Dimerization of Native and Proteolytically Modified Neurophysins As Monitored by Proton Magnetic Resonance Spectroscopy: Proximity of Tyrosine-49 to the Subunit Interface†

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ABSTRACT: Neurophysin is a self-associating protein in which peptide–hormone binding and dimerization are thermodynamically linked. The structural basis of the linkage is unknown. We have studied the dimerization of bovine neurophysin I and two proteolytically modified derivatives by proton nuclear magnetic resonance spectroscopy in order to identify residues at the intersubunit contact regions and to evaluate the origin of the reported loss of dimerization associated with tryptic excision of residues 1–8. The concentration dependence at neutral pH of the spectra of native neurophysin and des-90–92-neurophysin demonstrated a finite set of dimerization-sensitive resonances that included the ring protons of Tyr-49. Using these to monitor dimerization, we confirmed predictions of a large increase in the dimerization constant associated with carboxyl protonation. By the same criteria, dimerization of the des-1–8 protein, in disagreement with earlier reports, was found to be undiminished relative to that of the native protein. However, spectral changes in the Tyr-49 ring ortho proton region associated with dimerization of the des-1–8 protein differed significantly from those in the native protein and indicated an altered conformation of the des-1–8 dimer apparently restricted to the vicinity of Tyr-49. The results are shown to place Tyr-49 adjacent to both the intersubunit contact region and the 1–8 sequence in the native protein, loss of stabilizing interactions with 1–8 leading to altered interactions of Tyr-49 with the subunit interface. Because Tyr-49 is also close to the peptide-binding site, this arrangement spatially links the peptide-binding and dimerization sites of neurophysin. The results suggest a model in which bound peptide is stabilized by supporting interactions from both subunits, providing a potential explanation of the stronger binding of peptide to the dimer than to the monomer.

The binding of peptide hormones and smaller peptides to the pituitary protein neurophysin is linked to changes in neurophysin self-association. Unliganded neurophysin is present in monomer–dimer equilibrium (Breslow et al., 1971); occupancy by peptides of the principal hormone-binding sites leads to a shift in this equilibrium toward dimer and is associated with marked changes in conformation (Cohen et al., 1979; Rholam et al., 1982; Virmani-Sardana & Breslow, 1983). Regions of the protein participating in self-association are unknown. Additionally, relatively little is known about the peptide-binding site. Early and recent studies provide increasing evidence for the proximity of the single neurophysin tyrosine, Tyr-49, to the peptide site (Balaram et al., 1973; Abercrombie et al., 1982b; Peyton & Breslow, 1985); Glu-31 has also been implicated (Walter & Hoffman, 1973). An unsolved question is the relative role of neurophysin's internally duplicated sequences (12–31 and 60–75) in binding and self-association (Breslow, 1979; Cohen et al., 1979).

Neurophysin is an interesting candidate for nuclear magnetic resonance (NMR)[1] analysis. With a chain length of approximately 95 residues, the monomer ⇌ dimer system is slightly above the molecular weight of proteins typically accessible to detailed study by NMR spectroscopy. We have investigated aspects of the binding of peptides and peptide hormones to neurophysin by proton NMR and have made selected proton resonance assignments via comparison of native and proteolytically modified neurophysins [e.g., see Balaram et al. (1973) and Sardana & Breslow (1984)]. Among the proteolytically modified neurophysins studied by NMR and other techniques has been des-1–8-neurophysin, representing tryptic excision of the first eight residues (Abercrombie et al., 1982a; Breslow et al., 1982). Although by many criteria the conformation of this protein is the same as that of native neurophysin, its affinity for dipeptides is only $^1/_{50}$th that of the native protein; direct interactions between the 1–8 segment of the native protein and bound dipeptide are not evident and therefore do not appear to account for this effect (Sardana & Breslow, 1984). Additionally, dimerization ability has been reported to be lost or vastly diminished in the des-1–8 protein as judged by affinity chromatography criteria (Abercrombie et al., 1982a); preliminary NMR studies also appeared consistent with some reduction of self-association in the trypsin-modified protein (Sardana & Breslow, 1984). The functional effects of excision of residues 1–8, which have been assigned principally to the loss of Arg-8 (Breslow et al., 1982), therefore provide a potential approach to a more specific understanding of the relationship between conformation, binding, and self-association in neurophysin.

In the present study, in order to further evaluate the role of residues 1–8 in affecting protein self-association, we initiated a more detailed investigation of the dimerization properties of native and des-1–8-neurophysins, studying the concentration dependence of the NMR spectra of both proteins. These studies differ from the earlier NMR investigation of this system (Sardana & Breslow, 1984) in the use of a broad range of protein concentrations, which in turn permitted spectra of the native protein to be obtained under conditions ranging from

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[1] Abbreviations: NMR, nuclear magnetic resonance; TSP, sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate; CD, circular dichroism; EDTA, ethylenediaminetetraacetic acid.

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Ala-Val-Leu-Asp-Leu-Asp-Val-Arg-Thr-Cys-Leu-Pro-Cys-Gly-Pro-Gly-Gly-Lys-Gly-Arg-Cys-Phe-Gly-Pro-
 1                              8   9                                                      22

Ser-Ile-Cys-Cys-Gly-Asp-Glu-Leu-Gly-Cys-Phe-Val-Gly-Thr-Ala-Glu-Ala-Leu-Arg-Cys-Gln-Glu-Glu-Asn-
25                                      35

Tyr-Leu-Pro-Ser-Pro-Cys-Gln-Ser-Gly-Gln-Lys-Pro-Cys-Gly-Ser-Gly-Gly-Arg-Cys-Ala-Ala-Ala-Gly-Ile-
49

Cys-Cys-Ser-Pro-Asp-Gly-Cys-His-Glu-Asp-Pro-Ala-Cys-Asp-Pro-Glu-Ala-Ala-Phe-Ser.
73                              80                                      91
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FIGURE 1: Amino acid sequence of bovine neurophysin I, residues 1–92 (Breslow, 1979). This is the principal neurophysin I isolated under standard conditions. Numbers are in part to delineate residues discussed in the text.

essentially pure monomer to pure dimer. Among the particular questions investigated were the identity of residues normally participating in self-association, whether any of these can be assigned to the 1–8 sequence, and, of course, the relative ease of self-association in both proteins. As an aid to the interpretation of the spectra, the properties of carboxypeptidase A treated neurophysin (Sardana & Breslow, 1984), which lacks the three carboxyl-terminal residues, were also investigated. The results surprisingly demonstrate that residues 1–8 do not significantly contribute to the thermodynamic stability of the neurophysin dimer but singularly alter the response of Tyr-49 to dimerization. The effects of dimerization on Tyr-49 in the native and modified proteins are shown to place Tyr-49 proximal to the subunit interface, a location with significant implications for neurophysin structure–function relationships. The results also place Tyr-49 proximal to the 1–8 sequence and, in so doing, provide the first insights into how neurophysin is folded. For reference in interpreting these studies, the amino acid sequence of bovine neurophysin I is shown in Figure 1.

Experimental Procedures

Preparation of Native and Modified Neurophysins. Bovine neurophysin I and carboxypeptidase A treated bovine neurophysin I were prepared as previously described (Carlson & Breslow, 1981). Des-1–8-neurophysin I was prepared by treating bovine neurophysin I with trypsin also as previously described (Breslow et al., 1982; Sardana & Breslow, 1984), but employing a slight modification of the original isolation procedure. In the original procedure, the product of the controlled digestion of the native protein with trypsin was subjected to affinity chromatography (Rabbani et al., 1982). This product gave two general fractions—a weakly binding product (protein eluting at pH 6.2) representing neurophysin from which residues 1–8 had been excised and a strongly binding product (eluting at pH 2) which represented the native protein. The entire weakly binding product was used for NMR studies but was rechromatographed to remove a small fraction of completely nonbinding protein when thermodynamic studies were carried out. In the present study, the completely nonbinding protein was eliminated in a single affinity chromatography run by discarding all protein eluting at the column void volume at pH 6. The resultant weakly binding product showed complete loss of residues 1–8 on amino acid analysis as found previously and was completely devoid of internal tryptic clips as judged by carboxypeptidase B end-group analysis (Breslow et al., 1982; Sardana & Breslow, 1984); this product was used for the NMR studies reported here. Comparison of the completely negative carboxypeptidase B end-group analysis of this product with that of the unfractionated des-1–8 protein (Sardana & Breslow, 1984) indicates that the small but finite percentage of internal clips in the unfractionated protein are contained in the completely nonbinding component and represent cleavage at Lys-18 and Arg-43.

NMR Studies. Proton NMR studies were conducted at 24 °C in D_2O using the 300-MHz Rockefeller Nicolet instrument as previously described (Virmani-Sardana & Breslow, 1983; Sardana & Breslow, 1984) with the exception that EDTA was omitted from all samples. Earlier NMR studies had suggested that EDTA might react with neurophysin (Sardana & Breslow, 1984). More recent results suggest that the spurious peaks in EDTA-containing samples represent traces of metal–EDTA complexes (D. Peyton and E. Breslow, unpublished results). The presence or absence of EDTA has been shown not to alter neurophysin behavior (Sardana & Breslow, 1984).

Protein samples were typically prepared for NMR analysis by repeated lyophilization of a weighed quantity of protein from 99.8% D_2O. The deuterated protein was then dissolved in a measured volume of 99.996% D_2O and adjusted to the desired pD (uncorrected pH meter reading) with NaOD and/or DCl; the uncorrected meter reading is hereafter referred to as pH. Because the final salt concentration of the different samples was not necessarily uniform, control studies of the effects of added 50 mM NaCl to samples at both low and high protein concentration were performed. No spectral changes resulted from the increased NaCl. All spectra were obtained in 5-mm tubes and enhanced by a 2-Hz exponential factor to increase the signal:noise ratio. Low-concentration spectra were collected in double precision. At the lowest concentration (0.02 mM), 4000 scans were collected.

Results

Concentration-Dependent Resonances in Spectra of Native Neurophysin I and Carboxypeptidase A Treated Neurophysin I at pH 6. Figures 2 and 3 show the aliphatic and aromatic proton NMR spectra, respectively, of native neurophysin I at concentrations of 0.2 mg/mL (2×10^{-5} M), 2 mg/mL (2×10^{-4} M), and 30 mg/mL (3×10^{-3} M) at pH 6.2. From the published dimerization constant for neurophysin I under similar conditions, $\sim 7 \times 10^3$ M^{-1} (Nicolas et al., 1980), the weight concentration of dimer is expected to increase from ~5% at the lowest concentration to 90% at the highest concentration. Arrows indicate regions in which large, reproducible, and systematic concentration-dependent changes are seen. In the aliphatic region, these are ~0.6, 1.1–1.4, 1.7, 2.47, and 3.8–3.9 ppm. Since most of the concentration-dependent resonances cannot be assigned unambiguously, they are used here largely as empirical indicators of dimerization. It is pertinent, however, that some of the protons in the 1.1-1.4 ppm region have been assigned to the $-CH_3$ protons of Thr-9, Ala-89, and Ala-90 and that the relatively sharp tip of the peak at 3.8 ppm in the high-concentration spectrum has been assigned to the β-CH_2 of Ser-92 (Sardana & Breslow, 1984). The question therefore arises as to whether changes in these regions reflect changes at the carboxyl terminus or at Thr-9. Studies of the concentration dependence of the spectra of the carboxypeptidase-treated protein, which lacks residues 90–92 and in which the Ala-89 resonance is sharpened (Sardana & Breslow, 1984), indicate that none of the concentration-dependent changes reflect the 89–92 region of the sequence (data not shown). The possible role of Thr-9 will be discussed below.

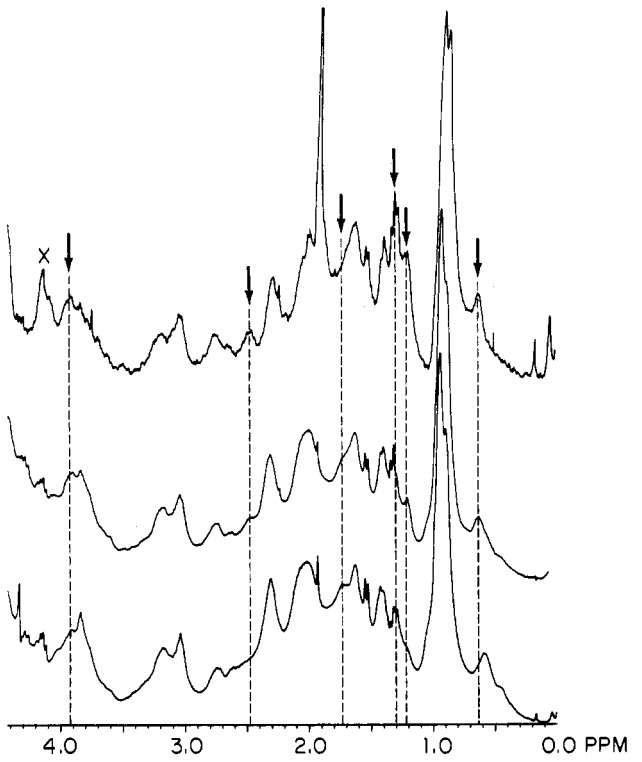

FIGURE 2: Aliphatic proton resonances of native neurophysin I at pH 6.2 as a function of protein concentration. Abscissa is in ppm downfield from TSP. Neurophysin concentration from top to bottom: 2×10^{-5}, 2×10^{-4}, and 3×10^{-3} M. Arrows point to concentration-sensitive resonances, with vertical lines drawn at constant chemical shift. The peak at 1.9 ppm is acetate. The peak marked by X is an artifact.

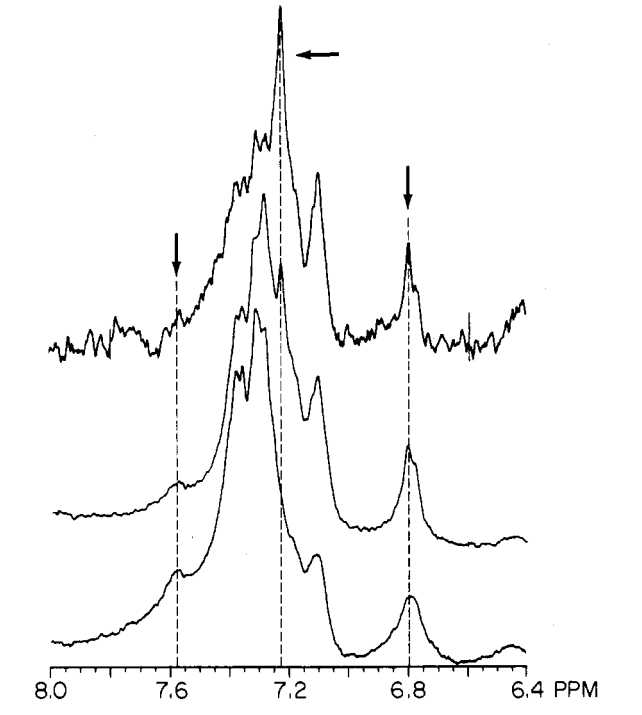

FIGURE 3: Aromatic proton resonances of native neurophysin I at pH 6.2 as a function of protein concentration. Neurophysin concentration from top to bottom: 2×10^{-5}, 2×10^{-4}, and 3×10^{-3} M. Other details as in Figure 2.

In the aromatic proton region of the native protein (Figure 3), three concentration-dependent changes are clearly and reproducibly seen at pH 6. With increasing concentration, these are the following: (1) marked broadening of the previously assigned Tyr-49 ortho ring proton peak at 6.79 ppm (Balaram et al., 1973) from a half-height width of ~19 Hz to ~30 Hz; (2) disappearance of a sharp peak at 7.22 ppm; and (3) increased resolution of a peak at 7.58 ppm, both previously assigned (Sardana & Breslow, 1984) to Phe. The data shown exclude the poorly resolved His-80 C-2 proton which is located near 8.5 ppm at this pH. The His-80 C-4 proton in native neurophysin I can be shown at pH 6.2 to be located at 7.27 ppm and is not the source of the low-concentration 7.22 ppm band at this pH [e.g., see Sardana & Breslow (1984)].

The Phe proton changes at 7.22 ppm are an extension of those seen earlier over a more limited concentration range and tentatively assigned to either Phe-22 or Phe-35 (Sardana & Breslow, 1984). The same changes with concentration at 7.22 ppm can be shown to occur in the carboxypeptidase-treated protein, which lacks Phe-91, confirming the assignment of the 7.22 ppm peak to either Phe-22 or Phe-35. The 7.58 ppm peak seen at high concentration (Figure 3) has also been previously assigned to Phe-22 and/or Phe-35 (Sardana & Breslow, 1984), but its concentration dependence had not previously been observed. Similarly, the spectral broadening of the Tyr ortho protons with increased concentration had not previously been reported, although small effects on Tyr-49 protons with more limited changes in concentration have been seen at 600 MHz (A. A. Bothner-By, P. K. Mishra, and E. Breslow, unpublished results).

The above changes are saturable, specific effects of concentration on molecular structure and, for the most part, do not represent nonspecific effects such as viscosity-induced broadening. This is shown in Figure 4 where changes in the 0.6 ppm proton region and at 7.22 ppm are plotted as a function of concentration. In analyzing these changes, it is relevant that those in the 0.6 ppm region occur in the slow to intermediate rate range on the NMR time scale. Thus, two peaks of equal intensity, centered at 0.57 and 0.65 ppm, can be seen at 1 mM neurophysin concentration (data not shown), and this phenomenon is also evident in the des-1–8 protein (vide infra). Accordingly, changes in the 0.6 ppm methyl proton region were analyzed as the change in the ratio of the 0.58 and 0.64 ppm intensities. Changes at 7.22 ppm were analyzed as the intensity at this chemical shift normalized to the area of the Tyr-49 ortho ring protons. The spectral changes are seen to be the most strongly concentration dependent below 1 mM and do not increase remarkably as the concentration is increased above 2 mM; the apparent midpoint at $\sim 3 \times 10^{-4}$ M is in reasonable agreement with the reported dimerization constant of $\sim 7 \times 10^{3}$ M^{-1} (Nicolas et al., 1980). They are therefore assigned to the neurophysin monomer $\rightleftharpoons$ dimer equilibrium.

Altered Concentration Dependence of the NMR Spectrum of Native Neurophysin I at pH 3. Effects of protein concentration in the 0.1–3 mM range are markedly reduced at pH 3. Figures 5 and 6 compare spectra of native neurophysin I at 0.1 and 3 mM at pH ~3 with spectra at 3 mM at pH 6.2. In the dimerization-sensitive regions near 0.6, 1.2, and 1.7 ppm, both concentrations at pH 3 resemble the high-concentration pH 6 spectrum (Figure 5). Observation of the other aliphatic proton peaks that change with concentration at pH 6 cannot be made at pH 3 because of titration-shifted resonances. However, in the aromatic proton region (Figure 6), Phe ring protons are seen to be independent of concentration at pH 3, and only subtle changes are seen in Tyr ortho protons. The latter are of uncertain significance since the low-concentration Tyr spectra at pH 3 are identical with the high-concentration spectra at pH 6 (Figure 3).

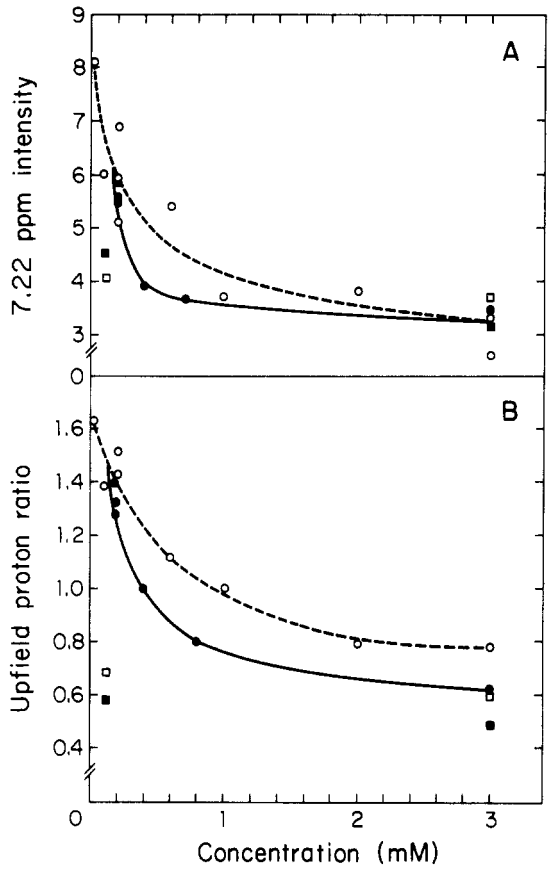

FIGURE 4: Concentration dependence of the 7.22 ppm resonance and the 0.6 ppm proton resonance bands in native and des-1–8-neurophysins I. The intensity of the 7.22 ppm resonance is given as the height of the band at 7.22 ppm normalized to constant area of the Tyr-49 ortho ring protons. Parameters for the 0.6 ppm region are the ratio of the intensity at 0.64 ppm to that at 0.58 ppm ("upfield proton ratio"): (○) native neurophysin I, pH 6.2; (●) des-1–8-neurophysin I, pH 6.2; (□) native neurophysin I, pH 3; (■) des-1–8-neurophysin I, pH 3. Dashed and solid lines are approximate fits of the pH 6.2 data for native neurophysin and the des-1–8 protein, respectively.

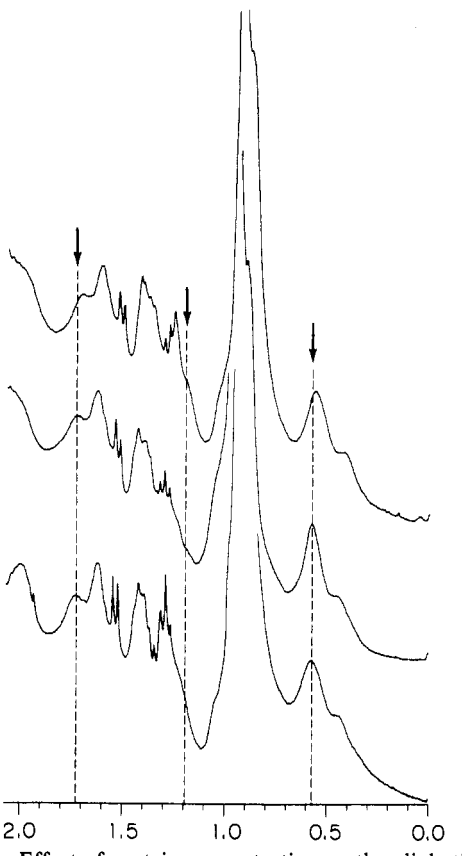

FIGURE 5: Effect of protein concentration on the aliphatic proton resonances of native neurophysin I at pH 3. (Top spectrum) 1×10^{-4} M protein, pH 3; (middle spectrum) 3×10^{-3} M protein, pH 3; (bottom spectrum) 3×10^{-3} M protein, pH 6. Resonances in the 0.8–1 ppm region are incompletely shown to allow stacking of the spectra; these resonances were the same in all spectra. Arrows and lines delineate regions that are concentration sensitive at pH 6. Other details as in Figure 2.

In Figure 4, the concentration dependence of dimerization-sensitive peaks at pH 3 is compared with that at pH 6. The data argue for an approximately 10-fold increase in the dimerization constant between pH 6 and pH 3.[2] CD studies have previously demonstrated a carboxyl pK_a in both bovine neurophysins I and II that increased with protein concentration (Breslow & Gargiulo, 1977; Sur et al., 1979). The increase in protein dimerization between pH 6 and 3 seen here appears to be the thermodynamically demanded parallel of the same phenomenon. It is relevant that we have previously also noted a decrease in dimerization constant as the pH is raised above pH 6.2 to pH 8.3 (Sardana & Breslow, 1984), and this observation has been confirmed in these studies. This decrease might represent the tail of the carboxyl titration curve or an additional effect of the titration of the single neurophysin I histidine (His-80) on dimerization.

Effects of Concentration on Des-1–8-neurophysin I. Figures 7 and 8 compare the spectra of des-1–8-neurophysin I at 0.2 and 3 mM concentration near pH 6. In the aliphatic proton region (Figure 7), spectra at high concentration are the same as those reported previously (Sardana & Breslow, 1984) with the exception of very subtle differences in the 1.6–1.9 ppm region; the latter can be shown to be due to elimination from these spectra of the earlier small contribution of internally clipped (completely nonbinding) protein (Experimental Procedures).[3] The high-concentration aromatic region spectra (Figure 8) are also the same as those previously reported except for the absence of the small broad shoulder seen earlier near 7.2 ppm and attributed to the concentration-dependent 7.22 ppm Phe protons. Note that a well-resolved peak at 7.17 ppm in the high-concentration spectrum here is clearly different from the concentration-dependent 7.22 ppm Phe peak (which can be seen in the low-concentration spectrum of the des-1–8 protein) and is assigned in this spectrum to the C-4 of His-80 based on the chemical shift of the accompanying His-80 C-2 proton.[4]

[2] An alternative but less probable explanation of the low-pH data is that conformational changes within the monomer on lowering the pH spectrally mimic the effects of dimerization. We consider this unlikely because of the number of different resonances that appear to be comparably affected by dimerization and by lowering the pH.

[3] We have carried out preliminary proton NMR studies of the internally clipped protein. Its spectrum is largely similar to that of des-1–8-neurophysin lacking internal clips but differs significantly in the 1.6–1.7 ppm region. These differences probably directly reflect the fact that the internal clips are at Lys-18 and Arg-43. Protons from both Lys-18 and Leu-42 can be expected (Sardana & Breslow, 1984) to occur in the 1.6–1.7 ppm region.

[4] The C-2 proton is located at 8.55 ppm at pH 6.2 (Sardana & Breslow, 1984) and shifted downfield approximately 1.28 ppm from the C-4 proton at this pH which can be shown to be located near 7.3 ppm. Small increases in pH above 6.2 lead to the expected upfield shifts in both the C-2 and C-4 protons. In the high-concentration spectrum in Figure 8, the C-2 proton was located at 8.38 ppm, corresponding to a pH of ~6.4, explaining the C-4 proton at 7.17 ppm. The 7.17 ppm peak is lost at lower pH. Similar pH effects are seen in the native protein.

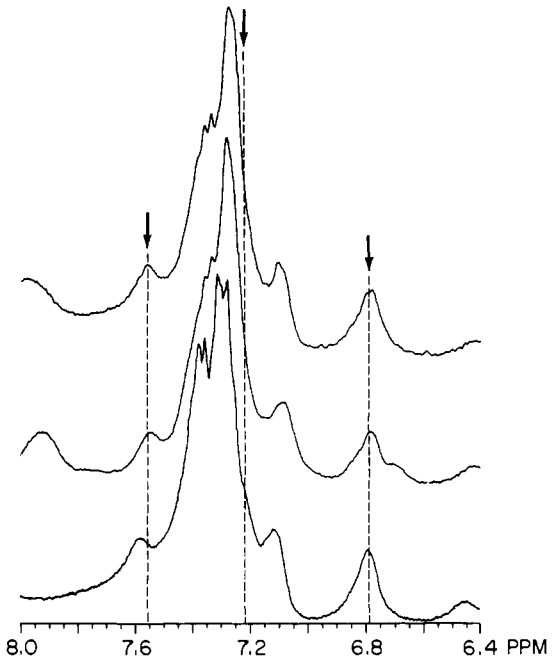

FIGURE 6: Effect of protein concentration on the aromatic proton resonances of native neurophysin at pH 3. (Top spectrum) 1×10^{-4} M protein, pH 3; (middle spectrum) 3×10^{-3} M protein, pH 3; (bottom spectrum) 3×10^{-3} M protein, pH 6. Arrows and lines delineate regions that are concentration sensitive at pH 6. Other details as in Figure 2.

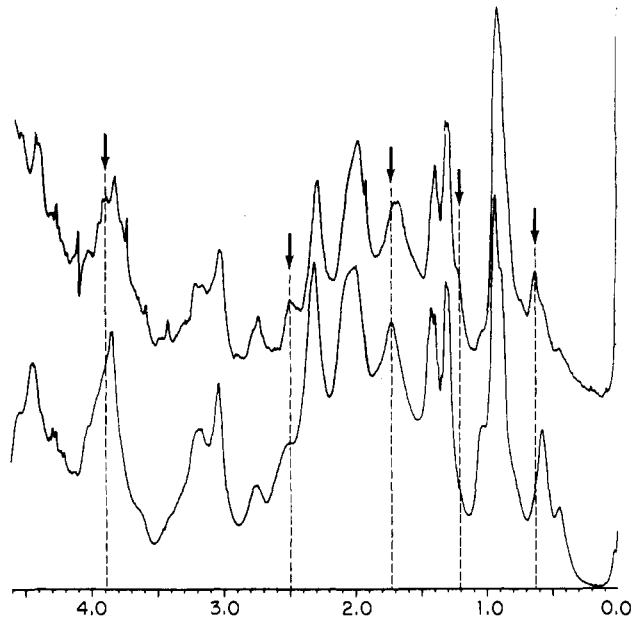

FIGURE 7: Aliphatic proton resonances of des-1–8-neurophysin I at low and high protein concentrations at pH 6.2. (Top spectrum) 2×10^{-4} M protein; (bottom spectrum) 3×10^{-3} M protein. Other details as in Figure 2.

As seen in Figures 7 and 8, spectra of the des-1–8 protein are concentration dependent, indicating the presence of self-association. Essentially all of the concentration-dependent changes seen in the native protein (Figures 2 and 3) are in fact present in the trypsin-treated protein. Even the slow exchange rate of the upfield aliphatic peak (vide supra) can be seen in the 0.2 mM spectrum in Figure 7 as a 0.55 ppm shoulder on the 0.63 ppm peak, the shoulder having the same chemical shift as the principal upfield peak at 3 mM concentration. Of the other concentration-dependent changes in the native protein, the separation of the 1.64 ppm peak into 1.73 and 1.63 ppm peaks at higher concentration (Figure 2) cannot be seen be-

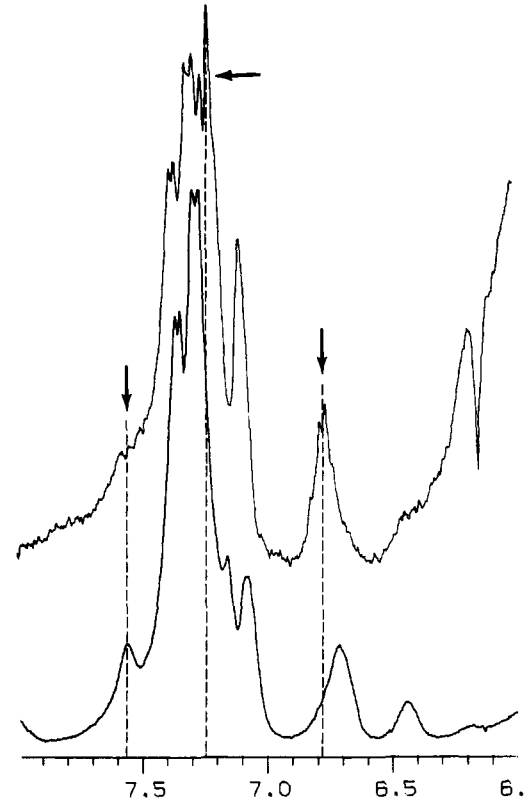

FIGURE 8: Aromatic proton resonances of des-1–8-neurophysin I at pH ~6.2 at low and high protein concentration. (Top spectrum) 2×10^{-4} M protein; (bottom spectrum) 3×10^{-3} M protein. The region below 6.3 ppm in the upper spectrum is HDO. Other details as in Figure 2.

cause the des-1–8 protein lacks the 1.63 ppm peak, which has been assigned to the 1–8 sequence (Sardana & Breslow, 1984). However, the 1.70 ppm peak in the low-concentration des-1–8 spectrum shifts to 1.73 ppm at higher concentration, a shift in the right direction to account for the changes in the native protein. Additionally, the broadening with increased concentration of a native protein 1.21 ppm peak (Figure 2) appears also to occur in the des-1–8 protein, although it is less evident because of the dominant peak at 1.31 ppm assigned to Thr-9 in the modified protein (Sardana & Breslow, 1984). The 1.21 ppm peak is of particular interest, since it is close to the position assigned to Thr-9 in the native protein. However, the fact that small concentration-dependent changes can be seen at 1.2 ppm in the des-1–8 protein, coupled with the fact that concentration-dependent changes at 1.31 ppm in the des-1–8 protein are not greater than in the native protein, suggests the lack of involvement of Thr-9 in self-association.

In Figure 4, changes at 7.22 ppm and in the 0.6 ppm proton region of the des-1–8 protein as a function of concentration are compared with those in the native protein. The results indicate a transition midpoint for the des-1–8 protein at a concentration equal to or less than that of the native protein. Therefore, by these criteria, the dimerization constant of the des-1–8 protein is at least as great as that of the native protein.

While dimerization in the des-1–8 protein therefore closely resembles that in the native, one central difference is present. As seen in Figure 8, the ortho protons of Tyr-49 in des-1–8-neurophysin shift from 6.77 ppm at 0.2 mM to 6.71 ppm at 3 mM. These values compare with the 6.79 ppm location of this peak in the native protein at both low and high concentrations. Moreover, the line width of the Tyr ortho protons is approximately 20% greater in the des-1–8 protein than in the native. Differences from the native protein in Tyr ortho

proton position and line width at high concentration were previously noted (Sardana & Breslow, 1984), but spectra were not available at sufficiently low concentration to permit recognition of the strong concentration dependence of the abnormal chemical shift. The present results demonstrate that the environment of Tyr-49 is significantly altered by dimerization in the des-1–8 protein and that this environment change is different from that in the native protein (Figure 3) where no concentration-dependent change in chemical shift occurs. Significantly, the concentration dependence of the Tyr ortho protons in the des-1–8 protein is not matched by comparable changes in Tyr meta protons (7.09 ppm at 3 mM in Figure 8). These are relatively less broadened and shifted at high concentration, differing only slightly from their position in the native protein. It is also relevant to note the appearance of a significant (methyl) shoulder at 1.02 ppm in the des-1–8 protein at high concentration (Figure 7) which is markedly more pronounced than in the native protein (Figure 2). However, the des-1–8 protein contains only two-thirds of the 1 ppm $-CH_3$ protons present in the native protein, allowing the possibility that the increased resolution reflects the presence of fewer overlapping resonances.

We have also studied the effects of concentration on the des-1–8 protein spectrum at pH 3. As with the native protein, only small effects of concentration are seen over the spectral range 0.1–3 mM, with spectra at both concentrations typically resembling that of the dimer. The concentration dependence at pH 3 of the 7.22 ppm and upfield methyl regions is shown in Figure 4. The only significant difference in the dimer spectrum between pH 6 and 3 was in the Tyr-49 ortho ring protons (data not shown) which were located at ~6.76 ppm at both high and low protein concentration, closer to their position in the native protein under these conditions (6.79 ppm) and clearly different from their 6.71 ppm position in the pH 6 dimer. These results suggest the influence of a titratable carboxyl on the environment of Tyr-49 in the des-1–8 dimer.

Discussion

The present results provide new insights into conformation and dimerization in native and des-1–8-neurophysins. With respect to dimerization in the native protein, the results confirm the participation of Phe-22 or -35 (Sardana & Breslow, 1984) and indicate additional concentration-sensitive resonances. These include Tyr-49 ring protons and represent a distinct subset of the total proton population, broadening significantly more than other resonances and, in most instances, also shifting with change in concentration. Studies elsewhere have presented evidence that neurophysin dimerization represents the side-by-side association of two asymmetric monomers, unaccompanied by significant conformational change (Rholam & Nicolas, 1981; Rholam et al., 1982). Accordingly, we assign the concentration-sensitive resonances to protons directly at or adjacent to the subunit interface. Further studies are necessary to assign all of these to individual residues. However, they are useful probes of self-association and, in this context, have permitted the demonstration of at least a 10-fold increase in neurophysin I dimerization between pH 6 and pH 3. These results are in accord with prediction (Results) and with preliminary ultracentrifuge studies as a function of pH in this laboratory (Carlson & Breslow, 1981) but differ from studies elsewhere (Nicolas et al., 1980) reporting only small effects of pH on neurophysin II self-association. We cannot yet explain these differences. It is possible (Results) that an effect on dimerization of His-80 titration (absent in neurophysin II) enhances the effects of pH on the dimerization of neurophysin I relative to neurophysin II.

We find no evidence for direct participation of residues 1–8 in dimerization; i.e., essentially all of the concentration-dependent changes in the native protein are paralleled by similar changes in the des-1–8 (and des-90–92) proteins. This information is insufficient to completely preclude effects of dimerization on residues 1–8. However, the 1–8 sequence does not contribute a net thermodynamic stabilization of the dimer; i.e., the concentration dependence of the spectrum of the des-1–8 protein demonstrates that it dimerizes with a dimerization constant equal to or greater than that of the native protein. This finding is in disagreement with the conclusion by Abercrombie et al. (1982a) that the des-1–8 protein did not dimerize significantly, the latter based ultimately on its lack of retardation by a native neurophysin-linked affinity column. While earlier NMR results had appeared consistent with some reduction in dimerization in the des-1–8 protein (Sardana & Breslow, 1984), this initial analysis can be shown to be due to the failure to adequately distinguish the concentration-dependent 7.22 ppm peak from adjacent protons. The observed lack of binding of the des-1–8 protein to a native neurophysin-linked affinity column might signal that self-association by the des-1–8 protein is stronger than its ability to bind to the native protein. Alternatively, the disparity points to potential differences between neurophysin–neurophysin interactions in solution and on an affinity support.

We observe one unambiguous difference in dimerization between native and des-1–8 proteins, that of the effect of dimerization on the environment of Tyr-49. In our earlier NMR investigation of the des-1–8 protein (Sardana & Breslow, 1984), the significant upfield shift of the des-1–8 Tyr-49 ortho ring protons at high protein concentration relative to their position in the native protein had also been observed, but the role of dimerization in producing this shift was unappreciated. The large dimerization-induced changes in both line width and chemical shift of Tyr-49 in the des-1–8 protein provide strong support for the concept that Tyr-49 is proximal to the subunit interface. In particular, the pronounced effects of dimerization on the ortho ring protons relative to the meta most directly argue that the former are relaxed in the des-1–8 dimer by specific dipolar interactions with interface protons from the adjacent subunit.[5]

The concept of proximity of Tyr-49 to the subunit interface is consistent with the observed effect of pH on dimerization. The concentration-dependent neurophysin carboxyl (Results) is detectable by its effect on the CD spectrum of nitrated Tyr-49 (Breslow & Gargiulo, 1977), suggesting that both are together at the subunit interface. The change in Tyr-49 chemical shift in the des-1–8 dimer upon carboxyl protonation (Results) supports this model. It is relevant, however, that earlier UV absorption (Nicolas et al., 1978) and fluorescence intensity (Sur et al., 1979) studies of the native protein had not demonstrated significant effects of dimerization on the properties of the Tyr-49 ring[6] and that NMR data here also

[5] An alternative possibility should also be considered. In principle, dimerization-induced changes in Tyr-49 in the des-1–8 protein might reflect a dimerization-induced rearrangement, absent in the native protein and representing residues distant from the interface. We consider this unlikely. First, the singularity of the differences between native and des-1–8 proteins with respect to Tyr-49 makes it unlikely that this reflects long-range conformational effects, transmitted by and involving a number of residues, rather than a specific interface change. Second, conformational changes are energetically uphill. A significant dimerization-induced conformational change in the des-1–8 protein, not present in the native, should be accompanied by a reduced dimerization constant, which is not observed.

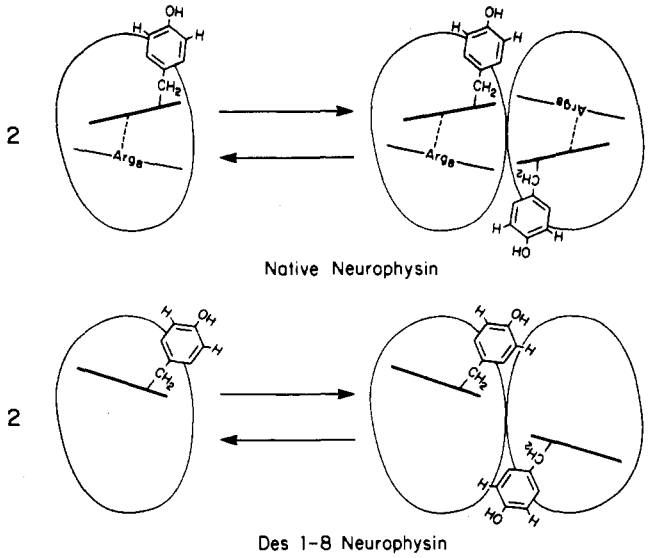

FIGURE 9: Proposed relationships among Tyr-49, Arg-8, and the subunit interface in native and des-1–8-neurophysins. Note that, in this model, Arg-8 is suggested to be proximal to Tyr-49 of the same subunit. Results presented here do not preclude the possibility that Tyr-49 is stabilized by Arg-8 from the adjacent subunit. However, the fact that the des-1–8 monomer also has diminished affinity for peptide (Sardana & Breslow, 1984) can be shown to suggest an intrasubunit interaction.

show no dimerization-induced change in chemical shift (as opposed to line width) of Tyr-49 in the native protein. Thus, in the native protein, the Tyr-49 ring per se probably does not provide a direct site of contact with the adjacent subunit of the dimer, but is suggested to flip over to do so in the des-1–8 protein.

The present results also provide strong evidence that the effects of excision of residues 1–8 on Tyr-49 are mediated by proximity. Although the des-1–8 protein is not grossly unfolded (Abercrombie et al., 1982a; Breslow et al., 1982), its reported loss of dimerization (Abercrombie et al., 1982a) left open the possibility of long-range conformational differences between native and modified proteins. The normal ability of des-1–8-neurophysin to dimerize reduces the likelihood of such conformational differences. Additionally, with the possible exception of small dimerization-induced differences between native and des-1–8 proteins near 1 ppm (Results), differences in Tyr-49 remain [cf. Sardana & Breslow (1984)] the only resolvable effects of loss of residues 1–8. Accordingly, the most direct interpretation of the change in Tyr-49 protons upon excision of residues 1–8 is that, in the native protein, Tyr-49 and/or its immediate neighbors interact directly with the 1–8 sequence, loss of stabilizing interactions from 1–8 leading to altered interactions of Tyr-49 with the intersubunit contact region. The specific functional effects of modification or cleavage of Arg-8 (Breslow et al., 1982) suggest that this is the residue that directly interacts with the Tyr-49 segment. Figure 9 shows the suggested relationships among Arg-8, Tyr-49, and the subunit interface.

The effects of excision of residues 1–8 on Tyr-49 are potentially sufficient to account for the diminished peptide-binding ability of the des-1–8 protein. Tyr-49 has had an uneven history with respect to its proposed relationship to the peptide-binding site [e.g., see Balaram et al (1973) and Lord & Breslow (1980)], but NMR and affinity-labeling evidence now clearly supports its proximity to this site (Abercrombie et al., 1982b; Peyton & Breslow, 1985). Accordingly, conformational alterations in the vicinity of Tyr-49 have the potential for significant repercussions on binding affinity. Incidentally, the demonstrated proximity between Tyr-49 and bound peptide also supports the concept of proximity of Tyr-49 to the 1–8 sequence. In particular, residue 3 of bound peptides has been shown by spin-label NMR (Lord & Breslow, 1980; Sardana & Breslow, 1984) and ring current shift evidence (D. Peyton, V. Sardana, and E. Breslow, unpublished results) to be near the 1–8 sequence. Therefore, bound peptide is adjacent to both Tyr-49 and the 1–8 sequence, consistent with proximity between Tyr-49 and 1–8.

The evidence that Tyr-49 is adjacent both to bound peptide and to an intersubunit contact region raises the possibility that, in the neurophysin dimer, each bound peptide is adjacent to an intersubunit contact region and interacts with both subunits. Although the neurophysin monomer is capable of binding peptide, binding by the dimer is significantly stronger (Cohen et al., 1979). Moreover, although the second binding constant of the dimer is higher than the first, both the first and second binding constants are stronger than that of monomer (Cohen et al., 1979). Conventional allosteric models, such as that represented by the myoglobin–hemoglobin comparison, predict a higher binding affinity of monomer for peptide than of dimer for the first bound peptide. While other models are possible, stronger binding of the first peptide by the dimer can be explained by the concept that supporting interactions for the binding of each peptide originate from both subunits. In this context, it is relevant that most of the resonances identified here as probable participants in intersubunit contacts have previously been shown to be perturbed by peptide binding (Sardana & Breslow, 1984). This is seen, for example, by the dimer 7.58 ppm Phe resonance which shifts upfield on peptide binding, a direction opposite to the shift associated with dimerization. Such results may signify a modification of intersubunit contacts when peptide is bound or, as suggested here, a direct effect of bound peptide on subunit interface protons.

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[6] Rholam and Nicolas (1985) have also concluded from fluorescence studies that the segmental flexibility of the local environment of Tyr-49 in native neurophysin is unaffected by dimerization, a result not directly reconcilable with the dimerization-induced broadening of Tyr-49 ring protons seen here. This difference may reflect the use in the Rholam and Nicolas studies of 80% glycerol–20% phosphate buffer as a solvent in studying the concentration dependence of the local motions of Tyr-49. High concentrations of glycerol alter the environment of Tyr-49 (Sur et al., 1979). Moreover, phosphate can increase the dimerization constant of neurophysin (Tellam & Winzor, 1980; Whittaker & Allewell, 1984) so that the concentration range studied (which appeared to be based on dimerization constants in acetate) might not have reflected significant differences in the relative content of monomer and dimer.

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DNA Degradation by Bleomycin: Evidence for 2′*R*-Proton Abstraction and for C–O Bond Cleavage Accompanying Base Propenal Formation†

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ABSTRACT: Reaction of poly(dA-[2′*S*-^{3}H]dU) with activated bleomycin yields [^{3}H]uracil propenal that completely retains the tritium label. In contrast, we have previously shown that reaction of poly(dA-[2′*R*-^{3}H]dU) with activated bleomycin affords unlabeled uracil propenal [Wu, J. C., Kozarich, J. W., & Stubbe, J. (1983) *J. Biol. Chem. 258*, 4694–4697]. We have also prepared both *cis*- and *trans*-thymine propenals by chemical synthesis and have observed that the trans isomer is the exclusive product of the bleomycin reaction. Moreover, the cis isomer was found to be stable to the conditions of bleomycin-induced DNA degradation. Taken together, these results establish that the formation of *trans*-uracil propenal occurs via an anti-elimination mechanism with the stereospecific abstraction of the 2′*R* proton. The question of phosphodiester bond cleavage during base propenal formation has also been addressed by the analysis of the fate of oxygen-18 in poly(dA-[3′-^{18}O]dT) upon reaction with activated bleomycin. The 5′-monophosphate oligonucleotide ends produced from thymine propenal formation have been converted to inorganic phosphate by the action of alkaline phosphatase, and the phosphate has been analyzed for ^{18}O content by ^{31}P NMR spectroscopy. The oxygen-18 is retained in the inorganic phosphate, establishing that the formation of thymine propenal by activated bleomycin proceeds with C–O bond cleavage at the 3′-position.

Studies from a number of laboratories indicate that the cytotoxicity of bleomycin (Umezawa et al., 1966) may be related to its ability to degrade DNA, and hence, the mechanism of nucleic acid destruction has been extensively investigated by a number of laboratories (Sausville et al., 1976; Burger et al., 1980, 1981; Kuramochi et al., 1981; Oppenheimer et al., 1981; Giloni et al., 1981; Rodriguez & Hecht, 1982; Wu et al., 1983, 1985a,b; Sugiyama et al., 1985; Murugesan et al., 1985). Their collective results have demonstrated that reaction of Fe(II), O_2, reductant, and bleomycin results in the production of a species that catalyzes the degradation of DNA to the monomeric products, base propenal[1]

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[1] Trivial names and abbreviations: base propenal, the products of bleomycin-induced degradation, 3-(uridin-1′-yl)-2-propenal and 3-(thymin-1′-yl)-2-propenal; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-pressure liquid chromatography; TMS, trimethylsilyl; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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