement system provide a major nonadaptive immune defense mechanism for its host (Reid, 1986; Law & Reid, 1988). These are activated in response to the challenge of foreign material in plasma. Complement activation proceeds through a series of limited proteolytic steps in one of two largely independent pathways, the classical and alternative pathways. The latter operates via a C3 "tick-over" mechanism, where the molecular structure of the target cell initiates an amplification loop (Lambris, 1990). Properdin is involved in the regulation of the alternative pathway by binding to and stabilizing the C3/C5 convertase complexes, C3b·Bb and $C3b_n$·Bb (Fearon & Austen, 1975; Medicus et al., 1976; Farries et al., 1987), and also by inhibiting the cleavage of C3b by factor I in the presence of factor H as a cofactor by interfering with the ability of factor I to bind C3b (Farries et al., 1988a). Properdin binds to the C-terminal region of the α-chain of C3 between residues 1402 and 1435 (Lambris et al., 1984; Daoudaki et al., 1988). There is also evidence that properdin binds to the Ba domain of factor B in order to facilitate the cleavage by factor D to form the C3 convertase complex, with the concomitent release of Ba (Farries et al., 1988b).

Mouse and human properdin each consist of a single polypeptide chain of 441–442 amino acids (Goundis & Reid, 1988; Nolan & Reid, 1990; Nolan et al., 1991a,b) with an apparent molecular weight of 55 000 when examined under dissociating conditions with or without reduction of disulfide bridges. Human properdin also contains up to 10% by weight of complex-type N-linked carbohydrate, corresponding to one or two oligosaccharides per monomer (Minta & Lepow, 1974; Farries

* To whom correspondence should be addressed.
‡ Royal Free Hospital School of Medicine.
§ University of Oxford.

0006-2960/91/0430-8000$02.50/0