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Nuclear Magnetic Resonance Studies of the Interaction of Peptides and Hormones with Bovine Neurophysin†

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ABSTRACT: The interaction of bovine neurophysin-II with lysine-vasopressin and with di- and tripeptide analogs of vasopressin and oxytocin was studied by nuclear magnetic resonance. Slow exchange between free and bound vasopressin at neutral pH allowed little information to be obtained about the hormone–protein reaction by the techniques used. However, fast exchange between free and bound forms of the smaller peptides was demonstrated and gave the following information. The protons on the aromatic ring in position 2 of most bound peptides are differentially broadened by binding, indicating dipolar relaxation of these protons by proximal residues on neurophysin, and confirming participation of this residue in the binding process. Differential line broadening on binding was also demonstrated for protons on the side chains of residues 1 and 3 of the bound peptide, suggesting, in agreement with conclusions drawn elsewhere, that these side chains also participate in binding. The pH dependence of line broadening in Ala-Tyr-PheNH$_2$ was shown to be in agreement with the contention that binding involves a bond between a protonated α-amino of the peptide and an unprotonated side-chain carboxyl of the protein. However, a conformational change in the protein at low pH and an altered form of some of the complexes at low pH are suggested. Nuclear Overhauser effects (NOE) were successfully applied to the study of peptide–neurophysin interaction and indicated that the aromatic ring in position 2 of the bound peptide is in close proximity to the single tyrosine of the protein and to two other classes of protons. The observed NOE effects were negative and an explanation for the direction of the effects as well as for their apparent distribution among both free and bound forms of the peptide are given in an Appendix.

The posterior pituitary gland contains the polypeptide hormones oxytocin and vasopressin in noncovalent association with a group of closely related proteins, the neurophysins (Acher *et al.*, 1955; Sawyer, 1961). Two principal bovine protein components, neurophysin-I[1] and neurophysin-II, which differ in amino acid composition but display similar hormone binding properties have been characterized (Rauch *et al.*, 1969; Breslow *et al.*, 1971; Breslow and Walter, 1972). Binding studies using hormone analogs have led to the conclusion that a primary electrostatic attraction between the protonated α-amino group of the hormones and a carboxylate group on the protein is enhanced by a nonpolar environment created by hydrophobic interactions between residues at positions 2 and 3 of the hormones and unspecified residues on the protein (Stouffer *et al.*, 1963; Ginsburg and Ireland, 1964; Breslow and Abrash, 1966; Hope and Walti, 1971). Breslow *et al.* (1971) have demonstrated that the principal qualitative features of the neurophysin-II–hormone interaction are preserved in the binding of tripeptides containing only the three N-terminal residues of the hormones and have also shown that the side chain in position 1 of the binding peptide is important in binding to neurophysin. With a view toward investigating further the molecular details of the protein–hormone inter-

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[1] Abbreviations used are: NP, bovine neurophysin; *S*-Me-Cys-Phe-IleNH$_2$, *S*-methyl-L-cysteinyl-L-phenylalanyl-L-isoleucinamide; *S*-Me-Cys-Tyr-PheNH$_2$, *S*-methyl-L-cysteinyl-L-tyrosyl-L-phenylalaninamide; Ala-Tyr-PheNH$_2$, L-alanyl-L-tyrosyl-L-phenylalaninamide; Met-TyrNH$_2$ L-methionyl-L-tyrosinamide; NOE, nuclear Overhauser effect; LVP, lysine-vasopressin; indor, internuclear double resonance; SSB, spinning side bands.

action, a nuclear magnetic resonance (nmr) study has been undertaken.

The use of nmr in studying the binding of small molecules to nucleic acids and proteins has been reviewed (Sheard and Bradbury, 1970). Direct nmr methods based on the observation of changes in the relaxation times or chemical shifts for nuclei on the small molecule on binding to the macromolecule have been used to monitor the binding process. Evidence for the nature of the groups at the macromolecular binding site is not generally obtained by these methods but, in exceptional cases, observation of unambiguously identifiable peaks in the protein nmr spectrum has resulted in more detailed descriptions of the protein binding site (Meadows *et al.*, 1969). The aim of this paper is twofold. First, we wish to report the use of conventional nmr techniques to map some of the sites of interaction of lysine-vasopressin and its peptide fragments with bovine NP-II. Second, we will report on the use of a negative nuclear Overhauser effect to help characterize further the hormone–protein interaction. The observation and significance of negative intra- and intermolecular nuclear Overhauser effects were first reported recently (Balaram *et al.*, 1972a,b) and used to localize the single tyrosine of bovine NP-II at the hormone-binding site (Balaram *et al.*, 1972a,b); such effects are potentially powerful tools for characterizing the nature of the amino acid residues at binding sites on proteins. In this manuscript we will amplify on our original observations and (in the Appendix) elaborate further on the theory of negative nuclear Overhauser effects in general.

Materials and Methods

Spectra were recorded at 250 MHz using the MPC-HF spectrometer described by Dadok *et al.* (1970) and at 100 MHz using a Varian HA-100 spectrometer. Most spectra were time averaged using a Northern Scientific, NS-544 digital memory oscilloscope to enhance the signal to noise ratios. Rapid scans, ~6 Hz/sec, were used without extensive loss of resolution (~1–2 Hz). Residual HDO in the samples was used as an internal locking signal. A high sensitivity proton probe yielding a signal to noise ratio of ~200:1 on a standard 1% ethylbenzene sample was used. Variable-temperature measurements were done at 100 MHz using ethylene glycol as a temperature standard. High-temperature experiments at 250 MHz were attempted by heating the spinner air prior to its entry into the probe assembly. Extensive radiation losses and the very slow approach to equilibrium temperatures made these experiments unfeasible. In all experiments, values of δ are reported as parts per million downfield from 2,2-dimethylsilapentane-5-sulfonate.

All double-resonance experiments were performed at 250 MHz. Two radiofrequency synthesizers were used to provide the H_1 and H_2 radiofrequency fields. The spectrometer is equipped with a single-coil probe and is operated in the time sharing mode using a pulse generator unit, with a pulse repetition frequency of 20 kHz. Typical pulse duration times for the double irradiation pulse range from 6 to 14% of the duty cycle. The weak observing pulse (H_1) times were ~1% of the total duty cycle.

Bovine neurophysin-II was prepared and purified by the method of Breslow *et al.* (1971). Nitrated NP-II was prepared by the method of Furth and Hope (1970). Peptide and hormone samples were those previously described (Breslow and Weis, 1972). *Stock protein solutions, containing 10 mg/ml of NP-II, were prepared by dissolving the dry lyophilized powder in 0.16* M *KCl–D_2O, at pH 3.0.* The protein is insoluble at this concentration in the region pH 4–5.5 and at higher pH the rate of solution is slow. Protein concentrations were measured using a molar extinction coefficient of 2300 at 280 nm. Protein concentrations in the nmr samples ranged from 1 to 4 mg per ml (mol wt 10,000). Peptide concentrations between 5×10^{-4} and 5×10^{-3} M were used. pH was adjusted using aliquots of NaOD and DCl and the volume of the sample was not significantly affected in the process. pH was measured on a Sargent Model S-30005-10 pH meter equipped with Sargent Model S-30070-10 miniature electrode and reported values are uncorrected for the deuterium isotope effect. Prolonged storage of neurophysin solutions at pH 6.0 at 4° led to denaturation; protein samples were therefore stored at pH 3 or as a lyophilized solid.

Results

Lysine-Vasopressin–Neurophysin-II Interaction. Figure 1a shows the 250-MHz spectrum of the aromatic protons of LVP. Addition of 0.14 equiv of NP-II to a solution of 1×10^{-3} M LVP at pH 6.8 did not cause any appreciable line broadening. This suggests that either the exchange rate between complexed and noncomplexed forms of the hormone is slow or that there is no detectable spectral change on binding. The fact that slow exchange is responsible for the lack of any visible effect is demonstrated in Figure 1b. Addition of 0.5 equiv of NP-II allows signals from both bound and free forms of the hormone to be observed, as sharp peaks from the latter superimposed on broad peaks from the former. (NP-II possesses only a single tyrosine and does not contribute significantly enough to the intensities of the resonances in the tyrosine region to confuse this interpretation. The extensive changes in the phenylalanine peaks are a consequence of the contributions of the three Phe residues on the protein.) Further evidence for the slow exchange rate was provided by variable-temperature measurements at 100 MHz. A distinct broadening of the hormone Tyr peaks in the presence of 0.14 equiv of protein could be discerned at 60°, but the poor signal to noise ratio at this lower frequency precluded a quantitative study. We attribute this to the increased rate of exchange at the higher temperature. Diminishing the pH also seems to increase the exchange rate. Figure 1c shows the effect of lowering the pH of an LVP solution containing 0.5 equiv of NP-II. At pH 1.5 the sharp peaks from the free hormone are not detectable, indicating an averaging process due to faster exchange. This pH dependence of the dissociation rate has been further confirmed by line broadening of the LVP aromatic resonances at pH values below 3.5 even at lower protein concentrations. It is of interest that Ca^{2+} ion, even at concentrations as high as 0.1 M, did not have any effect on the nmr spectra of NP-II–LVP mixtures. This is in agreement with results obtained by Breslow (1970) and at variance with the reported inhibition of LVP binding to porcine neurophysin by Ca^{2+} (Ginsburg *et al.*, 1966).

Tripeptide–Neurophysin-II Interaction. The basic features of the protein–hormone interaction are displayed by tripeptides containing residues 1–3 of the hormones (Breslow *et al.*, 1971). With a view toward attaining faster exchange rates at normal probe temperatures (25–31°), a study of the tripeptide–protein system was initiated.

As reported in a preliminary communication (Balaram *et al.*, 1972b), addition of NP-II to a solution of Ala-Tyr-Phe-NH_2 causes the Tyr ring protons ortho to the hydroxyl group (Tyr ortho) to broaden dramatically relative to the Tyr meta peaks, while the Phe ring protons are comparatively un-

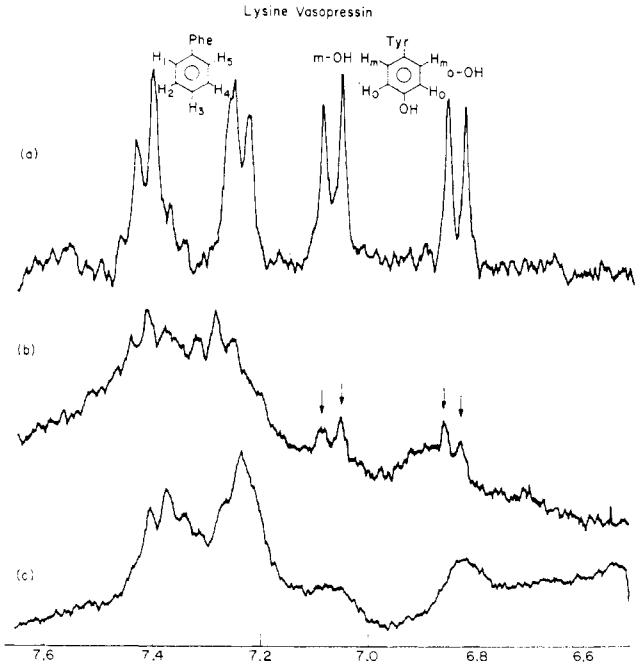

FIGURE 1: Aromatic protons of 1×10^{-3} M LVP at 250 MHz: (a) free hormone, pH 5.6; (b) 0.5 equiv of NP-II added, pH 6.8; (c) 0.5 equiv of NP-II added, pH 1.5. Abscissa is parts per million downfield from 2,2-dimethylsilapentane-5-sulfonate.

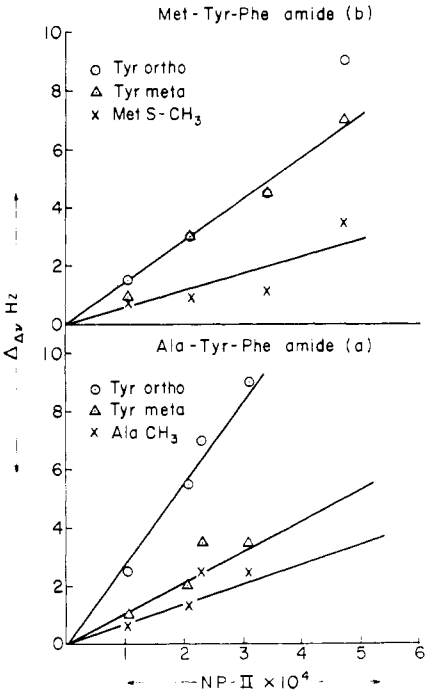

FIGURE 2: Plot of peptide proton line widths as a function of protein concentration: (a) Ala-Tyr-PheNH$_2$; (b) Met-Tyr-PheNH$_2$. Peptide concentrations were 2.2×10^{-3} and 2.0×10^{-3} M, respectively.

affected. Figure 2a shows the dependence on protein concentration of the line widths of the Ala methyl and Tyr ring resonances of Ala-Tyr-Phe-NH$_2$; the data illustrate the differential broadening of the Tyr ring protons and also suggest that the side chain in position 1 of the peptide is affected by binding. Additionally it was demonstrated that the Tyr β-CH$_2$ resonances in Ala-Tyr-Phe-NH$_2$ are broadened by NP more than are the Phe β-CH$_2$ resonances. An unambiguous assignment of the β-CH$_2$ protons was possible using double irradiation methods. Irradiation of the Tyr ring meta peak (591 Hz downfield from residual HDO) results in a sharpening of the Tyr β-CH$_2$ lines due to a collapse of unresolved long-range spin coupling; irradiation of the Phe ring resonances, 631 Hz downfield from residual HDO, produces a corresponding sharpening of the Phe β-CH$_2$ peaks. Interestingly, the β-CH$_2$ assignments are the same as those made for LVP and its peptide fragments in Me$_2$SO-d_6 (Von Dreele *et al.*, 1971). The Ala α-CH peak in Ala-Tyr-Phe-NH$_2$ could be detected and showed both a broadening and a small upfield shift in the presence of NP-II, but problems due to overlap of HDO spinning side bands hampered detailed study.

In the case of the Tyr ring protons the observed broadening can, in principle, arise either from slow exchange between two chemically shifted environments or dipolar relaxation. The nonequivalent broadening of the ortho and meta Tyr protons of Ala-Tyr-Phe-NH$_2$ argue for a dipolar contribution to broadening (Balaram *et al.*, 1972b). Moreover, addition of 0.3 equiv of NP-II to Ala-Tyr-Phe-NH$_2$ can be shown to result in an upfield shift of only $\sim$8 Hz of the Tyr ortho protons. The Tyr meta protons move $\sim$6 Hz downfield. These shifts are small and it can therefore be shown that exchange at a rate comparable to this frequency difference is insufficient to account for the observed line broadening. Furthermore, the line widths of the Ala methyl and Tyr ring resonances in Ala-Tyr-Phe-NH$_2$ and of the *S*-methyl group of *S*-Me-Cys-Tyr-Phe-NH$_2$ did not show any frequency dependence on varying the operating frequency between 250 and 100 MHz in the presence of protein. Our evidence therefore suggests a fast exchange between bound and free peptide in solution with relatively little exchange contribution to the line width. Further support for the contention that the broadening arises from dipolar relaxation will be provided by the double-resonance studies below.

Figure 2b shows the effect of varying the NP-II concentration on the line widths of the *S*-methyl and Tyr ring protons of Met-Tyr-Phe-NH$_2$. It is significant that, unlike Ala-Tyr-Phe-NH$_2$, the Tyr ortho and meta protons of Met-Tyr-Phe-NH$_2$ are affected to the same extent by binding, suggesting an effect of the side chain in position 1 on the orientation of the side chain in position 2 in the bound state. A role of the side chain in position 1 in binding is also suggested by the observed broadening of the *S*-CH$_3$ (Figure 2b) and the fact (Figure 3) that the β-CH$_2$ Met protons are perturbed more than the γ-CH$_2$ Met protons. (The Phe ring and β-CH$_2$ protons of Met-Tyr-Phe-NH$_2$ were relatively unaffected by NP-II, as previously observed with Ala-Tyr-Phe-NH$_2$.)

The effect of NP-II on the line widths of *S*-Me-Cys-Tyr-Phe-NH$_2$ was also studied. In this case, the Tyr ortho protons again appeared to be broadened slightly more than the meta protons, although not as much so as with Ala-Tyr-Phe-NH$_2$. Broadening of the *S*-methyl protons was also observed. Experiments with this peptide were carried out at pH 3.5 due to the insolubility of the complex in the pH range 3.8–7.5 at the concentrations used.

The Tyr residue at position 2 of the binding peptides can be substituted by Phe, and the Phe residue in position 3 can be substituted by Ile with almost no effect on binding (Breslow and Abrash, 1966; Breslow and Weis, 1972). Addition of NP-II to a solution of *S*-Me-Cys-Phe-Ile-NH$_2$ resulted in the following order of broadening of the various nonaromatic protons: Ile β-CH$_3$ $\sim$ S-CH$_3$ > Cys β-CH$_2$ > Ile γ-CH$_3$ (Figure 4). Furthermore, the Ile β-CH proton was broadened more than the γ-CH$_2$ protons by addition of protein. In addition to protons of residues 1 and 3, the Phe ring protons were *mark-*

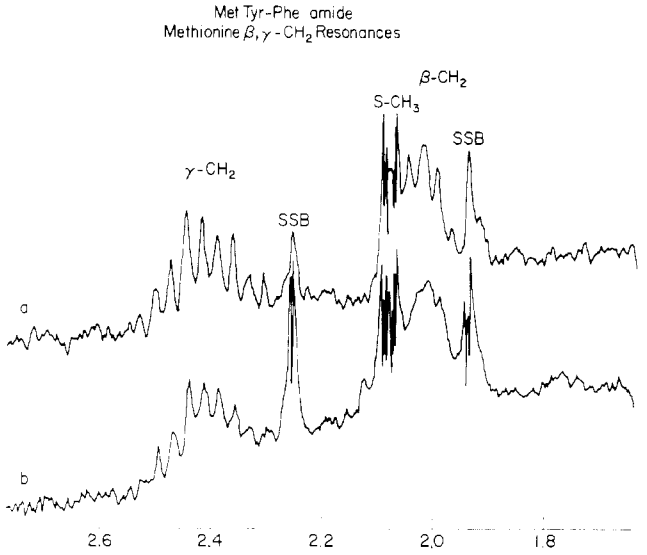

FIGURE 3: β- and γ-methylene protons of methionine in Met-Tyr-PheNH$_2$: (a) 2×10^{-3} M peptide, pH 5.9; (b) 0.03 equiv of NP-II added, pH 6.3.

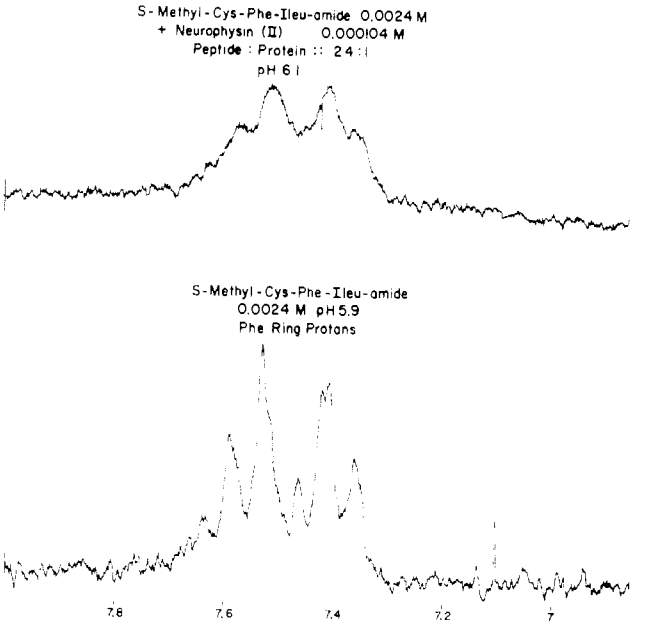

FIGURE 5: Aromatic protons of *S*-Me-Cys-Phe-IleNH$_2$ in the absence and presence of NP-II. Note that integrated intensities shown are 25% less in the presence than in the absence of peptide; this is due solely to the conditions chosen for computer display.

edly broadened (Figure 5), but the multiplets arising from the 1,2 and 3,4,5 protons overlapped sufficiently to preclude precise conclusions as to differences in broadening among them. The latter observations indicate that the aromatic ring of the binding peptide is principally affected when it is in position 2 since Phe aromatic protons are almost unaffected when Phe is in position 3 (Balaram *et al.*, 1972b).

A binding constant of 2×10^3 was calculated at pH 6.2 for Ala-Tyr-Phe-NH$_2$ from the nmr data, in good agreement with results from other measurements (E. Breslow and J. Weis, unpublished observations). No effort was made to evaluate quantitatively binding constants for the other peptides from the nmr results. However, qualitatively the nmr results indicate the following order of binding affinities: Ala-Tyr-Phe-NH$_2$ < Met-Tyr-Phe-NH$_2$ ~ *S*-Me-Cys-Phe-Ile-NH$_2$, in fair agreement with data elsewhere (Breslow *et al.*, 1971). There was no observable broadening of the resonances of Gly-Tyr-Phe-NH$_2$ on addition of NP-II, in agreement with the earlier report that Gly-Tyr-Phe-NH$_2$ binds very weakly to NP-II (Breslow *et al.*, 1971).

Figure 6 shows the line widths of Ala-Tyr-Phe-NH$_2$ as a function of pH in the presence of NP-II. The protons shown all exhibit curves of similar shape, demonstrating that binding is maximal in the vicinity of pH 6. Line widths seen at pH 2 and 10 are essentially identical to those of the uncomplexed peptide. This is in agreement with the suggestion cited above that a principal contribution to the binding energy arises from an electrostatic attraction between a positively charged α-amino group and a negatively charged carboxyl group. Corresponding studies with *S*-Me-Cys-Phe-Ile-NH$_2$ on the other hand (not shown) exhibit vastly different pH profiles for the *S*-CH$_3$ and Ile β- and γ-CH$_3$ protons. There is also evidence for incomplete dissociation, even below pH 2.5, in the form of line broadening by NP-II of the Phe ring and Ile β-CH$_3$ protons of the peptide at this low pH. The persistence of binding at low pH has already been noted for LVP in this study, as well as in a circular dichroism study (E. Breslow and J. Weis, unpublished observations). These results suggest somewhat different forms of binding of *S*-Me-Cys-Phe-Ile-NH$_2$ over the entire pH range. As a result, the change in line widths with pH cannot be taken as representative of only the change in binding affinity, but is also a measure of the reorientation of residues, either on the peptide or on the protein, resulting from a protonation or deprotonation of charged centers.

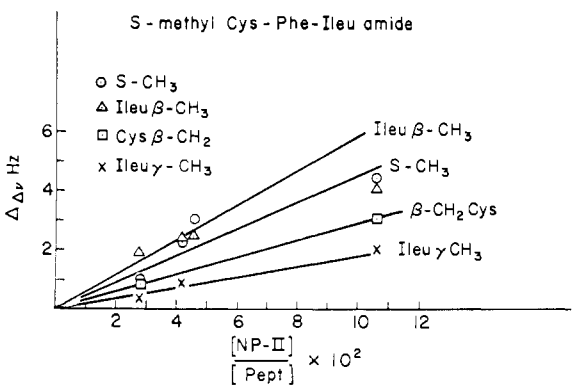

FIGURE 4: Plot of *S*-Me-Cys-Phe-IleNH$_2$ alkyl proton line widths as a function of relative NP-II concentration.

Nuclear Overhauser Effects. The differential broadening of the tyrosine ortho and meta protons of Ala-Tyr-Phe-NH$_2$ suggests different dipolar contributions from protons on NP-II to broadening of peptide tyrosine protons (Balaram *et al.*, 1972b). This has been discussed above and is amplified further in the Appendix. Dipolar interactions are very short range, diminishing with the 6th power of the internuclear distances inovlved (see Appendix). Therefore, the apparently close spatial proximity of groups on NP-II to the aromatic residue of the peptide in position 2 suggested that the NOE, a direct and unambiguous method of establishing the spatial proximity of two magnetic nuclei (Noggle and Schirmer, 1971), could be used to identify groups in NP-II which interact with the aromatic ring at position 2 of the peptide. In particular, saturation of specific resonances on NP-II and observation of intensity changes in the resonances of the peptide would allow interacting resonances to be identified. As previously indicated (Balaram *et al.*, 1972a) for NOE effects between like spins (as between two protons) on molecules with long correlation

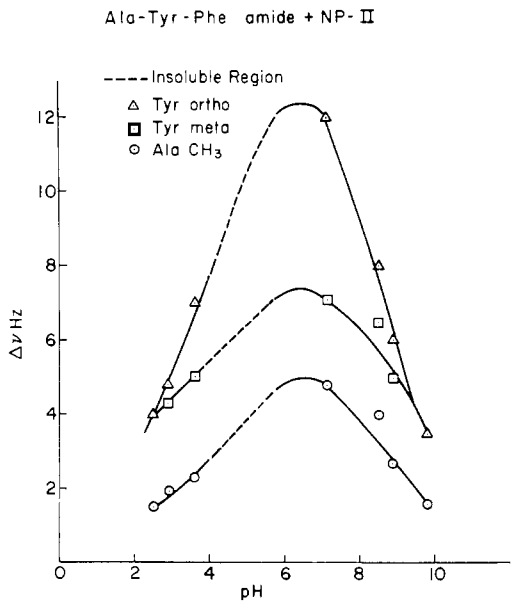

FIGURE 6: pH dependence of the line widths of 2.3×10^{-3} M Ala-Tyr-PheNH$_2$ containing 0.14 equiv of NP-II.

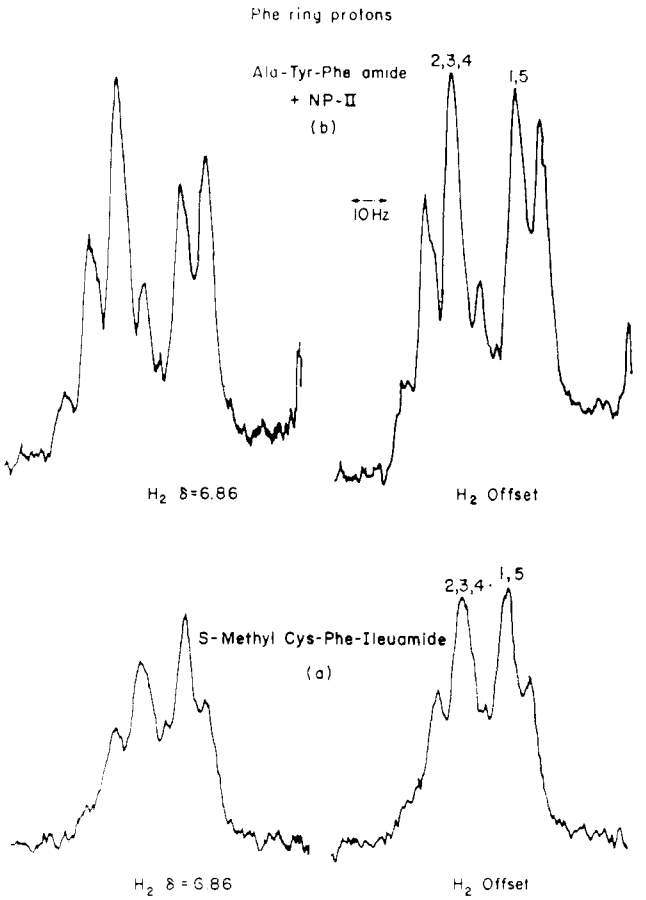

FIGURE 7: Phenyl proton resonances with and without a strong irradiating field at δ 6.86: (a) *S*-Me-Cys-Phe-IleNH$_2$, 4.9×10^{-3} M; NP-II, 2.3×10^{-4} M, pH 6.5; (b) Ala-Tyr-PheNH$_2$, 4.5×10^{-3} M; NP-II, 3×10^{-4} M, pH 6.5.

times, the NOE effect will lead to a decrease in intensity. This is not necessarily the case for situations involving unlike spins (see Appendix), but such a situation is not relevant to the present studies.

NOE effects between NP-II and bound peptide were demonstrated by irradiating a system containing a large excess of peptide to protein with a strong irradiating field H_2 and recording the peptide spectrum at different values of H_2. Irradiation of a mixture of NP-II and *S*-Me-Cys-Phe-IleNH$_2$ with a strong radiofrequency field (H_2) resulted in large decreases in intensity of the phenyl resonances of the peptide when the strong irradiating field was in regions centered at δ 1.9, 3.1, and 6.86 ppm from 2,2-dimethylsilapentane-5-sulfonate (*i.e.*, −710, −410, and +540 Hz from the internal HDO lock signal). In the absence of protein there was no observable effect of the second radiofrequency field (H_2) on the peptide phenyl resonances. No effects could be detected on the *S*-methyl and isoleucine β- and γ-methyl protons of the peptide even in the presence of protein. Figure 7 shows the effect of irradiating the protein spectrum at δ 6.86 ppm on the phenylalanine ring resonances of *S*-Me-Cys-Phe-IleNH$_2$ and Ala-Tyr-PheNH$_2$. The lower field component of the phenyl multiplet has an intensity corresponding to three protons and is assigned to protons 2, 3, and 4. The high-field component has an intensity corresponding to two protons and is assigned to the 1,5 protons. The 2,3,4 Phe protons of *S*-Me-Cys-Phe-IleNH$_2$ show a large decrease in intensity (22%) while the corresponding Phe ring protons of the Ala-Tyr-PheNH$_2$ are unaffected. The 1,5 Phe ring proton regions of both peptides also show a slight decrease on irradiation at 6.86 ppm but this is due, at least in part, to the fact that the Phe resonances of the protein underlie this region and are themselves decreased by irradiation at 6.86 ppm even in the absence of peptide. (Note, for example, that the "base line" immediately upfield of the 1,5 protons of Ala-Tyr-PheNH$_2$ is lowered on irradiation.) In any event, the irradiation-induced decrease in intensity of the 1,5 Phe ring protons of *S*-Me-Cys-Phe-IleNH$_2$ is clearly less than that of the 2,3,4 Phe ring protons of the same peptide while this is not true for the Ala peptide. This differential effect on the 2,3,4 Phe ring protons of the two peptides demonstrates that the double resonance effect is observable chiefly when the phenyl ring is at position 2 on the tripeptide. Line broadening of the phenyl resonances on binding also occurs principally when the phenyl ring is at position 2 (and, as indicated above for Ala-Tyr-Phe-NH$_2$, is greatest for the ortho protons of the Tyr ring which are analagous to Phe ring protons 2 and 4). These data strongly implicate the negative Overhauser effect as a direct result of the binding process.

Smaller differences between the two sets of Phe ring protons in *S*-Me-Cys-Phe-IleNH$_2$ were observed upon irradiation at δ 1.9 and 3.1. The effect of irradiating the alkyl resonances of NP-II is a broad intensity decrease in the peptide resonances. The low selectivity at high H_2 power levels and the width of the protein resonances involved could lead to this broad effect. A definite assignment of the protein resonances at δ 1.9 and 3.1 cannot be made at this time. However, the effect of δ 6.86 ppm can be unambiguously assigned to the ring protons ortho to the hydroxyl group of the sole tyrosine residue (position 49) in NP-II and indicates the proximity of the ortho protons of Tyr-49 to position 2 of the bound peptide. (The assignment of the 6.86-ppm resonance will subsequently be seen clearly in Figure 10 where the aromatic ring protons of NP-II are shown.)

Figure 8 shows the results of similar double-resonance experiments on the Tyr ortho protons of Ala-Tyr-Phe-NH$_2$. The overlap of the peptide and protein resonances prevented the double irradiation of the protein tyrosine peaks; however, intensity decreases were observed on irradiating the system at δ 1.9 and 3.10 and this effect occurred only in the presence of protein. No effects could be detected on the Ala methyl or

TABLE I: pH Dependence of the Negative NOE[a] in a Solution of *S*-Me-Cys-Phe-IleNH$_2$[b] and NP-II.

pH:	Peaks Observed 2.5		3.6		6.2		7.4		8.4	
ν_{H_2}[c]	2,3,4	1,5	2,3,4	1,5	2,3,4	1,5	2,3,4	1,5	2,3,4	1,5
−710	9	5	29	19	21	14	17	14	9	5
−410	12	6	24	22	25	20	16	14	12	6

[a] The intensity decreases on irradiating the protein Tyr ortho protons are not reported. This is because in the course of the experiment the residual HDO signal grew more intense. Spurious instrumental effects become more serious as the internal lock signal grows stronger. Qualitatively the effects followed the same trend as those reported in Table III. [b] [Peptide] = 5.1×10^{-3} M; [NP-II] = 2×10^{-4} M. [c] Hz upfield from HDO.

the phenyl protons. The Tyr meta protons showed smaller intensity decreases than the ortho protons in the presence of protein and, as already noted, these protons also broaden to a lesser degree on interaction with the protein. *S*-Me-Cys-Tyr-PheNH$_2$ behaved in similar fashion, with larger negative NOE's observed on the Tyr ortho protons relative to the meta protons. Figure 9 shows an indor spectrum obtained by holding H_1 stationary at the Tyr ortho resonance of the peptide and sweeping the strong H_2 field through the protein spectrum. Two negative peaks can be observed in the region δ 1.5–3.5 ppm.

The Tyr ring protons in Met-Tyr-PheNH$_2$ are broadened to the same extent on adding NP-II (Figure 2). Significantly the negative NOE effects on Tyr ortho and meta protons in this peptide are somewhat more equal than in Ala-Tyr-Phe-NH$_2$ (Balaram *et al.*, 1972b).

The double-resonance effects outlined so far are dependent on pH as well as protein concentration. Table I summarizes the pH dependence of the negative NOE's in the *S*-Me-Cys-Phe-IleNH$_2$. It must be stressed that overlap of the Phe 2,3,4 and 1,5 multiplets renders accurate evaluation of NOE magnitudes difficult. The numbers quoted are therefore only very approximate and can be used only to point out general trends. However, it is apparent that the negative NOE's fall off at high and low pH, again implying their close connection with the binding process.

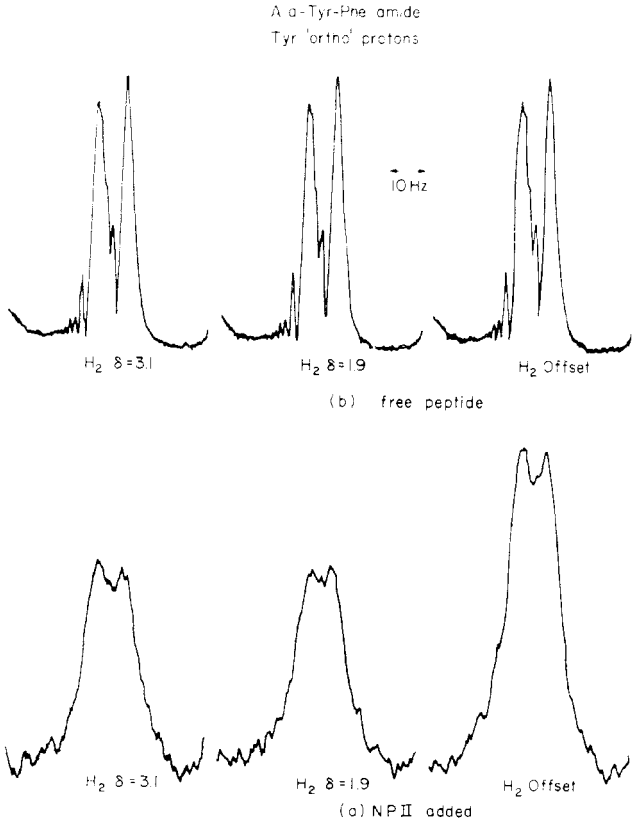

FIGURE 8: Tyr ortho protons of Ala-Tyr-PheNH$_2$ with and without strong double irradiation: (a) [peptide] = 4.5×10^{-3} M, [NP-II] = 3×10^{-4} M, pH 6.5, (b) free peptide, 2×10^{-3} M, pH 6.5.

The double-resonance effects show concentration dependence at low protein concentrations (3% of peptide molecules are complexed) but reach a plateau when 5% or more of the complex is present. The data available so far cannot be subjected to any rigorous quantitative treatment as errors in estimating NOE magnitudes are large. However, a steep dependence on protein concentration at high peptide to protein ratios is observed. This suggests that in small molecule–protein systems the dipolar relaxation effects are distributed over all the small molecules by chemical exchange (see Appendix).

Direct Observation of Protein Resonances. In order to clarify the role of the lone protein tyrosine, a set of experiments were carried out in which the aromatic protons of NP-II were directly observed. Figure 10 shows the aromatic protons of NP-II at pH 6 in the absence and presence of added peptides. In the absence of added peptide (10c), peaks at approximately 6.8 and 7.1 ppm can only be assigned to the ortho and meta protons, respectively, of the protein tyrosine when compared with known (Roberts and Jardetzky, 1970) amino acid chemical shifts; however, they are shifted 15–25 Hz upfield from Tyr ring protons of simple peptides containing Tyr as the sole aromatic amino acid (Balaram, 1972) suggesting that they are in a shielded environment. The large peak near 7.25 ppm can only represent the ring protons of the three phenylalanines.

Addition of 2 equiv of *S*-Me-Cys-Phe-IleNH$_2$ to a 4×10^{-4} M solution of NP-II at pH 6.7 (Figure 10b) results in a downfield shift of approximately 20 Hz and a slight narrowing ($\Delta_{\Delta\nu}$ = 6 Hz) of the protein Tyr ortho protons, while the Tyr meta protons move downfield under the phenyl resonances. These results clearly demonstrate that the environment of the protein tyrosine is changed by binding, in agreement with conclusions reached from spectrophotometric and circular dichroism studies of mononitrated NP-II (Furth and Hope, 1970; Breslow and Weis, 1972). Specifically the nmr changes seen in the tyrosine on binding suggest that it becomes more deshielded and experiences a greater freedom of motion (or is subject to fewer dipolar interactions) in the complex. In either event, these changes in the protein tyrosine nmr cannot be due only to a general conformational change on binding, since the NOE experiments above support the thesis that the

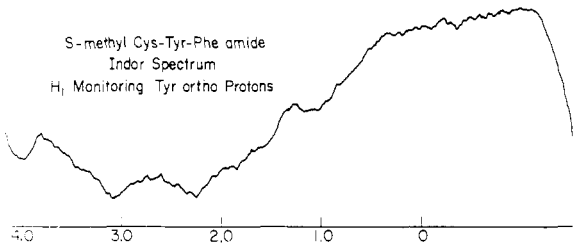

FIGURE 9: Indor spectrum obtained by monitoring Tyr ortho protons of *S*-Me-Cys-Tyr-PheNH_2 and sweeping the irradiating frequency through the protein spectrum. [peptide] = 5.4×10^{-3} M; [protein] = 3×10^{-4} M, pH 3.5.

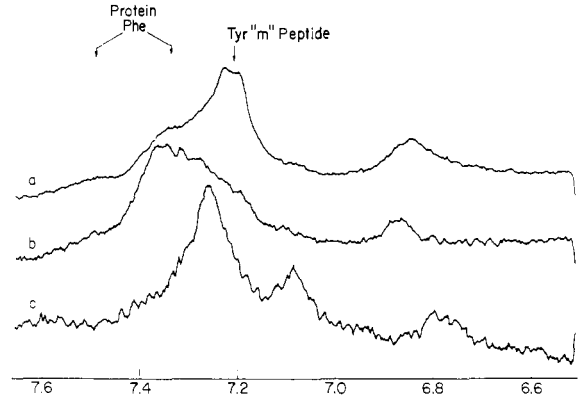

FIGURE 10: Aromatic region of NP-II, pH 6.7: (a) in presence of 3 equiv of Met-TyrNH_2; proton assignments shown here are tentative; (b) in presence of 2 equiv of *S*-Me-Cys-Phe-IleNH_2; (c) protein alone. Protein concentration = 4×10^{-4} M.

protein Tyr residue is spatially proximate to the aromatic ring at position 2 of the bound peptide.

In addition to effects on the NP-II tyrosine, binding may also perturb the protein Phe resonances. With *S*-Me-Cys-Phe-Ile-NH_2, overlap of protein and peptide phenyl peaks obscures this effect. However, in a preliminary experiment, addition of the binding dipeptide Met-Tyr-NH_2 (Figure 10a) suggested that one or more of the protein Phe ring resonances are shifted downfield by binding, either by a long range conformational change or by direct participation in binding.

Between pH 6 and 2.5 the nmr spectrum of NP-II is altered. Changes are seen in both the alkyl proton and aromatic regions over this pH interval, just as circular dichroism changes over the same interval have been reported (Breslow and Weis, 1972). The data indicate that a conformational change occurs.

Studies of Nitrated NP-II. Since the Tyr ortho protons of NP-II have been assigned as the origin of the δ 6.86-ppm negative NOE, a study of nitrated NP-II (NP-NO_2) was carried out. In this derivative, one of the ring protons ortho to the NP-II Tyr hydroxyl is replaced by a nitro group. The aromatic region of the nitrated protein spectrum at pH 7.7 contains the intense Phe ring peak and three weak resonances at 6.87, 7.62, and 7.86 ppm. The latter are assigned to the protons ortho to the hydroxyl (H_a), meta to the hydroxyl (H_b) and ortho to the nitro group (H_c) respectively, in agreement with reported shifts on going from phenol to nitrophenol (Castellano *et al.*, 1967a,b). H_a moved downfield under the Phe resonances as the pH was lowered from 7.7 due to protonation of the nitrotyrosine phenol (pK_a = 7.3) (Breslow and Weis, 1972). Addition of NO_2-NP to *S*-Me-Cys-Phe-IleNH_2 led to broadening of the Phe ring resonances of the peptide and large pH-dependent NOE effects between H_a and the Phe ring resonances of the peptide were observed, confirming that nitration does not block binding (Furth and Hope, 1970). Ionization of the protein hydroxyl (pK_a in complex = 6.8) (Breslow and Weis, 1972) has no appreciable effect on either the line widths of bound peptide or on the magnitude of the double-resonance effects; this indicates a nonessential role for the protein Tyr hydroxyl in the binding interaction, as also suggested by circular dichroism studies (Breslow and Weis, 1972).

Discussion

Although tripeptide analogs of residues 1–3 of the hormones appear to contain the principal binding sites of the hormones (Breslow *et al.*, 1971; Breslow and Weis, 1972), this study indicates a significant difference in exchange rate between the hormones and tripeptides with their neurophysin complexes. Assuming a bound line width of 40 Hz for the Tyr ortho protons of lysine vasopressin, an exchange rate considerably lower than 125 sec^{-1} is estimated for this hormone. Alternatively, the tripeptides exchange rapidly on an nmr time scale. Several reasons can be postulated for the difference in hormone and tripeptide exchange rate. First, it should be noted that the binding constant for tripeptides is approximately $^1/_{100}$ that of the hormones (Breslow *et al.*, 1973). If this difference in binding constant is entirely due to a decrease in the rate of dissociation of the hormone complex relative to that of the tripeptides it could account for the marked difference in exchange rate between the hormones and tripeptides. Alternate reasons for the marked difference in exchange rate between hormones and tripeptides might lie in different conformational accommodations made by the protein on binding hormones than tripeptides or the possible necessity for conformational changes in the hormone on binding.[2]

Despite any differences in exchange rate between the hormones and tripeptides, however, the present studies support the postulate that the tripeptides contain the principal binding features of the hormones in that they not only verify previous conclusions about tripeptide binding, but also support and explain previous data on hormone binding. For example, tyrosine-2 of both the hormones (Breslow and Abrash, 1966) and tripeptides (Breslow and Weis, 1972) has been shown to be a major participant in binding and is seen in the present studies to be broadened by dipolar relaxation and in sufficiently close proximity to several classes of protons on the protein to show NOE effects when the resonances of these protein protons are saturated. The demonstration that loss of the tripeptide tyrosine hydroxyl (*i.e.*, substitution of phenylalanine for tyrosine in position 2 of the binding tripeptide) does not diminish broadening of the aromatic ring in position 2 on binding is in keeping with the observation that oxytocin and 2-phenylalanine-oxytocin have very similar affinities for neurophysin (Breslow and Abrash, 1966) and, in this instance, has allowed the useful demonstration of an NOE between the single protein tyrosine and the peptide aromatic residue in position 2. The proximity of the protein tyrosine and the bound peptide is in keeping with the demonstration (Furth and Hope, 1970; Breslow and Weis, 1972) that the tyrosine of the protein is perturbed on binding and is lowered in pK_a by 0.6 pH unit. As stated elsewhere (Breslow and Weis, 1972)

[2] Recently evidence has been presented elsewhere (R. Alazard, P. Cohen, J. H. Griffin, and J. S. Cohen, manuscript in preparation) which suggests that the rate of LVP exchange may be faster than observed here; the basis for the dissimilar observations is uncertain.

the pK_a of the tyrosine is probably lowered on binding by its proximity to the protonated α-NH_3^+ of the bound peptide (whose influence in part is to neutralize the negative charge on a nearby carboxyl). However, it is tentatively unlikely that the aromatic ring in position 2 of the peptide and that of the protein tyrosine stack with each other in the bound state. Cohen *et al.* (1972) have shown that the tyrosine ring protons of oxytocin are not shifted upfield on binding and have used this as an argument against stacking. Similar conclusions are suggested by Figure 5 which shows no significant shift of the Phe ring protons of *S*-Me-Cys-Phe-IleNH$_2$ on binding and from the fact that the protein tyrosine ring protons shift downfield on binding.

The nmr data also offer partial explanation of the observation (Breslow and Abrash, 1966) that substitution of position 3 of oxytocin by glycine leads to reduction in binding to about 3% of its original value, while substitution by phenylalanine (as found in LVP) is almost without effect on binding. The data here indicate that the isoleucine β-CH and β-CH_3 protons in position 3 of *S*-Me-Cys-Phe-IleNH$_2$ are broadened more than the γ-CH_2 and γ-CH_3 protons of the same chain. When these data are considered together with the fact that the Phe ring protons in peptides containing Phe in position 3 experience almost no broadening on binding, they suggest that the side chains of position 3 of the hormones contribute to binding principally by interactions at the β-carbon atom or at groups directly attached to the β carbon. Thus, differences between phenylalanine and isoleucine at positions beyond the β carbon may be of less importance than the fact that both contain a β carbon.

The present data also confirm the participation of the side chain in position 1 in binding, significant broadening of the *S*-CH_3 protons of *S*-Me-Cys-Phe-IleNH$_2$ and of protons attached to the β carbon of position 1 being observed with all peptides studied. In addition, the greater broadening of the methionine β-CH_2 protons relative to the γ-CH_2 protons in Met-Tyr-PheNH$_2$ suggests a closer contact of the former as compared to the latter on the protein. As with the side chain in position 3, it is not strictly possible to prove that this differential broadening is due to different degrees of intermolecular dipolar relaxation of the two protons rather than differences in rotation time between the C_α-C_β bond and the C_β-C_γ bond of the bound peptide. However, it is relevant to note that neither oxytocin nor vasopressin contain a γ-CH_2 in position 1 and that addition of such a residue, as in 1-hemihomocysteine-oxytocin, leads to no change in binding affinity to neurophysin (Breslow and Abrash, 1966).

Another interesting feature of the interactions of the side chain in position 1 is that the *S*-methyl protons of *S*-Me-Cys-Phe-IleNH$_2$ are broadened far more than the *S*-methyl protons of Met-Tyr-PheNH$_2$. For a peptide to NP-II ratio of ~9.5, at a peptide concentration of 2×10^{-3} M, $\Delta_{\Delta\nu}S_{CH_3} = 0.9 \pm 0.2$ Hz for Met-Tyr-Phe-NH$_2$ at pH 6.5 and 4.4 ± 0.5 Hz for *S*-Me-Cys-Phe-IleNH$_2$ under essentially the same conditions. Since the difference in the magnitude of these effects is not due to differences in binding constants of the two peptides (Brewlow and Weis, 1972), two possible explanations can be invoked. First, because the CH_3 of the *S*-Me-Cys peptide is in a position roughly comparable to that of the disulfide sulfur in residue 6 of the hormones, it is possible that it is broadened by dipolar relaxation from groups on the protein normally interacting with the sulfur in position 6. Alternatively it is possible that the *S*-CH_3 of the *S*-Me-Cys peptide is broadened because it is more sterically hindered than that of the methionyl peptide when bound to the protein; thus, broadening may occur *via* more restricted rotation about the *S*-CH_3 bond in the *S*-methylcysteinyl peptide.

Finally additional comment should be made about the pH dependence of binding of peptides and hormones to NP-II. Data for Ala-Tyr-Phe-NH$_2$ are in keeping with the suggestion that binding involves interaction of a protonated α-NH_3^+ and a carboxyl residue and also support the thesis (Breslow *et al.*, 1971; Breslow and Weis, 1972) that the carboxyl involved is a side-chain carboxyl, since dissociation of this peptide under the conditions studied is almost complete at pH 4—indicating that the carboxyl involved has a $pK_a > 4$. Therefore, the increased rate of exchange between free and bound LVP at low pH is undoubtedly a reflection of weaker binding at this pH (Ginsburg and Ireland, 1964)—the weaker binding presumably resulting in part from competition between protons and the protonated α-NH_2 of the hormone for a particular carboxyl on the protein. However, it should also be noted that there is a distinct possibility that the observed increased exchange rate for LVP at pH 1.5 is also a reflection of an altered mode of binding at lower pH. This is not only suggested by the effect of pH on line broadening of *S*-Me-Cys-Phe-IleNH$_2$ noted above, but is also suggested by CD data (Breslow and Weis, unpublished observations) which tentatively indicate that the spectra of LVP and oxytocin complexes of NP-II seen at pH 2 *qualitatively* differ from those seen at pH 6. Such a difference may reflect a form of the complex in which interactions between the protonated α-NH_3^+ and carboxyl are rearranged (by carboxyl protonation) but in which binding at other sites remains. The relatively smooth pH dissociation of Ala-Tyr-Pye-NH$_2$ in contrast to that of LVP or *S*-Me-Cys-Phe-IleNH$_2$ may reflect the absence of sufficient secondary interactions at position 1 (no sulfur or *S*-CH_3) in this peptide to allow any altered binding of significant strength in the presence of an altered amino-carboxyl site.

In conclusion therefore, the present nmr studies confirm and extend many theses regarding NP–hormone binding arrived at by other methodology and, most importantly, have allowed the deduction that the single tyrosine of NP-II is at the hormone-binding site. Moreover, these studies tentatively suggest that the protein phenylalanine residues are perturbed by binding; further studies in this area are in progress.

Acknowledgments

The authors are particularly grateful to Miss Jane Weis for the preparation of all neurophysin samples used here and to Dr. Jose Dadok for many stimulating discussions.

Appendix

The dipolar interaction that leads to line broadening is very short range in nature and for $\omega_0\tau_c \ll 1$ (where ω_0 is the Larmor precession frequency and τ_c is the correlation time)

$$\frac{1}{T_{1M}} = \frac{1}{T_{2M}} = \sum_{ij}\frac{3}{2}\hbar^2\gamma_p^4\frac{\tau_{ij}}{r_{ij}^6} \qquad (1)$$

Here, the summation is over all pairs of nuclei contributing to the relaxation, γ_p is the magnetogyric ratio for protons, τ_{ij} is the effective correlation time for the internuclear vector, r_{ij} is the internuclear distance and $1/T_{1M}$ and $1/T_{2M}$ are the spin–lattice and spin–spin relaxation rates, respectively. The same correlation time must determine the reorientation of all

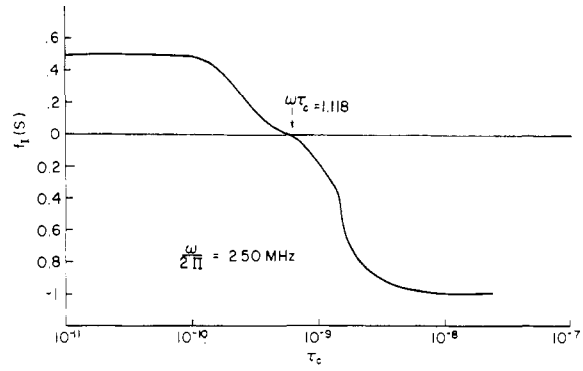

FIGURE 11: Plot of the Overhauser enhancement $f_I(S)$ *vs.* the correlation time (τ_c) modulating the dipolar interaction.

protons on the aromatic ring. Moreover, the Tyr ortho and meta protons do not relax only under the influence of their mutual dipolar interaction. If this were so, their line widths ($1/\pi T_{2M}$) would be the same. The differential broadening can therefore be explained only by invoking different dipolar contributions from protons on NP-II.

The use of the NOE to identify resonances contributing to intermolecular dipolar relaxation in a small molecule–macromolecule interaction is subject to three limitations. (1) In the absence of any prior information about the binding site, the search for an NOE is likely to be long and tedious. (2) High radiofrequency amplitudes may be required to saturate protein resonances, which are likely to be broad, spread over a range of resonance frequencies, or both. (3) Once observed, the assignment of the protein resonance causing the NOE to specific residues on the protein may be ambiguous. The first limitation is largely experimental and can be circumvented in part, by using indor, techniques. In this method the frequency of the weak observing field H_1 is centered on the small molecule resonance while the frequency of the strong irradiating field H_2 is swept through the protein spectrum. Changes in the intensity of the observed resonance signal are then recorded as a function of the H_2 frequency. The use of this technique is complicated by the stringent requirement of field homogeneity stability. The slower but more reliable method of recording the peptide spectra at different H_2 frequencies has been primarily used in this study. The second limitation is not very serious as large H_2 levels are initially used to detect the effects and greater selectivity is then achieved at lower radiofrequency power levels. The third limitation is inherent in any nmr study of macromolecules and may sometimes be overcome by resort to the techniques of specific deuteration or selective chemical modification.

For a system of two spins (I, S) the dipolar transition probabilities have been calculated by Solomon (1955). In the case of like spins (Balaram *et al.*, 1972a), *i.e.* $(\omega_I - \omega_S)^2\tau_c^2 \ll 1$, the fractional enhancement of the I resonance $f_I(S)$ when the S resonance is saturated is given by

$$f_I(S) = \frac{W_2 - W_0}{W_0 + 2W_1 + W_2}\frac{S_0}{I_0} = \frac{5 + \omega^2\tau_c^2 - 4\omega^4\tau_c^4}{10 + 23\omega^2\tau_c^2 + 4\omega^4\tau_c^4} \tag{2}$$

W_i's are the transition probabilities for the i-quantum dipole–dipole transitions and ω is again the Larmor precession frequency. For a two-proton system the effect of increasing the correlation time (τ_c) modulating the dipolar interaction on the sign and magnitude of the NOE is shown in Figure 11. $f_I(S) = {}^1/_2$ for $\omega\tau_c \ll 1$; $f_I(S) = 0$ for $\omega\tau_c = 1.118$ and, for large $\omega\tau_c$, $f_I(S) = -1$ corresponding to complete saturation of the observed signal. Bloembergen *et al.* (1948) in their classic paper on nuclear relaxation showed that for $\omega\tau_c \ll 1$, $T_1 = T_2$; T_1 passes through a minimum for $\omega\tau_c \approx 1$ and increases at longer τ_c. T_2 decreases monotonically with increasing τ_c and the theory is no longer applicable for $\omega\tau_c \approx 1/T_2$ *i.e.*, for very slow motion. Therefore T_1 becomes greater than T_2 for very slow motion. It is immediately apparent that the observation of a negative NOE would imply that $T_1 > T_2$. The negative NOE is a manifestation of the predominance of the W_0 transition over the W_2 transition. For the homonuclear case (like spins) the expressions for the probabilities are

$$W_0 = \frac{\tau_c k^2}{10\hbar^2}$$
$$W_2 = \frac{6\tau_c k^2}{10\hbar^2}\frac{1}{1 + 4\omega^2\tau_c^2} \tag{3}$$

where $k = \hbar^2\gamma_I\gamma_S/r^3$, r is the internuclear distance. For the heteronuclear case (unlike spins)

$$W_0 = \frac{\tau_c k^2}{10\hbar^2}\frac{1}{1 + (\omega_I - \omega_S)^2\tau_c^2}$$
$$W_2 = \frac{6\tau_c k^2}{10\hbar^2}\frac{1}{1 + (\omega_I + \omega_S)^2\tau_c^2} \tag{4}$$

Equations 4 must be used to calculate the dependence of the sign and magnitude of the heteronuclear NOE as a function of the correlation time.

For a $^{13}C(I)$–$^1H(S)$ system at H_0 = 58.7 kG, ω_I = 62.8 MHz, ω_S = 250 MHz.

At $\tau_c = 10^{-8}$ sec, $W_2 - W_0 = 0.334$.

For a $^{31}P(I)$–$^1H(S)$ system at 58.7 kG, ω_I = 101.2 MHz, and ω_S = 250 MHz.

At $\tau_c = 10^{-8}$ sec, $W_2 - W_0 = 0.139$. The heteronuclear NOE in these cases would therefore show no sign reversal even in macromolecular systems. Doddrell *et al.* (1972) have explored the dependence of the ^{13}C–^1H NOE on τ_c in detail and obtained a limiting value of $f_I(S) = 0.153$.

The possibility that in small molecule–protein systems the dipolar relaxation effects can be distributed over all the small molecules by chemical exchange can be readily explained. Consider the equilibrium reaction

$$E + S \rightleftharpoons ES$$

where E represents the protein; S, the peptide; and ES the complex. If the average z component of magnetization of peptide protons in the free state is m_f, then peptide enters the complex with this average magnetization. During the time of its average residence in the complex, τ_b, the magnetization will change as a result of two processes: (1) cross-relaxation with the proton on the protein which has been saturated will "heat" the peptide spin system; (2) dipolar relaxation with other nuclei will "cool" it. The behavior of the system may be approximately represented by the coupled Bloch eq 5 and 6

$$b\frac{dm_b}{dt} = b\frac{m_{b\infty} - m_b}{T_b} - b\frac{m_b}{\tau_b} + f\frac{m_f}{\tau_f} \tag{5}$$

$$f\frac{dm_f}{dt} = f\frac{m_{f\infty} - m_f}{T_f} - f\frac{m_f}{\tau_f} + b\frac{m_b}{\tau_b} \tag{6}$$

where f and b are the fractions of peptide bound and free, m_b and m_f are the z components of magnetization of peptide protons in n moles of peptide in the bound and free states, respectively (n is the total concentration of peptide), $1/T_b$ is the rate of approach of m_b to its equilibrium value $m_{b\infty}$, and $1/T_f$ is the corresponding relaxation rate of protons in the free peptide. The last two terms in eq 5 and 6 account for the exchange between bound and free states.

In the steady state, eq 5 and 6 may be equated to zero. Defining the total magnetization, m_t, according to eq 7

$$m_t = fm_f + bm_b \tag{7}$$

and taking τ_b to be independent of concentration, algebraic manipulation yields

$$m_t = \frac{fT_b m_{f\infty} + bT_f m_b + f\tau_b(fm_{f\infty} + bm_{b\infty})}{fT_b + bT_f + f\tau_b} \tag{8}$$

For fast exchange, the usual case, this reduces to

$$m_t = \frac{fT_b m_{f\infty} + bT_f m_{b\infty}}{fT_b + bT_f} \tag{9}$$

The dependence of signal strength, which is proportional to m_t, on fraction bound, thus gives information about the relative sizes of T_b and T_f. The data may be analyzed in various ways; it is sufficient to note here that $m_t = m_f$ (maximum effect) when $b = 1$; and $m_t = (1/2)(m_{f\infty} + m_{b\infty})$ when $T_b/T_f = b/f$.

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