Slowly Interchanging Conformers of Bovine Neurophysin-I in the Unliganded Dimeric State†

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ABSTRACT: The effect of neurophysin dimerization on Tyr-49, a residue adjacent to the hormone-binding site, was investigated by proton NMR in order to analyze the basis of the dimerization-induced increase in neurophysin hormone affinity. Dimerization-induced changes in Tyr-49 resonances, in two unliganded bovine neurophysins, suggested that Tyr-49 perturbation is an intrinsic consequence of dimerization, although Tyr-49 is distant from the monomer–monomer interface in the crystalline liganded state. To determine whether this perturbation reflects a conformational difference between liganded and unliganded states that places Tyr-49 at the interface in the unliganded state, or a dimerization-induced change in secondary (2°) or tertiary (3°) structure, the more general structural consequences of dimerization were further analyzed. No change in 2° structure upon dimerization was demonstrable by CD. On the other hand, a general similarity of regions involved in dimerization in unliganded and liganded states was indicated by NMR evidence of participation of His-80 and Phe-35 in dimerization in the unliganded state; both residues are at the interface in the crystal structure and distant from Tyr-49. Consistent with a lack of direct participation of Tyr-49 at the monomer–monomer interface, dimerization induced at least two distinct slowly exchanging environmental states for the 3,5 ring protons of Tyr-49 without significantly increased dipolar broadening relative to the monomer. Two environments were also found in the dimer of des-1–8 neurophysin-I for the methyl protons of Thr-9, another residue distant from the monomer–monomer interface and close to the binding site in the liganded state. These results, and the sensitivity of the chemical shift of Tyr 3,5 ring protons to the presence of binding site residue Arg-8 only in the dimer, suggest that dimerization of unliganded neurophysin is accompanied by subtle changes in 3° structure that involve the binding site, that lead to conformational heterogeneity at this site and that play a potential role in the stronger binding of peptide by dimer than by monomer.

The dimerization of neurophysin facilitates its interactions with ligand hormones and related peptides by an unknown mechanism [e.g., see Nicolas et al. (1980) and Breslow and Burman (1990)]. Potentially relevant to this mechanism is an observation from 300-MHz nuclear magnetic resonance (NMR)[1] studies that, in unliganded bovine neurophysin-I, ring proton signals from the sole tyrosine, Tyr-49, were among those selectively broadened or shifted by dimerization (Peyton et al., 1986); Tyr-49 is adjacent to the peptide-binding site (Chen et al., 1991). Still more pronounced effects of dimerization on Tyr-49 in the des-1–8 derivative of unliganded neurophysin-I were observed and, together with related results, suggested proximity of Tyr-49 both to Arg-8 and to the monomer–monomer interface (Peyton et al., 1986). The recently solved crystal structure of a peptide complex of bovine neurophysin-II confirmed the relationship of Arg-8 to Tyr-49, but placed these residues on the opposite side of the molecule from the monomer–monomer interface, the latter principally comprising β-sheet interactions involving residues 32–38 and 77–81 (Chen, 1991; Chen et al., 1991). Although Tyr-49 is at the center of a dimer–dimer interface in the crystal structure, ultracentrifuge studies argue against the presence of species higher than dimer in solution at the concentrations used for NMR (Nicolas et al., 1980).

The mechanism by which Tyr-49 is perturbed by dimerization in the unliganded state has potential significance for factors underlying the stronger binding of peptide to dimer than to monomer. One obvious candidate for a mechanism involves differences between the unliganded dimer and monomer in intrasubunit conformation near Tyr-49. However, neither hydrodynamic nor fluorescence polarization studies provided evidence of dimerization-mediated changes in secondary (2°) or tertiary (3°) structure (Rholam & Nicolas, 1981). Alternatively, since neurophysin conformation is significantly altered by peptide binding [e.g., see Rholam et al. (1982)], the relationship of Tyr-49 to the subunit interface might differ in unliganded and liganded (crystal structure) states. The present studies were undertaken to identify the origin of the dimerization-induced changes in the behavior of Tyr-49.

There are two closely related bovine neurophysins, with very similar physical–chemical properties, designated neurophysin-I and -II, respectively [reviewed in Breslow and Burman (1990)]. Amino acid sequences of both are given in Figure 1. Despite differences between the two in the 77–81 interface region and in other amino-terminal and carboxyl-terminal residues, their CD spectra and binding properties are almost identical (Breslow & Burman, 1990), and they have been demonstrated to form mixed dimers by affinity chromatography both in the unliganded, in the semiliganded, and in the liganded states [e.g., see Abercrombie et al. (1982) and Ando et al. (1987)]. Historically, most neurophysin NMR

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[1] Abbreviations: CD, circular dichroism; NMR, nuclear magnetic resonance; TSP, sodium 3-(trimethylsilyl)propionate-d_4; 2D, two dimensional; COSY, 2D correlated spectroscopy; NOESY, 2D nuclear Overhauser and exchange spectroscopy.

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                    10                  20                  30
NP-I  AVLDLDVRTCLPCGPGGKGRCFGPSICCGDELGCF
NP-II ---MS------E L---Q-----------------------------------------
                                                          |____

                40                  50                  60                  70
NP-I  VGTAEALRCQEENYLPSPCQSGQKPCGSGGRCAAA
NP-II ----------------------------------------------------------
      ____ _|

                        80                  90
NP-I  GICCSPDGCHEDPACDPEAAFS
NP-II ----------NDES----VTE----E---REGIVG---PRRV
      |_|       |_____|
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FIGURE 1: Amino acid sequences of bovine neurophysin-I and -II [reviewed in Breslow and Burman (1990)]. Neurophysin-II sequences are given only where different from neurophysin-I. Residues at the monomer–monomer interface in the crystal are bracketed.

studies have utilized bovine neurophysin-I because of the greater solubility of its complexes. However, the crystal structure of the liganded state was elucidated using bovine neurophysin-II, which is the only bovine neurophysin crystallized so far. Accordingly, selected NMR studies of bovine neurophysin-II are given in the present work to demonstrate that the perturbation of Tyr-49 upon dimerization of the unliganded state is also a property of this protein.

It is relevant that complete proton resonance assignments for neurophysin have not yet been achieved. This reflects the largely dimeric state of the protein at high concentrations in normal solvents (dimer M_r = 20 000) and broad line widths (30–35 Hz) for α-proton resonances in the central core of the molecule under these conditions (e.g., Figure 4) that have so far precluded observation of many COSY cross-peaks. This situation is further complicated by interchange between monomer and dimer, which is slow on the NMR time scale (Pearlmutter, 1979; Peyton et al., 1986), so that contributions from both states are seen in the spectra. In this paper, we present evidence that the dimer of the unliganded protein in solution itself consists of at least two conformationally distinct species, giving rise to a further multiplicity of signals.

MATERIALS AND METHODS

Proteins. All protein concentrations are given in terms of the concentration of monomer units ($M_r \simeq 10\,000$). Bovine neurophysins-I and -II and the mononitrated and des-1–8 derivatives of bovine neurophysin-I were prepared as described elsewhere (Lord & Breslow, 1979; Peyton et al., 1986; Sardana et al., 1987). The monocarboxymethylated derivative of bovine neurophysin-I (representing monocarboxymethylation of His-80) was prepared by reaction of 1 mM protein (in 3 mL of 0.1 M ammonium acetate/0.02% sodium azide) with sodium iodoacetate in the dark at pH 7. The iodoacetate was added in increments of 24 μmol every 12 h for 8 days, to give a final concentration of 0.13 M. Protein was then separated from other components of the reaction mixture by gel filtration and lyophilized. Purification of the monocarboxymethylated product (in 3-mg lots) was achieved by anion-exchange HPLC with a DEAE 5PW column (7.5 mm × 7.5 cm, Waters), using solvent systems (A) 0.05 M ammonium acetate/0.02% sodium azide, pH 7.0, and (B) 0.5 M ammonium acetate/0.02% sodium azide, pH 7.0, with a gradient of 75% A, 25% B to 25% A, and 75% B in 40 min. The most retarded peak was collected, dialyzed to remove salt, and further purified by affinity chromatography (Rabbani et al., 1982). Amino acid analysis confirmed the absence of both His and the dicarboxymethylated product in the purified protein.

Circular Dichroism Studies. CD[1] studies were conducted using a Jobin-Yvon Mark 5 circular dichroism spectrometer

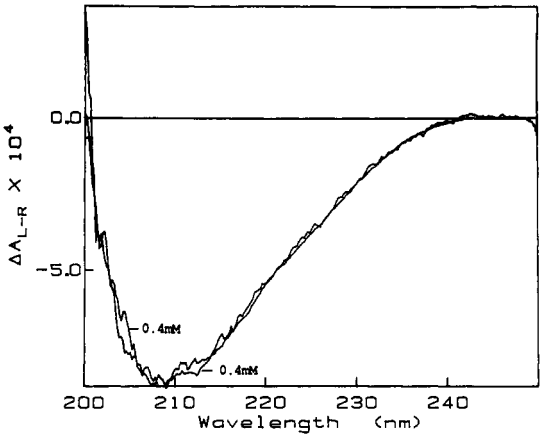

FIGURE 2: Normalized far-UV circular dichroism spectra of des-1–8 bovine neurophysin-I at pH 6.2 in 0.1 M KCl at 0.4 and 0.04 mM concentration. Values of ΔA shown are those of the 0.4 mM sample in a 0.022-cm path-length cell. The spectrum of the 0.04 mM sample was obtained in a 0.05-cm cell, and the data were normalized to the concentration and cell path length of the 0.4 mM sample.

at 25 °C. Cell path lengths were calibrated using *d*-10-camphorsulfonic acid, and the results reported at different concentrations are corrected for absolute cell path.

NMR Studies. The 500-MHz spectra at 25 °C were taken in D_2O using facilities at The Rockefeller University. Samples were prepared as described earlier (Peyton et al., 1986), and results are reported as ppm downfield from the reference sodium 3-(trimethylsilyl)propionate-d_4 (TSP). Spectra at 620 MHz were obtained with the 620-MHz spectrometer at the NMR Facility for Biomedical Studies at Carnegie-Mellon University. One-dimensional Fourier-transform spectra were typically obtained using a 6-μs pulse and a 2.4-s acquisition time, collecting 30 016 points over a sweep width of 6200 Hz. A total of 1024 transients were accumulated and zero-filled to 65 536 points; a 0.3-Hz line broadening was applied and transformed to give the spectrum. 2D COSY spectra were obtained using 90° pulses of 8.5 μs, a sweep width of 6200 Hz, and acquisition times of 0.083 s, collecting 1024 data points. A relaxation delay of 1.5 s was allowed. A total of 512 increments were used, and the final data matrix was zero-filled to 2048 × 2048. Standard phase cycling was used, and moderate Lorentzian–Gaussian filtering was applied. The transformation was made in the absolute-value mode. NOESY spectra were acquired with similar parameters, allowing mixing times from 0.10 to 0.8 s, with the principal information used obtained from the shorter mixing times. Spectra were normally recorded at 20.3 °C; measurements on temperature dependence were performed over the range 10–42 °C. Because of small differences (0.03–0.04 ppm) in chemical shifts measured at 500 and 620 MHz, which in part reflect differences in the references used, we have normalized all data to the 6.44 ppm peak discussed in the text.

RESULTS

Circular Dichroism Studies of the Effects of Dilution on Bovine Neurophysins. Figure 2 shows the effects on far-UV ellipticity of dilution of des-1–8 neurophysin-I from 0.4 to 0.04 mM at pH 6. Note that neurophysins have weak CD signals in the far-UV (Breslow & Weis, 1972) and the wavelength region below 200 nm was not accessible because of noise. Although the weight fraction of dimer decreases from ~60% to 20% over this concentration range (vide infra), no significant CD changes occur upon dilution. The far-UV

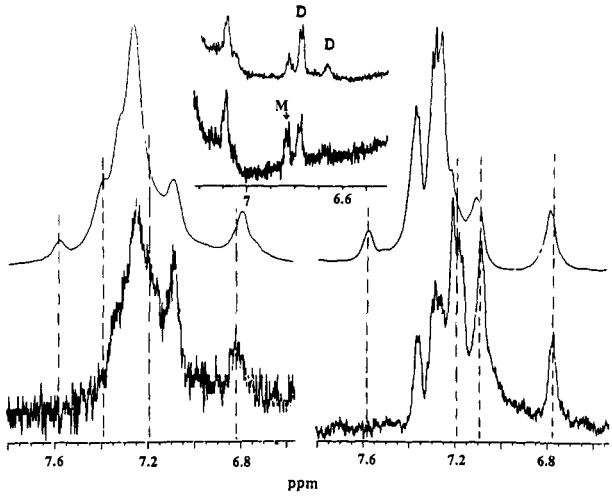

FIGURE 3: Effect of concentration on aromatic protons of bovine neurophysin-I and -II. Main figure: 500-MHz spectra of neurophysin-II (left) and neurophysin-I (right) at concentrations of 2 mM (upper spectra) and 0.02 mM (lower spectra), pH ~6.1; weight fractions of dimer are ~80% and 15% at the higher and lower concentrations, respectively. Tyr-49 3,5 and 2,6 ring protons are those respectively located at ~6.8 and 7.1 ppm. Inset: 620-MHz spectra of neurophysin-II at pH 3 at 1 mM (upper spectrum, ~75% dimer by weight) and 0.2 mM (lower spectrum, 50% dimer by weight) concentrations. Abscissas are ppm downfield from TSP.

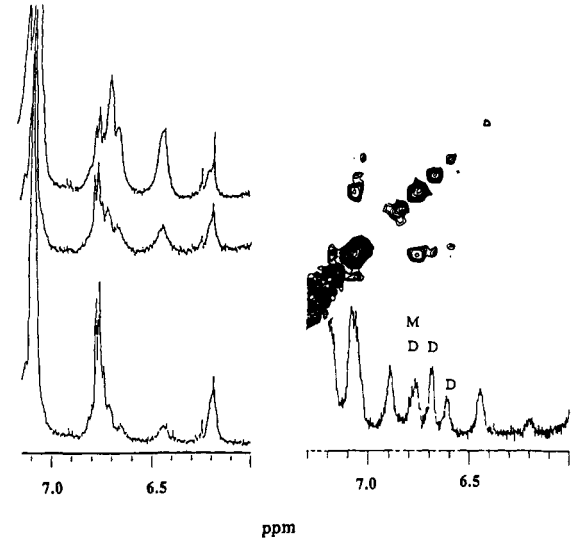

FIGURE 4: Effect of concentration on Tyr-49 3,5 ring protons and downfield α-protons of des-1–8 bovine neurophysin-I. Left: 500-MHz spectra at concentrations of 0.05 mM (lower spectrum, 20% dimer by weight), 0.25 mM (middle spectrum, 42% dimer), and 1 mM (upper spectrum, 65% dimer), pH 6.2. Resonances in the 6.55–6.8 ppm region represent Tyr 3,5 ring protons; signals at 6.44 and 6.2 ppm represent dimer and monomer forms of the same downfield α-proton (see text). Right: 620-MHz spectra at pH 8, ~2 mM. Protein is 75% dimer by weight as measured by intensities at 6.44 and 6.2 ppm. Tyr 3,5 resonances assigned to dimer (D) and monomer (M) are indicated; note the dual assignment at 6.76 ppm. The signal at 6.9 ppm represents the C-4 proton of His-80, which moves to this position with deprotonation of the imidazole ring. The superimposed COSY spectrum confirms that peaks between 6.55 and 6.8 ppm arise from the 3,5 ring protons of Tyr-49.

spectra of both native neurophysin-I and native neurophysin-II were also independent of dilution over this concentration range (data not shown). In the near-UV, average reductions of ~10% in disulfide ellipticity occurred with dilution, consistent with involvement of Cys-34 and -79 in dimerization as found in the crystal (data not shown). The results suggest that no significant changes in secondary structure occur upon dimerization.

Comparison of Dimerization-Induced Changes in NMR Signals of Aromatic Protons in Bovine Neurophysin-I and -II. As shown below, Tyr-49 and Phe ring protons are affected by dimerization in both bovine neurophysins. It is noteworthy that these effects are seen at both pH 3 and pH 6 in neurophysin-II, since neurophysin-II conformation differs at these two pH values (Lord & Breslow, 1979; unpublished 620-MHz studies).

Figure 3 demonstrates the concentration dependence of the 500-MHz aromatic proton spectra of the two neurophysins at pH 6, and of the neurophysin-II 620-MHz Tyr proton spectra at pH 3; relative weight fractions of monomer and dimer under the different conditions are given in the figure legend. As previously reported at lower field (Peyton et al., 1986), neurophysin-I dimerization is accompanied by a marked change in Phe proton signals, manifest by the appearance of a peak at 7.57 ppm and changes in the 7.2–7.4 ppm region; integration of the 7.57 ppm peak in the present studies indicates that it represents one proton in the dimer. These changes have been assigned to either Phe-22 or Phe-35, since they are independent of excision of Phe-91 (Sardana & Breslow, 1984; Peyton et al., 1986). The results in Figure 3 show that dimerization of neurophysin-II at pH 6 is accompanied by changes at 7.57 ppm identical to those of neurophysin-I and that other Phe ring protons are also shifted, albeit not quite identically to those of neurophysin-I.

For Tyr-49 of neurophysin-I, as also reported earlier, signals at 6.77 and ~7.1 ppm in the monomer (3,5 and 2,6 ring protons, respectively) broaden with dimerization at pH 6, with very small shifts now evident at the higher field. For neurophysin-II at pH 6, dimerization-induced changes both in Tyr ring proton line shape and in chemical shift (signals at 6.82 and ~7.1 ppm in the monomer) are evident at 500 MHz. Of particular interest are the 620-MHz spectra of neurophysin-II at pH 3, which clearly show a shift in 3,5 Tyr ring protons from 6.82 ppm in the monomer to 6.76 ppm in the dimer, without a significant change in line width, and a dimerization-induced increase in the fractional intensity of a peak at 6.65 ppm. COSY studies at pH 3 show that the 6.65 ppm component is coupled to protons at 7.1 ppm and this peak accordingly is also assigned to Tyr 3,5, ring protons in the dimer. The properties of the multiple 3,5 Tyr ring proton resonances in the dimer are explored further below in neurophysin-I derivatives.

Multiple Conformational States of Tyr-49 Induced by Dimerization of Des-1–8 Bovine Neurophysin-I. Studies at 300 MHz had indicated a marked dimerization-induced broadening and upfield shift of the 3,5 ring protons of Tyr-49 of des-1–8 bovine neurophysin-I at pH 6.2 (Peyton et al., 1986). When the system is observed at 500 or 620 MHz (Figure 4), the dimerization-induced changes are shown to result from the presence of multiple upfield shifted peaks in the dimer. At the lowest concentration (5×10^{-5} M), a single main resonance for the 3,5 ring protons, located at 6.76 ppm, is seen. Approximately 80% of the chains are monomers under these conditions (vide infra), and this peak is assigned to monomer. With increasing concentration, multiple upfield peaks appear and are assigned to dimer; there is no evidence of higher oligomers under these conditions for unmodified neurophysin (Nicolas et al., 1980), which is shown below to behave analogously. The presence of resolved coexistent monomer and dimer peaks is in agreement with the slow rate of monomer–dimer equilibrium on the NMR time scale (Pearlmutter, 1979). Also shown in Figure 4 are 620-MHz 1D and COSY studies of the des-1–8 protein at pH ~8,

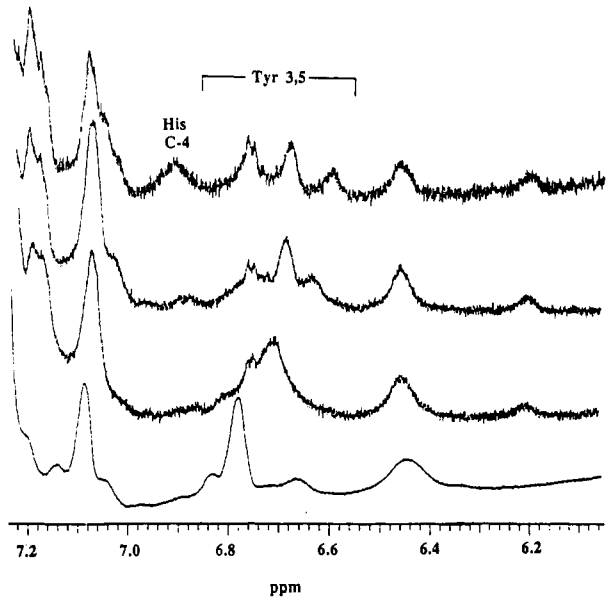

FIGURE 5: Effect of pH on Tyr ring protons and downfield α-protons of des-1–8 bovine neurophysin-I at 620 MHz. Concentrations ~2 mM. pH values from top to bottom are 7.8, 6.6, 5.8, and 2.8. An increase in the fractional content of monomer with increase in pH is evidenced by the relative intensities at 6.44 and 6.2 ppm and the increased intensity of the sharp Tyr-49 monomer resonance at 6.76 ppm.

representing 75% dimer. At pH 8, the different dimer 3,5 ring proton resonances show increased resolution relative to pH 6 (vide infra). 2D COSY analysis confirms that the multiple peaks originate from the 3,5 Tyr-49 ring protons, with each coupled solely to Tyr 2,6 ring protons giving peaks in the 7.0–7.1 ppm region.[2] The chemical shifts of the different 3,5 ring proton resonances in the dimer at this pH are 6.60 and 6.68 ppm, with an additional dimer component merging with the monomer at ~6.76 ppm. The line widths at half-height of the resolved dimer components are ~19 Hz, essentially the same as that observed for these protons in the monomer in spectra at low concentrations. The presence of different resolved dimer peaks indicates that the different components of the dimer are in slow exchange on the NMR time scale.

Also seen in Figure 4 are concentration-dependent peaks at 6.44 and 6.2 ppm. Like the 7.57 ppm peak, the 6.44 ppm peak increases with increasing concentration to a limiting intensity representing one proton, while that at 6.2 ppm decreases in intensity with increased concentration. The 6.44 and 6.2 ppm peaks are assigned to dimer and monomer, respectively. The ratio of these intensities as a function of concentration correlates well with known dimerization constants [e.g., see Nicolas et al. (1980) and Peyton et al. (1986)] and can be used in any spectrum to calculate the fractional

[2] More highly expanded COSY spectra also indicate the presence of a Phe proton in the 7.1 ppm peak, not coupled to Tyr 3,5 protons. Studies of mononitrated neurophysins confirm the absence of contributions from Phe protons in the 6.6–6.9 ppm region.

[3] The 6.44 ppm peak was assigned earlier to a nonexchangeable–NH (Virmani-Sardana & Breslow, 1983). The failure to observe loss of this proton in D_2O under any conditions obtained so far, together with the absence of any COSY connectivities to aromatic protons, leads to its reassignment as an α-proton. In confirmation of this assignment, COSY connectivity to 3.4 ppm has recently been demonstrated (Peyton and Breslow, unpublished results). The earlier observation that this proton is broadened by binding is also not supported by studies at higher field. However, on binding peptides with a tyrosine in position 2, a ring proton signal from this tyrosine can be demonstrated to superimpose on the 6.44 ppm peak (V. Sardana and E. Breslow, unpublished observations).

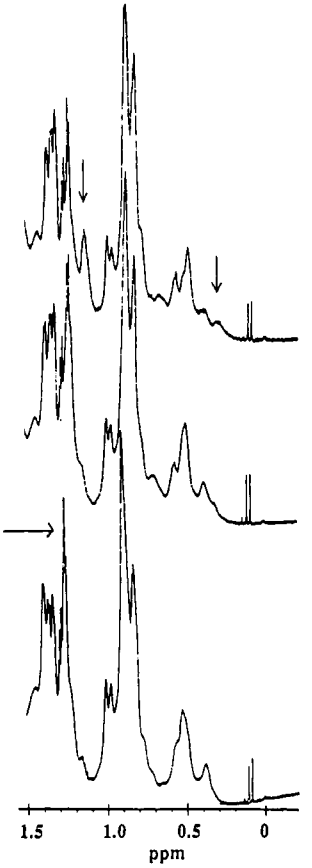

FIGURE 6: Effect of pH on upfield aliphatic protons of des-1–8 bovine neurophysin-I at 620 MHz. Conditions are the same as in Figure 5 except that the spectrum at pH 2.8 is deleted for simplification. pH values from top to bottom are 7.8, 6.6, and 5.8. Arrows point to the position of Thr-9 methyl signals under conditions in which the α-amino is completely protonated (pH 5.8) and largely deprotonated (pH 7.8).

content of dimer and monomer. Both peaks are assigned to the same downfield α-carbon proton, as evidenced by a chemical exchange cross-peak between the two, visible in NOESY spectra (data not shown).[3] The degree of dimerization, as evidenced by the intensity of the 6.44 ppm peak, also correlates with the loss of the sharp Tyr 3,5 ring proton signal assignable to monomer.

The increased resolution of Tyr 3,5 ring protons between pH 6.2 and 8 (vide supra) reflects one of at least two pH-dependent transitions for these protons in the dimer. Figures 5 and 6 show the 620-MHz spectra of ~2 mM des-1–8 neurophysin-I at several pH values for the Tyr-49 and upfield aliphatic regions, respectively. Above pH 5.7, there is an upfield shift with increased pH of the Tyr peak ultimately located at 6.60 (±0.01) ppm at and above pH 8 (Figure 5). The 6.68 ppm dimer peak is independent of pH between 6.6 and 8, but shifts downfield below pH 6 to its ultimate low-pH location at 6.78 ppm. The presence of two overlapping transitions prevents calculation of either of the two pK_a values involved, although the low-pH transition almost certainly involves carboxyl titration, since the pK_a of His-80 is ~7 [this study and Griffin et al. (1975)]. Consideration of the ~2:1 ratio of relative intensities of the resolved dimer peaks at 6.60 and 6.68 ppm indicates that these cannot be accounted for solely by potential nonequivalence of the two 3,5 ring protons, but instead reflect two different slowly-equilibrating Tyr-49 conformers.

In contrast to the pH dependence of Tyr 3,5 ring protons in the dimer, the peak assigned to monomer does not shift

Table I: Comparisons of Chemical Shifts for Tyr 3,5 Ring Protons in Native and Des-1–8 Bovine Neurophysin-I at Representative pH Values[a]

	chemical shifts (ppm)			
	native protein		des-1–8 protein	
pH	monomer	dimer	monomer	dimer
8.2	6.77	6.62, 6.72, 6.77[b]	6.76	6.60, 6.68,* 6.76
7.8	6.77	6.62, 6.73, 6.78, 6.83	6.76	6.60, 6.68,* 6.76
6.6	6.76	6.64, 6.74?, 6.76,* 6.83, 6.87[d]	6.76	6.64, 6.68,* 6.76
5.9	c	6.73, 6.77,* 6.87[d]	c	6.71,* 6.76
2.8			c	6.66, 6.71?, 6.78,* 6.82
2.5	c	6.72, 6.82,* 6.87		

[a] Peak positions are assigned on the basis of 620-MHz data. The most dominant peak, where one is present, is indicated by an asterisk. Question marks indicate poorly resolved minor components. The presence of dimer protons at the same chemical shift as monomer protons is deduced from the line shape at high protein concentrations. [b] An additional downfield component might be present, but is masked by the His C-4 proton. [c] The chemical shift of monomer protons is uncertain at this pH because of the increased dimerization constant. [d] Not clearly seen at 500 MHz.

between pH 6 and 8 (e.g., Figure 5). Studies of monomer protons at low concentrations, where dimer is absent, also do not indicate any pH-dependent shifts in this pH region. [Monomer protons were not studied at low pH because of the increased dimerization constant of neurophysin-I and its derivatives at low pH (Peyton et al., 1986).] Thus, dimerization places Tyr-49 ring protons in two environments that differ from that of monomer and at least one of which has a pH sensitivity different from that of monomer.

The titration specifically responsible for the shift in Tyr 3,5 ring protons in the dimer between pH 6 and 8 is manifest by few other discernible NMR changes and is difficult to assign because of its uncertain pK_a. Changes occur in the 0.5–0.6 ppm region (Figure 6), but these can be shown (from changes at 6.44 and 6.2 ppm) to reflect a small decrease in the dimerization constant over this pH interval and the known differences (Peyton et al., 1986) in the contributions of monomer and dimer to this region. Both His-80 and the α-NH_2 of Thr-9 (vide infra) titrate within this pH range. However, the transition can be shown to occur in the same pH region in unmodified bovine neurophysin-I, eliminating the α-NH_2 of Thr-9 as the responsible titration, and we have failed to obtain convincing evidence that the Tyr-49 transition correlates with His-80 titration. Significantly, however, the Tyr ring protons of neurophysin-II do not shift between pH 6.2 and 8, suggesting that this transition in neurophysin-I might reflect titration of a group unique to this neurophysin.

Dimerization-Induced Changes in Unmodified Bovine Neurophysin-I. At pH 6.2, dimerization induces broadening of the 3,5 ring proton signal of Tyr-49 in native neurophysin-I, with almost no change in the average chemical shift (Peyton et al., 1986; Figure 3). However, a subset of the dimer protons in the native protein, like those of the des-1–8 protein, undergo pH-dependent shifts between pH 6 and 8, giving rise to multiple components in slow chemical exchange (Table I). Identification of these protons as Tyr 3,5 ring protons was confirmed by 2D COSY (as with the des-1–8 protein) and by the loss of these peaks upon nitration of Tyr-49 (data not shown). Differences in chemical shifts and fractional concentrations of the different components in native and des-1–8 proteins (Table I) can be shown to account for the differences between the two dimeric proteins observed at 300 MHz (Peyton et al., 1986). Note that the chemical shift of Tyr 3,5 ring protons in the *monomer* is essentially identical for the modified and native proteins (6.77 ± 0.01 ppm) and, for both proteins, is independent of pH in this pH range.

The Tyr 3,5 ring protons of the native protein also exhibit pH-dependent changes below pH 6, but these are more modest than in the des-1–8 protein (e.g., Table I). This suggests that these changes might result from titration of Glu-47, a residue that interacts with Arg-8 in the crystal (Chen et al., 1991) and which has been suggested to potentially influence the spectroscopic properties of Tyr-49 (Sur et al., 1979). The Tyr 3,5 ring protons of bovine neurophysin-II also exhibit smaller pH-dependent shifts below pH 6 than the des-1–8 derivative of neurophysin-I (data not shown), consistent with this interpretation.

One question is whether the different Tyr ring proton peaks at pH 8 are related to those at pH 6 by real differences in the chemical shift of the different components, or solely by pH-dependent differences in exchange rate among multiple components. The data particularly suggested the possibility of moderate exchange rates among multiple components at pH 6 giving rise to a single broadened peak, with slow exchange at pH 8 allowing resolution of the different components. Studies of the effects of temperature over the interval 10–37 °C in the pH 6–8 region indicated complex effects, but were inconsistent with this possibility (data not shown). First, at fixed pH, the different components typically exhibited increased resolution with increased temperature, indicating that the single peak at pH 6 cannot result simply from an increased exchange rate. Instead, the increased resolution with increased temperature in the pH 6–7 range suggested that the group responsible for the transition exhibited a decreased pK_a with increased temperature. Second, the principal Tyr ring protons exhibited temperature-dependent changes in chemical shift, additionally suggesting that temperature influences the relative distribution of different conformers of Tyr-49.

Multiple Environments for Thr-9 in Dimers of Des-1–8 Neurophysin. Residue 9, like Tyr-49, is close to the peptide-binding site and distant from the monomer–monomer interface in the crystal (Chen et al., 1991). Like Tyr-49, it also appears to be affected by dimerization in the unliganded state and to have two environments in the dimer.

The chemical shift of the methyl protons of Thr-9 in des-1–8 bovine neurophysin-I at pH 6 has been assigned as 1.3 ppm, via comparison of spectra of the native and modified proteins (Sardana & Breslow, 1984). At this pH, the α-amino group of Thr-9 in the des-1–8 protein is almost completely protonated, and the methyl protons are unaffected by dimerization (Peyton et al., 1986). Titration of the amino group can be followed by a shift from 1.29 to 1.13 ppm (e.g., Figure 6) and shows an apparent pK_a of ~7. In the present studies, a peak is seen at ~0.35 ppm that increases in intensity coincident with deprotonation of the α-NH_3^+ of Thr-9 (Figure 6); this peak decreases in intensity upon dilution, indicating that it is a component of the dimer (data not shown). COSY analysis at 620 MHz (Figure 7) shows that the peak at 0.35 ppm and that at 1.13 ppm are both coupled to a proton at 3.95 ppm, the latter accordingly also assigned to the Thr-9 β-proton. The results indicate two slowly exchanging environments for the Thr-9 methyl group of the des-1–8 dimer when the α-amino group is deprotonated. The fact that the β-proton appears to have the same chemical shift in both conformers is probably best explained by sufficiently small differences between the two forms in its chemical shift that rapid equilibration is seen on the NMR time scale. It is relevant that spectra of the native protein also show a small dimer peak at ~0.35 ppm, but one that is pH-independent (data not shown). This is

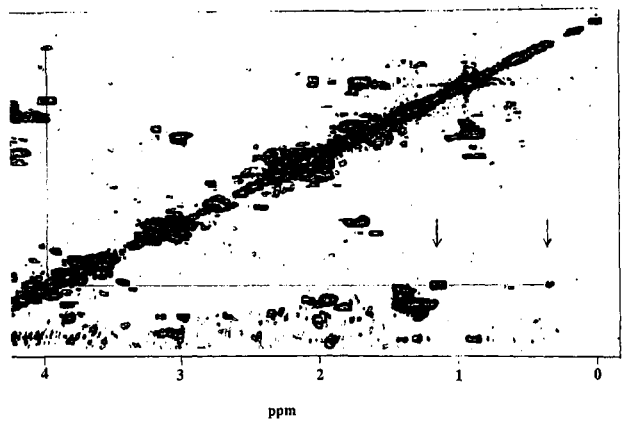

FIGURE 7: 620-MHz COSY spectra of ~2 mM des-1–8 neurophysin at pH 8. Arrows show peaks that are absent or shifted in the unmodified protein and assigned to Thr-9. Note that connectivities between the assigned Thr-9 β-proton and the corresponding α-proton are unclear.

consistent with a similar conformer of Thr-9 methyl protons in the native protein.

Partial Identification of Residues Participating in Dimerization in Unliganded Bovine Neurophysin-I. Since neither Tyr-49 nor residue 9 is close to the monomer–monomer interface in crystals of liganded bovine neurophysin-II (Chen et al., 1991), one question is whether there are fundamental differences in this interface in liganded and unliganded states. Studies of pressure-induced subunit dissociation have in fact demonstrated significant differences in interface properties between the two states (Breslow et al., 1991). However, this is more likely to reflect an effect of ligand binding on the nature or number of interactions at the interface, rather than a fundamental change in interface location. Liganded and unliganded states form mixed dimers with dimerization constants intermediate between those of the two states [e.g., see Ando et al. (1987)], and studies of ostrich neurophysin dimers, in which interface residue Phe-35 of the crystal is substituted by Tyr, indicate virtually no effect of ligand on the optical activity of Tyr-35 (Breslow et al., 1992). In the present study, the extent to which protons known to be at the monomer–monomer interface in the crystal are perturbed by dimerization in the unliganded state was probed.

Interface residue Val-80 of liganded bovine neurophysin-II is substituted by His in bovine neurophysin-I (Figure 1). 220-MHz NMR studies of His-80 had demonstrated its complex behavior as a function of pH, reflecting the presence of more than one conformer (Griffin et al., 1975). At higher field, the C-2 proton of His-80 is virtually impossible to detect unless completely in the protonated or deprotonated form because of exchange broadening; this appears to be true for both the monomer and dimer, indicating that this complexity in part reflects intrachain events (data not shown). Such studies are additionally complicated by the presence of a frequent 8.48 ppm contaminant. However, under optimal conditions, we detect a minor 8.39 ppm peak in high-field pH 6.2 spectra of the des-1–8 derivative that linearly increases in intensity with the weight fraction of monomer and that shifts with pH in the region of His titration (Figure 8). The integrated intensity of this peak in the monomer at pH 6.2 leads to its assignment as the C-2 proton of His-80, the effects of concentration indicating that its chemical shift changes with dimerization. Additionally, studies of the effects of monocarboxymethylation of His-80 (Materials and Methods) indicate that this modification leads to a 60% reduction in the dimerization constant above pH 6 (Figure 8). In most other respects, 1D spectra

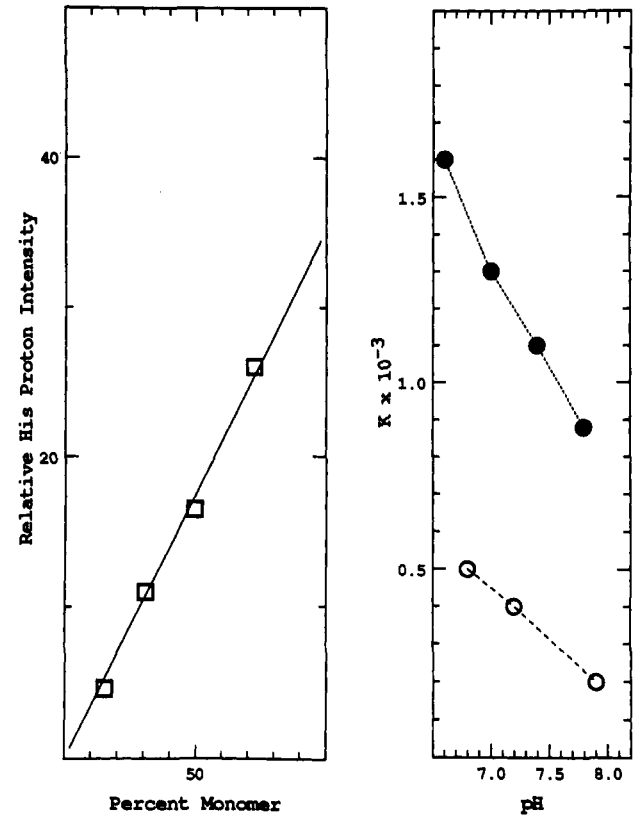

FIGURE 8: Role of His-80 in dimerization. Left: Intensity of the 8.39 ppm peak of des-1–8 bovine neurophysin-I relative to that of total Tyr 3,5 ring protons as a function of the weight fraction of monomer at pH 6.2. Data represent 500-MHz spectra and were calculated from the integrated intensities of the 8.39 ppm and Tyr 3,5 ring proton peaks and the integrated intensities of the 6.44 and 6.2 ppm peaks. Results are in good agreement with a model in which the 8.39 ppm protons represent the C-2 proton from protonated His-80 of the monomer, assuming a pK_a for His-80 in the monomer of 6.6. Right: Comparison of apparent dimerization constants of native (●) and monocarboxymethylated (○) bovine neurophysins as a function of pH. Data were calculated from the ratio of 6.44 to 6.2 ppm peaks in samples of known concentration and pH.

of the carboxymethylated and native proteins are similar. The sensitivity of His-80 protons to dimerization and the effect of modification of His-80 on dimerization are consistent with, albeit not proof of, the proximity of His-80 to the monomer–monomer interface in the unliganded protein.

In the liganded state, Phe-35 is at the center of a different segment of the monomer–monomer interface than that containing His-80, and its ring is in close proximity to Phe-35 from the partner chain (Chen, 1991); Phe-22 does not participate in dimerization. Of particular interest here is evidence suggesting that the 7.57 ppm dimer ring proton (Figure 3) and 6.44 ppm dimer α-proton (Figure 4) are part of the same Phe system. The 7.57 ppm proton shows COSY and NOESY connectivities to Phe protons at 7.38 ppm and NOESY connectivity to Phe protons at 7.28 ppm, allowing assignment of the 7.57, 7.38, and 7.28 ppm protons to protons 4, 3(5), and 2(6), respectively, of the same Phe residue. This residue either is the same as that containing the 6.44 ppm proton or is immediately adjacent to it, since a 1D NOE was demonstrated between the 6.44 ppm proton and protons at 7.38 and 7.29 ppm. [Recent NOESY studies give similar results (Peyton and Breslow, unpublished observations).] Moreover, since the 6.44 ppm peak is unaffected by peptide binding,[3] assignment to Phe-22 is improbable; the α-proton of Phe-22 is ~4 Å from the aromatic ring in position 2 of bound peptides (Chen et al., 1991). The results are therefore

consistent with assignment of the dimerization-sensitive Phe protons to Phe-35. This is supported by the fact that the 7.57 ppm band is absent in ostrich neurophysin dimers and the 6.44 ppm band is replaced by one at ~6.39 ppm (unpublished results), both the expected consequences [e.g., see Bundi and Wuthrich (1979)] of substitution of Phe-35 by Tyr in this protein. The results give a self-consistent picture of the involvement of Phe-35 at the interface in both liganded and unliganded states.

DISCUSSION

The present high-field NMR studies permit a closer look at the dimerization-induced change in behavior of Tyr-49 ring protons than obtained earlier. The results demonstrate that the dimerization-induced increase in signal line width seen at lower field primarily reflects heterogeneity in the environment of Tyr ring protons in the dimer, not increased dipolar broadening, and that this heterogeneity is present in dimers of both the native and des-1–8 proteins. Dimerization-induced differences between native and des-1–8 proteins are demonstrated to represent chemical shift differences between the two in their different conformers. The lack of significantly increased dipolar broadening of Tyr 3,5 ring protons in the dimer relative to the monomer weakens the argument that interactions of the ring across the monomer–monomer interface are responsible for the Tyr proton line width in the dimer and that Tyr-49 is close to the interface (Peyton et al., 1986). Also, the fact that perturbation of Tyr-49 protons correlates with the concentration of dimer as measured by the 6.44 ppm peak (vide supra) confirms that the perturbation under these conditions does not result from dimer–dimer interactions, such as those in the crystal (Chen et al., 1991), in which the Tyr-49 ring is a direct participant. The NMR data moreover are consistent with the concept that regions of the protein that participate in the monomer–monomer interface in the crystalline liganded state do so in the unliganded state; i.e., evidence for participation of the regions around residues 35 and 80 is found. Accordingly, it seems most likely, if not strictly established, that the dimerization-induced perturbation of Tyr-49, and also of Thr-9, reflects dimerization-induced conformational change and not the direct participation of these residues at or close to the monomer–monomer interface.

We have also considered the possibility that there are two equilibrating forms of the dimer in the unliganded state: one with a monomer–monomer interface analogous to that of the liganded state (primarily residues 32–38 and 77–81) and the other with an interface at Tyr-49, analogous to the dimer–dimer interface of the liganded state. This would place Tyr-49 at an interface without requiring its proximity to residues of the other interface. This scenario can be ruled out for two reasons. First, in the absence of other constraints, it suggests that unliganded neurophysin should be an indefinitely associating system, while ultracentrifuge studies indicate monomer–dimer equilibria (Nicolas et al., 1980). Most importantly, perturbation of Tyr-49 3,5 ring protons by dimerization parallels the intensity of both the 6.44 ppm α-proton and the 7.57 ppm Phe dimer peaks (e.g., Figure 4). When these peaks both reach their limiting values of one proton in the dimer, no Tyr-49 monomer protons are seen. These data can only be explained if all peaks assigned to dimer originate from a single dimer, albeit one with different conformers.

The proposed dimerization-induced conformational change in the unliganded state represents changes only in 3° structure, as evidenced by the CD results, but has a large impact on the peptide-binding site as evidenced by its effects on both Tyr-49 and Thr-9, which bracket this site in the crystal (Chen et al., 1991). This effect clearly has implications for the mechanism by which dimerization enhances peptide affinity. One consequence of dimerization is to make Tyr-49 sensitive to the deletion of Arg-8. Native and des-1–8 proteins differ in the chemical shift of Tyr 3,5 ring protons only in the dimer; chemical shifts are the same in the monomer. In the liganded state, the side chains of Glu-47 and Arg-8 interact with each other within the same subunit, close to the backbone of Tyr-49 (Chen et al., 1991). Accordingly, we suggest that intrasubunit differences between unliganded monomer and dimer in the relative orientations of Tyr-49, Glu-47, and Arg-8 account for the effects of dimerization on Tyr-49 and the modulation of these effects by Arg-8. Such differences would contribute to differences between monomer and dimer in binding affinity if the relative orientation of these residues in the dimer was energetically more similar to that in the bound state than that in the monomer.

The presence of different dimer conformers has additional significance. There are at least three peaks assignable to the 3,5 ring protons of Tyr-49 in dimers of neurophysin-I and its derivatives. Allowing that two of these (i.e., the minor peaks) might result from hindered ring rotation and nonequivalence of the two protons in a single conformer, at least two conformations are represented, raising the possibility of the nonequivalence of dimer subunits. Two environments for Thr-9 methyl protons in the dimer are also suggested. In unliganded neurophysin-II, both earlier Raman evidence (Liu, 1975) and present data (Figure 3) indicate two environments for Tyr-49. In liganded bovine neurophysin-II, two environments for Tyr-49 were suggested by fluorescence polarization studies (Scarlata & Royer, 1986), although participation of Tyr-49 in dimer–dimer interactions, analogous to those of the crystal, is not precluded in this instance (Breslow et al., 1991). The present data are insufficient to establish whether the different unliganded conformers reflect true subunit nonequivalence or different conformations of the whole dimer. The slow exchange among the different conformers might be relevant, indicating an exchange rate of less than 15 s^{-1} that compares to a dimer dissociation rate of 12 s^{-1} (Pearlmutter, 1979). It is therefore possible that the dimer must dissociate in order for the tyrosine environment to change, suggesting that dimerization might lock in a particular conformation, each dimer fixed with its two tyrosines in nonidentical conformations.

In either event, it is plausible that the different Tyr-49 conformers reflect different arrangements of interactions at the monomer–monomer interface that are communicated to the peptide-binding site; only one conformer has at least so far been detected in the monomer.[4] Such different arrangements of interface residues might contribute to the relatively low volume change accompanying pressure-induced dimer dissociation in the unliganded state (Breslow et al., 1991), by allowing intermediate states during dissociation, thereby reducing the slope of the pressure dissociation curve and the calculated volume change. Different possible arrangements of interface residues, which were also suggested by the pressure studies, would account for the ability of liganded and unliganded chains to form mixed dimers, despite their conformational differences (Breslow et al., 1991). The model

[4] Strictly speaking, the possible presence of multiple rapidly interconverting monomer species or different monomer conformations with overlapping chemical shifts is not precluded by these data. However, in neurophysin-I derivatives, such conformers must differ from those of the corresponding dimers by their insensitivity to pH above pH 6.

suggested here for the interface of the unliganded state, however, differs from that based on studies of pressure-induced dimer dissociation, which suggested a loose and relatively flexible packing of interface residues. The present results suggest that the different conformations at the interface do not reflect simply loose packing of interface residues, but a finite number of defined arrangements of similar energy that are separated by a significant kinetic barrier.

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