Application of Peptide-Mediated Ring Current Shifts to the Study of Neurophysin–Peptide Interactions: A Partial Model of the Neurophysin–Peptide Complex†

David Peyton,‡ Vinod Sardana, and Esther Breslow*

Department of Biochemistry, Cornell University Medical College, New York, New York 10021

Received August 22, 1986; Revised Manuscript Received November 20, 1986

ABSTRACT: Perdeuteriated peptides were synthesized that are capable of binding to the hormone binding site of neurophysin but that differ in the position of aromatic residues. The binding of these peptides to bovine neurophysin I and its des-1–8 derivative was studied by proton nuclear magnetic resonance spectroscopy in order to identify protein residues near the binding site through the observation of differential ring current effects on assignable protein resonances. Phenylalanine in position 3 of bound peptides was shown to induce significant ring current shifts in several resonances assignable to the 1–8 sequence, including those of Leu-3 and/or Leu-5, but was without effect on Tyr-49 ring protons. The magnitude of these shifts was dependent on the identity of peptide residue 1. By contrast, the sole demonstrable direct effect of an aromatic residue in position 1 was a downfield shift in Tyr-49 ring protons. Study of peptide binding to des-1–8-neurophysin demonstrated similar conformations of native and des-1–8 complexes except for the environment of Tyr-49, confirmed the peptide-induced ring current shift assignments in native neurophysin, and indicated an effect of binding on Thr-9. These observations are integrated with other results to provide a partial model of neurophysin–peptide complexes that places the ring of Tyr-49 at a distance 5–10 Å from residue 1 of bound peptide and that places both the 1–8 sequence and the protein backbone region containing Tyr-49 proximal to each other and to peptide residue 3. The peptide–protein topographical relationships deduced from the ring current shift data support and extend the preliminary model suggested by spin-label data [Lord, S. T., & Breslow, E. (1980) *Biochemistry 19*, 5593–5602] and indicate that systematically introduced ring current shifts can be employed to provide a qualitative picture of protein topography.

A persistent problem in protein chemistry is defining the conformations and active sites of proteins for which X-ray crystallographic data are not available. Of techniques applied to this problem, nuclear magnetic resonance (NMR)[1] spectroscopy is probably the most promising, but is limited in its applicability to proteins beyond 10000 molecular weight, where resonances are poorly resolved and a high potential for ambiguity in spectral assignments exists. We have been attempting to define the conformation and active site of neurophysin, a protein of 10000 molecular weight that dimerizes to 20000 and which is the neurohypophyseal carrier protein for the hormones oxytocin and vasopressin [e.g., see Breslow (1979) and Cohen et al. (1979)]. This protein is of interest both because of its accessibility as a model for the study of peptide hormone–protein interactions and because its sequence raises resolvable questions about the evolution of protein structure–function relationships. For example, the exon arrangement of the neurophysin gene parallels the variable and conserved regions of the protein (Land et al., 1982), raising the question as to whether, in this instance, as has been suggested for some other proteins [e.g., see Craik et al. (1980)], there is a direct relationship between exon arrangement and functional components of the protein. In the present paper, we report the use of ring current shifts to further define structural relationships in neurophysin complexes.

The neurophysin residues central to this study are the single tyrosine, Tyr-49, which lies within the highly conserved region encoded by the second gene exon, and the relatively variable 1–8 sequence encoded by the first exon. Earlier spin-label NMR and affinity labeling studies had led to a potentially inconsistent picture of the relationship between Tyr-49 and residues 1 and 3 of bound peptide (Lord & Breslow, 1980; Abercrombie et al., 1982b) (see Discussion for details). The 1–8 sequence first became of interest with the demonstration that a spin-label in peptide position 3 broadened Ala-1 methyl protons (Lord & Breslow, 1980) and subsequently with the demonstration that excision of this sequence led to a marked reduction in dipeptide affinity, an effect attributed largely to loss of Arg-8 (Breslow et al., 1982; Abercrombie et al., 1982a). No evidence of direct interactions between the 1–8 sequence and bound dipeptides was found, the loss of function upon excision of 1–8 instead associated with a subtle conformational perturbation manifested only by NMR changes in Tyr-49 and suggesting proximity between Arg-8 and Tyr-49 (Sardana & Breslow, 1984; Peyton et al., 1986).

†Supported by NIH Grant GM-17528. The NMR facilities were purchased with funds from the National Science Foundation (PCM-7912083), the Camille and Henry Dreyfus Foundation, and the Fleischmann Foundation.

* Correspondence should be addressed to this author.

‡Present address: Department of Chemistry, University of California, Davis, CA 95616.

[1] Abbreviations: Leu-Phe-NH_2, L-leucyl-L-phenylalanine amide; Phe-Phe-NH_2, L-phenylalanyl-L-phenylalanine amide; Phe-Phe-Leu-NH_2, L-phenylalanyl-L-phenylalanyl-L-leucine amide; Phe-Phe-Phe-NH_2, L-phenylalanyl-L-phenylalanyl-L-phenylalanine amide; *S*-methyl-Cys-Tyr-Phe-NH_2, *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalanine amide; Met-Tyr-Phe-NH_2, L-methionyl-L-tyrosyl-L-phenylalanine amide; Met-Phe-Tyr-NH_2, L-methionyl-L-phenylalanyl-L-tyrosine amide; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide; DCC, dicyclohexylcarbodiimide; NMR, nuclear magnetic resonance; TSP, sodium 3-(trimethylsilyl)propionate-d_4; Boc, *tert*-butoxycarbonyl; TLC, thin-layer chromatography.

0006-2960/87/0426-1518$01.50/0

The use of spin-labeled ligands to measure distances between bound ligand and specific protein residues contains intrinsic uncertainties; e.g., the geometric relationship between the added nitroxyl group and the rest of the ligand is often not known, a particularly important problem when distances between the nitroxyl and the rest of the ligand are significant relative to nitroxyl–protein distances. Additionally, the spin-label might not position at the binding site identically with the analogous unlabeled ligand. These uncertainties were present in neurophysin spin-label studies. Accordingly, we were interested in different methods to probe the proximity between bound peptides and individual protein residues. For this purpose, we investigated the usefulness of NMR ring current effects induced by different residues of bound peptide on specific protein protons. Peptides that bind to the hormone binding site of neurophysin must contain an aromatic residue in position 2 but can contain either aromatic or aliphatic side chains in positions 1 and 3 (or lack position 3 altogether), such differences affecting only binding affinity [e.g., see Breslow (1979)]. By comparing the effects of aliphatic and aromatic residues in comparable positions of perdeuteriated peptides on individual neurophysin protons, we hoped to learn more of the topography of neurophysin–peptide complexes. This paper reports the effects of four perdeuteriated peptides, Leu-Phe-NH_2, Phe-Phe-NH_2, Phe-Phe-Leu-NH_2, and Phe-Phe-Phe-NH_2, on the proton NMR spectrum of bovine neurophysin I and its des-1–8 derivative, in addition to several studies with related nondeuteriated peptides. The results are correlated with spin-labeling and other studies to yield a self-consistent partial model of neurophysin–peptide complexes. To our knowledge, the use of ring current shifts to probe the conformation of proteins of completely unknown three-dimensional structure has not been significantly exploited elsewhere.

For reference in interpreting these studies, the 1–8 sequence of bovine neurophysin I is Ala-Val-Leu-Asp-Leu-Asp-Val-Arg [e.g., see Breslow (1979)].

Experimental Procedures

Protein and Peptides. Native bovine neurophysin I and its des-1–8 derivative were prepared and purified as described earlier (Sardana & Breslow, 1984; Peyton et al., 1986). The nondeuteriated peptides *S*-methyl-Cys-Tyr-Phe-NH_2 and Met-Tyr-Phe-NH_2 were those described elsewhere (Breslow et al., 1973). Nondeuteriated Met-Phe-Tyr-NH_2 was synthesized as described elsewhere (Sardana et al., 1987). The perdeuteriated peptides Leu-Phe-NH_2 and Phe-Phe-NH_2 were synthesized by using perdeuteriated L-Phe-d_8 (MSD Isotopes, 98 atom % D) and/or L-Leu-d_{10} (MSD Isotopes, 98 atom % D) also as described earlier (Peyton & Breslow, 1985).[2] Perdeuteriated tripeptides are described below.

Synthesis of Perdeuteriated Tripeptides. For the synthesis of perdeuteriated Phe-Phe-Leu-NH_2, the Boc derivative of L-Phe-d_8 was synthesized by the addition at 0 °C of 330 mg of BOC-On (Aldrich) to 200 mg of L-Phe-d_8 dissolved in 2 mL of H_2O/2 mL of acetone containing 255 μL of triethanolamine. The solution was stirred in an ice bath for 20 h, diluted with 3 mL of H_2O, and evaporated in vacuo. After being washed with ethyl acetate, the product was acidified with HCl and extracted into ethyl acetate in the presence of $MgSO_4$. The organic phase was filtered and the supernatant evaporated to yield the desired ninhydrin-negative product as an oil. The methyl ester of L-Phe-d_8 was synthesized as described earlier (Peyton & Breslow, 1985). Boc-Phe-d_8-Phe-d_8 methyl ester was synthesized by dissolving Boc-Phe (obtained above) in 2 mL of CH_2Cl_2/140 μL of triethanolamine. To 1.5 mL of this solution, 150 mg of L-Phe-d_8 methyl ester was added, followed by 225 mg of EDC. After being stirred at room temperature for 20 h, the solution was washed sequentially with H_2O, 0.1 N HCl, 0.1 N $NaHCO_3$, and H_2O and the organic phase evaporated to dryness. The methyl ester was then converted to Boc-Phe-Phe by dissolving it in a 50:50 mixture of LiOH (pH 12) and dioxane. After 30 min at 45 °C, the pH was lowered to 5 with HCl and product evaporated to dryness in vacuo. The residue was dissolved in 15 mL of H_2O (70 °C) at pH 8 and centrifuged to remove the precipitate; the pH was then lowered to 2.5 with HCl, and after being cooled, 170 mg of white product was separated by filtration and subsequent lyophilization. For the coupling of Boc-Phe-Phe to Leu-d_{10}-NH_2, the methyl ester of Leu-d_{10} was synthesized by dissolving 49 mg of L-Leu-d_{10} in 900 μL of CH_3OD containing 150 μL of $SOCl_2$; the mixture was allowed to stand for 90 min, at which time the reaction was judged complete by TLC. The product was evaporated to dryness under vacuum and combined with 80 mg of Boc-Phe-Phe in 2 mL of CH_2Cl_2 containing 40 μL of triethanolamine and 60 mg of EDC. After being stirred for 24 h, the reaction mixture was extracted sequentially with two 1-mL washings each of H_2O, 0.1 N HCl, 0.1 N $NaHCO_3$, and H_2O and the organic phase evaporated in vacuo to give an oil representing Boc-Phe-Phe-Leu methyl ester. This was converted to the amide by allowing it to stand for 1 day in CH_3OH presaturated with NH_3 and evaporating the solution. The Boc group was removed by allowing the protected ester to stand in ethyl acetate saturated with HCl for 2 h. The evaporated product was dissolved in 5 mM ammonium acetate, pH 5.6, and chromatographed on a 45-mL column of CM-cellulose using 10 mM ammonium acetate, pH 5.6, as the elution buffer. The main peak was pooled and lyophilized to give 60 mg of the desired product.

Perdeuteriated Phe-Phe-Phe-NH_2 was synthesized by the same route as used for Phe-Phe-Leu-NH_2, substituting L-Phe-d_8 for L-Leu-d_{10} in the final steps. The assigned structures of the final products were confirmed by amino acid analysis and UV absorption studies, and all products were homogeneous on silica gel TLC using a mixture of butanol/acetic acid/H_2O (4:1:1 v/v) as the moving phase and visualizing by ninhydrin, KI, and *o*-toluidine. The perdeuteriated tripeptides synthesized by this method contained only minor proton-containing contaminants when analyzed at high concentration by proton NMR spectroscopy. This result differs from the perdeuteriated dipeptides (Phe-Phe-NH_2 and Leu-Phe-NH_2) which were synthesized by using DCC as the coupling agent (Peyton & Breslow, 1985). As previously reported, these dipeptides required additional chromatographic purification to separate DCC-derived contaminants.

NMR Spectroscopy. Spectra were obtained at 25 °C by using the Rockefeller University NT-300W spectrometer as previously reported [e.g., see Peyton & Breslow (1985) and Peyton et al. (1986)]. All shifts are reported as ppm downfield from TSP. All samples were adjusted to pD 6.2 (uncorrected pH meter readings) prior to recording of the spectra.

Results

Ring Current Effects of Peptides on Tyr-49 Ring Protons. Figure 1 shows aromatic region spectra of neurophysin in the presence of saturating concentrations of perdeuteriated Phe-Phe-NH_2, Phe-Phe-Leu-NH_2, Phe-Phe-Phe-NH_2, and Leu-

[2] In a preliminary account (Peyton & Breslow, 1985), perdeuteriated leucine was erroneously listed as L-Leu-d_8. The correct formulation is L-Leu-d_{10}.

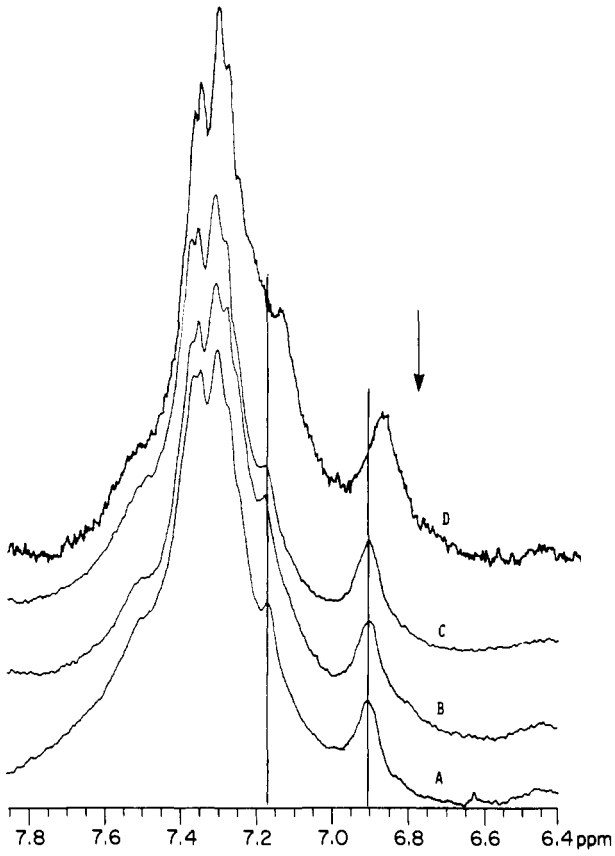

FIGURE 1: Aromatic proton NMR spectra of 2–3 mM native neurophysin I, pH 6, in the presence of the following perdeuteriated peptides: (A) ~4 mM Phe-Phe-NH_2; (B) 4 mM Phe-Phe-Leu-NH_2; (C) 4 mM Phe-Phe-Phe-NH_2; (D) 12 mM Leu-Phe-NH_2. Concentrations are sufficient to ensure ≥90% saturation with each peptide as estimated from known binding constants (Breslow et al., 1973). Note that spectrum D is 25% expanded vertically relative to the other spectra. Lines indicate the chemical shift of Tyr ortho protons (6.91 ppm) and meta protons (7.17 ppm) in spectra A–C. The arrow indicates the position of the Tyr ortho protons in the absence of peptide. Chemical shifts are given in ppm downfield from TSP.

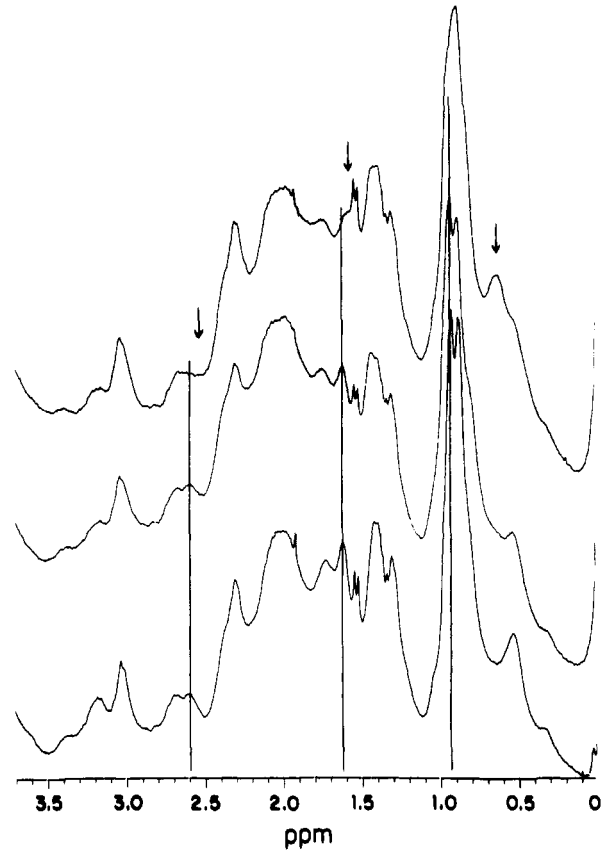

FIGURE 2: Aliphatic proton NMR spectra of 2–3 mM native neurophysin I, pH 6, in the presence of the following perdeuteriated peptides: (bottom spectrum) ~4 mM Phe-Phe-NH_2; (middle spectrum) 4 mM Phe-Phe-Leu-NH_2; (top spectrum) 4 mM Phe-Phe-Phe-NH_2. Protein is ≥90% saturated with each peptide. Lines delineate protons in complexes of Phe-Phe-NH_2 and Phe-Phe-Leu-NH_2 that are reproducibly shifted to the positions indicated by the neighboring arrows in the complex of Phe-Phe-Phe-NH_2.

Phe-NH_2 (in ascending order). A preliminary report of results with Leu-Phe-NH_2 and Phe-Phe-NH_2 has already appeared (Peyton & Breslow, 1985); these data are repeated here for comparison. The arrow in Figure 1 at 6.78 ppm points to the position of the ortho ring protons of Tyr-49 in the unligated state, originally assigned by Balaram et al. (1973) and specifically shown in Figure 4A. As also originally reported by Balaram et al. (1973), binding of peptides to neurophysin leads to a downfield shift in these protons. However, for peptides containing Phe in position 1, these protons are shifted to a final position of 6.90–6.91 ppm relative to their position at 6.85–6.86 ppm in the presence of peptides containing Leu or other nonaromatic residues in position 1 (Figure 1; Peyton & Breslow, 1985). These effects have been interpreted as indicating deshielding of Tyr-49 by Phe-1 of bound peptides (Peyton & Breslow, 1985). By contrast, when the data for tripeptides are compared with those for dipeptides, the spectra (Figure 1) demonstrate that the chemical shifts of Tyr-49 ring protons are unaffected by either the presence or the nature of a residue in position 3; i.e., in the presence of Phe-Phe-Leu-NH_2 and Phe-Phe-Phe-NH_2, the ortho protons are also located at 6.91 ppm.

Effects of Phe-1 on the meta ring protons of Tyr-49 (located at 7.10 ppm in the unbound state) are less clear than those on the ortho protons because the meta protons also shift downfield when peptide is bound and merge with the large asymmetric Phe ring proton peak centered near 7.3 ppm. However, a shoulder at 7.14 ppm in the presence of Leu-Phe-NH_2 and at 7.17 ppm in the presence of all the other peptides in Figure 1 is in the position expected for Tyr meta protons and suggests that these, like the ortho protons, are sensitive to an aromatic residue in position 1 of bound peptides and insensitive to such a substitution in position 3. In contrast with the Tyr ring protons, no differences in Phe ring protons are discernible among the different complexes at this field.

Ring Current Effects of Peptides on Native Neurophysin Aliphatic Proton Spectra. In a preliminary account of this work, we demonstrated that the effects of perdeuteriated Leu-Phe-NH_2 and Phe-Phe-NH_2 on the resolvable aliphatic protons of neurophysin were indistinguishable; these results provided evidence of similar conformations of both complexes and indicated that ring currents of peptide residue 1 were without effect on resolvable aliphatic protein resonances (Peyton & Breslow, 1985). With an aromatic residue in peptide position 3, the effects on protein aliphatic resonances are quite different. Figure 2 compares the aliphatic proton spectra of neurophysin saturated with perdeuteriated Phe-Phe-NH_2, Phe-Phe-Leu-NH_2, and Phe-Phe-Phe-NH_2 (in ascending order). No significant differences are seen between spectra obtained in the presence of Phe-Phe-NH_2 and Phe-Phe-Leu-NH_2. However, in the presence of Phe-Phe-Phe-NH_2, marked but localized and reproducible differences occur in four regions: (A) In the 2.5–2.7 ppm region, a twin peak is altered, reflecting changes particularly between 2.5 and 2.63 ppm. (B) In the 1.5–1.65 ppm region, a peak located at 1.62

Table I: Effects of Peptide Residue 3 on Leucine and Methyl Proton Resonances of the 1–8 Sequence of Native Neurophysin at pH 6

peptide	chemical shift (ppm)		
	Leu-3/Leu-5 β,γ-protons	residue 3 sensitive $-CH_3$ protons	Ala-1 $-CH_3$ protons[a]
—	1.62	0.93	1.53
Phe-Phe-NH_2	1.62	0.93	1.53
Phe-Phe-Leu-NH_2	1.62	0.93	1.53
S-CH_3-Cys-Tyr-Phe-NH_2	1.61	*b*	1.53
Met-Tyr-Phe-NH_2	1.60	0.73, 0.79	1.53
Met-Phe-Tyr-NH_2	1.60	~0.75 (2 unresolved components)	1.53
Phe-Phe-Phe-NH_2	~1.58 (sh)[c]	0.62	1.53

[a] Center of doublet. [b] Position uncertain. [c] Broad shoulder; true center cannot be accurately determined.

ppm in the presence of either Phe-Phe-NH_2 or Phe-Phe-Leu-NH_2 merges with the Ala-1 methyl doublet centered at 1.53 ppm. This merger is the result of an ~0.05 ppm average upfield shift in the 1.62 ppm peak; however, because the shifted peak also broadens, it is probable that the shifts of its individual protons are nonidentical. (C) In the 0.9 ppm region, the 0.93 ppm downfield peak component of the large methyl proton band is lost, and at 0.62 ppm, a broad peak, representing at least three protons, is evident that is absent from the other complexes. The 0.62 ppm peak does not appear to arise from a downfield shift in the 0.52 ppm peak present in the other complexes, since this can be seen as a shoulder in the complex with Phe-Phe-Phe-NH_2. Instead, it appears to represent an upfield shift in other methyl protons, most probably in part those at 0.93 ppm as discussed further below. Because of the discrete number of shifted peaks, we attribute these shifts to ring current effects of Phe-3.

The peak located at 1.62 ppm in the presence of Phe-Phe-NH_2 or Phe-Phe-Leu-NH_2, or in the absence of peptide (e.g., Figure 5), has been assigned earlier to the β- and γ-protons of Leu-3 and -5 (Sardana & Breslow, 1984); the results thereby demonstrate specific ring current effects of peptide residue 3 on the 1–8 segment of the protein sequence. As shown below, this assignment is confirmed by studies of des-1–8-neurophysin. Additionally, studies of the des-1–8 protein show that the observed changes in the 0.9 and 0.62 ppm regions and at least some of the changes near 2.6 ppm induced by Phe in peptide position 3 arise from effects on the 1–8 sequence.

Effect of Peptide Identity on Ring Current Shifts Induced by Residue 3 on Native Protein Aliphatic Protons. The magnitude of the ring current effects of peptide residue 3 on the 1–8 sequence is dependent on the identity of the tripeptide. We had previously reported differences near 0.9 ppm between the effects of Phe-Phe-NH_2 and two nondeuteriated peptides that contained Phe in position 3 (*S*-CH_3-Cys-Tyr-Phe-NH_2 and lysine vasopressin), although the assignment of these different effects to the 1–8 sequence could not be definitively made at that time (Peyton & Breslow, 1985). More significantly, effects elsewhere in the spectrum of a Phe in position 3 were too subtle to be reported without corroborative data since they were smaller than the effects reported here with Phe-Phe-Phe-NH_2. Accordingly, we examined the complexes of neurophysin with two other tripeptides containing an aromatic residue in position 3, Met-Phe-Tyr-NH_2 and Met-Tyr-Phe-NH_2. Both peptides bind to neurophysin with equivalent affinity, and although neither peptide was perdeuteriated, neither contains protons in the region upfield from 1.9 ppm in either the free or the liganded state. As seen in Figure 3, Met-Phe-Tyr-NH_2 (middle spectrum) led to a similar loss of the 0.93 ppm band as that induced by Phe-Phe-Phe-NH_2 (top spectrum), when compared to peptides not containing Phe in position 3 (bottom spectrum). Also, very small

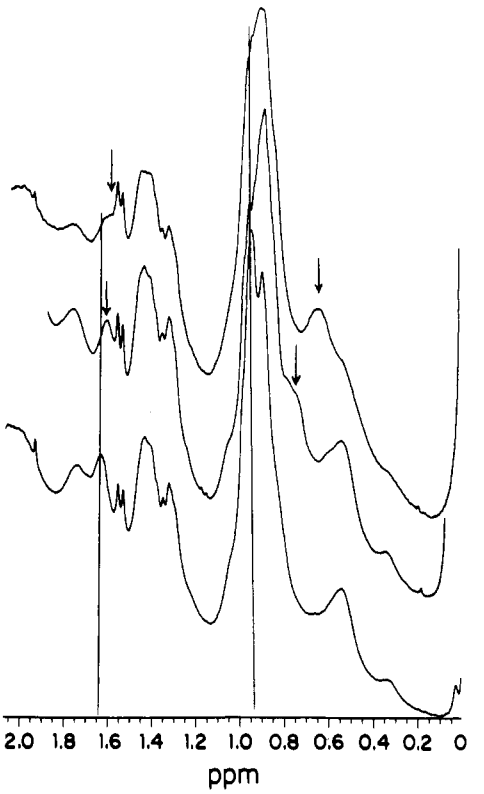

FIGURE 3: Aliphatic proton spectra of 2–3 mM native neurophysin I, pH 6, in the presence of the following peptides: (bottom spectrum) ~4 mM perdeuteriated Phe-Phe-NH_2; (middle spectrum) 6 mM Met-Phe-Tyr-NH_2; (top spectrum) 4 mM perdeuteriated Phe-Phe-Phe-NH_2. Protein is ≥90% saturated with each peptide. Lines delineate protons in the Phe-Phe-NH_2 complex that are shifted to positions indicated by the neighboring arrows in other spectra.

shifts were induced near 1.62 ppm and moderate shifts in the 0.5–0.8 ppm region of the spectrum, but both less than induced by Phe-Phe-Phe-NH_2. Met-Tyr-Phe-NH_2 produced shifts similar to, but not quite identical with, those of Met-Phe-Tyr-NH_2 in the 1.62 and 0.5–0.8 ppm regions (Table I). Complexes of Met-Phe-Tyr-NH_2 and Met-Tyr-Phe-NH_2 appeared also to differ from those of Phe-Phe-NH_2 in the 2.5–2.6 ppm region, but uncertainties exist in this region as to the presence of overlapping proton bands from bound peptide.

The effects of an aromatic residue in peptide position 3 in the different complexes can be systematically compared by examination of the chemical shifts of the Leu-3 and -5 β,γ-proton peaks and selected methyl proton peaks. With respect to the methyl protons, the most pronounced effects of an aromatic residue in position 3 are, in all cases, the loss of the 0.93 ppm methyl peak and the appearance of additional protons upfield from this position. Accordingly, we tentatively assume that the new upfield protons represent protons shifted from the 0.93 ppm position. In Table I, the chemical shifts

Table II: Comparison of Representative Peptide-Induced Chemical Shifts in Native and Des-1–8-neurophysin Dimers at pH 6

	chemical shift (ppm)			
	Tyr-49 ortho	Tyr-49 meta	3.4 ppm "bound" proton peak[a]	upfield shoulder
native neurophysin (3 mM)	6.79	7.10	~3.2	0.45
native neurophysin (3 mM) + Phe-Phe-NH_2 or Phe-Phe-Leu-NH_2 (saturated)	6.91	7.17	3.40	0.36
des-1–8-neurophysin (3 mM)	6.71–6.72	7.09	~3.2	0.45
des-1–8-neurophysin (3 mM) + 6.3 mM Phe-Phe-NH_2	6.86 ($\bar{\nu}$ = 0.74)[b]	7.20	3.36	0.38
des-1–8-neurophysin (2 mM) + ~10 mM Phe-Phe-Phe-NH_2	6.87 ($\bar{\nu}$ = 0.79)[b]	7.20	3.38	0.38
des-1–8-neurophysin (2 mM) + ~10 mM Phe-Phe-Leu-NH_2	6.89 ($\bar{\nu}$ = 0.90)[b]		3.40	0.38

[a] This peak is outside the range of data reported in Figure 5. It is seen at ~3.4 ppm only in the liganded state and appears from spectra (Peyton & Breslow, 1985) to represent a shift of protons from ~3.2 ppm in the unliganded state. It can be seen in Figures 2 and 6. [b] Moles of peptide bound per mole of des-1–8-neurophysin ($\bar{\nu}$) were calculated from the chemical shift of Tyr-49 ortho ring protons by using the relationship $\bar{\nu}$ = (observed chemical shift − 6.715)/(6.91 − 6.715). This relationship assumes fast exchange and a chemical shift at complete saturation of 6.91 ppm.

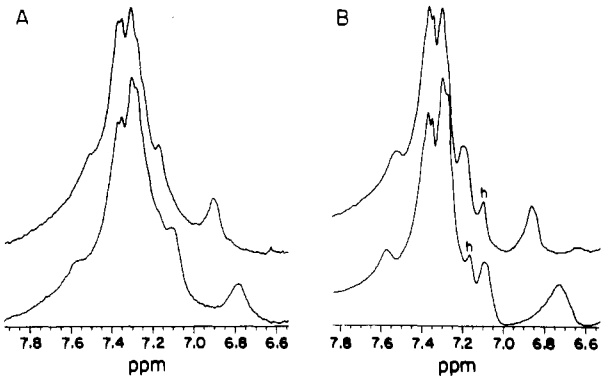

FIGURE 4: Effect of binding perdeuteriated Phe-Phe-NH_2 on the aromatic protons of native and des-1–8-neurophysin. (A) 3 mM native neurophysin in the absence (lower spectrum) and in the presence (upper spectrum) of ~4 mM Phe-Phe-NH_2, pH 6. (B) 3 mM des-1–8-neurophysin in the absence (lower spectrum) and in the presence (upper spectrum) of 6.3 mM Phe-Phe-NH_2, pH ~6.5. Peaks marked "h" represent the C-4 proton of His-80 which is shifted due to partial ionization at this pH.

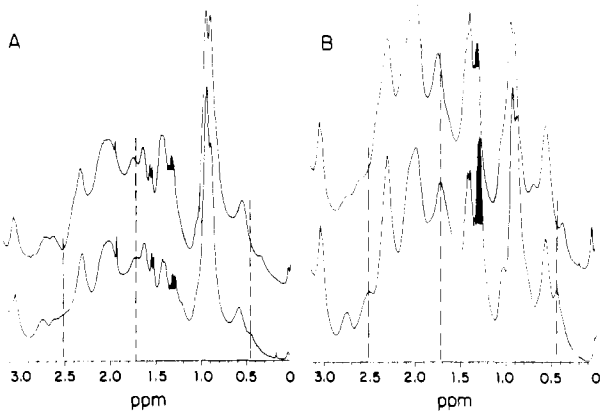

FIGURE 5: Effect of binding perdeuteriated Phe-Phe-NH_2 on the aliphatic protons of native and des-1–8-neurophysin. (A) 3 mM native neurophysin in the absence (lower spectrum) and in the presence (upper spectrum) of ~4 mM Phe-Phe-NH_2, pH 6. (B) 3 mM des-1–8-neurophysin in the absence (lower spectrum) and in the presence (upper spectrum) of 6.3 mM Phe-Phe-NH_2, pH 6.5. Lines delineate representative protons of native protein and des-1–8 protein that are comparably affected by binding. Shaded area delineates the 1.3 ppm peak in the two proteins. Note that spectra of des-1–8-neurophysin are vertically expanded relative to those of the native protein.

of the Leu-3 and -5 β,γ-peak band and the residue 3 sensitive methyl protons are shown for the different complexes investigated. The results indicate a parallel between the magnitude of the shift of the Leu β,γ-proton peak and that of the methyl proton peak. Because substitution of Phe for Tyr appears to have only trivial effects on the magnitude of the observed shifts (cf. Met-Tyr-Phe-NH_2 and Met-Phe-Tyr-NH_2), the factor most responsible for the differences among the peptides in the shifts induced by an aromatic residue in position 3 appears to be the identity of residue 1 (cf. Met-Phe-Tyr-NH_2 and Phe-Phe-Phe-NH_2). The results demonstrate that the orientation of the protein in the bound state relative to the peptide is dependent on the identity of the peptide, particularly of residue 1.

Binding-Induced Changes and Binding Affinity in Des-1–8-neurophysin. The availability of perdeuteriated ligand peptides permits the binding of peptides to des-1–8-neurophysin to be investigated by NMR for the first time, since the low affinity of this derivative precludes the use of saturating concentrations of nondeuteriated peptides without compromising the NMR spectrum. The properties of the bound state of des-1–8-neurophysin are of interest because they facilitate identification of residues in the native protein affected by binding and peptide ring currents and additionally have the potential to provide insights into the reason for the diminution in binding affinity associated with loss of residues 1–8.

The conformation of complexes of the des-1–8 protein appears to be similar to that of the liganded native protein with the exception of the environment of Tyr-49. Similar relationships had been found earlier for the relative conformations of the two unliganded proteins (Sardana & Breslow, 1984). This is seen in Figures 4 and 5 and Table II, which compare spectral effects of binding Phe-Phe-NH_2 to native and des-1–8-neurophysin. At the protein concentration used, both proteins are present largely as dimers (Peyton et al., 1986), so the changes induced by peptides are not compounded by potential binding-induced increases (Cohen et al., 1979) in the fraction of dimer. In the aromatic region, the data in Figure 4 demonstrate that, as in the native protein (Sardana & Breslow, 1984), the des-1–8 protein 7.57 ppm Phe protons move upfield and Tyr-49 ortho and meta ring protons (located at 6.71 and 7.1 ppm, respectively, in the unliganded state) move downfield when peptide is bound. The binding-induced changes in the aliphatic region (Figure 5, Table II) are also analogous to those in the native protein when differences in composition are considered (see also below). However, the chemical shift of Tyr-49 meta protons in the presence of peptide is 7.20 ppm for the des-1–8 protein compared to 7.17 ppm for the native protein (Figure 4) although both proteins have essentially the same meta proton chemical shift in the unbound state. The same discrepancy has been found for complexes of the two proteins with Phe-Phe-Phe-NH_2 (data not shown) and demonstrates that, as in the free state, loss of 1–8 selectively perturbs the Tyr-49 environment.

The magnitude of the chemical shift of Tyr-49 ring protons upon peptide binding to the des-1–8 protein can be used as an indicator of the amount of peptide bound. By contrast with the meta ring protons of Tyr-49, the chemical shift of Tyr-49 ortho ring protons in the unliganded des-1–8 dimer is abnormal

(Figure 4; Sardana & Breslow, 1984) but appears to normalize relative to the native protein in the liganded state. For example (Table II), the chemical shift of the des-1–8 protein ortho protons in the presence of Phe-Phe-Leu-NH_2 differs by only 0.02 ppm from that in the similarly liganded native protein, compared to the 0.08 ppm difference in the unliganded state. Using the assumptions of fast exchange[3] and that the chemical shift of Tyr-49 ortho protons in the completely liganded des-1–8 protein is the same as in the native protein (6.91 ppm), we can approximate the fraction of the des-1–8 protein bound in the spectra shown (Table II). For Phe-Phe-NH_2, this is ~0.75, which calculates to a binding constant of ~700 M^{-1} at 3 mM protein concentration. The extent of change in other binding-sensitive regions of the spectra (Table II) is also reasonably consistent with this estimate, suggesting that the assumptions are not too inappropriate. The calculated constant compares with the value of 200 M^{-1} for the same peptide at a protein concentration of 5×10^{-5} M at the same pH (Sardana & Breslow, 1984). The results indicate that, as with the native protein [e.g., see Cohen et al. (1979)], binding to the des-1–8 protein increases with increased protein concentration because of stronger binding by the protein dimer.

Comparison of the binding-induced changes in the des-1–8 protein aliphatic resonances with those of the native protein (Figure 5) indicates one significant difference that cannot be accounted for directly by differences in the protons contributed by the 1–8 sequence. At 1.3 ppm, there is a large binding-induced decrease in intensity in the des-1–8 protein which should be compared with much smaller changes in the native protein. In the unliganded state (Figure 5), the 1.3 ppm band of the des-1–8 protein is markedly increased in intensity relative to that in the native protein; the increased 1.3 ppm intensity has been assigned to shifted and sharpened Thr-9 protons (Sardana & Breslow, 1984). The large binding-induced decrease in this band in the des-1–8 protein therefore indicates an effect on Thr-9 methyl protons. Such an effect would not be observable in the native protein because of the poor resolution of Thr-9 protons in this state.

One other binding-induced change in the des-1–8 protein in Figure 5 is of interest. In particular, the data indicate that a shoulder at 2.51 ppm in the unbound state is both broadened and shifted to 2.61 ppm in the presence of peptide. Protons represented by the 2.51 ppm peak have been shown (Peyton et al., 1986) to be sensitive to dimerization in the unliganded state in both the native and des-1–8 proteins but are not well resolved at 300 MHz in the native protein dimer because of overlapping protons from the 1–8 sequence (cf. the 2.51 ppm region in the unliganded native and des-1–8 proteins in Figure 5). By analogy with the des-1–8 protein, we assume that the 2.60 ppm peak in the native protein complex with Phe-Phe-NH_2 (Figures 2 and 5) similarly arises at least in part from the 2.51 ppm concentration-dependent peak; preliminary studies at 500 MHz confirm this assumption. The binding sensitivity of the 2.51 ppm peak adds to the list of neurophysin resonances (Peyton et al., 1986) that are independently perturbed by both peptide binding and self-association.

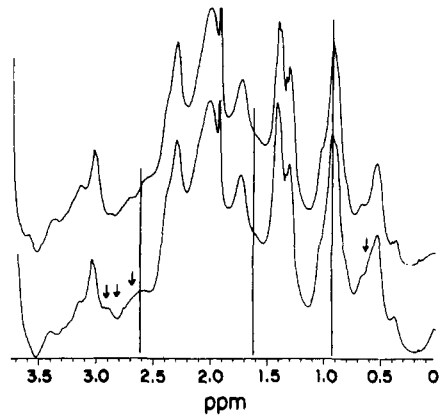

FIGURE 6: Aliphatic proton region of 3 mM des-1–8-neurophysin in the presence of ~10 mM Phe-Phe-Leu-NH_2 (lower spectrum) and ~10 mM Phe-Phe-Phe-NH_2 (upper spectrum), pH 6. Lines indicate the chemical shifts of protons in native neurophysin that are shifted in the complex of Phe-Phe-Phe-NH_2 relative to that of Phe-Phe-Leu-NH_2 (Figure 2). Arrows indicate protons of the des-1–8 complex of Phe-Phe-Leu-NH_2 which differ in chemical shift from those of the complexes of both Phe-Phe-NH_2 and Phe-Phe-Phe-NH_2.

Effects of Residue 3 on Des-1–8-neurophysin. Figure 6 compares the bound state of the des-1–8 protein in the presence of Phe-Phe-Leu-NH_2 and Phe-Phe-Phe-NH_2. From the chemical shift of Tyr-49 ortho ring protons, the protein is estimated as 90% and 80% saturated with peptide, respectively (Table II). No differences are seen in the 1.62 and 0.9 ppm regions, in which an aromatic residue in position 3 confers significant differences on complexes of the native protein. Similarly, the des-1–8 complexes of Phe-Phe-NH_2 (Figure 5) and of Phe-Phe-Phe-NH_2 (Figure 6) give identical spectra. These results confirm that the ring current shifts of peptide residue 3 on these protons in the native protein reflect effects on the 1–8 sequence. In addition, the 0.62 ppm peak seen in the native protein in the presence of Phe-Phe-Phe-NH_2 is absent in this complex of the des-1–8 protein; a much smaller peak at ~0.67 ppm, representing no more than one proton, is present in the complexes both of Phe-Phe-Phe-NH_2 and Phe-Phe-Leu-NH_2 (Figure 6) and of Phe-Phe-NH_2 (Figure 5) and hence is independent of peptide residue 3. These results indicate that the major component of the 0.62 ppm peak in the native protein complex with Phe-Phe-Phe-NH_2 also originates from the 1–8 sequence.

By contrast with the above, analysis of the spectra of des-1–8-neurophysin complexes, in order to assign the 2.6 ppm region shifts induced by peptide residue 3 in the native protein (Figure 2), leads to ambiguous conclusions. Spectra of the des-1–8 complexes of Phe-Phe-NH_2 (Figure 5) and of Phe-Phe-Phe-NH_2 (Figure 6) appear identical throughout the aliphatic region, strongly suggesting that the residue 3 induced shifts in the native protein near 2.6 ppm, as in the other regions, originate from the 1–8 sequence. This is consistent with the presence of two Asp residues in the 1–8 sequence with protons within this region. However, at least some of the 2.6 ppm protons in the complexes of Phe-Phe-NH_2 are common to both the native and des-1–8 proteins (vide supra). Additionally, comparison of the different des-1–8 complexes indicates subtle but reproducible differences in the 2.5–3 and 0.6 ppm regions between the complex of Phe-Phe-Leu-NH_2 on the one hand and those of both Phe-Phe-NH_2 and Phe-Phe-Phe-NH_2 on the other, as demonstrated in Figure 6. These results indicate specific perturbation by peptide Leu-3 of protons in the 2.5–3 ppm region that originate from the 9–92 sequence. We tentatively assume that these same perturbations

[3] The tyrosine of native neurophysin is in the intermediate exchange rate category with peptides such as Phe-Phe-NH_2 on the NMR time scale (Virmani-Sardana & Breslow, 1983). The exchange rate of the des-1–8 protein has not been thoroughly evaluated. However, at the high protein concentrations reported here, where the rate of monomer–dimer interconversion is not rate limiting in the binding process, we have observed only a single sharp peak for Tyr-49 at fractional saturation levels above 50%. Given the fact that the chemical shift change of the ortho protons of Tyr-49 in the des-1–8 protein undergoes a larger chemical shift on binding than those in native neurophysin (Results), reasonably fast exchange is suggested.

are present in the native protein but masked by the more dominant contributions from protons in the 1–8 sequence.

Discussion

For peptides bound to neurophysin, the results demonstrate that an aromatic side chain at position 1 induces specific shifts, best interpreted as ring current shifts (Peyton & Breslow, 1985), in Tyr-49 ring protons, while an aromatic side chain at peptide position 3 is without such an effect. Conversely, an aromatic side chain at peptide position 3, but not at position 1, specifically perturbs protons assigned to the neurophysin 1–8 sequence, most clearly those of Leu-3 and/or Leu-5; the magnitude of the latter effect is highly sensitive to the identity of the peptide. The results can be viewed within the context of earlier spin-labeling and affinity labeling studies of the relationship of bound peptide to individual neurophysin residues. Specifically, a ligand peptide containing a nitroxyl group in a position similar to, but not identical with, the side chain of peptide position 3 was found to have only a weak effect on the relaxation rate of the ortho ring protons of Tyr-49, indicating a distance between the nitroxyl and Tyr ortho protons $\geq$14 Å (Lord & Breslow, 1980). This same spin-label demonstrably broadened the methyl protons of Ala-1, indicating a distance in this case $\leq$12 Å; evidence of broadening of other resonances in the 1–8 sequence by this spin-label was subsequently obtained (Sardana & Breslow, 1984), although no distance estimates were possible. On the other hand, a spin-label placed on an extension of the side chain of peptide position 1 markedly increased the relaxation rate of Tyr-49 ring protons (calculated distances $\leq$10 Å), although the length of the arm to which the nitroxyl was attached rendered any conclusions about distances to the position 1 side chain itself ambiguous. In contrast to the spin-label data, which provided no evidence for very close proximity of peptide residue 3 to the Tyr-49 ring, photoaffinity labeling studies (Abercrombie et al., 1982b) indicated that an azido group in position 3 of bound peptides specifically labeled Tyr-49.

The magnitude and direction of ring current induced shifts are influenced by both distance and orientation [e.g., see Johnson & Bovey (1958)], precluding the use of such shifts for distance calculations in the absence of orientation information. Similarly, the significance of distances obtained from spin-label studies can be limited by lack of knowledge of the geometrical relationship of the nitroxyl to other segments of the bound ligand. For example, in studies of the peptide spin-labeled at position 1 (Lord & Breslow, 1980), the potential separation of the nitroxyl group from its point of attachment to the peptide was 7 Å. Given the difference in uncertainties influencing proximity estimates by the two methods, it is significant that the present results and spin-label studies are mutually consistent in supporting a model that places the ring of Tyr-49 close to the side chain of residue 1 and more distant from the side chain of residue 3, while the side chain of residue 3 is positioned close to the 1–8 sequence. In particular, the 0.05 ppm downfield shift in Tyr-49 ortho ring protons induced by the presence of an aromatic residue in peptide position 1 can be shown from theoretical ring current shift calculations [e.g., see Johnson & Bovey (1958) and Perkins & Wüthrich (1979)] to suggest *maximum* distances between the side chain of position 1 and Tyr-49 ortho protons that are clearly within the maximum distance suggested by the spin-label data (10 Å). Additionally, ring current effects of residue 3 on Leu-3/Leu-5 β,γ-protons and on $-CH_3$ protons from the same or other residues in the 1–8 sequence are of sufficient magnitude to place peptide residue 3 even closer to these residues than the distance obtained by spin-label measurements to Ala-1. Thus, Phe-3 of bound peptides leads to no shift of the Ala-1 methyl (1.53 ppm doublet in aliphatic region) while producing shifts that range up to 0.3 ppm in other protons of the 1–8 sequence (Table I). The lack of an effect on Ala-1 might partially reflect the motional or orientation properties of Ala-1 relative to Phe-3. However, the upfield methyl shifts of 0.3 ppm of other residues in the 1–8 sequence suggest that these are probably closer to Phe-3 than 7 Å [e.g., see Johnson & Bovey (1958) and Perkins & Wüthrich (1979)], closer than the maximum distance between residue 3 and Ala-1 obtained from spin-label studies. Such close proximity is also supported by the observation that the magnitude of the observed ring current shifts induced by peptide residue 3 is highly sensitive to what are presumably very subtle differences in peptide positioning. Effects of orientation or distance changes on ring current shifts become increasingly smaller as the distance between the ring and the proton under observation increases. For example, a decrease in shielding from 0.2 to 0 ppm would require approximately 7-Å movement (sum of movement in two directions) at an original above-ring distance of 6 Å, but only 1-Å movement at an original distance of 3 Å [e.g., see Johnson & Bovey (1958)].

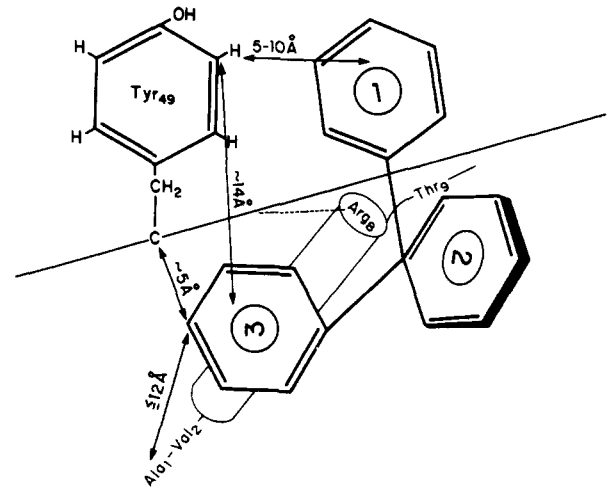

FIGURE 7: Proposed partial model of the complex of native neurophysin I with Phe-Phe-Phe-NH_2. Only the phenyl rings of the peptide, numbered for position, are shown. The model is not drawn rigorously to scale. The distance between the rings of Tyr-49 and peptide residue 2 is not specifically shown, but has been estimated as 7–10.4 Å [e.g., see Breslow (1984)], which can be shown to be compatible with the other distances.

In attempting to reconcile the close proximity between the side chain of peptide residue 3 and Tyr-49 suggested by affinity labeling studies and the longer distance suggested by spin-label and ring current effects, we have obtained evidence that the observed photoaffinity labeling of Tyr-49 by peptide residue 3 probably represents proximity of the residue 3 side chain to the backbone or ipso carbon region of Tyr-49, not to the outer segments of the ring (Sardana et al., 1987). In separate studies (Peyton et al., 1986), we have presented evidence of stabilizing interactions between Arg-8 and the immediate segment containing Tyr-49. These data, the spin-label results, and those in the present study can be incorporated into a model, containing self-consistent distances, showing the general relationships among individual segments of bound peptide, Tyr-49, and the 1–8 sequence (Figure 7). The model is additionally constrained by the fact that the rings of Tyr-49 and peptide residue 1 are not sufficiently close to permit dipolar broadening of the protons of Tyr-49 by the protons of peptide residue 1 (Peyton & Breslow, 1985) and that relatively ex-

tensive modification of both Tyr-49 ring ortho positions and the Tyr-49 hydroxyl can occur without impeding binding (Sardana et al., 1987). The model depicts the 3–8 sequence in α-helical configuration, as predicted by Chou–Fasman considerations,[4] although there is no experimental evidence confirming this assignment. The model neither represents nor precludes a direct binding role for Arg-8 and Thr-9. There is no evidence of direct interaction of Arg-8 with peptide residues 1 and 2 (Sardana & Breslow, 1984), and residue 9 is not conserved in evolution [e.g., see Breslow (1984)]. However, the experimental evidence that Thr-9 is perturbed by binding (Results) is consistent with reasonable proximity to bound peptide. The model can be shown to be consistent with fluorescence measurements of the probable distance (7–10.4 Å) between the Tyr-49 ring and the ring of peptide residue 2 (Sur et al., 1979; Breslow, 1984).

While available evidence mitigates against direct interactions between dipeptides and residues 1–8 (Sardana & Breslow, 1984), the proximity suggested here between peptide residue 3 and protein residues 1–8 raises the question as to whether the 1–8 sequence interacts directly with the side chain of peptide residue 3. Such a possibility has evolutionary implications. Oxytocin and vasopressin each share common precursors with their associated neurophysins and appear to interact intramolecularly with the neurophysin segment within the precursor in much the same manner as the intermolecular interactions studied here [e.g., see Land et al. (1982) and Chaiken et al. (1983)]. Despite remarkable conservation of the sequence 10–74 in all neurophysins, the 1–9 sequence and the carboxyl-terminal region show significant variability. Within this variability, however, there are a set of residues common to those neurophysins that share a common precursor with oxytocin, and a set of residues common to those that share a common precursor with vasopressin [for a review, see Breslow (1984)]. Oxytocin and vasopressin differ only in positions 3 and 8, the latter of which is not believed to participate in interactions with neurophysin. Given the likelihood that constraints on *intra*molecular binding are greater than those on the corresponding *inter*molecular reaction, the results here suggest that differences between oxytocin- and vasopressin-associated neurophysins in the 1–8 sequence may have arisen from the need to maximize tight *intra*molecular interactions with residue 3 of the hormones within the different precursors.

In sum, the present studies demonstrate that systematically introduced ring current shifts can be used to provide structural information that complements and extends information obtained from spin-label studies. The usefulness of the approach in the present system lies partially in the fact that simple peptides, rather than nitroxyl-derivatized peptides, can be used. This simplifies the chemistry of peptide synthesis, increases the diversity of peptide ligands that can be investigated, and permits the investigation of ligands that are closely related to the natural ligands. Additionally, the sensitivity of ring current shifts to orientation effects, a property which limits their usefulness for absolute distance calculations, can be exploited to probe subtle changes in protein conformation. In the present case, ring current shifts induced by residue 3 of bound peptides are seen to be highly sensitive to the identity of peptide residue 1, this effect both placing constraints on distance estimates and demonstrating subtle differences among the different complexes that were not previously appreciated and which would be difficult to detect by alternative solution methods.

[4] We acknowledge the calculations of neurophysin helix content, according to Chou–Fasman rules, independently by Dr. Susan Lord and by Drs. Barbara Whittaker and Norma Allewell.

ACKNOWLEDGMENTS

We thank Dr. David Cowburn of Rockefeller University for use of the NMR facilities at Rockefeller University.

REFERENCES

Abercrombie, D. M., Angal, S., Segueira, R. P., & Chaiken, I. M. (1982a) *Biochemistry 21*, 6458–6465.

Abercrombie, D. M., McCormick, W. M., & Chaiken, I. M. (1982b) *J. Biol. Chem. 257*, 2274–2281.

Balaram, P., Bothner-By, A. A., & Breslow, E. (1973) *Biochemistry 12*, 4695–4704.

Breslow, E. (1979) *Annu. Rev. Biochem. 48*, 251–274.

Breslow, E. (1984) in *Cell Biology of the Secretory Process* (Cantin, M., Ed.) pp 276–308, Karger, Basel, Switzerland.

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

Breslow, E., Pagnozzi, M., & Co, R. T. (1982) *Biochem. Biophys. Res. Commun. 106*, 194–201.

Chaiken, I. M., Tamaoiki, H., Brownstein, M. J., & Gainer, H. (1983) *FEBS Lett. 164*, 361–365.

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

Craik, C. S., Buchman, S. R., & Beychok, S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1384–1388.

Johnson, C. E., & Bovey, F. A. (1958) *J. Chem. Phys. 29*, 1012–1014.

Land, H., Schutz, G., Schmale, H., & Richter, D. (1982) *Nature (London) 295*, 299–303.

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

Perkins, S. J., & Wüthrich, K. (1979) *Biochim. Biophys. Acta 576*, 409–423.

Peyton, D., & Breslow, E. (1985) *Biochem. Biophys. Res. Commun. 128*, 1211–1218.

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

Sardana, V., & Breslow, E. (1984) *J. Biol. Chem. 259*, 3669–3679.

Sardana, V., Carlson, J., Breslow, E., & Peyton, D. (1987) *Biochemistry* (in press).

Sur, S. S., Rabbani, L. D., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026–1035.

Virmani-Sardana, V., & Breslow, E. (1983) *Int. J. Pept. Protein Res. 21*, 182–189.