Fluorescence Studies of Native and Modified Neurophysins. Effects of Peptides and pH†

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ABSTRACT: The effect of neurophysin–hormone interaction on the environment of the single tyrosine of bovine neurophysin (Tyr-49) and on that of the tyrosine of oxytocin and vasopressin was studied by fluorescence; tyrosine-free peptides were used to determine effects on Tyr-49, and acetylated neurophysin was used to determine effects on the hormone tyrosine. Binding increases the fluorescence intensity of Tyr-49 by 130% while the fluorescence of the hormone tyrosine is almost completely quenched. Correlation of these results with those obtained on binding oxytocin or vasopressin to native neurophysin indicates that in the hormone complexes less than half of the fluorescence of Tyr-49 is lost by Förster energy transfer to the quenched hormone tyrosine. These results support spin-label studies in indicating that the distance between Tyr-49 and the tyrosine of hormone bound to the strong hormone binding site is greater than 5 Å. In the absence of peptides, the fluorescence of Tyr-49 increases by 40% on lowering the pH from 6.2 to 2. Titration of the acid fluorescence transition in bovine neurophysins-I and -II, and in bovine neurophysin-II treated with carboxypeptidase B to remove the Arg-Arg-Val sequence at the carboxyl terminus, indicates that this transition is due to titration of a side-chain carboxyl with an intrinsic pK of 4.6. The effects of guanidine, glycerol, and disulfide cleavage on the magnitude of the acid transition indicate that the conformational information necessary for the transition resides within the amino acid sequence adjacent to Tyr-49. Accordingly, the fluorescence acid transition is attributed to decreased quenching by Glu-46 or Glu-47 upon protonation. Glycerol is shown to perturb the glutamate–tyrosine interaction in the absence of general conformational effects. Comparison of the fluorescence low-pH transition with that of the low-pH circular dichroism transition of nitrated neurophysins suggests that the fluorescence and CD transitions reflect related, but not necessarily identical, phenomena. In an appendix, evidence is presented which suggests that the products of carboxypeptidase digestion of bovine neurophysin-II are the same as two minor bovine neurophysin components, one of which is neurophysin-C.

Neurophysins are a family of highly homologous proteins which serve as carriers for the hormones oxytocin and vasopressin within the hypothalamo–neurohypophyseal tract. There are two principal bovine neurophysins, I and II, each of which is packaged within neurosecretory granules with a different hormone, but both of which in vitro can bind either oxytocin or vasopressin with comparable affinities (e.g., Breslow, 1974); additionally, there are several minor bovine neurophysins whose properties have not been extensively investigated (e.g., Rauch et al., 1969; Breslow, 1974). Each neurophysin contains a single tyrosine (Tyr-49) and three phenylalanine residues per polypeptide chain but no tryptophan (Rauch et al., 1969; Breslow, 1974; Chauvet et al., 1975); as such the neurophysins are fluorescence class A (Teale, 1960) proteins. Although the single tyrosine has been studied by NMR and UV absorption spectroscopy (Furth & Hope, 1970; Balaram et al., 1973; Griffin et al., 1973) and, when nitrated, by circular dichroism (Breslow & Weis, 1972), no fluorescence studies have been reported. The role of the tyrosine in binding is of particular interest because each neurophysin chain contains internally duplicated segments which flank the central nonduplicated region containing Tyr-49 (Capra et al., 1972) and each chain is capable of binding a second mole of hormone or peptide under selected conditions, although only a single site is most typically expressed (Nicolas et al., 1976; Glasel et al., 1976; Lord & Breslow, 1978; Nicolas et al., 1978). The question therefore arises as to the relative roles of the duplicated and nonduplicated regions in binding; Tyr-49 serves as a probe of the behavior of the nonduplicated region.

Tyr-49 has been shown to be markedly perturbed by binding oxytocin and vasopressin, or small peptide analogues of the hormones, to the hormone binding site (Furth & Hope, 1970, Breslow & Weis, 1972; Griffin et al., 1973); however, considerable uncertainty exists as to its exact function (cf. Balaram et al., 1973; Cohen et al., 1975; Lord & Breslow, 1978; Nicolas et al., 1978). Additionally, the tyrosine in position 2 of the hormones and peptides which bind to neurophysin has been shown to be a direct participant in the binding reaction (Breslow & Abrash, 1966; Breslow & Weis, 1972; Balaram et al., 1973; Griffin et al., 1973). One purpose of the present study is to use fluorescence spectroscopy to obtain additional insights into changes in both the neurophysin and hormone tyrosines attendant to binding. Two approaches are used to differentiate between effects on protein and hormone tyrosines. First, as in previous studies (Breslow & Weis, 1972; Griffin et al., 1973), binding-induced effects on the protein are isolated by use of peptides in which the tyrosine at position 2 is substituted by phenylalanine; this substitution has been shown to have no effect on binding affinity or on the spectral properties of the protein tyrosine (Breslow & Abrash, 1966; Breslow et al., 1973). Second, binding-induced changes in the hormone tyrosine are isolated by use of a derivative of neurophysin in which Tyr-49 is acetylated. O-Acetylated tyrosine is both nonfluorescent and unable to accept energy from unmodified tyrosine (cf. Cowgill, 1976); acetylated neurophysin has been shown to quantitatively retain the binding properties of native neurophysin (Furth & Hope, 1970; Fukuda et al., 1976) and is therefore assumed not to alter the mode of hormone binding. Comparison of the binding-induced changes in Tyr-49 and Tyr-2 with the effects of binding oxytocin or vasopressin to the native protein additionally allows an estimate to be made of the extent of Förster energy transfer between the two tyrosines in the 1:1 hormone–protein complexes.

On lowering the pH of neurophysin and mononitrated neurophysin below neutrality, CD[1] changes occur which, in

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part, involve the protein tyrosine (Breslow & Gargiulo, 1977). These changes are provocative because differences between different neurophysins in the apparent pK of the transition appear to parallel differences between neurophysins in the pH dependence of hormone binding (Camier et al., 1973; Breslow & Gargiulo, 1977; Klausner et al., 1978). Accordingly, we have also studied the fluorescence of the single tyrosine of native and modified neurophysins as a function of pH to learn more of the origins of tyrosine environmental changes at low pH. Fluorescence studies were supplemented by additional CD studies of the nitrated protein in order to compare the pH dependence of the acid transitions as observed by both technique.

In the course of these studies, we have prepared a new derivative of bovine neurophysin-II by removal of the Arg-Arg-Val sequence at the carboxyl terminus (Chauvet et al., 1975) by treatment with carboxypeptidase B. Selected spectral data on this derivative are reported in the main body of the paper. In an appendix, we present data which suggest that this derivative is the same as bovine neurophysin-C (Rauch et al., 1969) and that a related derivative in which only the last two carboxyl-terminal residues have been lost is analogous to another typical but unnamed component of bovine neurophysin preparations. These data complement demonstrations elsewhere (Wuu & Crumm, 1976) that two of the porcine neurophysins differ from each other only by removal in one of the carboxyl-terminal Arg-Arg-X sequence.

Materials and Methods

Protein Preparation and Modification. Bovine neurophysins-I and -II were purified as previously described (Breslow et al., 1971) with the modification that traces of UV-absorbing contaminants from pyridine–acetate chromatography were removed by chromatography on Sephadex G-50 in 0.1 N acetic acid. Neurophysin-II was approximately 95% pure as judged by electrophoresis. Neurophysin-I preparations obtained by this procedure were variably contaminated (10–25%) with neurophysin-C and traces of other neurophysin components. Accordingly, in some instances, bovine neurophysin-I was further fractionated to remove residual neurophysin-C by ion-exchange chromatography on Sephadex DEAE-50 in 0.1 M Tris-HCl buffer, pH 8.1, containing 0.27 M NaCl. The resultant protein was at least 90% pure as judged by amino acid analysis and gel electrophoresis. Fluorescence studies did not reveal any significant differences between the once-fractionated and refractionated proteins.

Acetylation and nitration of neurophysin were carried out as described by Furth & Hope (1970), with modifications introduced into the nitration procedure by Breslow & Gargiulo (1977). The completeness of acetylation was determined both by reaction with hydroxylamine (Furth & Hope, 1970) and by the residual tyrosine fluorescence (see also Results) and was typically 85–90%. Under the conditions used for fluorescence studies, no deacetylation occurred, as evidenced by constant fluorescence values over the time course of an experiment. Complete reduction and carboxamidomethylation of neurophysin-I were accomplished by addition of 20 mg of protein to 10 mL of 0.03 M dithiothreitol in 5 M guanidine containing 5×10^{-4} M EDTA, at pH 8. After 1 h, 10 mL of 1 M Tris buffer, pH 8.2, containing 310 mg of iodoacetamide and 5×10^{-4} M in EDTA was added. The solution was allowed to stir under N_2 for 2 h, the pH was then lowered to 3.6, and the solution was chromatographed on Sephadex G-25 in 0.1 M acetic acid to separate the protein and reagents; the protein peak was lyophilized. The reduced carboxamidomethylated protein was devoid of cystine as evidenced by the lack of the near UV ellipticity normally (Breslow et al., 1971) associated with neurophysin disulfides and by amino acid analysis; on polyacrylamide gel electrophoresis in a 7.5% gel at pH 9.5 it migrated at a rate approximately half that of native neurophysin-I. Amino acid analysis indicated that no residues other than half-cystine residues were altered by the reduction–carboxamidomethylation treatment.

Cleavage of the carboxyl-terminal residues of neurophysin-II was carried out with diisopropyl fluorophosphate treated carboxypeptidases A and B obtained from Worthington Biochemical Corp. Carboxypeptidase A digestion was carried out for 2 h at room temperature at a protein concentration of 15 mg/mL and enzyme concentration of 0.1 mg/mL in 0.2 M $NaHCO_3$, pH 7.8, containing 0.02 M NaCl. Carboxypeptidase B digestion was carried out for 20 h at room temperature at a protein concentration of 8 mg/mL and enzyme concentration of 0.25 mg/mL in 0.2 M $NaHCO_3$ buffer, pH 7.8, containing 0.18 M NaCl. After treatment with either carboxypeptidase A or B, digestion was terminated by lowering the pH to 3, and the reaction mixture was chromatographed on Sephadex G-50 in 0.1 N acetic acid. The entire column eluate, which emerged after the protein peak and the neurophysin peak, was lyophilized separately. The identity and quantity of the liberated amino acids were determined by amino acid analysis (without prior hydrolysis) of the eluate which followed the protein peak. Electrophoresis and peptide-binding studies of the protein products are reported in the Appendix. Nitration of the carboxypeptidase-treated protein was carried out using the same procedure as for native neurophysin-I.

Peptides and Hormones. All but one of the dipeptides were obtained from Vega-Fox Chemicals (Tucson, AZ). Methyl *N*-acetyl-L-tyrosyl-L-phenylalaninate had been obtained earlier from Cyclo Chemical Corp. Absorption and fluorescence properties of each were in accord with their amino acid content. Binding of each to the hormone binding site of nitrated neurophysin was determined by CD as previously described (Breslow et al., 1973). Binding constants for peptides containing a phenylalanine in position 2 were, within experimental error, the same as those previously reported (Breslow et al., 1973) for the corresponding peptides containing tyrosine in position 2. Lysine-vasopressin was an earlier gift from Dr. Vincent du Vigneaud. Oxytocin was generously supplied by Dr. Jan Mulder of Ferring Laboratories, Sweden, or was purchased from Vega-Fox Chemicals. 2-Phenylalanine-oxytocin was also purchased from Vega-Fox Chemicals. All hormones were checked for purity by amino acid analysis and, prior to use, were refractionated on Sephadex G-25 in 0.1 N acetic acid to remove oligomeric material.

Spectroscopic Studies. Fluorescence measurements were carried out using a Perkin-Elmer Model MPF-4 or MPF-3 spectrofluorometer in the ratio mode. A 1-cm cell in a temperature-controlled cell holder was used for all studies. Unless otherwise stated, all studies were carried out at 25 °C by use of an excitation wavelength of 277.5 nm (see Results) and 6-nm slit widths for both exciting and emitted radiation. All data are corrected for the Raman scattering band of water which coincides with the emission spectrum of tyrosine upon excitation at 277.5 nm; weak fluorescence from occasional impurities in peptides is also subtracted. Unless otherwise

[1] Abbreviations used: CD, circular dichroism; NP, neurophysin; Ac-NP, acetylated neurophysin; Phe-TyrNH_2, L-phenylalanyl-L-tyrosinamide; LVP, lysine-vasopressin; Phe-PheNH_2, L-phenylalanyl-L-phenylalaninamide.

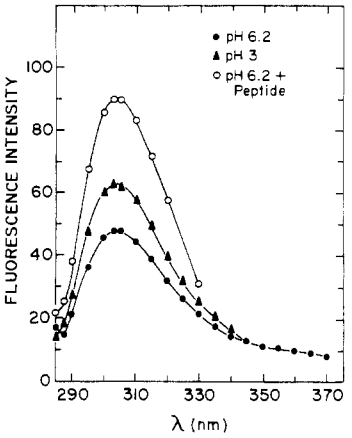

FIGURE 1: Effect of pH and peptides on the emission spectrum of neurophysin-II. (●) pH 6.2, (▲) pH 3 and (○) pH 6.2 in the presence of 2.5×10^{-3} M L-norleucyl-L-phenylalaninamide. Protein concentration = 5×10^{-5} M in 0.16 M KCl; excitation wavelength = 277.5 nm. Spectra are uncorrected for absorbance. On the same scale the uncorrected fluorescence of 5×10^{-5} M tyrosine at pH 6 is 320 at 303 nm.

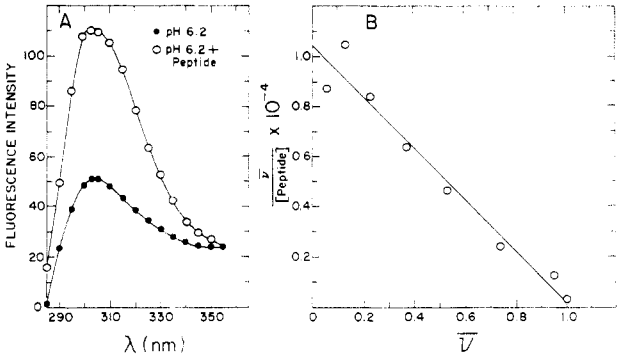

FIGURE 2: Binding of peptides to neurophysin-I. (A) Emission spectra of neurophysin-I at pH 6.2 in the absence (●) and presence (○) of saturating concentrations (3.5×10^{-3} M) of Phe-PheNH$_2$. Protein concentration = 5×10^{-5} M in 0.16 M KCl. Spectra are uncorrected for absorbance. On the intensity scale reported, the uncorrected emission at 303 nm of 5×10^{-5} M Tyr, pH 6, equals 330. Emission above 330 nm in this study appears to originate from an impurity picked up during refractionation (see Materials and Methods). (B) Scatchard plot of binding of Phe-PHeNH$_2$ to NP-I. Successive increments of Phe-PheNH$_2$ were added to a solution of 5×10^{-5} M protein in 0.16 M KCl, pH 6.2, and the fluorescence intensity was determined. All intensities were then corrected for absorbance (see Materials and Methods). For each addition of peptide, the number of moles bound per mole of protein ($\bar{\nu}$) was calculated from the observed fluorescence relative to that in the absence of peptide and in the presence of saturating peptide concentrations by use of the same equations as for CD binding studies (cf. Breslow & Gargiulo, 1977) but substituting fluorescence intensities for ellipticities. One peptide binding site per mole (10000 g) was assumed.

reported, all fluorescence intensities, F, are calculated from emission at 303 nm and are also corrected for absorption of exciting light and reabsorption of emitted light (303 nm) using the relationship (Weill & Calvin, 1963; Kirby, 1971)

$$F_{cor} = F_{obsd}(\text{antilog } \tfrac{1}{2}A_{277.5})(\text{antilog } \tfrac{1}{2}A_{303})$$

where A is the absorbance in a 1-cm cell. Absorbance at 303 nm was due almost exclusively to neurophysin disulfides (Breslow et al., 1971) and introduced only a trivial correction factor in all studies reported. The validity of the absorption correction factor was confirmed by studies of the effect of concentration on the fluorescence of the amino acid tyrosine; the corrected fluorescence was linearly related to concentration over the range 10^{-5}–10^{-3} M, the upper concentration of which represents an absorbance of 1.5 at the exciting wavelength. In neurophysin studies, absorbance at 277.5 nm was typically 0.15 (5×10^{-5} M protein) except in the presence of the hormones where absorbance values increased to a maximum of 0.27 or in the presence of Phe-TyrNH$_2$ where the higher protein and peptide concentrations gave an absorbance of 0.5 at the exciting wavelength. In studies of different phenylalanine-containing peptides, a fluorescent impurity emitting very weakly near 303 nm was occasionally observed and its fluorescence was routinely subtracted from all spectra. For studies in the presence of glycerol, spectral quality glycerol (Eastman) was used and was free of fluorescent impurities.

Circular dichroism studies were carried out as previously described (Breslow & Gargiulo, 1977). UV-absorption measurements were made with a Gilford Model 222-A spectrophotometer equipped with Beckman DU optics.

General Methods. Amino acid analyses were carried out as previously described (Breslow & Gargiulo, 1977). Polyacrylamide gel electrophoresis was carried out typically by use of a 7.5% gel and running pH of 9.5 as previously described (Breslow et al., 1971); in occasional studies an 11% gel and running pH of 7.5 (Williams & Reisfeld, 1964) were used. Protein concentrations are reported from the weight of lyophilized powder used (cf. Breslow & Gargiulo, 1977).

Results

Fluorescence Spectra of Neurophysin as a Function of Concentration, pH, and Bound Tyrosine-Free Peptides. Figure 1 shows the emission spectra of solutions of 5×10^{-5} M neurophysin-II in 0.16 M KCl at pH 3 and pH 6.2 in the absence of peptide and at pH 6.2 in the presence of the peptide L-norleucyl-L-phenylalaninamide (2.5×10^{-3} M). The spectra were obtained by excitation at 277.5 nm, which was shown to be the excitation maximum upon monitoring emission at 303 nm; the emission and excitation maxima are typical of tyrosine (cf. Longworth, 1971). The spectra shown in Figure 1 are not corrected for self-absorption of exciting or emitted radiation but can be directly compared since all the solutions had the same absorbance above 275 nm. From the binding constant of L-norleucyl-L-phenylalaninamide (1.5×10^3 M^{-1}), the peptide concentration used in Figure 1 was sufficient to give 77% saturation of the peptide binding site. Results from these and related studies indicate that decreasing the pH from 6 to 3 leads to a 40% (±5%) increase in protein tyrosine fluorescence while binding of peptide increases protein tyrosine fluorescence by 130%. Results identical with these obtained with neurophysin-II were obtained for the effect of pH (vide infra) and of peptide binding (Figure 2) on the fluorescence of neurophysin-I. The effect of binding peptides which do not contain tyrosine can be used to measure binding constants (Figure 2), and the values so determined are in good agreement with values obtained by CD by using the same peptides and the nitrated protein. Within experimental error, binding of 2-phenylalanine-oxytocin to neurophysins-I and -II produced the same change in Tyr-49 fluorescence intensity at saturation as did the smaller tyrosine-free peptides.

Figure 3 shows the effect of concentration on the emission of neurophysin-II at 303 nm when corrected for self-absorption as described under Materials and Methods. The concentration range studied is one in which the absorbance of neurophysin at 277.5 nm (1-cm cell) changes from 0.034 to 0.516, but application of the correction factor yields a linear plot within experimental error, as found for the free amino acid tyrosine (see Material and Methods). Neurophysin-II reversibly dimerizes with a dimerization constant of approximately 5×10^3 M^{-1} (Breslow et al., 1971; Nicolas et al., 1976) and, near pH 6, the weight fraction of the dimer form increases from

Table I: Representative Fluorescence Studies of Neurophysin in the Presence of Tyrosine-Containing Peptides[a]

protein	peptide	pH	fraction protein bound[b]	fraction peptide bound[b]	corrected fluorescence (obsd)	theoretical fluorescence[c] model A	theoretical fluorescence[c] model B
5×10^{-5} M NP-II	–	5.8	–	–	63		
–	4×10^{-5} M LVP	5.8	–	–	36		
5×10^{-5} M NP-II	4×10^{-5} M LVP	5.8	0.57	0.66	103	146	127
–	8×10^{-5} M LVP	5.8	–	–	66		
5×10^{-5} M NP-II	8×10^{-5} M LVP	5.8	0.79	0.52	137	194	166
1×10^{-4} M NP-II	–	6.2	–	–	102		
–	2×10^{-4} M Phe-TyrNH$_2$	6.2	–	–	214		
1×10^{-4} M NP-II	2×10^{-4} M Phe-TyrNH$_2$	6.2	0.55	0.275	321	389	–
4.5×10^{-5} M NP-II	–	6.0	–	–	69		
–	5.5×10^{-5} M oxytocin	6.0	–	–	73.5		
4.5×10^{-5} M NP-II	5.5×10^{-5} M oxytocin	6.0	0.70	0.57	156	205	167
–	1.1×10^{-4} M oxytocin	6.0	–	–	153		
4.5×10^{-5} M NP-II	1.1×10^{-4} M oxytocin	6.0	0.87	0.34	218	300	252
5×10^{-5} M NP-I	–	5.6	–	–	54		
–	5×10^{-5} M oxytocin	5.6	–	–	53		
5×10^{-5} M NP-I	5×10^{-5} M oxytocin	5.6	0.64	0.64	111	152	125
5×10^{-5} M NP-I	–	5.6	–	–	59		
–	2.25×10^{-5} M LVP	5.6	–	–	20		
5×10^{-5} M NP-I	2.25×10^{-5} M LVP	5.6	0.34	0.75	89	105	93
–	4.5×10^{-5} M LVP	5.6	–	–	40		
5×10^{-5} M NP-I	4.5×10^{-5} M LVP	5.6	0.60	0.67	112	145	124
–	9×10^{-5} M LVP	5.6	–	–	80		
5×10^{-5} M NP-I	9×10^{-5} M LVP	5.6	0.83	0.45	147	203	174

[a] All fluorescence values are reported at 303 nm, are corrected for absorbance, and are reported on a scale on which the absorbance-corrected emission of 5×10^{-5} M tyrosine is 350. The validity of the absorbance correction was additionally confirmed by showing that, under nonbinding conditions (low pH), the absorbance-corrected fluorescence of mixtures of protein and peptide was equal to the sum of that of its isolated components. [b] The degree of binding was calculated by assumption of a 1:1 interaction in each case. This has been demonstrated for the binding of Phe-TyrNH$_2$ (Lundt, 1978) and oxytocin (cf. Camier et al., 1973), and the most recent data (Nicolas et al., 1976) indicate that secondary binding sites are also insignificant in the binding of vasopressin to neurophysin-II under the conditions used. For the binding of vasopressin to neurophysin-I (Nicolas et al., 1976), the occupancy of secondary sites may be significant. Binding constants used were 1.2×10^4 M^{-1} for Phe-TyrNH$_2$ (Breslow & Gargiulo, 1978) and 1×10^5 M^{-1} for the hormones at pH 5.6 (Nicolas et al., 1976) corrected to 7×10^4 M^{-1} at pH 6.0 (cf. Breslow et al., 1973). [c] Values are calculated by use of models A and B as described in the text. Binding was assumed to increase the intrinsic protein tyrosine fluorescence to a value 230% of its value in the unbound state. For (B) the fluorescence intensity of bound hormone was assumed to be 20% that of unbound hormone except in the case of oxytocin and neurophysin-II where a value of 8% was used. The theoretical fluorescence, F_t, was calculated for each model as F_t = fraction protein bound (bound protein fluorescence) + fraction protein unbound (unbound protein fluorescence) + fraction peptide bound (fluorescence of bound peptide) + fraction peptide unbound (fluorescence of unbound peptide).

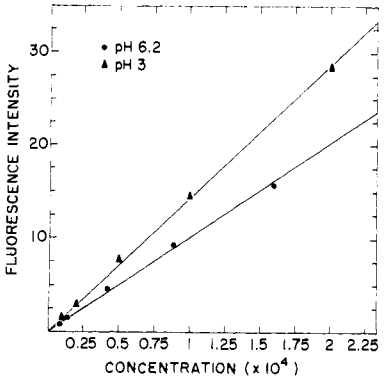

FIGURE 3: Effect of concentration on the absorbance-corrected fluorescence intensity at 303 nm of neurophysin-II. Concentration is expressed in molarity. Solvent = 0.16 M KCl. (●) pH 6.2; (▲) pH 3. Fluorescence intensity is reported in arbitrary units.

10 to 50% over the concentration range studied in Figure 3. The linearity of the fluorescence plot in Figure 3, together with the fact that no change in spectral shape was observed over the concentration range studied, suggests that the single tyrosine is not directly involved in the dimerization process—in agreement with recent observations elsewhere (Nicolas et al., 1978). Correlation of the absorption-corrected fluorescence of neurophysin II with that of the amino acid tyrosine at pH 6 on the same fluorometer, by assuming a quantum yield for tyrosine at pH 6 of 0.14 (Chen et al., 1969), gives an approximate quantum yield for the neurophysin-II tyrosine at pH 6 of 0.025. This value is within the range of tyrosine quantum yields frequently observed in proteins and peptides (Cowgill, 1976).

Effect of Binding Tyrosine-Containing Peptides to Native and Acetylated Neurophysin. The increase in fluorescence at 303 nm when peptides containing phenylalanine in position 2 are bound to neurophysin clearly reflects the change in neurophysin tyrosine fluorescence since phenylalanine is too weak a chromophore to make significant fluorescence contributions and does not absorb at the exciting wavelength (277.5 nm). However, on addition to native neurophysin of peptides which contain tyrosine in position 2, under conditions representing significant binding, the observed fluorescence is approximately the sum of that of the individual components, as shown in Table I for the binding of Phe-TyrNH$_2$, oxytocin, and vasopressin. Data here are presented solely as the fluorescence emission at 303 nm since the shape of the emission spectrum is unaffected by binding. The deviation of the observed fluorescence intensities from those calculated (model A, Table I) by assumption of a 130% increase in the

Table II: Calculated Energy Transfer in Neurophysin-Hormone Complexes

system	energy transfer,[a] efficiency ± AD	tyrosine-tyrosine distance (Å)		
		$\kappa^2 = 0.07$	$\kappa^2 = 0.667$	$\kappa^2 = 4$
neurophysin-I + oxytocin	0.19 ± 0.004	6.9	9.9	13.4
neurophysin-I + lysine-vasopressin	0.19 ± 0.06	6.9	9.9	13.4
neurophysin-II + oxytocin	0.12 ± 0.08	7.6	11.0	14.8
neurophysin-II + lysine-vasopressin	0.34 ± 0.06	6.1	8.8	11.8

[a] Calculated from the difference ($\Delta F_{303\ nm}$) between the theoretical fluorescence intensity of mixtures of protein and hormone (see model B, Table I) and the observed fluorescence intensity by use of the relationship: energy transfer efficiency = $\Delta F_{303\ nm}/([F_{2(calcd)}][(1 - Q_1)/Q_2])$, where $F_{2\ (calcd)}$ is the calculated fluorescence intensity of Tyr-49 in the complex in the absence of energy transfer and Q_1 and Q_2 are the quantum yields respectively of the hormone tyrosine and Tyr-49 in the complex. The second term in the denominator is a minor correction term which allows for the fact that energy transferred from Tyr-49 in the complex to the hormone tyrosine is not completely lost but is emitted with the quantum yield of the hormone tyrosine.

fluorescence intensity of Tyr-49 and no change in the peptide tyrosine in the bound state suggests that the quantum yield of the peptide tyrosine must be reduced on binding. Note that the failure to observe a significant fluorescence increase on binding cannot be attributed to potential errors in self-absorption corrections; while these corrections are relatively large in the case of Phe-TyrNH_2 shown, they are very small in the LVP and oxytocin studies.

The extent of quenching of the peptide tyrosine on binding was determined from the effect of acetylated neurophysin on the fluorescence of oxytocin and vasopressin. At pH 6 the fluorescence of acetylated neurophysin alone was approximately 10–15% that of native neurophysin, in keeping with the completeness of the acetylation reaction as determined by the hydroxylamine test (see Methods and Materials). The residual fluorescence of the acetylated protein was shown to be associated with native neurophysin by the fact that it doubled in the presence of 5×10^{-3} M Phe-PheNH_2. Figure 4 shows the effects of increasing concentrations of acetylated neurophysins-I and -II on the fluorescence of oxytocin and vasopressin; the data are corrected for the presence of residual unmodified protein as described in the figure legend. The results indicate that binding leads to an 80–95% decrease in the quantum yield of the hormone tyrosine. Small differences between the different hormone–protein complexes in the extent of hormone quenching are tentatively assumed to be real since they roughly parallel differences in CD changes at 280 nm accompanying formation of the different hormone–protein complexes (cf. Cohen et al., 1975; Lundt, 1978).

Calculation of Energy Transfer from Tyr-49 to Tyr-2 in Hormone–Neurophysin Complexes. In the hormone–protein mixtures shown in Table I, with the possible exception of mixtures of vasopressin and neurophysin-I, only a single hormone binding site is occupied to a statistically significant extent (cf. Glasel et al., 1976; Nicolas et al., 1976). In principle therefore it should be possible to ascertain the distance between Tyr-49 and the hormone tyrosine in the 1:1 complex from the degree of Förster energy transfer between the two tyrosines (e.g., Dale & Eisinger, 1975). In the present instance, the net effect of such energy transfer should be a reduction in overall quantum yield of the complex relative to that predicted on the basis of the separate binding-induced changes in Tyr-49 and the hormone tyrosine, since energy transferred from the intrinsically more fluorescent protein tyrosine would be emitted with the quantum yield of the quenched hormone tyrosine (e.g., Cowgill, 1976). In model B, Table I, we calculated theoretical intensities for mixtures of hormone and unmodified protein, assuming that the fluorescence of Tyr-49 increases by 130% in the bound state while that of Tyr-2 decreases to the same extent as observed

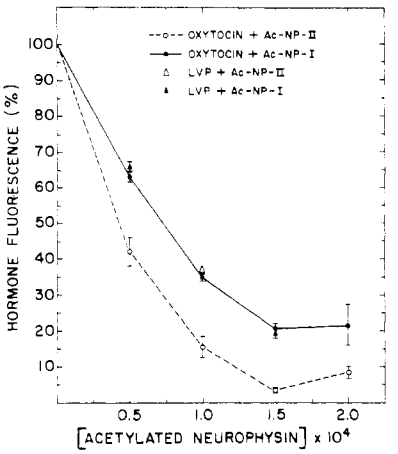

FIGURE 4: Changes in hormone fluorescence accompanying binding to acetylated neurophysin. Results were obtained from the fluorescence at 303 nm of 5×10^{-5} M hormone (pH 5.8, 0.16 M KCl) in the presence of increasing concentrations of acetylated neurophysin. The absorbance-corrected fluorescence of the acetylated protein (see Methods and Materials) was subtracted from the absorbance-corrected fluorescence of the protein–hormine mixture to give the apparent hormone fluorescence, F', at each protein concentration. The value of F' at each protein concentration was corrected to an intrinsic fluorescence, F^0, to allow for the fact that a small fraction of bound hormone (10–15%) was bound to the nonacetylated protein (see Methods and Materials) and therefore behaved as if its fluorescence (and that of the protein to which it was bound) was unchanged (see Results). The last correction was applied using the relationship: $[F^0$(no protein present) $- F'$(presence of protein)$]/$(fraction of protein that is acetylated) $= [F^0$(no protein present) $- F^0$(presence of protein)$]$. The results are expressed in the figure as the F^0 percentage at each protein concentration of the value of F^0 observed in the absence of protein.

upon its binding to the acetylated protein. Comparison of these values with the observed fluorescence intensities (Table I) indicates that the latter are slightly but consistently lower than predicted by model B. Assuming that this discrepancy represents only Förster energy transfer, we calculated the apparent efficiency of energy transfer and it is shown in Table II. The significance of the data is that they indicate that Förster energy transfer must be inefficient. Although the calculated efficiency underestimates the true transfer efficiency by neglecting back transfer from the hormone tyrosine, this approximation introduces only a trivial error because the quantum yield in the complex of the hormone tyrosine is only a small fraction ($^1/_{15}$) of that of Tyr-49. Note that the calculated efficiencies are also not significantly affected by reasonable disparities between assumed and effective binding constants.

The efficiency of energy transfer between two chromophores is a function of interchromophore distance and the Förster

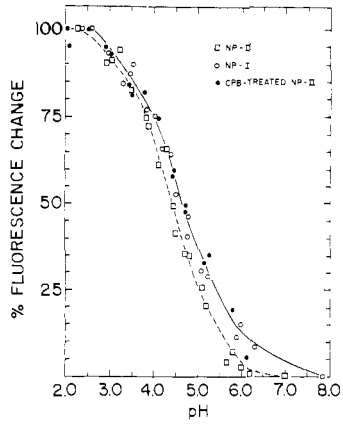

FIGURE 5: Fluorescence titration of the acid transition of different neurophysins. Conditions: 5×10^{-5} M protein in 0.16 M KCl containing 0.01 M NaOAc; 25 °C. Data are plotted by using the emission intensity at 303 nm. (--□--) Neurophysin-II; (–○–) neurophysin-I; (●) carboxypeptidase B treated neurophysin-II. Note that lines have no theoretical significance. Derived pK values are given in the text and in Table IV.

critical distance, R^0, for energy transfer (e.g. Dale & Eisinger, 1975). The value of R^0 depends on the overlap integral between donor emission and acceptor absorption spectra, the fluorescence lifetime of the donor in the absence of acceptor, and the orientation factor, κ^2, between donor and acceptor. We have used the overlap integral calculated for tyrosine–tyrosine energy transfer by Perlman et al. (1968) and an expected lifetime of 1.4 ns for Tyr-49 in the complex as estimated from its quantum yield relative to tyrosine (cf. Gauduchon & Wahl, 1978) to calculate values of R^0 = 5.5, 7.9, and 10.65 Å corresponding to values of κ^2 = 0.07, 0.667, and 4, respectively. Using these R^0 values, the apparent distance, r, between Tyr-49 and the hormone tyrosine in the complex has been calculated from the relationship

$$\text{efficiency of energy transfer} = \frac{1}{1 + (r/R^0)^6} \qquad (1)$$

The calculated values or r are also shown in Table II and will be discussed further below.

Fluorescence Titrations. In order to determine the origin of the low-pH neurophysin fluorescence transition the fluorescence titration curves of neurophysins-I and -II were determined (Figure 5 and Table IV). These titrations were carried out in the presence of 0.01 M acetate as buffer, but control studies indicated that this low acetate concentration was without effect on neurophysin tyrosine fluorescence at either low or neutral pH. The data in Figure 5 indicate that the neurophysin transition occurs in the region of side-chain carboxyl titration and is shifted to slightly higher pH in neurophysin-I relative to neurophysin-II. The midpoints of the two titration curves are 4.65 for neurophysin-I and 4.40 for neurophysin-II. The apparent ΔH of ionization associated with the fluorescence titrations was approximated from the effect of temperature on the titration midpoints over the interval 12–40 °C as −1.4 ± 1.4 kcal for neurophysin-I and −2.9 ± 1 kcal for neurophysin-II, the average values being slightly more negative than typically (cf. Steinhart & Beychok, 1964) associated with carboxyl ionizations. Attempts to analyze the individual titration curves in terms of a single pK_a indicated that the apparent pK_a for each transition decreased slightly with decreasing pH. This behavior can be explained by assumption of an effect of the change in net protein charge on apparent pK_a values over the pH interval of the fluorescence titration or by assumption of the direct influence of more than one titratable group on tyrosine fluorescence. Analysis of the individual titration curves to account for effects of net protein charge, by use of the Linderstrom–Lang model (cf. Steinhardt & Beychok, 1964) and net charge values calculated from potentiometric titration data (Breslow et al., 1971), gave intrinsic pK values for the carboxyl groups responsible for the fluorescence change of neurophysin-I and neurophysin-II of 4.61 and 4.59, respectively (Table IV). These were calculated by a least-squares straight line plot of the apparent pK at each pH, calculated from the percentage fluorescence change, vs. the net protein charge. The average standard error of the intercept within each titration and the standard error of the average intrinsic pK when different titrations were compared were ±0.07 pH units. Apparent electrostatic interaction factors ("w" values) were in good agreement with those calculated for carboxyl groups from potentiometric titration studies (Breslow et al., 1971). These results suggest that the fluorescence transition represents the titration of the same carboxyl in both proteins, the difference between the two proteins in titration midpoint reflecting differences in protein net charge, i.e., neurophysin-II has two additional positive charges near pH 4.5 (Breslow et al., 1971). This conclusion is supported by the fact that removal of the carboxyl-terminal Arg-Arg-Val sequence of neurophysin-II by treatment with carboxypeptidase B (see Materials and Methods and Appendix), which alters the neurophysin-II net charge to that of NP-I, also results in a shift in its titration curve to that of neurophysin I (Figure 5). The intrinsic pK_a of the carboxyl responsible for the fluorescence titration suggests its identity as a glutamate side-chain carboxyl, the normal intrinsic pK_a values of aspartate and glutamate side chains being 4.0 and 4.6, respectively (Nozaki & Tanford, 1967).

Role of Sequence and Conformation in the Acid Transition. Effects of Guanidine, Disulfide Cleavage, and Glycerol. Collisional quenching of tyrosine by carboxylic acid anions is well known and tyrosine quenching by spatially proximate carboxylates in proteins has also been demonstrated (cf. Teale, 1960; Longworth, 1971; Cowgill, 1976); protonated carboxyls are markedly less effective as quenchers than the anions (Feitelson, 1964). Thus the fluorescence transition might reflect the titration of a spatially proximate carboxylate. Alternatively, since tyrosine quantum yield is sensitive to environment in the absence of carboxylate quenching (cf. Cowgill, 1976; Gauduchon & Wahl, 1978), the transition might represent a carboxyl-controlled conformational change. In order to determine the role of conformation in the transition, either in bringing together Tyr-49 and the carboxylate or in changing with pH, the effect of denaturation on the transition was studied. Table III shows the effect of 5 M guanidine on the magnitude of the pH transition in native neurophysin and in neurophysin in which all seven disulfides have been reduced and carboxamidomethylated. Guanidine, which was without significant effect on the fluorescence of the free amino acid tyrosine [in confirmation of results elsewhere (cf. Cowgill, 1976)] or on the fluorescence of the peptide norleucyl-tyrosinamide, halves the magnitude of the pH transition in the native protein largely by increasing fluorescence at pH 6. Since neurophysin is denatured at this concentration of guanidine (Menendez-Botet & Breslow, 1975), conformational factors are implicated. However, reduced carboxamidomethylated neurophysin behaves identically with native neurophysin with respect to the magnitude of the pH transition and the effects of guanidine; the absolute magnitude of its intrinsic fluorescence differs only slightly from that of native neurophysin. This is significant since even partial reduction

Table III: Effect of pH and Guanidine on the Fluorescence of Native and Reduced Carboxamidomethylated Neurophysin-I[a]

protein	solvent	pH	fluorescence (303 mm)	fluorescence, pH 3/pH 6	
				KCl	guanidine
native	0.16 M KCl	6.2	54		
native	0.16 M KCl	3	79	1.46	
native	5 M guanidine	6.2	65		
native	5 M guanidine	3	78		1.20
reduced CM[b]	0.16 M KCl	6.2	62		
reduced CM	0.16 M KCl	3	88	1.40	
reduced CM	5 M guanidine	6.2	74		
reduced CM	5 M guanidine	3	90		1.22

[a] Values are normalized to a protein concentration of 5×10^{-5} M and reported on a scale such that the same concentration of tyrosine at pH 6, on the same fluorometer during the same study, gave a fluorescence intensity at 303 nm of 350. [b] Reduced carboxamidomethylated protein.

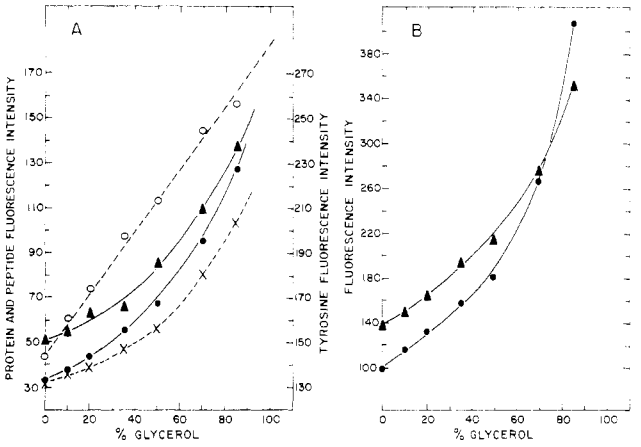

FIGURE 6: Effect of increasing glycerol concentrations on the fluorescence emission of neurophysin and other tyrosine-containing systems. Glycerol concentration is expressed as weight percent; the second component is aqueous KCl. The concentration of each fluorophore is 5×10^{-5} M at each glycerol concentration. Fluorescence intensities are corrected for absorbance and are reported on an arbitrary scale which is internally consistent within each diagram. (A) In this study, the KCl concentration was 0.16 M in the absence of glycerol but progressively decreased with increasing glycerol concentrations to a concentration of 0.02 M at the highest glycerol concentration. (●) Neurophysin-II, pH 6.2; (▲) neurophysin-II, pH 3; (○) tyrosine, pH 6; (×) *N*-acetyl-L-tyrosyl-L-phenylalanine methyl ester. (B) In this study, the concentration of KCl was 0.16 M in the absence of glycerol and was adjusted as glycerol was added such that it did not decrease below 0.11 M. (●) Neurophysin-II, pH 6.2, (▲) neurophysin-II, pH 3.

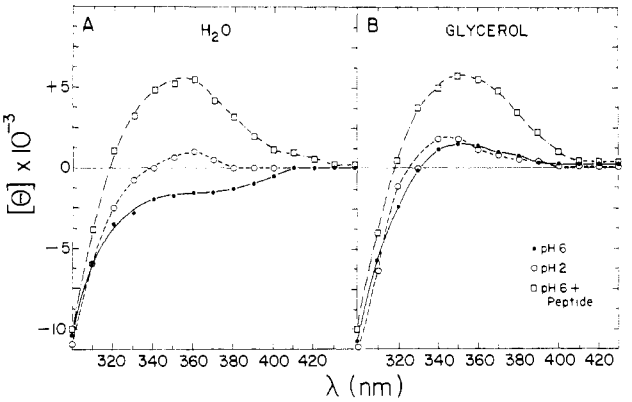

FIGURE 7: Effect of glycerol on the CD spectra of nitrated neurophysin-II. Results are reported as molar ellipticities. (●) pH 6.2, (○) pH 2, and (□) pH 6.2 in the presence of saturating concentrations of Phe-TyrNH_2. (A) Spectra in 0.16 M KCl. (B) Spectra obtained in the presence of 75% glycerol (by weight). KCl concentration = 0.05 M. Note that, in aqueous solution, CD spectra are independnt of KCl concentration.

and carboxamidomethylation of neurophysin has been shown to lead to markedly altered physical–chemical properties, reflecting major changes in conformation (Menendez-Botet & Breslow, 1975); in the present studies, the completely reduced carboxamidomethylated protein, like the partially reduced carboxamidomethylated protein, behaved on gel electrophoresis as if it were markedly unfolded or aggregated (see Materials and Methods) and exhibited absolutely no fluorescence increase upon addition of concentrations of Phe-PheNH_2 sufficient to saturate native neurophysin. Thus, cleavage of the disulfide bridges destroys the principal conformational features of the native protein but is without significant effect on those structural features which determine fluorescence behavior. These results strongly suggest that, in the absence of peptides, the conformational information necessary for the pH dependence of neurophysin tyrosine fluorescence is contained within the disulfide free sequence of nine amino acids (Capra et al., 1972) adjacent to the tyrosine. This sequence contains only two carboxyl groups, Glu-46 and Glu-47. We therefore assign the fluorescence transition to the titration of either Glu-46 or Glu-47 and suggest that the participating carboxyl, when ionized, directly quenches Tyr-49. Effects of guanidine are viewed as disrupting the local conformation favorable to carboxylate–tyrosine interaction.

The role of strictly local conformation in the fluorescence acid transition can also be seen by observations, made during the course of preliminary fluorescence polarization studies, of the effects of glycerol. High concentrations of glycerol (75% by weight) were found to be without effect on the integrity of the near UV neurophysin disulfide CD spectrum, an indicator of the integrity of the overall neurophysin conformation (Menendez-Botet & Breslow, 1975), and did not decrease the affinity of neurophysin for peptides. Nonetheless, as shown in Figure 6, the magnitude of the fluorescence acid transition diminishes with increasing concentrations of glycerol as evidenced by the gradual merger of the pH 6 and 3 fluorescence intensities of Tyr-49. The nonlinear response of the fluorescence of Tyr-49 to increasing concentrations of glycerol, relative to the linear response of the amino acid tyrosine, probably in part reflects decreased peptide-bond quenching of tyrosine in nonaqueous solvents (cf. Cowgill, 1976). Thus a nonlinear response to glycerol of the tyrosine fluorescence of the peptide methyl *N*-acetyl-L-tyrosyl-L-phenylalaninate also occurs (Figure 6A), but the nonlinearity of the Tyr-49 response at pH 6 is more marked. The data suggest a special effect of glycerol on the environment of Tyr-49 at pH 6.

The existence of a local effect of glycerol on the environment of Tyr-49 was confirmed by CD studies of nitrated neurophysin in the presence of glycerol; the results indicated a loss also of the low-pH CD transition of nitrated Tyr-49 at high glycerol

concentrations. Figure 7 compares the CD spectra of nitrated Tyr-49 in water and 75% glycerol as a function of pH and peptide. In glycerol, the CD spectrum is virtually constant between pH 6 and pH 2, at both pH values being similar to but not quite identical with that in H_2O at pH 2. However, addition of peptide to the nitrated protein in both the absence and presence of glycerol produces the same enhanced nitrotyrosine spectrum, indicating both that peptide binding occurs in glycerol and that the high-glycerol refractive index does not in itself perturb the CD spectrum. [It is relevant to note here that the tyrosine of the complexed protein appears to be completely solvent exposed (Griffin et al., 1973).] The results indicate a local effect of glycerol on nitrotyrosine conformation in the absence of peptide and, in accord with the fluorescence data, suggest that this effect is to alter the environment of Tyr-49 at pH 6 toward that of the pH 2 environment.

The failure to observe a low-pH transition in glycerol is not due to a change in the practical significance of pH measurements in glycerol. We have compared the *apparent* pK values (i.e., the pH meter readings using a glass electrode) of a series of buffers at 50% ionization in 0.16 M KCl with those in 75% glycerol (0.05 M in KCl) and observed no shifts greater than 0.3 pH units. The buffers included acetate, phosphate (second pK_a), Tris, and *p*-nitrophenol; additionally, the apparent hydroxyl pK of the nitrated neurophysin tyrosine, as measured spectroscopically (Breslow & Weis, 1972), was shifted upward by no more than 0.2 pH units in glycerol, and large pH-dependent differences in the fluorescence of the free amino acid tyrosine are unaffected by glycerol. Therefore, *if* the lack of a pH transition in glycerol results from a large change in one or more pK values, this change must result from highly selective solvent interactions at sites adjacent to Tyr-49. In either event the results indicate that local effects on the conformation of Tyr-49, in the absence of general conformational changes, can abolish the acid transition of Tyr-49.

Comparison of Fluorescence and Circular Dichroism Titrations. The similar effect of glycerol on both the low-pH fluorescence and CD transitions argues for a similar origin of both transitions and it would in any event be surprising if the optical activity of Tyr-49 was not sensitive to the titration of a neighboring group. Nonetheless, we present here data which argue against a strict identity of both transitions. As shown below, differences between nitrated neurophysins-I and -II in their CD transition pK values (Breslow & Gargiulo, 1977) are not matched by corresponding differences in the fluorescence titrations of the unmodified proteins. (Note that fluorescence titrations cannot be performed with the nitrated protein, which is nonfluorescent, and that CD titrations cannot be accurately carried out with unmodified neurophysin.) Additionally, carboxypeptidase B digestion of neurophysin-II, in contrast to its effects on fluorescence titration, does not bring the CD titration curves of neurophysin-I and -II into superposition.

The relationships among the fluorescence and CD titrations are shown more specifically in Table IV where the intrinsic pK values for each titration, calculated as above with the aid of potentiometric titration data, are tabulated. The assumptions were made that the potentiometric titrations of the nitrated and native proteins are the same below pH 6 and that carboxypeptidase treatment does not alter the potentiometric titration of neurophysin-II below pH 6 (except for a change in net charge). Each CD titration could also be described in terms of a single pK with average standard errors comparable to those calculated above (± 0.07 pH units) for the fluorescence titrations of neurophysin-I and -II.[2] There are

Table IV: Intrinsic pK Values of Fluorescence and CD Titrations[a]

protein	fluorescence pK°, 5×10^{-5} M protein	CD pK° 2×10^{-5} M protein	CD pK° 5×10^{-5} M protein
neurophysin-I	4.61	4.84	5.05
neurophysin-II	4.59	4.51	
carboxypeptidase B treated neurophysin-II	4.69	4.41	

[a] Calculated to correct for changes in net charge during the course of the titration as described in the text.

no significant fluorescence pK differences among the three proteins, but the CD titration of nitrated neurophysin-I differs significantly from that of the others as well as from the fluorescence titration of unmodified neurophysin-I; differences between the CD and fluorescence titrations of the carboxypeptidase-treated protein also appear significant, but different protein concentrations were used (vide infra).

In comparing fluorescence and CD titrations, it is relevant to point out that the CD titration has been shown to shift to higher pH with increasing protein concentration (Breslow & Gargiulo, 1977). Technical problems have prevented a similar study of the concentration dependence of the fluorescence titration. It is therefore possible that the differences between fluorescence and CD titrations and among the different CD titrations represent nitration-induced differences in dimerization or other properties. Alternatively the data may signify that the CD transition involves residues different from or additional to those responsible for the fluorescence titration (see Discussion).

Discussion

The fluorescence increase of Tyr-49 attendant to binding peptides which do not contain tyrosine parallels other observations which indicate that peptide binding alters the environment of Tyr-49. Protein tyrosines are quenched by interactions with peptide bonds and can also be quenched by carboxylates and disulfides (cf. Cowgill, 1976); in the present instance, in addition to peptide-bond quenching, carboxylate quenching is likely (vide supra), but there is no indication of important disulfide quenching since reduction and carboxamidomethylation have little effect on fluorescence. Conformational changes attendant to binding must therefore reduce the quenching effect of peptide bonds and/or carboxylates. Since the magnitude of the peptide-mediated fluorescence increase considerably exceeds that of the pH-mediated increase, the results indicate that a large fraction of the peptide-mediated fluorescence increase is due to reduction of peptide-bond quenching. This conclusion is supported by the fact that the quantum yield of Tyr-49 in the bound state can be shown to be markedly greater than that of analogous tyrosine-containing oligopeptides. Two general mechanisms have been invoked for the diminution of peptide-bond quenching. One, an increase in the hydrophobicity of the environment (Cowgill, 1976), is unlikely here since UV difference absorption studies suggest that Tyr-49 moves to a more polar environment on binding (Griffin et al., 1973). The second, a change in tyrosine rotamer distribution (Gauduchon

[2] The standard error in the CD titrations is less when the data are corrected for the effects of changes in net charge using the Linderstrom–Lang model than when the data are analyzed, as initially (Breslow & Gargiulo, 1977) as the uncomplicated titration of a monobasic acid.

& Wahl, 1978), perhaps associated with a local change to the α-helical state (Cowgill, 1976), is attractive since it would account for the high optical activity induced in nitrated Tyr-49 when peptides are bound (Breslow & Weis, 1972).

The opposing effects of binding on the fluorescence of Tyr-49 and the hormone tyrosine is in accord with the observation (Griffin et al., 1973) that, in contrast to Tyr-49, Tyr-2 is transferred to an apolar environment on binding. Two possible sources of strong quenching in this environment would be proximity to the active site protein carboxylate (cf. Breslow, 1974) or hydrogen bonding of the phenolic hydroxyl. A distinction between these is not possible on the basis of the present data. Nonetheless, the quenching of Tyr-2 on binding provides a mechanism for probing the distance between Tyr-49 and Tyr-2 of hormones bound to the principal hormone binding site. This distance has been of particular interest since the demonstration of a nuclear Overhauser effect between Tyr-49 and residue 2 of bound peptides (cf. Balaram et al., 1973) suggested a close proximity between these two residues. However, recent spin-label studies (Lord & Breslow, 1978) have raised the possibility that the nuclear Overhauser effect may be mediated by a second very weak peptide binding site much closer to Tyr-49 than the principal site. Since steady-state energy transfer, as distinguished from spin-label proton relaxation, should only be affected by secondary binding sites to the extent that they are thermodynamically significant, and since such sites are not significant in most of the studies here (vide supra), the distances calculated in Table II are of particular interest since they indicate either that the distance between Tyr-49 and the strong site is relatively large or that the orientation factor, κ^2, is highly unfavorable for energy transfer. For example, distances calculated by use of $\kappa^2 = 4$ are maximum distances representing the theoretically most favorable orientation, those calculated by use of $\kappa^2 = 0.667$ assume random orientation between Tyr-49 and Tyr-2, and the value of $\kappa^2 = 0.07$ corresponds to a highly unfavorable orientation (cf. Dale & Eisinger, 1975). Although lower values of κ^2 and hence smaller calculated distances are theoretically possible, spin-label studies (Lundt, 1978; Lord & Breslow, 1978) strongly suggest that the distance between Tyr-49 and Tyr-2 at the strong site cannot be less than 5 Å. For example, the distance between the ortho ring protons of Tyr-49 and the unpaired electron of the spin-label 4-(glycyl-L-phenylalaninamido)-2,2,6,6-tetramethylpiperidinyl-1-oxy at the strong site was ≥15 Å. Since this electron is approximately 10 Å from the ortho ring protons of the spin-label phenylalanine, a 5 Å minimum distance between Tyr-49 and residue 2 of the spin-label is calculated (Lord & Breslow, unpublished results).

With reference to changes in neurophysin fluorescence properties between pH 6 and 3 in the absence of bound peptides, the lack of effect of disulfide cleavage and the localized effects of glycerol argue strongly that the transition results from decreased quenching by the carboxylate side chain of either Glu-46 or Glu-47 upon protonation. For intramolecular quenching, the ability of the carboxylate to contact the tyrosine side chain is important; e.g., the glutamate carboxyl of the peptide Glu-Tyr is not effective as an intramolecular quencher, and several proteins are known to undergo significant changes in the degree of carboxyl protonation without changes in tyrosine fluorescence (Cowgill, 1976). In this context, the effects of guanidine and glycerol on the acid transition, and of glycerol on nitrotyrosine ellipticity, are all explained as due to the disruption at neutral pH of the locally determined interactions between Tyr-49 and its neighboring carboxyl. The assignment of a direct quenching effect of Glu-46 or Glu-47

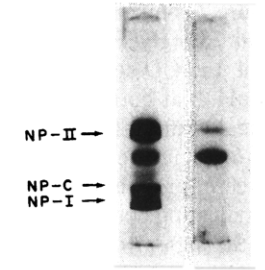

FIGURE 8: Gel electrophoresis at pH 9.5 of unfractionated bovine neurophysin (left) and the product of carboxypeptidase A digestion of neurophysin-II (right). Direction of migration is from top to bottom. The gel on the left is overloaded so that the minor components can be seen.

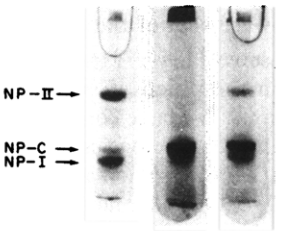

FIGURE 9: Gel electrophoresis at pH 7.5 of unfractionated bovine neurophysin (left), carboxypeptidase B treated neurophysin-II (center), and a mixture of crude neurophysin and carboxypeptidase B treated neurophysin-II (right). Direction of migration is from top to bottom. The center gel is overloaded. All components of crude neurophysin migrated 3% more slowly in the gel on the right than in the gel on the left. The mobility of neurophysin-C relative to that of neurophysin-II and neurophysin-I in crude neurophysin is 1.73 and 0.91, respectively. The mobility of carboxypeptidase B treated neurophysin-II relative to that of neurophysins-II and -I is 1.75 and 0.92, respectively.

on Tyr-49 is also in accord with the elevated pK_a of the phenolic hydroxyl in nitrated neurophysin (Furth & Hope, 1970; Breslow & Weis, 1972). It should be stressed, however, that the above arguments do not rule out the possibility of conformational changes mediated by titration of Glu-46 or Glu-47; the slightly abnormal value of the ΔH of the fluorescence titration (vide supra) is compatible with an associated conformational change of low energy and, to the extent that the carboxyl–tyrosine interactions might involve weak hydrogen-bond formation, reorientation of the glutamate and tyrosine side chains upon carboxyl protonation would be expected.

A surprising feature of the data is the failure of the CD transition of the nitrated protein to correspond with the fluorescence titration of the native protein. A direct or indirect effect of nitration on the glutamate pK is possible, but, to account for the data, this effect would have to be different for different neurophysins. An alternative explanation however is that, while the principal factor controlling the CD transition is protonation of the neighboring carboxyl, the CD of the nitrotyrosine is also sensitive to a pH-dependent transition elsewhere in the protein, which is not identical in the different neurophysins; this transition would be assumed to introduce a relatively small perturbation in the CD titration but one significant enough to generate an apparent pK shift. The greater sensitivity of the CD spectrum of the protein nitrotyrosine to the conformation of the entire protein than that of the fluorescence spectrum is actually demonstrable by the fact that the nitrotyrosine CD spectrum is lost when the disulfides are cleaved or rearranged (Menendez-Botet & Breslow, 1975) but the fluorescence properties are unaltered (vide supra). In earlier studies (Breslow & Gargiulo, 1977), the possibility was raised that the carboxyl responsible for the

low-pH CD transition might be the active site carboxyl. While all the factors contributing to the CD transition have yet to be defined, the present studies indicate that the carboxyl responsible for the low-pH *fluorescence* transition is unlikely to be the active site carboxyl. Thus, differences between native neurophysins-I and -II in the pH dependence of binding in the carboxyl p*K* region (Camier et al., 1973; Klausner et al., 1978) are not paralleled by the same differences in fluorescence titration; additionally, affinity-labeling studies (Walter & Hoffman, 1973; Capra & Walter, 1975) have led to tentative identification of the active site carboxyl as either Asp-30 or Glu-31.

Acknowledgments

We are particularly grateful to Dr. Martin Sonenberg of Sloan-Kettering Institute and to Dr. Arleen Rifkind of the Department of Pharmacology for donating time on the Perkin-Elmer spectrofluorometers used for our fluorescence studies. We are also grateful to Dr. Daniel Wellner of this department for all amino acid analyses and to Erin Lawton for very capable technical assistance.

Appendix

The carboxyl terminal sequence of bovine neurophysin-II is Arg-Arg-Val (Chauvet et al., 1975). Amino acids liberated on treatment with carboxypeptidase A were found to be arginine and valine in the ratio of 0.7:1; amino acids liberated by digestion with carboxypeptidase B were arginine and valine in the ratio 1.9:1. Gel electrophoresis patterns of the carboxypeptidase A treated protein are shown in Figure 8, together with that of crude neurophysin for comparison. The slowest moving component of the crude protein is neurophysin-II (Breslow et al., 1971) and the component moving just ahead of it has not been identified. Data in Figure 8 show that the carboxypeptidase A treated protein has a minor component which migrates like neurophysin-II and a major component which moves as the unidentified component of the crude protein. The relative quantities of the two components are in accord with the amino acid analysis which indicates that removal of the penultimate arginine has not proceeded to completion; the faster component represents protein which has lost the Arg-Val sequence.

The ratio of Arg/Val released by treatment with carboxypeptidase B indicates that this treatment liberates the three carboxyl-terminal residues from neurophysin-II. Figure 9 shows electrophoretic patterns at pH 7.5 of the carboxypeptidase B treated protein, crude neurophysin, and a mixture of the carboxypeptidase B treated protein and crude neurophysin. The second most rapidly moving major component in crude neurophysin is neurophysin-C (Rauch et al., 1969; Breslow et al., 1971). The results show that carboxypeptidase B treatment results in a relatively homogeneous protein which comigrates with neurophysin-C. Comigration of the product of carboxypeptidase digestion and neurophysin-C was also observed on electrophoresis at pH 9.5.

The above results, in themselves, do not prove the identity of the carboxypeptidase products of neurophysin-II digestion with the different crude neurophysin components. However, when considered together with the data of Wuu & Crumm (1976), which indicate that, in the pig, one of the neurophysins is derived from another by removal of the carboxyl-terminal Arg-Arg-X tripeptide, the results are strongly suggestive of such a relationship. This conclusion is supported by the amino acid composition of bovine neurophysin-C (cf. Rauch et al., 1969; Breslow, 1974) which is within experimental error of that of bovine neurophysin-II except that neurophysin-C contains two less arginine residues and one less valine than neurophysin-II. Additionally, De Mey & Vandesande (1976), on the basis of immunochemical studies, have concluded that neurophysin-C and neurophysin-II are very closely related.

We have also studied the binding of peptides to carboxypeptidase B treated neurophysin-II and its circular dichroism spectrum. CD spectra were typical of those seen for native neurophysin-II and binding properties of the nitrated protein were identical with those (Breslow & Gargiulo, 1977) of the nitrated native protein. The results indicate that the carboxyl-terminal three residues do not play a significant role in binding to the principal peptide site [which is the only site demonstrable in the nitrated protein (Cohen et al., 1975)] although it remains possible that they affect the availability of the secondary hormone sites (cf. Camier et al., 1973; Glasel et al., 1976; Nicolas et al., 1976) in the unmodified protein.

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Folylpoly-γ-glutamates as Cosubstrates of 10-Formyltetrahydrofolate:5′-Phosphoribosyl-5-amino-4-imidazolecarboxamide Formyltransferase†

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ABSTRACT: N^{10}-Formyltetrahydropteroylpoly-γ-glutamates (N^{10}-formyl-H_4PteGlu$_n$) having n = 1, 3, 4, 5, 6, and 7 glutamyl residues have been tested as cosubstrates of the purine biosynthesis enzyme 10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase (AICAR transformylase) of chicken liver. The cosubstrates were synthesized by solid-phase synthesis, reduced catalytically, and formylated; a purified enzyme preparation was used and assayed spectrophotometrically following the ΔOD at 298 nm resulting from conversion of the formylated folate to the free tetrahydro form. K_m values and V_m values determined at saturating concentrations of AICAR and at 25 and 150 mM KCl were used to calculate the relative specificity constants V_m/K_m for the N^{10}-formyl-H_4PteGlu$_n$. At physiologic [K^+] (150 mM) they were 1.0, 52, 250, 93, 120, and 59 and at the lower (25 mM) [K^+] the relative specificity constants were 1.0, 64, 78, 34, 48, and 37 for n = 1, 3, 4, 5, 6, and 7, respectively. The poly-γ-glutamates are clearly the preferred cosubstrates, particularly when tested at physiologic [K^+]. The maximal relative specificity constant observed with N^{10}-formyl-H_4PteGlu$_4$ supports the hypothesis that regulation of certain pathways of one-carbon metabolism may operate via alterations of the poly-γ-glutamyl chain length. No inhibition by the unnatural (*d*) isomers of the N^{10}-formyl-H_4PteGlu$_n$ was observed.

The enzyme 10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase (EC 2.1.2.3), from here on referred to as AICAR transformylase, was originally purified by Flaks and co-workers and Warren and co-workers (Flaks et al., 1957a,b; Warren et al., 1957). This enzyme catalyzes the transfer of the formyl moiety from N^{10}-formyltetrahydropteroylglutamate (N^{10}-formyl-H_4PteGlu) to the 5-amino position 5′-phosphoribosyl-5-formamido-4-imidazolecarboxamide (AICAR) to yield 5′-phosphoribosyl-5-formamido-4-imidazolecarboxamide (formyl AICAR) and tetrahydropteroylglutamate (H_4PteGlu).

We have chosen to investigate AICAR transformylase in order to determine enzyme–cosubstrate specificity with respect to the poly-γ-glutamyl moiety of the folate cosubstrate (N^{10}-formyl-H_4PteGlu$_n$; n = 1, 3, 4, 5, 6, and 7). It is now well established that pteroylpoly-γ-glutamates are the naturally occurring forms of the folate cosubstrates (Baugh & Krumdieck, 1971). One of us (C.L.K.) has postulated that the poly-γ-glutamyl chain serves a role in the regulation of one-carbon metabolism (Krumdieck et al., 1977). An important mechanism which would fulfill this role would be the regulation of folate-dependent pathways via enzyme–cosubstrate specificity mediated by alterations in the poly-γ-glutamyl chain length. In this paper we report the kinetic parameters V_m and K_m and the ratio V_m/K_m which we use as a measure of the enzyme–cosubstrate specificity. The kinetic parameters and the specificity constants, V_m/K_m, were determined at low (25 mM KCl) and physiologic (150 mM KCl) potassium ion concentration. These studies were conducted by use of a partially purified chicken liver enzyme and a new spectrophotometric assay for AICAR transformylase.

Experimental Procedures

Materials

Substrates. Pteroylpoly-γ-glutamates were synthesized by the method of Krumdieck & Baugh (1969) and were reduced to their 5,6,7,8-tetrahydro forms (H_4PteGlu$_n$; n = 1, 3, 4, 5, 6, and 7) by the method of Hatefi et al. (1960). N^5,N^{10}-Methenyltetrahydropteroylpoly-γ-glutamates (N^5,N^{10}-

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