Effect of Low pH on Neurophysin–Peptide Interactions: Implications for the Stability of the Amino–Carboxylate Salt Bridge†

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ABSTRACT: The optical activity and peptide affinity of bovine neurophysin and of bovine neurophysin mononitrated at its single tyrosine have been studied between pH 6.2 and pH 1; several studies of neurophysin dinitrated at its single tyrosine are also reported. The circular dichroism spectra of both neurophysin and mononitrated neurophysin change between pH 6 and pH 2 in a manner indicative of a change in the environment of tyrosine and of at least one other chromophore; the pH of the transition midpoint is approximately 4.25 for neurophysin II and 4.85 for neurophysin I. The peptide-binding affinity of neurophysin diminishes between pH 6 and pH 2 in a manner consistent with the dependence of binding on the presence of an ionized side-chain carboxyl of normal pK_a with which the protonated α-NH_2 of the peptide forms a salt bridge. However, significant binding occurs below pH 2 and binding affinity is virtually constant between pH 2 and pH 1. Using the assumption that the pH-independent region of binding below pH 2 represents binding in the absence of salt-bridge formation, the difference in binding affinity between pH 6 and pH 1, corrected for changes in protein net charge and binding isotherm shape over this pH interval, has been used to assign a tentative value of −2.4 kcal per mol at 25 °C to the free energy of salt-bridge formation. However, deaminooxytocin does not effectively compete with oxytocin at low pH, indicating either that significant conformational differences exist between oxytocin and deaminooxytocin or that binding at low pH is still dependent on salt-bridge formation. In view of the latter alternative and the effect of pH on the CD spectrum of the unliganded protein, the possibility is considered that the decrease in binding affinity between pH 6 and pH 2 may be the consequence of a carboxyl-dependent change in protein conformation rather than the consequence of protonation of the salt-bridge carboxyl, but such a possibility is held to be remote.

The interaction of neurophysin with oxytocin, vasopressin, and small peptide analogues of the hormones is thought to include formation of a salt bridge (ion pair) between the protonated hormone or peptide α-amino and an unprotonated side-chain carboxyl of neurophysin (cf., Breslow, 1974). The concept of salt-bridge formation derives principally from the pH dependence of hormone–neurophysin interaction (Ginsburg and Ireland, 1964), analogous changes in proton equilibria attending the interaction (Breslow et al., 1971) and the marked loss in binding affinity resulting from replacement of the hormone α-amino by a hydrogen (Stouffer et al., 1963) or by a hydroxyl group (Hope and Walti, 1971). In fact, the affinity of neurophysin for analogues containing the latter replacements, deaminooxytocin and hydroxymercaptopropionic oxytocin, respectively, has yet to be determined, although NMR evidence suggests some interaction between deaminooxytocin and neurophysin at low pH (Glasel et al., 1973).

We have been interested in assigning the thermodynamic parameters associated with formation of the different types of noncovalent bonds involved in neurophysin–hormone interaction; although parameters for some noncovalent bonds have been estimated from nonprotein models, there are compelling reasons for questioning the extent to which these numbers realistically describe the thermodynamics of noncovalent bond formation within proteins and protein complexes (Jencks, 1975). In order to calculate the contribution of a given bond to the stability of a complex, it is necessary that the energy of formation of the complex be calculated both in the presence and absence of the bond in question (Jencks, 1975) and preliminary estimates of the free energy contributions of individual apolar interactions accompanying neurophysin–peptide interaction have been obtained in this manner (Breslow, 1975). However, an exact estimate of the free energy of formation of the neurophysin–hormone salt bridge has been hindered by the lack of a binding constant for analogues lacking the α-amino group. On the other hand, in preliminary studies (cf., Breslow, 1974), we noted incomplete dissociation of neurophysin–oxytocin complexes at very low pH which suggested that binding of oxytocin to neurophysin at low pH might involve a species in which the key neurophysin carboxyl is protonated and in which the salt bridge might therefore be absent. If this deduction is correct, then calculation of the difference in binding affinities between neutral and low pH would yield a direct estimate of the contribution of the salt bridge to the stability of the complex, provided that no other significant changes occurred within the complex, hormone, or protein over the same pH interval. Interestingly, preliminary studies (Breslow and Weis, 1972) have also suggested that conformational changes within neurophysin may occur between pH 6 and 3. In order to probe this effect further and to attempt an estimate of the free energy contribution of the salt-bridge to neurophysin–hormone interaction, a quantitative study of the effects of pH on neurophysin and its interactions with peptides which are known to bind to the principal hormone-binding site (Breslow et al., 1973) was undertaken. Most of these studies utilize neurophysin that has been mononitrated at its single tyrosine and which has been previously shown to retain the oxytocin-binding properties of the unmodified protein (Furth and Hope, 1970); the neurophysin nitrotyrosine residue serves as a convenient reporter group of both conformation and binding (Breslow and Weis, 1972). However, we have observed that previous nitrated neurophysin II samples

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were contaminated in varying degree with neurophysin in which the single tyrosine was dinitrated. Because the ionization of 3,5-dinitrotyrosine near pH 3 might potentially confuse the interpretation of other changes that occur in this pH region, we also report here purification procedures used to resolve the mono- and dinitrated neurophysins, and some properties of the dinitrated protein; to our knowledge, this is the first study of a dinitrated tyrosine residue in a protein.

Materials and Methods

Protein Preparation. Bovine neurophysins I and II were prepared as previously described (Breslow et al., 1971). Nitration of the single neurophysin tyrosine was accomplished using the method of Furth and Hope (1970). The properties of nitrated neurophysin II so prepared were generally those previously described (Breslow and Weis, 1972); on amino acid analysis, more than 70% of the tyrosine was recovered as 3-nitrotyrosine and no unmodified tyrosine was detected. However, the visible absorption spectra of occasional preparations, in addition to the single 352-nm peak previously seen below pH 6, showed a minor peak at 449 nm characteristic of an ionized nitrotyrosine side chain; this suggested that some fraction of the tyrosine ionized with a pK_a considerably below the value of 7.45 previously observed for the mononitrated neurophysin II tyrosine and that it might be dinitrated. Accordingly, preparations of nitrated neurophysin II were routinely fractionated on a 3 × 36 cm column of DEAE[1]-Sephadex A-50 in pH 5.9 pyridine–acetate buffer (0.823 M pyridine–0.158 M acetic acid), monitored by Folin analysis and by absorption at 350 and 440 nm. An asymmetric peak (I), which generally accounted for more than 90% of the recovered protein, was eluted between 250 and 350 mL and showed a visible absorption maximum at 360 nm at pH 5.9, characteristic of 3-nitrotyrosine; amino acid analysis confirmed that approximately 90% of tyrosine in this component was present as 3-nitrotyrosine and that no 3,5-dinitrotyrosine was present. A second very small peak (II), which typically accounted for less than 10% of the recovered protein (but occasionally for 20%), was eluted between 500 and 600 mL and showed a visible absorption maximum at 449 nm at pH 5.9, similar to that of 3,5-dinitrotyrosine; amino acid analysis using 3,5-dinitrotyrosine as a standard confirmed that the tyrosine in this peak was present as 3,5-dinitrotyrosine. Peak I (mononitrated protein) was subdivided into its leading shoulder (IA) which accounted for approximately 30% of the recovered protein and a major component (IB) which contained approximately 60% of the recovered protein. IB behaved as mononitrated protein of greater than 90% purity on gel electrophoresis in a 7.5% gel at a running pH of 9.5, moving slightly ahead of native neurophysin II under the same conditions. IA behaved electrophoretically as approximately 70% nitrated protein and 30% of a component that had the same mobility as the unnitrated protein. All the pooled fractions from pyridine–acetate chromatography were lyophilized and the dinitrated protein was studied without further purification. Both lyophilized fractions containing mononitrated protein (IA and IB) were then individually chromatographed on a 1.6 × 58 cm column of Sephadex G-50 in 0.1 N acetic acid to remove traces of polymeric material and of UV absorbing contaminants from the pyridine–acetate, and relyophilized. [It is relevant that very little polymeric protein was noted on G-50 fractionation of protein that had previously been fractionated on DEAE-Sephadex. However, crude nitrated neurophysin II showed a significant quantity of polymeric material on G-50, in accord with the observations of Wolff et al. (1975). This suggests that the DEAE-Sephadex fractionation alone removes most of the polymeric species.] Studies of mononitrated neurophysin II were generally performed using the purer fraction of the mononitrated protein (IB), although control studies indicated only trivial differences in the spectroscopically derived binding constants between the two fractions.

Nitrated neurophysin I was also prepared according to the method of Furth and Hope (1970). Spectrophotometric studies of the few nitrated neurophysin I preparations made did not reveal any significant quantities of dinitrated neurophysin I so this protein was used without additional purification.

Circular Dichroism Studies. These were performed on a Cary 60 spectropolarimeter equipped with a Model 6001 circular dichroism attachment. Except as noted, studies were performed at 24 ± 1 °C. For studies at 13 and 37 °C, water was circulated around a temperature-controlled sample cell holder from a Lauda K_4/RD temperature-controlled bath; the temperature within the sample cell at temperature equilibrium was determined prior to the CD scan using a small-bulbed thermometer inserted into the sample. For studies at concentrations near 2 mg/mL protein, a 1-cm light path was used. For studies at 0.4 and 0.14–0.2 mg/mL protein, 5- and 10-cm light paths were used, respectively. No significant differences in the spectra obtained with the different light paths were noted.

Binding isotherms for the interaction of hormones and peptides with the mononitrated protein were determined by monitoring the CD spectrum of the protein between 500 and 290 nm as a function of hormone or peptide concentration; the intensity of the 350-nm neurophysin nitrotyrosine band has previously been shown to be a sensitive indicator of binding to the hormone-binding site (Breslow et al., 1973). To a solution of known volume and protein concentration, measured quantities of peptide or hormone were sequentially added either as solid or as small aliquots of a solution of known concentration; the pH was adjusted with HCl or NaOH after each addition and the CD spectrum determined. Transfers of solution between cuvette and titration vessel were performed so as to minimize volume loss and evaporation; the volume of the solution was checked at the end of the binding study to ascertain that significant evaporation or loss of solution had not occurred. For each binding study, ellipticity values characteristic of the unliganded protein, $[\theta]_{protein}$, were determined on the same protein sample at the beginning of the study and of the fully liganded protein, $[\theta]_{complex}$, from the saturated protein at the end of the study. For those peptide-binding studies at pH 2, in which the 10-cm light path cuvette was used, the quantity of peptide necessary to achieve saturation was very large and, for these studies, the ellipticity characteristic of the fully liganded protein was determined at pH 3 (where binding is stronger) at the end of the study. It is relevant that ellipticity values characteristic of complete saturation in general appeared 10% greater at and below pH 3 than at pH 6. Calculations used in analysis of the binding data are those previously described (Breslow et al., 1973), but are repeated here for convenient reference. The number of moles of peptide bound per 10 000 g of protein ($\bar{\nu}$) was calculated from the relationship

$$\bar{\nu} = \frac{[\theta]_{350nm}\,(\text{obsd}) - [\theta]_{350nm}\,(\text{protein})}{[\theta]_{350nm}\,(\text{complex}) - [\theta]_{350nm}\,(\text{protein})} \quad (1)$$

The unbound peptide concentration was determined by cor-

[1] Abbreviations used: DEAE, diethylaminoethyl; CD, circular dichroism; Phe-Tyr-NH_2, L-phenylalanyl-L-tyrosine amide; UV, ultraviolet; NMR, nuclear magnetic resonance.

recting the total peptide concentration for that removed by binding according to the relation:

$$[\text{peptide}]_{\text{unbound}} = [\text{peptide}]_{\text{total}} - \bar{\nu}\,[\text{protein}]_{\text{total}} \quad (2)$$

Protein molar concentrations were calculated using the monomer molecular weight of 10 000 and were determined from the weight of the lyophilized powder corrected for a water content of 7%. As before, binding of 1 mol of hormone or peptide per neurophysin II monomer was assumed responsible for the nitrotyrosine ellipticity change; equilibrium dialysis studies (Wolff et al., 1975) and gel-filtration binding data (M. Oldewurtel and E. Breslow, unpublished observations) confirm the presence of one peptide-binding site per nitrated neurophysin monomer. Binding studies with the dinitrated protein were conducted as with the mononitrated protein, but CD changes were monitored at 440 nm. For binding studies with both the mononitrated and dinitrated proteins, under the experimental conditions used, the 1-millidegree reproducibility of the spectrometer specified by the manufacturer places an absolute uncertainty of ±0.025 in each calculated value of $\bar{\nu}$.

For determination of the effect of pH on peptide-binding constants, complete binding isotherms for mononitrated neurophysin II were determined in 0.16 M KCl at pH 6.2, 3.0, 2.0, 1.5, and 1.0. Control studies indicated that the slightly higher ionic strength per se at low pH was without significant effect on binding. Additionally, binding constants relative to those at pH 6.2 were determined at selected pH values using single protein solutions partially saturated with peptide at pH 6.2 and monitoring the change in CD as the pH was progressively lowered. Binding constants were calculated as above from these data, taking into account the CD changes of the fully liganded and unliganded protein with change in pH. Good agreement was obtained between relative binding constants calculated in this way and those obtained from complete binding isotherms when the same pH was studied by both techniques. Relative binding constants in the pH region 3.5–5 could only be determined using very dilute protein solutions (1.4×10^{-5} M) because the peptide complexes become less soluble in this pH region.

Peptides, Hormones, and Amino Acids. Oxytocin was a gift from Dr. Jan Mulder of Ferring Laboratories, Sweden. Deaminooxytocin was a gift from Dr. Victor Hruby. All peptides were purchased from either Cyclo Chemical Corp. or Vega-Fox Chemicals, Tucson, Ariz., and were checked for purity by amino acid analysis, UV absorption studies, and thin-layer chromatography. 3-Nitrotyrosine was purchased from Cyclo Chemicals and 3,5-dinitrotyrosine was obtained from ICN Pharmaceuticals, Inc.

General Methods. Amino acid analyses were performed according to the method of Spackman et al. (1958) using a Durrum-500 amino acid analyzer. Gel electrophoresis was performed as previously described (Breslow et al., 1971). Deionized water was used for all studies.

Results

Properties of Mononitrated and Dinitrated Neurophysin II. The general properties of the purest mononitrated neurophysin II fractions are those previously reported for unfractionated nitrated neurophysin II (Breslow and Weis, 1972) except that the intensities of the NO_2–tyrosine absorption and ellipticity bands are approximately 15% higher in the fractionated material and the 352-nm band of the unfractionated protein at pH 6 shifts to 360 nm after purification, a wavelength virtually identical with the corresponding absorption maximum of 3-nitrotyrosine. It is relevant that Wolff et al. (1975) reported a 352-nm absorption maximum for nitrated neurophysin II purified by procedures that probably did not remove dinitrated protein.

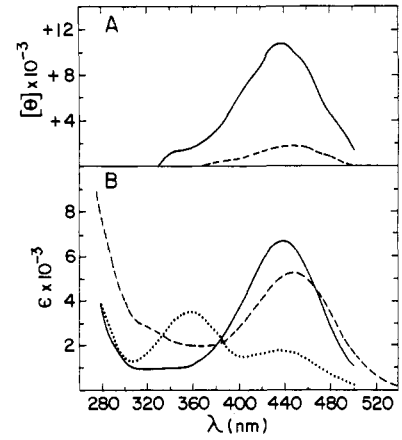

FIGURE 1: Absorption and circular dichroism spectra of dinitrated neurophysin II. (A) Visible CD spectrum of dinitrated neurophysin II at pH 6.2 in the absence (- - -) and in the presence (—) of 10^{-3} M Phe-Tyr-NH_2. Ellipticity values (deg cm^2 dmol^{-1}) are calculated per mol of neurophysin II. (B) Absorption spectra of dinitrated neurophysin II at pH 6.2 (- - -) and of 3,5-dinitrotyrosine at pH 6.2 (—) and pH 3 (· · · ·).

The visible and near-UV absorption and CD spectra of dinitrated neurophysin II at pH 6.2 are shown in Figure 1 and compared with the absorption spectra of 3,5-dinitrotyrosine at pH 3 and 6.2. The dinitrophenol group of 3,5-dinitrotyrosine ionizes with an apparent pK of 3.3; the longest wavelength absorption band of the protonated dinitrophenol occurs at 359 nm and that of the deprotonated dinitrophenol at 440 nm. The longest wavelength band of dinitrated neurophysin II occurs at 449 nm at pH 6.2 and can be shown to diminish in intensity and shift to shorter wavelengths with an apparent pK of 3.2. The 449-nm location of the nitrotyrosine absorption band in dinitrated neurophysin II at pH 6.2 is paralleled by appearance of a weak 449-nm-centered CD band at this pH. On addition of peptides that bind to the hormone-binding site of neurophysin II, the 449-nm CD band shifts to 440 nm and is markedly intensified (Figure 1). The shift to shorter wavelengths on binding can also be observed by absorption spectroscopy. These results indicate that dinitration of the neurophysin tyrosine does not prevent binding of peptide and parallel the observation of Wolff et al. (1975) that the long wavelength absorption band of the mononitrated protein is shifted to shorter wavelengths on hormone-binding. Peptide-binding studies at pH 6.2 (see Materials and Methods) indicate that the dinitrated protein binds with an affinity one-half that of the mononitrated protein at the same pH; however, because the tyrosine of the mononitrated protein is protonated at pH 6.2 and because tyrosine ionization leads to a fourfold increase in binding affinity (Breslow et al., 1973), the intrinsic affinity of the dinitrated protein for peptides is probably only one-eighth that of the mononitrated protein.

Effect of Low pH on Neurophysin and Mononitrated Neurophysin. Lowering the pH of solutions of neurophysin from 6.2 to 3 produces subtle but reproducible and reversible changes in both near- and far-UV ellipticity spectra. For neurophysin II (Figure 2) negative ellipticity increases between 330 and 290 nm, while ellipticity becomes more positive between 290 and 240 nm as well as in the far-UV. Interestingly, these effects show a marked similarity to CD changes observed on binding peptides to neurophysin II that contain a phenyl-

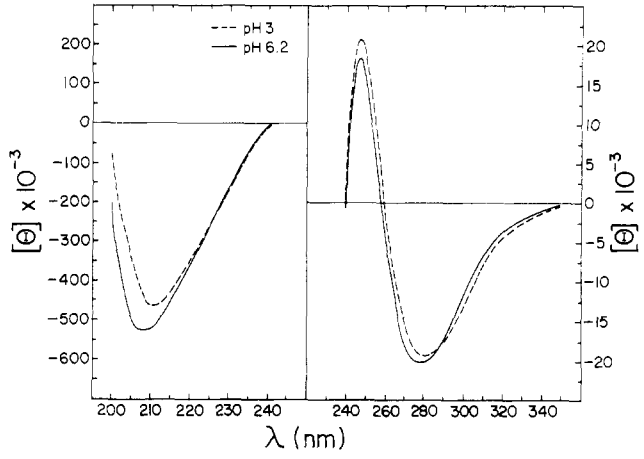

FIGURE 2: Effect of pH on the circular dichroism spectrum of neurophysin II in 0.16 M KCl. Ellipticity values are calculated per mol of neurophysin II. (—) pH 6.2; (- - -) pH 3.

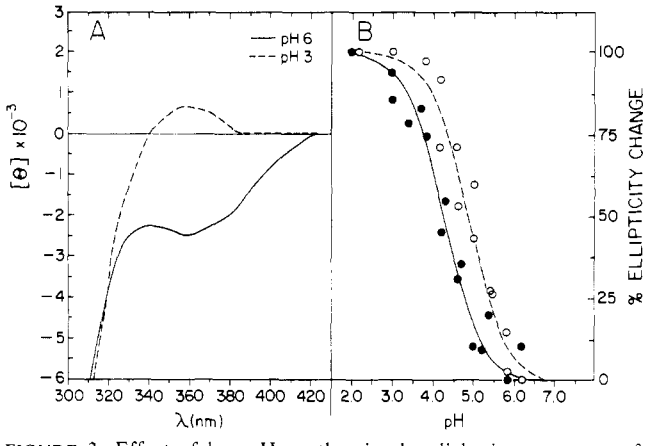

FIGURE 3: Effect of low pH on the circular dichroism spectrum of mononitrated neurophysin. (A) CD spectra of mononitrated neurophysin II at pH 6 and pH 3. Ellipticity values are expressed per mol of neurophysin II. (B) Percentage of the total ellipticity change as a function of pH for 1.4×10^{-5} M mononitrated neurophysin II (●) and 1.4×10^{-5} M mononitrated neurophysin I (○). (—) Calculated CD titration curve for a single acid with $pK_a = 4.25$; (- - -) calculated CD titration for a single acid with $pK_a = 4.85$.

alanine residue in position 2 (Breslow and Weis, 1972) and which, therefore, in the near-UV, reflect principally binding-induced changes in the protein; in fact, changes above 270 nm appear identical in the two situations, although the precision of measurement of such small CD perturbations must be recognized as low.

The pH of the midpoint of the change in CD spectrum can only be approximated using the native protein because the CD changes are small. However, the CD change is more marked in mononitrated neurophysin II which exhibits a large change with pH of the 360-nm nitrotyrosine ellipticity band (Figure 3A). Determination of the extent of the CD change in mononitrated neurophysin II as a function of pH at a protein concentration of 1.4×10^{-5} M indicates that the change approximates the titration of a group with an apparent pK_a of 4.25 (Figure 3B); the midpoint of the transition appears to be concentration dependent and to shift to approximately pH 4.8 at a protein concentration of 2×10^{-4} M.[2] The CD spectra of neurophysin I and nitrated neurophysin I also change on lowering the pH; at a concentration of 1.4×10^{-5} M the nitrated neurophysin I ellipticity change occurs with an apparent pK_a of 4.85 (Figure 3B). Differences between the two proteins in apparent pK_a are judged significant since the standard deviation of the individual data points from the average pK_a (considering data points between 20 and 80% ellipticity change) is 0.14 pH unit for nitrated neurophysin II and 0.18 pH unit for nitrated neurophysin I. As with neurophysin II, the pK_a of the neurophysin I transition increases with increasing protein concentration.

It is relevant to note that the CD changes accompanying the low pH transition suggest that more than one chromophore is involved. Thus, effects on nitrotyrosine clearly indicate that the tyrosine environment is altered and, by arguments previously advanced for CD changes attending peptide binding (Breslow and Weis, 1972), those occurring above 300 nm and near 250 nm in the unmodified protein probably reflect alterations in disulfide transitions. Similarly, far-UV changes in the unmodified protein appear to be marked at wavelengths not typically (Wetlaufer, 1962) associated with electronic transitions of tyrosine and probably reflect changes in chromophores other than tyrosine.

Qualitative Evidence Supporting pH-Independent Binding of Hormones and Peptides to Neurophysin below pH 2. The affinity of neurophysin for posterior pituitary hormones (Ginsburg and Ireland, 1964; Camier et al., 1973) and for small peptide analogues of the hormones (vide infra) decreases sharply below pH 5, the change in binding constant with pH suggesting that the effect is due to titration of a side-chain carboxyl. That the decrease in binding affinity of oxytocin and small peptides for neurophysin II and nitrated neurophysin II does not continue significantly below pH 2 is indicated by the following studies. Figure 4 shows the near-UV ellipticity spectra of neurophysin II in the presence of oxytocin and deaminooxytocin. When neurophysin II is mixed with 1.4 equiv of deaminooxytocin at pH 1.5, the observed CD spectrum is clearly the sum of the two constituents, suggesting no significant interaction between the two, in keeping with the low affinity of deaminooxytocin for neurophysin. However, on mixing the same concentration of neurophysin II with 1 equiv of oxytocin at pH 1.5, changes in CD spectra occur that are indicative of binding; these changes are unaffected by lowering the pH to 0.6, indicating no decrease in binding in this pH interval, but are magnified by raising the pH to 6.2, in keeping with the increased binding at higher pH. The same pattern of CD spectra are obtained if the samples are mixed at pH 6.2 and the pH successively lowered; comparison with the deaminooxytocin data indicates that low pH results are not optical artifacts. These effects are seen more clearly using mononitrated neurophysin II (Figure 5). Figure 5A shows the nitrotyrosine 360-nm CD band of mononitrated neurophysin II in the presence of oxytocin. At pH 3, the intensity of the CD band indicates approximately 90% saturation of the hormone-binding site under the conditions used (see Materials and Methods). On lowering the pH to 2, binding is reduced to 30% of saturation; no significant further reduction in ellipticity occurs on lowering the pH to 1. Figure 5B shows the CD spectrum near 360 nm of mononitrated neurophysin II in the

[2] The concentration dependence of the CD transition is puzzling inasmuch as it suggests that the neurophysin dimer has a higher proton affinity than the monomer and, by the usual principles of linked equilibria, that dimerization should be more favored at low pH than at pH 6. However, as indicated in the text, comparison of neurophysin properties at pH 6 and at pH 2 shows no such trend, although sedimentation equilibrium studies as a function of pH have yet to be performed. It is possible that below pH 3 there is a second phase of proton uptake, which is not reflected in the CD spectrum, and which is more favored in the monomer than in the dimer, tending to cancel the effects on dimerization of the CD-active transition.

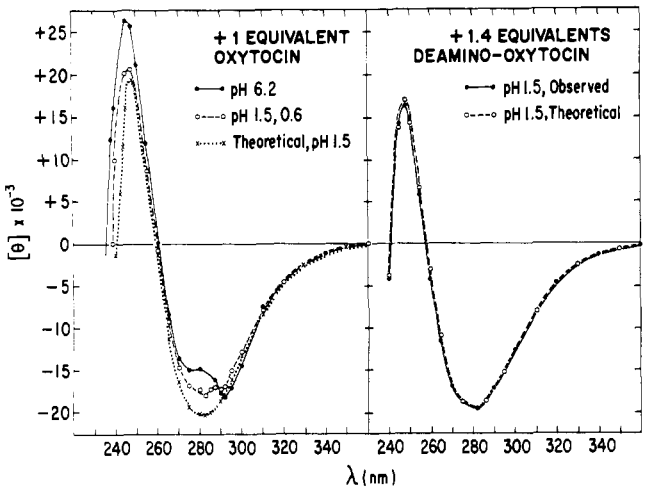

FIGURE 4: Circular dichroism spectra of neurophysin II in the presence of oxytocin and deaminooxytocin. Conditions: 2×10^{-4} M neurophysin II in 0.16 M KCl. Ellipticity values are expressed per mol of neurophysin II. Theoretical spectra shown were calculated as the summed spectra of the individual components at pH 1.5; other spectra are those *observed* for the mixtures at the pH values indicated.

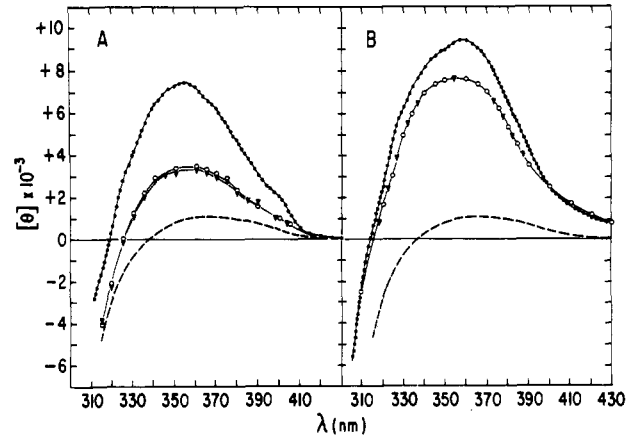

FIGURE 5: Circular dichroism of mononitrated neurophysin II in the presence of oxytocin and Phe-Tyr-NH_2 at low pH. Ellipticity values are calculated per mol of neurophysin II. (- - -) Protein alone, pH 1-3; (●) + peptide (or hormone), pH 3; (○) + peptide (or hormone), pH 2; (▼) + peptide (or hormone), pH 1. (A) Protein, 4×10^{-5} M, in the absence and presence of 2.8×10^{-4} M oxytocin in 0.16 M KCl; (B) 1.8×10^{-4} M protein in the absence and presence of 4.5×10^{-2} M Phe-Tyr-NH_2 in 0.16 M KCl.

presence of Phe-Tyr-NH_2, a peptide known to bind to the hormone-binding site (Breslow et al., 1973). The spectrum at pH 3 is characteristic of complete saturation of the hormone-binding site; lowering the pH to 2 reduces the degree of saturation to 80% and no further reduction in ellipticity occurs on lowering the pH further to 1. The fact that the ellipticity is constant between pH 2 and 1 irrespective of the extent to which the protein is saturated with ligand at pH 2 (which can be demonstrated over a wide degree of protein saturation) indicates that the constancy of ellipticity between pH 2 and 1 is not the result of an increase in the ellipticity of the fully saturated species below pH 2 compensating for a decrease in the fraction of protein that is bound. Moreover, we have observed no changes in the CD spectrum of the unliganded or of the completely saturated protein between pH 2 and pH 1. Additionally, it is relevant that CD spectra are time-independent and protein-peptide complexes exposed to pH 1 can be dissociated by gel filtration or electrophoresis to give protein indistinguishable from native protein, indicating that no covalent changes are responsible for the low pH phenomena. Therefore, the data indicate in the most general sense that, irrespective of the nature of the change in neurophysin which leads to a decrease in binding below pH 5, the fully altered protein retains a lesser but finite affinity for peptides and for oxytocin.[3]

Effect of pH on Peptide-Binding Isotherms. In order to determine the difference between the free energy of peptide binding at neutral pH and that in the low pH region where binding appears to become pH independent, isotherms for binding of peptides to mononitrated neurophysin II were obtained at two different protein concentrations at pH 6.2 and 2 and at a single protein concentration at other selected pH values. Phe-Tyr-NH_2 was the peptide chosen for principal study, because its binding constant is sufficiently great to allow high levels of protein saturation to be achieved without exceeding the solubility limit of the peptide; this is particularly important at low pH values where binding affinities are much lower than at neutrality. Selected studies with oxytocin were also carried out, but the lesser availability of oxytocin than of the smaller peptides necessitated that the number of these studies be kept to a minimum. The choice of pH 6.2 as the pH for comparison with low pH data was dictated by the fact that NP complexes become less soluble as the pH is lowered below 6 and that binding is affected at higher pH by deprotonation of both the α-NH_2 of the peptide and the nitrotyrosine of the protein; at pH 6.2, only trivial corrections are needed to convert apparent binding constants for Phe-Tyr-NH_2 to intrinsic constants that describe the affinity for the protonated peptide (Breslow et al., 1973). pH 2 was the low pH selected for the most detailed investigation because of the initial observation that binding is pH independent below pH 2 and because of the possibility that exposure of the protein to lower pH for the length of time needed for a complete binding study might be destructive.

Figure 6 shows the binding of Phe-Tyr-NH_2 to mononitrated neurophysin II at protein concentrations of 1.8 and 0.18 mg/mL and pH 6.2 plotted according to the method of Scatchard and Black (1949), where $\bar{\nu}$ is the number of moles of peptide bound per 10 000 g of protein (monomer molecular weight). Binding is clearly dependent on protein concentration, a fact also observed recently (Nicolas et al., 1976) for the binding of oxytocin to the unnitrated protein within a similar protein concentration range. Additionally, despite a large degree of scatter at low values of $\bar{\nu}$, average binding isotherms at pH 6.2 are nonlinear, showing evidence of a low degree of positive cooperativity virtually identical with that seen for binding of oxytocin to the unnitrated protein in the same pH region (Hope et al., 1975; Nicolas et al., 1976). The scatter at low values of $\bar{\nu}$ at the higher protein concentration arises at least in part from the uncertainty in measurement of small ellipticity changes (see Materials and Methods) which is magnified in the case of strongly binding peptides such as Phe-Tyr-NH_2 because it generates uncertainty in values of both $\bar{\nu}$ and free peptide concentration; i.e., at the higher protein concentration, a very large fraction of the total Phe-Tyr-NH_2

[3] Strictly speaking, it is possible that the apparent plateau in binding constant below pH 2 results from two compensatory phenomena: (A) a continued decline in binding affinity due to the continued decrease in concentration of the neutral pH species below pH 2 and (B) an increase in concentration of a new binding species as the pH is lowered. The titration properties of bovine neurophysin II appear normal (Breslow et al., 1971); it is therefore unlikely that any group titrates below pH 2 and it is also difficult to conceptualize what type of protonation would lead to an increased binding affinity. We consider this possibility unlikely.

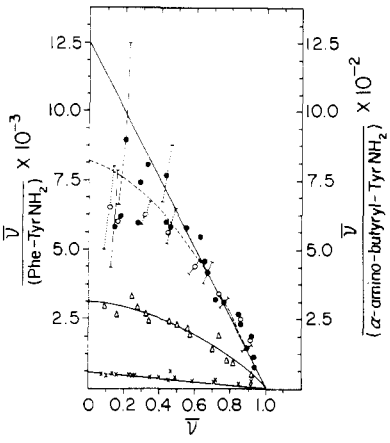

FIGURE 6: Binding of peptides to mononitrated neurophysin II at pH 6.2 and pH 3. (●) Binding of Phe-Tyr-NH_2 to 1.8×10^{-4} M protein, pH 6.2; data are results of three studies with three different protein preparations. The solid line through these data is an approximate straight line fit ignoring data at low values of $\bar{\nu}$; the dashed line is a theoretical curve corresponding to weak cooperativity as described in the text. (○) Binding of α-amino-butyryl-Tyr-NH_2, pH 6.2 at 1.8×10^{-4} M protein. (△) Binding of Phe-Tyr-NH_2 to 1.8×10^{-5} M protein, pH 6.2. (×) Binding of Phe-Tyr-NH_2 to 1.8×10^{-4} M protein, pH 3. Bars through selected data points show the estimated uncertainty in each point due solely to the estimated error (see Materials and Methods) in spectrometer readings; solid bars are for Phe-Tyr-NH_2 and dashed bars are for α-aminobutyryl-Tyr-NH_2. Lines through the data points at the lower protein concentration and at pH 3 serve only to delineate the data.

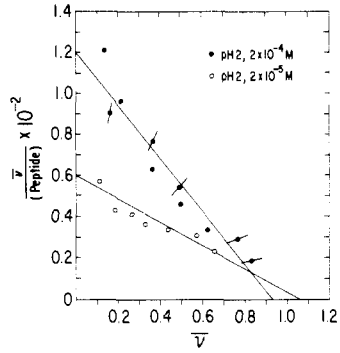

FIGURE 7: Representative binding isotherms for Phe-Tyr-NH_2 to mononitrated neurophysin II at pH 2 at two protein concentrations. Note that protein concentrations listed in the figure are approximate; exact protein concentrations are 1.8×10^{-4} M (●) and 1.8×10^{-5} M (○). Data at the higher protein concentration represent two studies; in one of these studies, spectrometer tracings were noisy and the average of results at both 350 and 360 nm are plotted as single points with error bars representing the limits of the difference in results at the two wavelengths. Lines through the data at both protein concentrations represent the least-squares fit.

added is bound to the protein, making estimates of free peptide concentration potentially unreliable. Accordingly, we also studied the binding of a more weakly binding peptide, L-α-amino-butyryl-L-tyrosine amide, under the same conditions. This peptide binds with exactly one-tenth of the affinity of Phe-Tyr-NH_2 and the fraction of peptide bound is low over the course of the binding isotherm. The binding isotherm for this peptide is also shown in Figure 6 and has the same shape as that for Phe-Tyr-NH_2.

Native neurophysin II dimerizes with a dimerization constant (K_D) of 5×10^3 M^{-1} (Breslow et al., 1971). Nicolas et al. (1976) accounted for the dependence of binding on protein concentration in the range 5×10^{-6} to 3×10^{-4} M by assuming a fivefold greater affinity of oxytocin for each dimer site than for the corresponding monomer site; however, this assumption by itself accounted for only a small fraction of the observed cooperativity in the binding isotherms. Hope et al. (1975) attributed the observed cooperativity at concentrations of 1×10^{-4} M solely to cooperative interactions between the two sites on the dimer such that occupancy of the first site in the dimer doubled the affinity for the second hormone molecule bound; good agreement between theory and experiment was obtained with this assumption. Data for the nitrated protein shown here support the conclusion that binding by the dimer is significantly stronger than that by the monomer since binding increases with increasing protein concentration; preliminary analysis of the magnitude of the concentration dependence indicates that the affinity of the dimer is at least fivefold that of the monomer. Additionally, the data can be shown to support the presence of weak cooperative interactions within the dimer at pH 6.2. Although the dimerization constant for nitrated neurophysin II has not yet been precisely determined, sedimentation velocity data previously published for unfractionated nitrated neurophysin II (Breslow et al., 1973) and recently confirmed with the more purified nitrated protein used here (P. Gargiulo and E. Breslow, unpublished observations) indicate that the dimerization constant of the nitrated protein is at least as high as that of the native protein. Thus, at concentrations of 2×10^{-4} M, at least 50% by weight of the unliganded protein is in the dimer form and, under these conditions, the amount of positive cooperativity introduced into a Scatchard plot by preferential binding to dimer can be shown to be relatively small. For example, the reader is referred to the theoretical Scatchard curves of Nicolas et al. (1976) which were calculated assuming a fivefold greater hormone affinity for the dimer than for the monomer; similar calculations in our hands assuming that binding occurred exclusively to dimer also did not generate a curve of analogous shape to that in Figure 6. On the other hand, a theoretical curve is plotted in Figure 6 calculated using the intradimer cooperativity model of Hope et al. (1975), ignoring small potential contributions of binding to monomer and similarly assuming only that binding to the first site on a dimer doubles the affinity for the second site; this curve fits the data at 2×10^{-4} M protein well within the experimental uncertainties. Note that an identical theoretical curve can be generated using the Adair equation (Adair, 1925) and assuming that $K_1/K_2 = 2$ instead of the value of 4 statistically predicted for two equivalent independent sites. In either event, at 2×10^{-4} M protein, the binding affinities for Phe-Tyr-NH_2 at pH 6.2 are calculated to be 8×10^3 M^{-1} and 1.6×10^4 M^{-1} to the first and second dimer sites, respectively. To the extent that the unliganded protein is not 100% in the dimer form at this concentration, these values are both somewhat lower than the true dimer affinities.

Also shown in Figure 6 are the binding data at pH 3 at the higher protein concentration. Binding at pH 3 is markedly weaker than at pH 6.2, indicating that the decline in binding constant for the hormones within this pH region is also seen with small peptides that bind to the hormone-binding site, in keeping with the previously demonstrated displacement of protons by such peptides on binding to neurophysin II at pH 4 (Breslow et al., 1971) and their general behavior as hormone analogues in their interactions with neurophysin (Breslow et al., 1973). Although not particularly evident because of the scale of the presentation of Figure 6, binding isotherms at pH 3 are relatively linear. The change in isotherm shape between pH 6.2 and 3 prevents a simple overall comparison of binding at the two pH values, but if the average slopes between 20 and 80% saturation are compared in both instances, the binding affinity at pH 3 is approximately 1/20 that at pH 6.2.

Figure 7 shows the binding data for Phe-Tyr-NH_2 at pH 2.

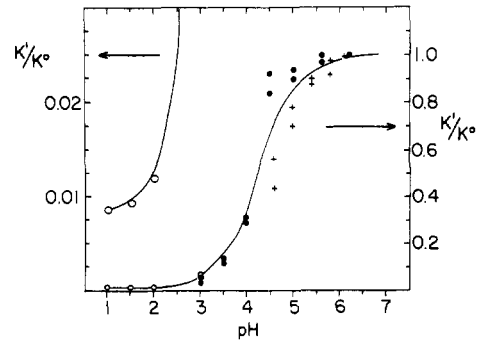

FIGURE 8: Effect of pH on the binding of Phe-Tyr-NH_2 plotted as the ratio of the apparent binding constant at any pH (K') to the binding constant at pH 6.2 ($K°$). At the lower protein concentration, $K'/K°$ was determined as described in the text. At the higher protein concentration, relative binding constants were determined using the average binding constant at pH 6.2, 1.2×10^4 M^{-1}, which is equivalent to the constant calculated from a straight-line fit of the binding isotherm at pH 6.2 using values of $\bar{\nu}$ between 0.2 and 0.8. (●) Binding to mononitrated neurophysin II at 1.4×10^{-5} M protein; (○) binding to mononitrated neurophysin II at 1.8×10^{-4} M protein. (+) Binding to 1.4×10^{-5} M mononitrated neurophysin I. (—) Theoretical curve described by eq 4.

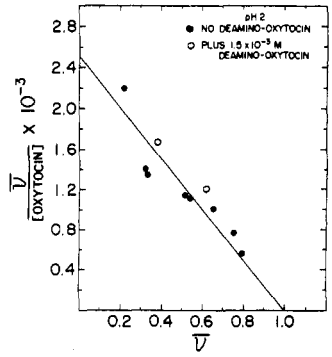

FIGURE 9: Binding of oxytocin to mononitrated neurophysin II at pH 2 in the absence and presence of deaminooxytocin (1.5×10^{-3} M). Protein concentration = 1.8×10^{-4} M.

At pH 2, as at pH 3, binding isotherms appear linear; however, the dependence of binding on protein concentration is only slightly less at pH 2 than at pH 6.2. The binding constant at pH 2 is approximately 1/5 the value at pH 3 and 1/100 the value at pH 6.2 if average slopes of the binding isotherms between 20% and 80% saturation are used for comparison. To determine whether binding constants below pH 2 were completely pH independent, as suggested by studies above, complete binding isotherms were also determined at pH 1 and 1.5 and compared, at the higher protein concentration, with the pH 2 isotherm obtained with the same stock protein preparation. The calculated binding constants at pH 1.5 and 1.0 were 78 and 70%, respectively, of the value at pH 2.0. These data therefore indicate a small decrease in binding affinity below pH 2, but the binding constant at pH 1 remains markedly greater than would be predicted (vide infra) for complete dependence of binding on the high pH species and appears to define a lower affinity limit.

The pH dependence of Phe-Tyr-NH_2 binding to mononitrated neurophysin II is shown in Figure 8. The data presented are those described above as well as data at several intermediate pH values obtained by following the effect of pH on the CD spectra of partially saturated mixtures of mononitrated neurophysin II (1.4×10^{-5} M) and peptide (see Materials and Methods). Note that precipitation of the peptide-protein complex between pH 4.5 and 4 prevents any data from being obtained in this pH region even at the lower protein concentration. Nonetheless, with the exception of some hypersharpening above pH 4.5, the data at 1.4×10^{-5} M protein approximate that of the titration of a single side-chain carboxyl group with an apparent pK_a of 4.25, thus resembling the pH dependence of the CD spectrum of the unliganded protein (vide supra). Data at the higher protein concentration could not be obtained over a sufficient pH range to determine whether there is a small concentration dependence of the pH dependence of binding.

If binding were completely dependent on the ionization of a group with $pK_a = 4.25$, the pH dependence of binding would be described by:

$$K' = \frac{K°}{1 + [H^+][10^{4.25}]} \qquad (3)$$

where $K°$ is the intrinsic affinity for the ionized species and K' is the apparent affinity. This relationship predicts that binding constants at pH 1 and 1.5 should be 10 and 32%, respectively, of that at pH 2 as compared with the observed values of 70 and 78%. A similar discrepancy exists between the observed ratio of the binding constants at pH 2 and pH 3 and that predicted from eq 3. The titration behavior of neurophysin II (Breslow et al., 1971) indicates that these discrepancies cannot be explained by major changes in effective pK_a with change in pH in this pH region. Alternatively, the data are readily explained by the assumption that the protonated species retains a binding affinity 8/1000 that of the unprotonated species. Thus, at any pH, the observed binding affinity (K') is predicted to be the sum of a pH-dependent constant, K'', and a pH-independent constant, K''', where K''' is equal to 8/1000 the maximum value ($K°$) of K'', as shown:

$$K' = K'' + K''' = \frac{K°}{1 + [H^+][10^{4.25}]} + 0.008K° \qquad (4)$$

This relationship fits the data well, as shown in Figure 8.

Because of the resemblance between the effect of pH on binding affinity and on the CD spectrum of the unliganded native protein, and because the effect of pH on unliganded neurophysin I differs from its effect on unliganded neurophysin II, studies of the effect of pH on binding to nitrated neurophysin I were initiated. Unfortunately, neurophysin I-peptide complexes were insufficiently soluble between pH 3.1 and 4.5 to allow any data to be obtained over this relatively broad pH region. However, the limited data that were obtained (Figure 8) suggest that the pK of the carboxyl on which binding to nitrated neurophysin I is dependent is higher than that in neurophysin II. Camier et al. (1973) have observed a similar difference in the pH-dependence of oxytocin binding to unnitrated neurophysin I and neurophysin II; in fact, the magnitude of the difference they observed (0.6 pH unit) is identical with the difference we observe in the pH dependence of the CD spectra of the unliganded proteins and tentatively suggests, but does not prove, a relationship between the carboxyl(s) responsible for the pH dependence of binding and the carboxyl(s) responsible for the pH dependence of the CD spectrum of the unliganded protein.

Binding Studies with Oxytocin and Deaminooxytocin at Low pH. Figure 9 shows the binding of oxytocin to mononitrated neurophysin II at pH 2. The apparent binding constant for oxytocin at this pH, 2.5×10^3 M^{-1}, is 1/50 to 1/250 of values which can be estimated from existing data (Breslow et al., 1973; Hope et al., 1975; Nicolas et al., 1976) as representative of the intrinsic binding constant to neurophysin at neu-

tral pH of that species of oxytocin in which the α-amino is protonated. Thus, the change in neurophysin affinity for oxytocin upon protonation of the key neurophysin carboxyl is similar to the change in affinity for Phe-Tyr-NH_2, but further studies are needed to ascertain whether the effects of pH on peptide and hormone are identical.

Mixing of deaminooxytocin with unnitrated neurophysin II at low pH leads to no change in CD spectrum (vide supra) and a similar lack of change can be seen at neutral pH. Similarly, we have observed that concentrations of deaminooxytocin as high as 1.5×10^{-3} M lead to no CD change with the nitrated protein at pH 2, although comparable concentrations of oxytocin would be sufficient to nearly saturate the protein at this pH. This is relevant because, if oxytocin were binding largely without a salt bridge at pH 2, the deamino hormone might be expected to bind almost as strongly as oxytocin at this pH, although it binds much more weakly at neutral pH. In order to determine whether deaminooxytocin was binding to the hormone-binding site but not perturbing the CD spectrum (as could be explained if the protonated amino group were the cause of the CD perturbations), the effect of 1.5×10^{-3} M deaminooxytocin on binding of oxytocin at pH 2 was studied using the same stock protein preparation for comparison (Figure 9); no reduction in binding was seen. If we assume that a reduction in oxytocin-binding constant of 20% in the presence of the deamino hormone could have gone undetected (this is an overestimate of the possible error on a single preparation of protein), then, from the usual relationships for a competitive binding situation, a maximum binding constant for the deamino hormone at pH 2 of 1.3×10^2 M^{-1} can be calculated from the data. The results indicate that, even at very low pH, deaminooxytocin binds significantly more weakly than oxytocin to the oxytocin-binding site. A more detailed investigation of the effects of 1-hydroxymercaptopropionic oxytocin (E. Breslow and R. Walter, unpublished data) has similarly indicated that this analogue, which also lacks the α-NH_2, does not effectively compete with Phe-Tyr-NH_2 for the hormone-binding site at low pH.

Preliminary Studies of the Effect of pH on the Enthalpy and Entropy of Peptide Binding. Binding isotherms for Phe-Tyr-NH_2 to nitrated neurophysin II were also determined at 13 and 37.5 °C for comparison with room temperature (24 °C) data. At pH 6.2, extrapolating from the slope of the Scatchard plot data at values of $\bar{\nu} = 0.5$ and correcting for a change in the degree of ionization of Phe-Tyr-NH_2 over this temperature interval, binding constants were 1.8×10^4 and 5.6×10^3 M^{-1} at 13 and 37.5 °C, respectively. At pH 2.0, Scatchard plots were linear and binding constants were respectively 2.4×10^2 and 0.65×10^2 M^{-1} at 13 and 37.5 °C. Van't Hoff plots of the data at the three tempeatures were linear and indicated enthalpies of binding of −8.1 kcal/mol at pH 6.2 and −9.4 kcal/mol at pH 2. Entropies of binding at room temperature were calculated to be −8 eu and −21 eu at pH 6.2 and pH 2, respectively. Binding is therefore an enthalpy-driven process at both pH values.

Discussion

The above results indicate that binding of peptides to the principal hormone-binding site of nitrated neurophysin II below pH 7 is dependent on the ionization of a group with $pK_a = 4.25$ and that the protonated species is still capable of binding but with an affinity approximately 1/100 that of the unprotonated species. Because neurophysin II contains no histidines and its binding is unaffected by loss of either the amino terminus (Breslow et al., 1971) or the α-carboxyl (S. Sur and E. Breslow, unpublished observations), the group in question is clearly a side-chain carboxyl. The above results also indicate that, in the absence of bound hormone, protonation of the same carboxyl that determines binding affinity (or, one or more very similar to it) is accompanied by significant circular dichroism changes which may reflect a change in conformation. Two alternative general explanations can be advanced. The first assumes that the carboxyl whose protonation reduces binding affinity by a factor of 100 is the one with which the protonated α-amino of the hormone forms an ion pair at neutral pH. Binding at low pH is assumed to occur to a species in which the carboxyl is protonated and the ion pair, as such, no longer exists. CD changes in the unliganded protein at low pH are attributed to electronic perturbations or local low energy conformational changes accompanying protonation of the same, or a similar carboxyl. The second explanation assumes that the carboxyl that determines binding affinity is not the carboxyl with which the amino forms an ion pair, but instead represents a group whose protonation changes the conformation of the protein to one which has 1/100 the peptide affinity of the neutral pH conformation. In this instance, the carboxyl that participates in the ion pair is assumed to be an abnormal carboxyl with $pK_a < 1$, to account for the virtual independence of binding between pH 2 and 1. Such a low pK_a could be accounted for by assuming that this carboxyl, in the unliganded protein, participates in an intramolecular ion pair or hydrogen bond and is displaced from this pairing by the hormone or peptide α-NH_3^+ in the liganded state.

Several arguments can be advanced in favor of the greater plausibility of the former explanation, although the second cannot yet be rigorously excluded. First, there is evidence that conformational changes in the unliganded proteins below pH 6 must be small. Analysis of neurophysin II titration data below pH 6 (Breslow et al., 1971) indicates that the titration curve shows no inflections such as would be expected if major changes in conformation, accompanied by changes in electrostatic factors or pK_a values, occurred; additionally, we have observed (E. Breslow, C. J. Menendez-Botet, and P. Gargiulo, unpublished) that the sedimentation velocities of neurophysin II and nitrated neurophysin II, over a range of protein concentration, are unchanged on lowering the pH from 6 to 2. Second, it is noteworthy that the pK_a of 4.25 is well within the range of a normal carboxyl ionization. The apparent proton affinity of a carboxyl whose protonation was linked to a conformational change that reduced peptide affinity by a factor of 100 would be expected to be 100-fold lower than that of a normal carboxyl.

The assumption that the carboxyl with $pK_a = 4.25$ is the ion-pair partner at neutral pH has, as its simplest corollary, the assumption that the low pH complex does not contain the amino–carboxylate salt bridge. Within this model, changes in binding affinity between pH 6 and 1 allow a tentative estimate of the thermodynamic contributions of the salt bridge to formation of the peptide–protein complex. This estimate is necessarily preliminary because it involves additional assumptions as to effects on binding affinity, of factors other than competition between protons and the peptide amino group for the salt-bridge carboxyl, which might contribute to apparent changes in binding affinity between neutral and low pH. Three such factors merit consideration. First, there is a decrease in the degree of cooperativity on going from pH 6 to pH 3 and below. Second, in the absence of binding, CD changes occur between pH 6 and 1; these may reflect low energy conformational alterations in the unliganded protein which in themselves affect binding. Finally, titration data (Breslow et al., 1971)

indicate that the net charge on neurophysin II increases from −2.5 at pH 6 to +10 at pH 1; thus, there will be greater electrostatic repulsion of a positively charged peptide at pH 1 than at pH 6. An argument can be advanced, as follows, that the change in cooperativity between neutral and low pH and the CD changes that occur in the unliganded protein over this same pH interval are related. The intradimer cooperativity seen at pH 6 reflects conversion of the dimer by the first peptide bound from a "low-affinity" to a "high-affinity" state. The loss of such cooperativity at low pH may reflect the inability of the "low-affinity" state to convert to the "high-affinity" state at the lower pH or it may indicate that the unliganded protein is predominantly in the "high-affinity" state at low pH. As indicated above, the CD changes within the protein which accompany peptide binding are similar in many respects to those seen on lowering the pH of the unliganded protein below 6. We therefore propose that the pH-dependent CD changes in the unliganded protein reflect its conversion from a "low-affinity" form at pH 6 to a "high-affinity" form at pH 3. Thus, at 2×10^{-4} M protein, the higher binding constant at pH 6.2 (1.6×10^4 M^{-1}) and the average binding constant at pH 1 (1×10^2 M^{-1}) are both assumed to reflect binding to the same "high-affinity" form; the difference in ΔG represented by these two values (−3 kcal) need be corrected solely for the increased electrostatic repulsion of the peptide at pH 1 to obtain the free energy of salt-bridge formation. It is useful to point out that the −3 kcal value of ΔG is relatively independent of the assumption of cooperativity at pH 6.2; i.e., the value of ΔG obtained from the difference between the *average* free energy of binding at pH 6.2 (as calculated from a straight line fit of the data between 20 and 80% saturation) and that at pH 1 is −2.85 kcal. Similarly, despite the fact that binding constants at both pH values are uncorrected for the percentage of monomer in the unliganded protein, the fact that the sedimentation velocity of the unliganded protein (vide supra) and the concentration dependence of binding appear similar at both neutral and low pH suggests that the difference between the two binding constants does not contain significant contributions from potential pH-dependent differences in monomer ⇌ dimer equilibria in the unliganded or liganded states. However, because the monomer contributes relatively little to binding, the value of ΔG is more certainly correct for the dimer than for the monomer.

A crude estimate of the electrostatic repulsion term (ΔG_{elect}) can be obtained, by analogy to calculations of protein charge effects on pK_a values, using the electrostatic interaction factor (ω) and the change in neurophysin II net charge between pH 6.2 and pH 1 ($\Delta\bar{z}$) derived from analysis of neurophysin II carboxyl titration behavior (Breslow et al., 1971); here, $\Delta G_{elect} = -RT \ln 0.868(\omega)(\Delta\bar{z})$, $\omega = 0.077$, and $\Delta\bar{z} = +12.5$. The value of ΔG_{elect} calculated by this approximation (1.2 kcal) is undoubtedly an overestimate since the value of ω derived for neurophysin carboxyls was uncorrected for differences between carboxyls in *intrinsic* proton affinity and accordingly overestimates the effects of charge on any single group. The single tyrosine of neurophysin is thought to be near the binding site (Balaram et al., 1972). A value for ω of 0.038 for this tyrosine can be calculated from comparison of tyrosine titration in nitrated neurophysins I and II (S. Lundt and E. Breslow, unpublished observations) if it is assumed that the 0.1 pH unit difference in pK_a between the two proteins solely reflects the difference of 3 in net charge. Using this value of ω to describe general electrostatic effects at the binding site, the calculated value of ΔG_{elect} is reduced to 0.6 kcal and the free energy of salt-bridge formation is estimated as −2.4 kcal per mol. Additionally, if the small difference in the enthalpy of binding at pH 6.2 and pH 2 is taken to reflect solely the different contribution of the salt-bridged species to the stability of the complex at the two pH values (from eq 4 this is estimated as 99 and 41% at pH 6.2 and pH 2, respectively) the approximate enthalpy and entropy of salt-bridge formation at 25 °C are estimated as +2 kcal and +22 eu per mol, respectively. The value of −2.4 kcal for the contribution of the salt bridge to the free energy of neurophysin-peptide interaction can be compared with the value of −2.9 kcal calculated (Fersht, 1971) for the free energy contribution of the amino-carboxylate salt bridge of δ-chymotrypsin to the stability of its active conformation. The positive enthalpy and entropy terms calculated for salt-bridge formation in neurophysin suggest that the large negative enthalpy term associated with the overall peptide-binding reaction derives from interactions other than salt-bridge formation.

The assumption that binding at low pH does not involve a salt bridge has additional implications when considered in the light of the deaminooxytocin data. The maximum binding constant for this analogue at pH 2 is 1.3×10^2 M^{-1} compared with a value of 2.5×10^3 M^{-1} for oxytocin under the same conditions. If oxytocin binding occurs principally without ion-pair formation under these conditions, why is binding of deaminooxytocin so much weaker? One factor to be considered is the relative conformations of oxytocin and deaminooxytocin at low pH. Arguments have been presented elsewhere (Breslow, 1975) to support the concept that the conformation of oxytocin contributes to the strength of its interaction with neurophysin. While the relative conformations of oxytocin and deaminooxytocin are incompletely understood, these two differ significantly in their CD properties and this difference is markedly enhanced in the pH region where the α-NH_2 of oxytocin is protonated (Beychok and Breslow, 1968; Urry et al., 1968). Additionally, Deslauriers et al. (1974) have presented NMR evidence suggesting that a conformational change accompanies protonation of the oxytocin α-NH_2. Therefore, it seems probable that the conformation of deaminooxytocin and protonated oxytocin differ; the fact that it is the protonated form of oxytocin which binds to neurophysin makes it likely that this conformational difference contributes in some measure to the observed differences between the two in binding to neurophysin.

However, alternate explanations for the failure of deaminooxytocin to bind at low pH can be invoked. For example, the possibility must be considered that the protonated α-NH_3^+ of oxytocin, at low pH, is capable of participating as a donor in a hydrogen bond with either the carboxyl with which it had been in ion-pair linkage at higher pH or an adjacent electronegative group; depending on the local environment in which the interaction occurred, an anion from the solvent would probably be needed to neutralize the positive charge on the α-NH_3^+. Deaminooxytocin would clearly be unable to participate in a comparable bonding interaction; nonetheless, we consider the possibility of such a hydrogen bond unlikely in view of the fact that oxytocin analogues in which the α-NH_2 has been substituted by a hydroxyl group also do not bind at low pH (vide supra). Alternatively it should be pointed out that differences between the binding of oxytocin and deaminooxytocin at low pH would indeed be expected if the decrease in binding below pH 6 were determined not by protonation of the ion-pair carboxyl but solely by a conformational change in the protein at low pH, i.e., the second general mechanism suggested above to account for the data. In fact, the failure of deaminooxytocin to bind as strongly as does oxytocin at low

pH is the most compelling argument in support of such a mechanism, despite its improbability based on other considerations. Further study of the nature of any conformational changes between pH 6 and 3 should help to resolve this residual uncertainty.

Acknowledgments

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Ficellomycin and Feldamycin; Inhibitors of Bacterial Semiconservative DNA Replication†

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ABSTRACT: The two peptide-like antibiotics ficellomycin and feldamycin impair semiconservative DNA replication but not DNA repair synthesis in bacteria. Specifically both antibiotics cause the accumulation of a 34S DNA species in toluenized *Escherichia coli* cells which lacks the capability of being integrated into larger DNA pieces and eventually the complete bacterial chromosome. Novobiocin, a known inhibitor of replicative DNA synthesis, was investigated for comparative purposes. The action of this latter antibiotic differs from the ones exerted by ficellomycin and feldamycin in that novobiocin appears to block an event associated with the initiation of Okazaki fragments. The fact that novobiocin impairs DNA gyrase suggests that this enzyme plays an essential role during the initiation of Okazaki pieces.

The three antibiotics ficellomycin, feldamycin, and nojirimycin are produced by and were isolated from the culture broth of *Streptomyces ficellus* (Argoudelis et al., 1976b,c). Ficellomycin and feldamycin are new antibiotics; nojirimycin has been described previously and was shown to be 5-amino-5-deoxy-D-glucose (Ishida and Kumagi, 1967).

Chemically both ficellomycin and feldamycin represent dipeptide-like structures. Ficellomycin is a basic antibiotic and possesses a molecular formula of $C_{13}H_{24}N_6O_3$ corresponding to a formula weight of 312. The compound contains one valine residue per molecule and an unknown amino acid. Feldamycin is an amphoteric dipeptide composed of one residue of *N*-methylhistidine and a new histidine-like amino acid designated feldamycic acid (Argoudelis et al., 1976a) (Figure 1). The formula weight of feldamycin amounts to 407.

Both antibiotics inhibit a variety of bacteria in vitro. However, their specific activities assayed in vitro against whole bacterial cells are rather low. Accordingly, their minimal inhibitory concentrations (MIC) amount to >1000 μg/mL against most organisms when determined in broth. Nevertheless, ficellomycin is effective for the treatment of experimental *Staphylococcus aureus* infections in mice; feldamycin lacks any in vivo activity in these tests (Argoudelis et al., 1976b,c).

Investigations of the mode of action of ficellomycin and feldamycin have shown that these agents selectively impair semiconservative DNA replication when studied in *Escherichia coli* cells deficient in DNA polymerase I and rendered permeable to nucleotides by toluene treatment. Specifically these

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