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Partial Digestion of Neurophysins with Proteolytic Enzymes: Unusual Interactions between Bovine Neurophysin II and Chymotrypsin†

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ABSTRACT: Bovine neurophysin II was partially digested by chymotrypsin and by chymotrypsin followed by carboxypeptidase B to produce large fragments collectively representing deletions of residues 1–5 and 91–95. All such fragments were capable of binding peptides to the principal hormone-binding site of neurophysin with normal or near-normal affinity, indicating that residues 1–5 and 91–95 do not directly participate in binding. In addition, preliminary results with thermolysin-derived fragments suggested that residue 6 does not participate in peptide binding. During the course of chymotrypsin studies, it was demonstrated that bovine neurophysin II behaves as a transient competitive inhibitor of chymotrypsin; for neurophysin–peptide complexes, $K_i \simeq 8 \times 10^{-6}$ M. This inhibition is dependent on neurophysin conformation and is relieved by the anomalous preferential splitting by chymotrypsin of Arg–Arg and Phe–Pro bonds near the carboxyl terminus of neurophysin II. It is suggested that this phenomenon might reflect the interaction of neurophysin II with a chymotrypsin-related enzyme in the pituitary. One approach used in the study of binding properties of proteolytically modified neurophysin was affinity chromatography; the preparation and properties of a conveniently prepared affinity column for neurophysin are described.

The noncovalent interaction of neurophysins with oxytocin and vasopressin involves binding of the amino-terminal three residues of the hormones to largely unassigned residues of the protein (Breslow, 1979). A question of particular interest is the extent to which the binding site on the protein involves its internally duplicated segments (residues 12–31 and 60–77) or the nonduplicated segments at the ends and central region of the polypeptide chain [cf. Capra et al. (1972), Camier et al. (1973), and Breslow (1979)]. One approach to identifying the hormone-binding region(s) of the protein is to examine the hormone-binding ability of polypeptide fragments derived from the native protein by partial proteolytic digestion. In the present study, we report on the isolation of such fragments, slightly smaller than native neurophysin, from the action of chymotrypsin and carboxypeptidase B on bovine neurophysin II and give some preliminary results from studies with thermolysin. Chymotrypsin was the most intensively studied enzyme because we were also interested in the ability of the neurophysins to inhibit the activity of trypsin and chymotrypsin. This interest was initially prompted by the fact that the neurophysins are particularly rich in disulfides, a property characteristic of several known inhibitors of trypsin and chymotrypsin [e.g., see Barker & Dayhoff (1976)]. In unpublished trial studies, we found that bovine neurophysin II did not inhibit the activity of trypsin. In the present study, we report a marked inhibition of chymotrypsin activity by bovine neurophysin II and demonstrate that this inhibition is associated with the anomalous affinity of chymotrypsin for peptide bonds at the carboxyl terminus of bovine neurophysin II.

During this work, we found it useful to assess the binding activity of neurophysin fragments by affinity chromatography. Accordingly, we developed a new affinity chromatographic procedure that makes use of the affinity of the principal hormone-binding site of neurophysin for the first two residues of oxytocin and vasopressin (Cys-Tyr). This procedure involves coupling L-cysteinyl-L-tyrosine amide to a Bio-Gel support by disulfide linkage; its advantage over other affinity chromatographic methods for neurophysin [e.g., see Chaiken (1979)] is the simplicity of column preparation.

Materials and Methods

Neurophysins, Peptides, and Enzymes. Bovine neurophysins I and II were prepared and quantitated as previously described (Breslow et al., 1971). Carboxypeptidase B treatment of native neurophysin II was performed with diisopropyl fluorophosphate (DFP)[1] treated carboxypeptidase B (Sur et al., 1979) and quantitatively liberated the three carboxyl-terminal residues.

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[1] Abbreviations: CD, circular dichroism; Phe-TyrNH$_2$, L-phenylalanyl-L-tyrosine amide; Phe-PheNH$_2$, L-phenylalanyl-L-phenylalanine amide; Leu-TyrNH$_2$, L-leucyl-L-tyrosine amide; Arg-Val, L-arginyl-L-valine; DFP, diisopropyl fluorophosphate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; NCDC, 2-nitro-4-carboxyphenyl *N*,*N*-diphenylcarbamate; GPNA, glutaryl-L-phenylalanyl-*p*-nitroanilide; NP, neurophysin; NaDodSO$_4$, sodium dodecyl sulfate.

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Carboxypeptidase A digestion of neurophysin II was performed with DFP-treated carboxypeptidase A as previously described (Sur et al., 1979) with the exception that a 25:1 ratio of neurophysin to enzyme was used and the incubation was carried out for 2 days. For preparation of reduced, alkylated neurophysin II, the disulfides were completely reduced with excess dithiothreitol and alkylated with iodoacetamide as previously described (Sur et al., 1979) except that guanidine, which was found to be unnecessary, was omitted; the modified protein was separated from excess reagent by chromatography on Sephadex G-25 in 0.1 M acetic acid, lyophilized, and found to have the same amino acid composition as the native protein with the exception of the expected cystine alkylation.

L-Phenylalanyl-L-tyrosine amide, L-phenylalanyl-L-phenylalanine amide, and L-argininyl-L-valine were obtained from Vega Biochemicals; their composition and homogeneity were confirmed by amino acid analysis and thin-layer chromatography (TLC). Oxytocin and vasopressin samples were those previously described (Sur et al., 1979). Two chymotrypsin preparations were used. These were Sigma 3× crystallized bovine pancreatic α-chymotrypsin (40–50 units/mg of protein), reportedly prepared free of autolysis products, and Sigma bovine pancreatic α-chymotrypsin (40–50 units/mg of protein) pretreated with N^{α}-*p*-tosyl-L-lysine chloromethyl ketone to inhibit trypsin; both preparations gave the same results. DFP-treated carboxypeptidases A and B were from Sigma. Thermolysin was type X protease from Sigma.

Preparation of Chymotrypsin-Modified and Thermolysin-Modified Neurophysin II. For large-scale preparation of the "final products" of chymotrypsin digestion of neurophysin II, native neurophysin II (4 mg/mL) and Phe-PheNH_2 or Phe-TyrNH_2 (1 mg/mL) in 0.05 M Tris buffer (pH 7.6, containing 0.02 M $CaCl_2$) were treated with chymotrypsin (0.25 mg/mL) for 24–28 h at room temperature. The pH was lowered to 3 and the mixture chromatographed on Sephadex G-50 in 0.1 M acetic acid. The protein peak (minus its leading edge which contains chymotrypsin) and all fractions containing released peptides and amino acids were separately pooled, lyophilized, and analyzed as reported in the text. The crude final protein product of chymotrypsin digestion was further fractionated by ion-exchange chromatography on a 1.6 × 70 cm DEAE-50 column at pH 5.9, 24 °C, in pyridine–acetate buffer (0.823 M pyridine and 0.158 M acetic acid). Two major peaks were obtained and lyophilized: peak A (140–200-mL elution volume) and peak B (310–480-mL elution volume). Treatment of lyophilized peak B from this fractionation (see Results) with carboxypeptidases A and B was carried out by using the same procedures as for the unmodified protein.

For preparation of the initial cleavage products of chymotrypsin digestion, the native protein (2 mg/mL) in the presence of Phe-TyrNH_2 (0.25 mg/mL) was treated with chymotrypsin (0.12 mg/mL) for 75 min at room temperature in the Tris–$CaCl_2$ buffer cited above; protein and peptides were separated as described above. Other conditions for chymotrypsin digestion are given in the text when appropriate. For studies of the effects of inhibitors on the course of chymotrypsin digestion, chymotrypsin was pretreated either with 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (NCDC) (Sigma) by using the procedure of Erlanger & Edel (1964) or with TLCK (by the manufacturer) or with 1,10-phenanthroline (5×10^{-4} M, pH 7.6, 1-h incubation at room temperature).

Thermolysin-digested fragments were prepared by incubating native neurophysin II (4 mg/mL) plus Phe-TyrNH_2 (0.3 mg/mL) in 0.05 M Tris buffer, pH 7.6, with thermolysin (0.2 mg/mL). Incubations were carried out for 24 h at room temperature. The reaction was stopped by lowering the pH to 3 and the mixture chromatographed on Sephadex G-50 and analyzed as described above for chymotrypsin digestions. The lyophilized protein peak from Sephadex G-50 was then further fractionated by chromatography on DEAE-Sephadex A-50 at pH 5.9, also as described above for chymotrypsin digestion products except that 1×10^{-4} M 1,10-phenanthroline was added to the equilibrating and eluting buffer to prevent further action of any residual thermolysin. The sharp leading peak of the chromatogram, representing the 99–147-mL effluent, was collected and lyophilized for the studies described in the text.

Affinity Column Preparations. Centrifuge-packed Affi-Gel 401 (5 mL) (Bio-Rad) was washed with 5 mL of 0.05 M dithiothreitol (in 0.1 M phosphate, pH 7.68, containing 0.001 M EDTA) to ensure that all the sulfhydryls attached to the agarose support were in the reduced form. The slurry was allowed to stand 30 min, and the gel and supernatant were separated by centrifugation. The Affi-Gel was then washed with several 5-mL rinses of 0.1 M phosphate buffer, pH 6.2, separating the gel and supernatant by centrifugation, until most of the supernatant sulfhydryls were removed as monitored by Ellman's reagent (Ellman, 1964). The gel was then mixed with 50 mg of L-cystinylbis(L-tyrosine amide) (Vega Biochemicals) dissolved in 5 mL of 0.1 M phosphate buffer, pH 7.6, a step which leads to disulfide interchange of peptide with the gel and also to formation of reduced peptide. The slurry was allowed to stand overnight and then poured into a 5-mL graduated pipet for use as a column. The column was washed with 0.1 M phosphate, pH 6.2, until all the reduced peptide was removed as evidenced by a negative Ellman's test on the column effluent.

For a typical chromatographic run, 1–15 mg of protein was dissolved in 1–2 mL of 0.1 M phosphate, pH 6.2, and applied to the column. The column was eluted with the same buffer, collecting 30 fractions of 1 mL each, after which the buffer was changed to 0.1 M phosphate, pH 2.1, and elution continued. Nonbinding protein emerged as a sharp peak centered at 6 mL of pH 6.2 buffer, and native protein eluted at 6 mL of pH 2.1 buffer. In several studies, elution with pH 6.2 buffer was continued for 50 mL, but native protein did not elute until 6 mL of pH 2.1 buffer. Chromatographic runs were monitored either by UV absorbance at 260 and 280 nm or by Coomassie Blue G-250 (Bradford, 1976; Sedmark & Grossberg, 1977).

Other Methods. Chymotrypsin activity was monitored spectrophotometrically at room temperature with 8×10^{-4} M glutaryl-L-phenylalanyl-*p*-nitroanilide (GPNA) as substrate (Erlanger et al., 1966). Polyacrylamide gel electrophoresis was performed by using a discontinuous 7.5% running gel system, with a running pH of 9.5, as previously described (Breslow et al., 1971) except that gels were stained with Coomassie Blue G-250 (Reisner et al., 1975). Standards of unfractionated neurophysin prepared as previously described (Breslow et al., 1971; Walter & Breslow, 1974) were electrophoresed with each run. $NaDodSO_4$ gel electrophoresis was carried out in 12.5% polyacrylamide gels with urea (Swank & Munkres, 1971); fluorescamine-labeled samples were pretreated with 8 M urea, 1% $NaDodSO_4$, and 1% mercaptoethanol at pH 6.8 at 100 °C for 5 min to ensure reduction of disulfides, and peptides and protein were monitored by both fluorescamine fluorescence and Coomassie Blue R stain. Amino acid analyses were performed with a Durrum automated analyzer; for hydrolyzed samples, hydrolysis was carried out for 24 h in 6 N HCl at 120 °C in vacuo. Automated Edman sequencing of component 3b (disulfides intact) was

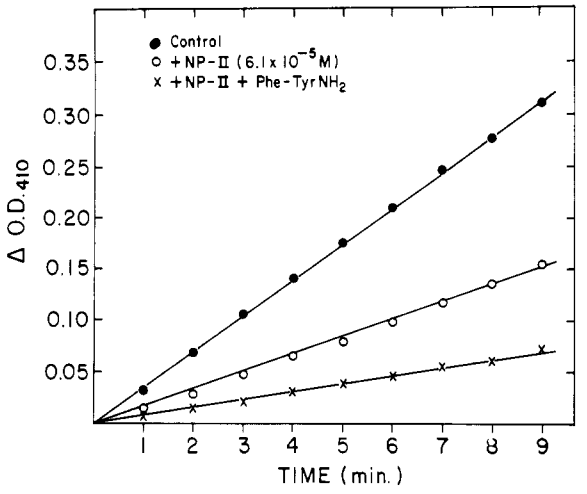

FIGURE 1: Time course of GPNA hydrolysis during typical chymotrypsin assays. Conditions were as follows: 8×10^{-4} M GPNA, pH 7.6, in 0.05 M Tris containing 0.04 M $CaCl_2$; room temperature; 0.083 mg/mL chymotrypsin. (●) No additions; (○) in the presence of 6.1×10^{-5} M neurophysin II; (×) in the presence of 6.1×10^{-5} M neurophysin II and 2.4×10^{-3} M Phe-TyrNH_2. Readings are begun immediately after introduction of the enzyme.

carried out by Dr. Gary Litman. Amino acid identification was made by high-pressure liquid chromatography (HPLC) of the phenylthiohydantoins and amino acid analyses of the back-hydrolysis products; Cys residues were identified by the absence of product formation at a given step. Circular dichroism studies were carried out by using a Cary 60 spectropolarimeter equipped with a Model 6001 CD attachment or a Jobin-Yvon Mark 5 dichrograph as previously described (Breslow et al., 1971, 1973). For determination of CD binding isotherms, protein samples were nitrated by the method of Furth & Hope (1970) and binding studies and calculations performed by the method of Breslow et al. (1973).

Results

Inhibition of Chymotrypsin Activity by Bovine Neurophysin II and Its Peptide Complexes. Figure 1 shows the time course of chymotrypsin substrate hydrolysis during a typical assay in the absence and presence of bovine neurophysin II. The concentration of neurophysin II used in this study produced 50% inhibition of enzyme activity. In the presence of both neurophysin and the peptide Phe-TyrNH_2, which binds to the hormone-binding site of neurophysin (Breslow et al., 1973), inhibition increases to 80% (Figure 1). Note that the concentration of Phe-TyrNH_2 used is sufficient to give approximately 85% neurophysin saturation at the pH of the assay (7.6) and that by itself Phe-TyrNH_2 leads to no inhibition of chymotrypsin activity. It is relevant that the source of the inhibition of chymotrypsin activity cannot be the binding of substrate (GPNA) to neurophysin since the concentration of neurophysin is less than one-tenth of the GPNA concentration.

Table I summarizes the results of a series of studies of the effects of bovine neurophysins I and II on chymotrypsin activity and of the effect of different peptides which bind to the hormone-binding site of neurophysin on chymotrypsin inhibition. Again, in no case did the peptides themselves inhibit chymotrypsin action on GPNA. The important result is that neurophysin II is a markedly more effective inhibitor than neurophysin I and that binding of all peptides produced the same increase in inhibitory behavior. Additionally (Table I), the inhibitory effect of neurophysin II is significantly reduced by reduction and alkylation, a modification that leads to protein unfolding (Menedez-Botet & Breslow, 1975).

Table I: Effects of Different Neurophysins and Neurophysin-Peptide Complexes on the Activity of Chymotrypsin toward GPNA

protein	peptide	% inhibition[a]
neurophysin II (6×10^{-5} M)		51
	Phe-TyrNH_2 (2×10^{-3} M)	0
neurophysin II (6×10^{-5} M)	Phe-TyrNH_2 (2×10^{-3} M)	76
neurophysin II (3×10^{-5} M)		38
	oxytocin (4×10^{-4} M)	0
neurophysin II (3×10^{-5} M)	oxytocin (4×10^{-4} M)	59
neurophysin II (3×10^{-5} M)		36
	lysine vasopressin (4×10^{-4} M)	0
neurophysin II (3×10^{-5} M)	lysine vasopressin (4×10^{-4} M)	51
neurophysin I (3×10^{-5} M)		0
neurophysin I (3×10^{-5} M)	oxytocin (4×10^{-4} M)	0
neurophysin I (6×10^{-5} M)		6
neurophysin I (6×10^{-5} M)	Phe-TyrNH_2 (2×10^{-3} M)	21
reduced, alkylated neurophysin II (6×10^{-5} M)		16
reduced, alkylated neurophysin II (6×10^{-5} M)	Phe-TyrNH_2 (2×10^{-3} M)	19

[a] Calculated as [1 − (observed rate)/(control rate)] × 100, where the control rate is that in the presence of substrate (GPNA) with no other added peptides or proteins. The concentrations of protein and peptide given are those present during the assay. In the presence of peptides, the native proteins are approximately 70% saturated with hormone or 80% saturated with Phe-TyrNH_2; reduced, alkylated protein does not bind peptide.

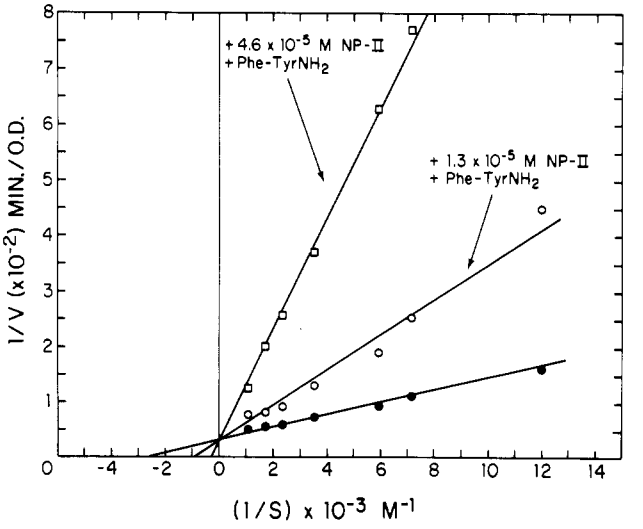

FIGURE 2: Lineweaver–Burk plot of the effect of the complex of Phe-TyrNH_2 with neurophysin II on chymotrypsin activity. Buffer conditions, enzyme concentration, and procedure are described in Figure 1. (●) No additions; (○) in the presence of 1.3×10^{-5} M neurophysin II and 2.3×10^{-3} M Phe-TyrNH_2; (□) in the presence of 4.6×10^{-5} M neurophysin II and 2.3×10^{-3} M Phe-TyrNH_2.

Figure 2 shows a Lineweaver–Burk plot of the effect of different concentrations of neurophysin II, in the presence of nearly saturating concentrations of Phe-TyrNH_2, on chymo-

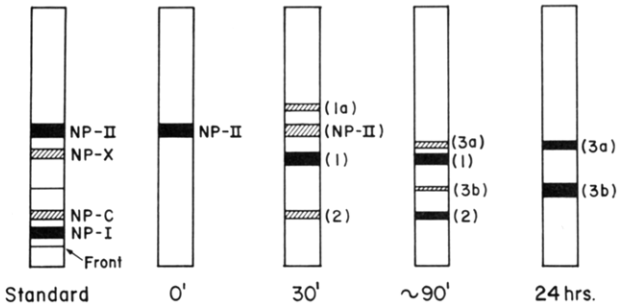

FIGURE 3: Schematic representation of gel electrophoresis patterns obtained during chymotrypsin digestion of bovine neurophysin II in the presence of peptide. Conditions were as follows: 7.5% gel, running pH 9.5, no $NaDodSO_4$, direction of migration is from top to bottom. Sample on far left (standard) is unfractionated neurophysin. Other samples represent purified neurophysin II incubated with chymotrypsin for the approximate time periods indicated in the presence of Phe-$PheNH_2$ or Phe-$TyrNH_2$, using the conditions described under Materials and Methods to obtain the "final products" of digestion. Solid bands indicate the most intensely staining components. Note that component 1a is only a trace component under these conditions and that 3a can often be detected as a minor component at 30 min but is omitted in the 30-min schematic for simplification.

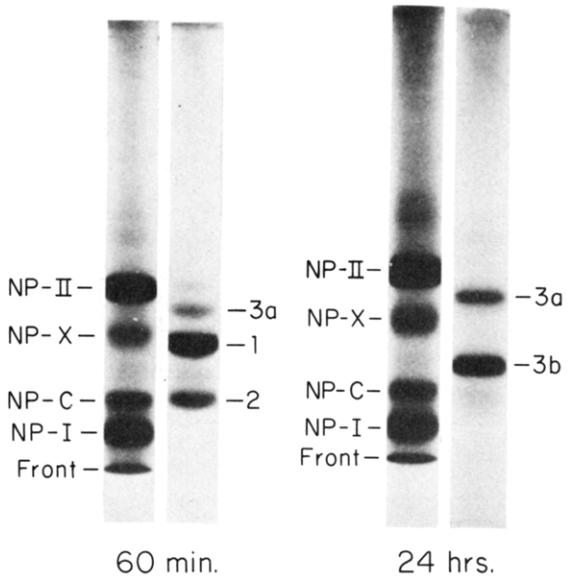

FIGURE 4: Gel electrophoresis patterns on neurophysin II incubated with chymotrypsin in the presence of peptide under different conditions. Gel conditions are those indicated in Figure 3. (Left panels) Neurophysin II was incubated with peptide and chymotrypsin as described in Table II. The sample electrophoresed here is the 60-min sample of Table II. A gel of unfractionated neurophysin, used as an electrophoresis standard, is shown for comparison. (Right panels) Neurophysin II was incubated with peptide and chymotrypsin for 24 h under conditions described under Materials and Methods to obtain the final digestion products. A standard gel of unfractionated neurophysin is shown for comparison.

trypsin activity. The results indicate that inhibition is largely competitive. From the data, an apparent K_i for the peptide complex of neurophysin II of 8×10^{-6} M can be calculated.

Proteolytic Degradation of Neurophysin II by Chymotrypsin. Chymotrypsin partially digests neurophysin II, in the absence or presence of peptides that bind to its hormone-binding site, as evidenced by changes in electrophoretic behavior. Figures 3 and 4 show the pattern of changes occurring with time for the complex of neurophysin II with Phe-$TyrNH_2$ incubated at pH 7.6 in the presence of chymotrypsin; identical patterns were obtained for the neurophysin complex with Phe-$PheNH_2$. The different components are numbered in order of their appearance. The final pattern shown is stable

Table II: Effect of Preincubation on the Effectiveness of Neurophysin II as a Chymotrypsin Inhibitor[a]

preincubation time (min)	rel inhibitory activity (%)
0	100
60	45 ± 5[b]
120	12 ± 9[b]
240	21 ± 5[b]

[a] Neurophysin II at a final concentration of 6×10^{-5} M was incubated at room temperature at pH 7.6 (0.05 M Tris buffer containing 0.02 M $CaCl_2$) in the presence of Phe-$TyrNH_2$ (1.7×10^{-3} M) and chymotrypsin (0.12 mg). After the incubation period listed above, 0.7 mL of the solution was mixed with 0.5 mL of 1×10^{-3} M GPNA and the rate of GPNA hydrolysis measured. The rate of hydrolysis was compared with that of an identically incubated control sample containing enzyme and Phe-$TyrNH_2$ but no neurophysin. For each sample, the percent inhibition was first calculated as in Table I; results are reported as "relative inhibitory activity" which is (% inhibition by sample)/(% inhibition by unincubated sample) × 100. [b] Range of values given reflects uncertainties in the control rates.

for many days under our conditions. In the absence of peptide, the initial electrophoretic changes and final electrophoretic state are the same as those in the presence of peptide, but components 3a and 3b were generated from intermediate components more rapidly than in the presence of peptide; additionally, a minor intermediate component (1a) that moves more slowly than the native protein and is barely visible in Figure 4 is more prominent in the unliganded protein than in the complex.

The mobility of the electrophoretic components relative to that of the components of unfractionated neurophysin used as a standard (see Materials and Methods) allows evaluation of their net charge relative to the native protein. In the standard (Figures 3 and 4), components designated NP-X and NP-C have been assigned respectively one and two more negative charges than native neurophysin II (Sur et al., 1979) while neurophysin I has three additional negative charges (Breslow et al., 1971). This indicates that chymotrypsin-generated products 1 and 2 probably differ from the native protein by addition of one and two negative charges, respectively,[2] while 3a and 3b behave as if they have zero to one and one to two additional negative charges, respectively. Component 1a behaves as if it is less negative than the native protein.

Effect of Proteolytic Digestion of Neurophysin II on Chymotrypsin Inhibition. Digestion of neurophysin II by chymotrypsin markedly reduces its ability to inhibit chymotrypsin. This can be shown by the decreased ability of the isolated final products of chymotrypsin digestion (vide infra) to inhibit chymotrypsin and also by following the effects of preincubation of neurophysin with chymotrypsin on inhibitory ability; in routine assays, neurophysin and chymotrypsin are not preincubated. Table II shows the loss of inhibition activity by a neurophysin–peptide complex after different preincubation times with neurophysin. The results indicate that the principle loss of inhibition occurs within the first hour of incubation.

[2] Electrophoretic component 1 moves just slightly faster than NP-X. In our earlier study (Sur et al., 1979), we reported that neurophysin II, from which the terminal Arg-Val had been removed by carboxypeptidase A, and which accordingly differed by one charge from the native protein, had a mobility identical with that of NP-X. We now find that the carboxypeptidase A product also migrates slightly faster than NP-X and identically with component 1. This does not affect the arguments here although it may alter our tentative assignment of NP-X (Sur et al., 1979) as neurophysin II from which the terminal Arg-Val has been removed.

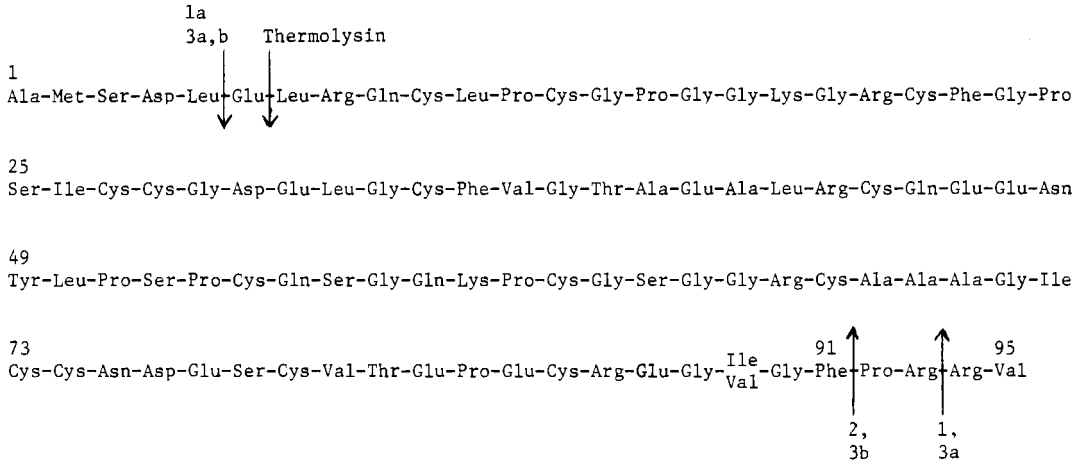

FIGURE 5: Primary structure of bovine neurophysin II. Arrows indicate assigned positions of chymotrypsin cleavage to generate components 1, 1a, 2, 3a, and 3b and assigned position of thermolysin cleavage to generate the slowest electrophoretic component.

Table III: Amino Acid Compositions of Final Products of Chymotrypsin Digestion and of Peptides Released by Digestion[a]

amino acid	control NP-II	released peptides (per mol of protein)[f]	unfractionated chymotrypsin-treated NP-II	fraction A (component 3a)	fraction B (component 3b)
Asp	5.0 (5)	1.2	4.2	4.0 (4)	3.9 (4)
Thr	2.0 (2)	0.1	2.0	1.7 (2)	1.8 (2)
Ser	5.9[b] (6)	1.0[b]	5.0[b]	4.9[b] (5)	4.9[b] (5)
Glu	13.9 (13)	0.4	12.6	13.9 (13)	13.3 (13)
Pro	8.0 (8)	0.7	9.2[c]	7.6 (8)	6.8 (7)
Gly	14-16[d] (15)	0.3	14.8	16.2 (15)	13.9 (15)
Ala	6.4 (6)	1.0	5.3	5.5 (5)	5.1 (5)
Cys	12.3[b] (14)	0	10.8[b]	10.7[b] (14)	9.5[b] (14)
Val	3.7 (4)[e]	1.0	2.9	2.7 (3)	2.7 (3)
Met	1.1 (1)	0.9	0.3	0.21 (0)	0.1 (0)
Ile	2.3 (2)[e]	0.2	2.3	2.4 (2)	2.0 (2)
Leu	6.0 (6)	1.3	4.7	5.1 (5)	5.0 (5)
Tyr	1.0 (1)	0	0.85	0.95 (1)	1.0 (1)
Phe	3.1 (3)	[14][f]	3.0	3.1 (3)	3.0 (3)
His	0 (0)	0	0	0 (0)	0 (0)
Lys	2.0 (2)	0	2.0	2.0 (2)	2.0 (2)
Arg	7.0 (7)	1.4	5.3	6.3 (6)	5.0 (5)

[a] Results for the protein fractions are expressed as moles of amino acid per mole of polypeptide chain normalized to give a value of two Lys residues per chain; there are two Lys residues per chain in the native protein, and none are lost by treatment with chymotrypsin under our conditions. Values in parentheses next to each observed value are theoretical values. For the control sample, these are calculated from the amino acid sequence [e.g., see Chauvet et al. (1977)]; for components 3a and 3b, these are calculated from their structures assigned in the text. [b] There is relatively reproducible 20% destruction of serine and a variable degree of cystine destruction under our hydrolysis conditions. Values shown are corrected for serine destruction but not for cystine destruction. [c] Unreliable value. [d] Values for glycine of 15 ± 1 are regarded as normal. [e] The correct isoleucine content should be slightly greater than 2 residues per mole, and that for valine should be slightly less than 4 residues per mole. Position 89 is heterogeneous (Chauvet et al., 1977), being largely valine and occasionally isoleucine. [f] This represents the composition of the peptide-containing fractions that elute from Sephadex G-50 after the digested protein; the phenylalanine is from the peptide Phe-PheNH$_2$ which was present in a 7:1 molar ratio to the protein during chymotrypsin digestion.

From Figure 4, which shows the gel electrophoresis pattern obtained at 1-h preincubation, this time period corresponds to the disappearance of the native protein and the formation of component 1 and, to a lesser extent, component 2. These results and the competitive nature of the inhibition indicate that hydrolysis of the peptide bond(s) involved in the conversion of neurophysin II to components 1 and 2 diverts chymotrypsin from its assay substrate.

Identification of the Final Products of Chymotrypsin Digestion of Neurophysin II. The primary structure of bovine neurophysin II is shown in Figure 5. The large number of disulfide bonds (all Cys are half-cystine residues) indicates that internal peptide bonds could be hydrolyzed with retention of fragments by disulfide bonds. In the gel electrophoretic system used, which runs at pH 9.5, products containing such "internal clips" should have an increased negative charge because the α-carboxyl and α-NH_2 so generated would normally be negatively charged and neutral, respectively (Steinhardt & Beychok, 1964). Thus, the change in electrophoretic mobility associated with chymotrypsin digestion could result from the loss of charged amino acids or internal clips.

To ascertain whether chymotrypsin was leading to the loss of peptide fragments external to the disulfide bonds and/or was producing internal clips, samples of neurophysin II in the presence of Phe-PheNH$_2$ were digested for 24 h with chymotrypsin by using conditions which generate the final products (principally 3a and 3b) shown in Figures 3 and 4. The digestion mixture was chromatographed on Sephadex G-50 (see Materials and Methods) to separate the protein from Phe-PheNH$_2$ and any released peptides. The total amino acid compositions of the protein and low molecular weight fractions are shown in Table III. The composition of the low molecular weight fractions indicates the release from the protein of approximately one residue each of Ala, Met, Ser, Asp, Leu, Val, and Pro, approximately 1.5 Arg residues, and fractional amounts of Gly and Glu; no Cys is released. Correspondingly, the composition of the isolated protein largely confirms the loss of these residues. Since the nonprotein peak contains

Table IV: Effect of Limited Digestion on the Ability of Neurophysin II To Inhibit Chymotrypsin[a]

protein	peptide	% inhibition
native neurophysin II (6×10^{-5} M)		54 ± 4
native neurophysin II (6×10^{-5} M)	Phe-TyrNH$_2$ (2.5×10^{-3} M)	76 ± 3
carboxypeptidase B digested neurophysin II (6×10^{-5} M)		0
carboxypeptidase B digested neurophysin II (6×10^{-5} M)	Phe-TyrNH$_2$ (2.5×10^{-3} M)	11
carboxypeptidase A digested neurophysin II (6×10^{-5} M)		18
carboxypeptidase A digested neurophysin II (6×10^{-5} M)	Phe-TyrNH$_2$ (2.5×10^{-3} M)	35
component 3b (6×10^{-5} M)		14
component 3b (6×10^{-5} M)	Phe-TyrNH$_2$ (2.5×10^{-3} M)	21
component 3a (6×10^{-5} M)	Phe-TyrNH$_2$ (2.5×10^{-3} M)	27

[a] The rate of hydrolysis of GPNA by chymotrypsin was measured in the absence of any additions and in the presence of protein and peptide at the final concentrations indicated. Percent inhibition was calculated as in Table I. Native neurophysin II was run as a control with each set of partial digestion products. Because results with the native protein showed little daily variation, they are presented as average values.

Phe-PheNH$_2$, the possible release of the Phe from the protein can only be determined from the composition of the isolated protein; this indicates no loss of Phe.

For the purification of components 3a and 3b, the isolated digested protein was fractionated on DEAE-Sephadex A-50 to give two peaks, A and B (see Materials and Methods). On electrophoresis, peak A was identified as component 3a contaminated with approximately 10% component 1a; peak B behaved as purified 3b. Both peaks (Table IV) showed a marked reduction relative to native neurophysin II in chymotrypsin inhibition. Amino acid compositions of peaks A and B are shown in Table III. Both peaks have lost one residue each of Ala, Met, Ser, Asp, Leu, and Val when compared with the native protein. However, peak A has lost one Arg while peak B shows the loss of two Arg and probable loss of Pro. The difference in Arg content of the two peaks is sufficient to explain their differences in electrophoretic mobility.

The amino acids released on proteolysis, viewed in the context of the primary structure of neurophysin II, argued that component 3a represents deletion of residues 1–5 (Ala, Met, Ser, Asp, and Leu) and 94–95 (Arg and Val) while component 3b represents deletion of residues 1–5 and 92–95 (Pro, Arg, Arg, and Val). Loss of the first five residues in 3b was confirmed by automated Edman analysis which gave the sequence Glu (probable)-Leu-Arg-Gln-Cys-X-Pro-Cys-Gly for the first nine residues. Amino acid analyses (Table III) were somewhat ambiguous as to whether Pro_{92} was deleted in component 3b. This ambiguity was removed by carboxypeptidase digestion of component 3b. One mole of phenylalanine (Phe_{91}) as the sole amino acid was released by carboxypeptidase B, demonstrating that the C-terminal residue of component 3b is Phe_{91}. Carboxypeptidase A quantitatively released 1 mol of Phe and also small amounts of Gly (0.24 mol per mol of protein) and Val and Ile (0.11 mol combined per mol of protein), the latter amino acids immediately preceding Phe_{91} (Figure 5). The carboxypeptidase results both confirm the identity of the C terminus of 3b and argue that chymotrypsin did not introduce internal clips into the protein. The lack of internal clips was also supported by NaDodSO$_4$ gel electrophoresis in the presence of mercaptoethanol (see Materials and Methods); the mobilities of 3a and 3b were 15% *lower* than that of the native protein.

The assignment of the composition of components 3a and 3b is in agreement with their electrophoretic mobilities in Figure 3; i.e., 3a and 3b should have the same formal net charge as the native protein and NP-X, respectively, but should migrate faster because of their shorter length. However, the assigned compositions do not explain the appearance of Gly and Glu in the hydrolyzed low molecular weight fraction obtained from chymotrypsin digestion (Table III). Both Gly and Glu will be seen below to be particularly significant in the low molecular weight fraction obtained during the initial phases of chymotrypsin digestion (vide infra). We have no explanation for the appearance of Gly, having ruled out its origin in chymotrypsin itself, and tentatively consider it an artifact. However, we consider it likely that Glu originates from an ancillary chymotrypsin cleavage point between Glu_6 and Leu_7 and that components that have lost this Glu are not electrophoretically resolved in our gel system.[3]

Demonstration That the Initial Point of Chymotrypsin Attack Occurs Principally at Arg-93. The identification of component 3a as residues 6–93 implies that chymotrypsin hydrolyzed the Arg–Arg bond between residues 93 and 94 (Figure 5). In fact, the pattern of electrophoretic changes with time during chymotrypsin digestion strongly suggests that this is the principle initial point of chymotrypsin attack and one of the principal bonds involved in chymotrypsin inhibition. Thus, component 1, the first significant digestion product, has a mobility similar to that of NP-X; we have previously shown that removal of Arg_{94} and Val_{95} by carboxypeptidase A generates a product with this mobility (Sur et al., 1979).[2] Identification of component 1 as protein from which Arg_{94} and Val_{95} have been deleted and identification of components 3a and 3b as above also allow a simple interpretation of the appearance of different electrophoretic components with time (Figure 3). Thus, component 1 could be converted to component 2 by cleavage at Phe_{91} (losing a positive charge) or to component 3a by cleavage of residues 1–5 (gaining a positive charge); component 2 in turn could be converted to 3b by cleavage of residues 1–5. The minor component 1a, which does not play a significant role here, would be generated directly from the native protein by loss of the amino-terminal five residues without loss of the -Arg-Val carboxyl terminus, accounting for its more positive charge. It is relevant that identification of component 2 as representing only the deletion of the terminal -Pro-Arg-Arg-Val sequence is in agreement with its electrophoretic mobility being identical with that of NP-C (Figures 3 and 4) which in turn has the same mobility as neurophysin II from which the -Arg-Arg-Val sequence has been removed by carboxypeptidase B (Sur et al., 1979).

To confirm that the first clip leads to loss of the carboxyl-terminal Arg-Val dipeptide, neurophysin II, in the presence of Phe-TyrNH$_2$, was digested by chymotrypsin for a limited

[3] Components from which residues 1–6 have also been deleted would probably migrate similarly to 3a if the Pro-Arg-Arg-Val tetrapeptide were also deleted and similarly to 1a if the terminal Arg-Val dipeptide was absent. Components in which only residues 1–6 were deleted should, we believe, migrate similarly to the slow component from thermolysin digests (see text).

Table V: Comparison of Peptides Released by the Early Action of Chymotrypsin on Neurophysin II[a]

amino acid	mol released/ mol of protein	amino acid	mol released/ mol of protein
Asp	0.28	Met	0.18
Thr	0.04	Ile	0.03
Ser	0.30[b]	Leu	0.24
Glu	0.22	Tyr	[3.4][c]
Pro	0.18	Phe	[3.4][c]
Gly	0.28	His	0.05
Ala	0.29	Lys	0.04
Cys	0.05	Arg	0.96
Val	0.82		

[a] Native neurophysin II was incubated for 75 min with chymotrypsin in the presence of Phe-TyrNH$_2$ as described under Materials and Methods and the partially digested protein separated from the released peptides also as described under Materials and Methods. [b] Corrected for 20% loss of serine during hydrolysis. [c] Phenylalanine and tyrosine are from the Phe-TyrNH$_2$ in the incubation mixture.

time period (see Materials and Methods) such that the principal digestion product was component 1; small amounts of residual native protein and components 2 and 3a, and a trace of 1a, were also present. The protein and nonprotein components were separated on Sephadex G-50 as above, but in this case, the nonprotein components were subjected to amino acid analysis both before and after acid hydrolysis. With the exception of traces of Gly, no free amino acids were found in the nonprotein fractions before acid hydrolysis; however, two ninhydrin-positive peaks, not corresponding in position to known amino acids, were observed. The largest of these (approximately 3× the ninhydrin intensity of the smaller peak) moved slightly behind free Arg on the analyzer column in a position identical with that separately determined for the synthetic peptide Arg-Val. After acid hydrolysis, neither of the above peaks was present, confirming that they are peptides. The composition of the acid hydrolysate of the nonprotein peak is shown in Table V. The predominant amino acids are Arg and Val in slightly greater than a 1:1 Arg:Val ratio, with lesser quantities of amino acids representing the amino-terminal five to six residues, Ala-Met-Ser-Asp-Leu-Glu, which were presumably contained in the smaller unhydrolyzed peptide peak seen on the analyzer; small quantities of Pro, presumably representing the Phe-Pro carboxyl-terminal clip, and unexplained Gly are also present. It is of interest that the ratio of Glu to Ala is higher in the early stages of digestion than in the final stages (cf. Tables III and V), suggesting that the exact position of the amino-terminal clip changes during the course of hydrolysis.

We also examined the products of the earliest stages of chymotrypsin digestion obtained in the absence of peptide and in the presence of the neurophysin ligand Leu-TyrNH$_2$. In all cases, the principal products were Arg and Val in ~1:1 ratio. To confirm that the Arg_{93}–Arg_{94} bond (and the other bonds) was being hydrolyzed by chymotrypsin and not by a contaminant in chymotrypsin preparations, we carried out a number of control studies. Pretreatment of chymotrypsin with TLCK to inhibit trypsin had no effect on the course of neurophysin digestion or on the inhibition of chymotrypsin by neurophysin. Treatment of chymotrypsin with 5×10^{-4} M 1,10-phenanthroline to inhibit any contaminating carboxypeptidase also had no effect on the digestion products. The only treatment that slowed the rate of proteolytic degradation was treatment with NCDC, a specific chymotrypsin inhibitor (Erlanger & Edel, 1964). Pretreatment of chymotrypsin with NCDC to give 90–100% inhibition of hydrolysis of typical

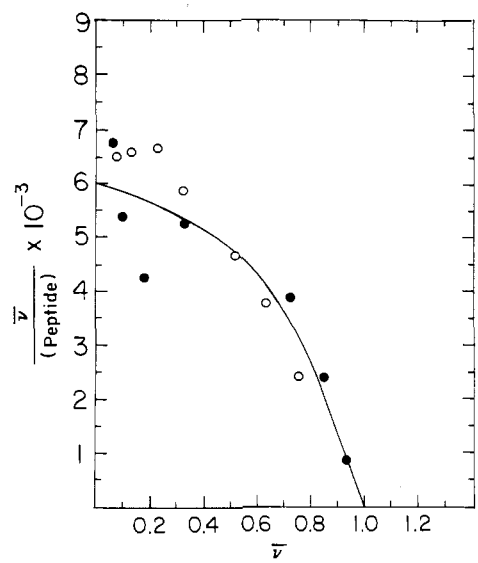

FIGURE 6: Scatchard plots of the binding of Phe-TyrNH$_2$ to nitrated native neurophysin II (○) and to nitrated component 3b (●). Conditions were as follows: pH 6.2 in 0.005 M sodium citrate containing 0.16 M KCl, 24 °C; protein concentration = 1.9×10^{-4} M; $\bar{\nu}$ = moles of peptide bound per neurophysin polypeptide chain.

chymotrypsin substrates (e.g., of GPNA) also slowed digestion of neurophysin II, as monitored electrophoretically,[4] by more than 95%.

Confirmation of the Role of the Carboxyl Terminus of Neurophysin II in Chymotrypsin Inhibition. The role of the C terminus in chymotrypsin inhibition was confirmed by treating native neurophysin II with carboxypeptidase B to remove the terminal -Arg-Arg-Val (Sur et al., 1979); no other residues are affected by this treatment. The isolated product of this digestion, which is electrophoretically homogeneous, was almost completely inactive in inhibiting chymotrypsin (Table IV). In fact, it is less active than components 3a and 3b, a fact possibly attributable to impurities, particularly in component 3a. We also attempted to remove only the terminal -Arg-Val sequence by using carboxypeptidase A as previously reported (Sur et al., 1979). However, our more recent carboxypeptidase A preparations yielded a product contaminated with approximately 20% other components, including approximately 10% native protein. This product (Table IV) also showed reduced inhibition relative to the native protein but was significantly more inhibitory than the carboxypeptidase B generated product, a fact either reflecting the impurities or signifying that the presence of Arg_{93} contributes to inhibition.

Peptide-Binding Ability of Chymotrypsin-Generated Fragments of Neurophysin II. The ability of the isolated fragments 3a and 3b to bind peptides to the principle hormone-binding site was studied by CD. The near-UV circular dichroism spectra of 3a and 3b at pH 6 and pH 3 were identical with that of native bovine neurophysin II, and upon addition of Phe-TyrNH$_2$ at pH 6.2, the identical CD changes occurred as with the native protein (Breslow et al., 1973). The fact that the binding affinity of component 3b at pH 6.2 is identical with that of native neurophysin II was confirmed by nitrating component 3b (see Materials and Methods) and comparing its binding isotherm with that of the nitrated native protein (see Figure 6). While these results signify that residues 1–5 and 92–95 play no direct role in binding, it is relevant that CD studies at pH 3 suggest that 3b actually has a higher

[4] It is relevant that excess NCDC from the inhibited enzyme reacts with Coomassie G-250 and migrates as a deeply staining band at the buffer front in our electrophoresis system.

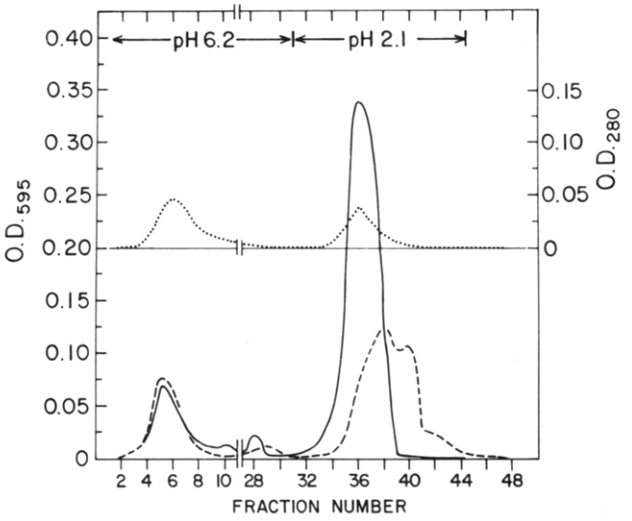

FIGURE 7: Affinity chromatography of native and modified bovine neurophysin II. (···) Mixture of native and reduced, alkylated neurophysin II monitored at 280 nm; the first peak is reduced, alkylated protein, and the second peak is native protein. (—) Product of carboxypeptidase B digestion of component 3b monitored by Coomassie Blue G-250. (---) First peak from ion-exchange chromatography of thermolysin-digested neurophysin II monitored by Coomassie Blue G-250.

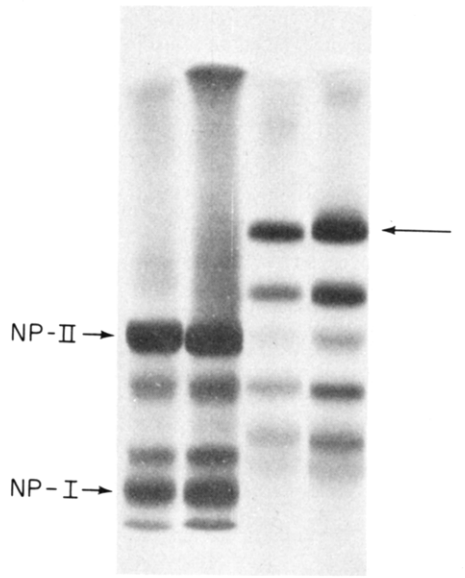

FIGURE 8: Electrophoresis of products of thermolysin digestion. Gel conditions are the same as those in Figures 3 and 4. Gels on left are unfractionated neurophysin. Gels on right are native neurophysin II (2 mg/mL) incubated with Phe-TyrNH_2 and themolysin (0.05 mg/mL) in 0.05 M Tris buffer, pH 7.6, for 4 h at room temperature. Arrow indicates the low-mobility component ultimately isolated by ion-exchange and affinity chromatography (text).

affinity for peptide at this pH than the unmodified protein; i.e., the spectral changes observed when 3b and peptide are mixed at pH 3 are larger than those observed with the native protein. The stronger binding by the modified protein at low pH, where binding is weaker than at neutral pH because of carboxyl protonation [e.g., see Camier et al. (1973) and Breslow & Gargiulo (1977)], may reflect changes in carboxyl pK_a leading to a shift in the pH dependence of binding. In either event, because deletion of the -Arg-Arg-Val carboxyl terminus of nuerophysin II does not in itself significantly affect binding at low pH [Sur et al. (1979) and unpublished experiments], the change in low pH binding for 3b probably reflects the loss of residues 1–5.

Carboxypeptidase B digestion of component 3b quantitatively liberates Phe_{91} (vide supra), allowing us to evaluate the role of Phe_{91}. For this purpose, we studied binding by using the affinity column prepared (see Materials and Methods) for isolating proteins with the peptide specificity of neurophysin. Neurophysin binds to this column at pH 6.2; at pH 2, the affinity for peptide is decreased [e.g., see Camier et al. (1973) and Breslow & Gargiulo (1977)], and the bound protein is released. Figure 7 compares the chromatographic behavior of native and denatured (reduced, alkylated) neurophysin and of the carboxypeptidase B product of component 3b. The main component of the carboxypeptidase product behaved identically with the native protein, eluting from the column only at pH 2.1; denatured protein elutes at pH 6.2. Upon isolation from the column, the amino acid composition of the main component from the proteolytically modified protein was redetermined and the loss of a single Phe confirmed. The results indicate that Phe_{91} is not essential to peptide binding.

Partial Digestion of Neurophysin II by Thermolysin. Incubation of bovine neurophysin II with thermolysin (see Materials and Methods) led to the formation of a number of proteolytically modified species (Figure 8). We were particularly interested in the least acidic principal component (indicated by the arrow) because its low mobility and the specificity of thermolysin for cleavage of bonds involving the amino group of hydrophobic residues, particularly leucine (Feder & Lewis, 1967), suggest that it represented cleavage between Glu_6 and Leu_7 with resultant loss of two negative charges.

Table VI: Effect of Thermolysin Digestion on the Amino Acid Composition of Neurophysin II[a]

amino acid	undigested (control) neurophysin II	released amino acids per mol of protein	fractionated active digested protein
Asp	5.0	1.4	*b*
Thr	2.0	0.6	*b*
Ser	5.9	1.6	*b*
Glu	13.9	1.5	13.6
Pro	8.0	1.2	8.5
Gly	14–16	2.1	14.6
Ala	6.4	1.8	5.5
Val	3.7	1.5	3.3
Met	1.1	0.9	0.2
Ile	2.3	0.45	1.9
Leu	6.0	1.1	4.8
Tyr	1.0	*c*	0.9
Phe	3.1	*c*	2.8
His	0	0	0
Lys	2.0	0.09	2.0
Arg	7.0	1.6	7.0

[a] Neurophysin II was incubated with thermolysin as described under Materials and Methods and the amino acid composition of the peptides released by this digestion (column 3) determined. The digested protein peak was fractionated by ion-exchange and affinity chromatography (see text). The amino acid composition of the fraction that was bound by the affinity column is given in the last column. Values for the undigested protein (column 2) are the same as in Table III. Values for cystine content are unreliable (Table III) and are not included here; only traces of cystine were found in the peptides released by digestion. Serine values are corrected as in Table III. [b] Values for these could not be individually determined because the salt content of this sample led to merger of these peaks on the analyzer column. [c] Values for Phe and Tyr are not included because these reflect the Phe-TyrNH_2 present during digestion.

The possibility that thermolysin was splitting the Glu_6–Leu_7 bond was supported by amino acid analysis of the peptides released by digestion; among the amino acids were those

representing residues 1–6 in approximately equimolar quantities (Table VI). Accordingly, digestion conditions (Materials and Methods) for obtaining the electrophoretically slow component in reasonable yield were determined, and this component was purified to 80% electrophoretic homogeneity by fractionation of DEAE-Sephadex A-50 at pH 5.9 (Materials and Methods). The partially purified electrophoretically slow component was then subjected to affinity chromatography (Figure 7); 75% of the material bound to the column and was actually slightly retarded relative to the native protein upon elution at low pH, suggesting that, at least at low pH, it was bound more tightly by the column than the native protein. On electrophoresis, the binding fractions were found to be the essentially homogeneous slow moving component with only traces (<5%) of another slow moving component. Amino acid analysis of the binding component (Table VI) indicated a significant difference between this component and the native protein only in the loss of residues from the amino-terminal pentapeptide and possibly fractional loss of one Val (presumably from the C terminus); results were ambiguous with respect to loss of a Glu, indicating the loss of 0.3 Glu residue. The ambiguity with respect to the loss of Glu_6 arises from the high Glu content; we continue to assign the slow component to neurophysin II from which the first six residues have been deleted because this assignment is the one most compatible with the specificity of the enzyme, the loss of other residues from the amino terminus, and the electrophoretic mobility. Interestingly, the retardation of this component on the affinity column relative to the native protein at low pH parallels the observation from CD studies (vide supra) that cleavage of residues 1–5 from the amino terminus leads to stronger binding near pH 3.

Discussion

Results with chymotrypsin-digested neurophysin II and its carboxypeptidase B product demonstrate that residues 1–5 and 92–95 do not play a direct role in the binding of peptides to the principal hormone-binding site and indicate that Phe_{91} is not essential for binding. Additionally, the thermolysin data argue against a role for Glu_6. One caveat about Phe_{91} is necessary. We observed that the fragment from which Phe_{91} has been removed, which also contains deletions of residues 1–5, behaves identically with the native protein on an affinity column. However, the conditions for elution from this column (pH 2) can be shown to be such that small decreases in binding to the column at neutral pH relative to the native protein would not have been detected in the present study. Moreover, there is reason to believe (see Results) that, at least at low pH, fragments representing deletions at the amino terminus should elute later than the native protein from the column at low pH, while the fragment from which Phe_{91} and residues 1–5 have been clipped behaves similarly to the native protein. It therefore remains possible that Phe_{91} contributes weakly, probably indirectly, to the binding process.

The nonessentiality of residues 1–6 and 91–95 is in accord with sequence comparison studies [e.g., see Chauvet et al. (1979)] which indicate that the amino-terminal and carboxyl-terminal regions of the protein are the least conserved during evolution and support the concept that the binding domain lies within the central invariant core of the protein consisting largely of residues 10–77. However, it should be noted that substitutions at the amino and carboxyl termini are relatively conservative so that the nonessentiality of these regions was not a foregone conclusion. This is particularly true for Asp_4 and Phe_{91} which are present in all neurophysins sequenced to date (Breslow, 1979; Chauvet et al., 1979) with the exception of one of the equine neurophysins which contains a Leu in position 91 (Chauvet et al., 1977). In this context, the effect of amino-terminal deletions on binding at low pH is of interest because it suggests that the amino region may lie close enough to the active site to modulate its properties.

The surprising result in this study is the unusual interaction between bovine neurophysin II and chymotrypsin. This interaction is particularly manifest by the preferential hydrolysis by chymotrypsin of a neurophysin II Arg–Arg bond, an atypical bond for chymotrypsin under most conditions [see Hirs (1975) and references cited therein] but one which in this instance is hydrolyzed with affinity high enough to successfully compete for chymotrypsin against the substrate GPNA. Moreover, the interaction of chymotrypsin with Arg_{93}–Arg_{94} exhibits a dependence on protein conformation opposite from that of typical proteolytic degradations since it is diminished by protein unfolding and enhanced by the conformational changes (Breslow, 1979; Cohen et al., 1979) associated with peptide binding. There are two potential explanations of these results. First, it is possible that the conformation of neurophysin II, and to a greater extent that of its peptide complexes, imposes on Arg_{93}–Arg_{94} a conformation favorable to chymotryptic attack with both a low K_m and low V_{max}, effectively tying up the enzyme. Alternatively, to account for the fact that the Phe_{91}–Pro_{92} bond is a second point of attack and also appears to contribute to inhibition, there may be an ancillary binding site for chymotrypsin on neurophysin II which orients the enzyme so that its active site is blocked by neurophysin residues 92–95, increasing selectivity for bonds in this region of the protein and hindering access of other substrates until residues 92–95 are proteolytically removed. The increased inhibitory behavior of neurophysin in the presence of peptide is attributed by this second model to an altered conformation that either binds chymotrypsin more tightly or alters the relative position of the carboxyl terminus. The fact that neurophysin I is not a very effective inhibitor is attributed by the second model to the absence of a chymotrypsin-binding site (the sequence differs significantly from that of neurophysin II in the first 8 and last 18 residues) or by either model to the fact that bovine neurophysin I, as isolated from acetone powders such as we use, terminates at residue 92 (Schlesinger et al., 1978).

There is now compelling evidence that the neurophysins are synthesized from higher molecular weight precursors that are probably the same as the precursors for oxytocin and vasopressin (Brownstein et al., 1980; Guidice & Chaiken, 1979; Nicolas et al., 1980; Schmale & Richter, 1981). The enzymes involved in precursor processing have not been isolated, but it has been suggested that the carboxyl terminus of neurophysin may represent a region of precursor cleavage (Drenth, 1981). Because an enzyme identical with pancreatic chymotrypsin is unlikely to occur in bovine pituitary neurosecretory granules, where precursor processing occurs, interactions between neurophysin II and chymotrypsin may be an accident of nature. However, enzymes nonidentical with chymotrypsin, but with chymotrypsin-like specificity, have been found in posterior pituitary preparations (North et al., 1977). It is therefore attractive to speculate either that this interaction is the reflection of a binding site on neurophysin for a chymotrypsin-related pituitary enzyme involved in precursor processing or that the extremely high concentrations of neurophysin within neurosecretory granules modulate proteolysis by serving as enzyme inhibitors.

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Characterization of a Highly Soluble Collagenous Molecule Isolated from Chicken Hyaline Cartilage†

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ABSTRACT: Recently, we have isolated a new collagenous molecule from chicken hyaline cartilage after limited pepsin digestion. This molecule, which contains interchain disulfide bonds, has been called the high molecular weight fraction or HMW [Reese, C. A., & Mayne, R. (1981) *Biochemistry 20*, 5443-5448]. We now present a detailed model for the structure of HMW, the model being derived from analyses both of components of HMW obtained after denaturation either with or without reduction and from electron microscopic observation of replicas of HMW obtained after rotary shadowing. We propose that HMW is a typical, triple-helical collagen molecule of length 134 nm, in which one of the chains has been cleaved at a distance of 96 nm from one end of the triple helix, while the other two chains remain uncleaved. The result of this cleavage is the appearance of a recognizable kink in molecules of HMW when visualized after rotary shadowing.

We have recently reported that chicken hyaline cartilage contains two collagenous molecules which we have called the high molecular weight (HMW) and low molecular weight (LMW) fractions, these collagens comprising approximately 5% of the total soluble collagen (Reese & Mayne, 1981). HMW and LMW are solubilized after limited pepsin digestion of chicken sterna and can be fractionated from type II collagen and the native forms of the 1α, 2α, and 3α chains (Burgeson & Hollister, 1979) by differential salt precipitation performed in acidic conditions, these molecules remaining in solution at 1.2 M NaCl-0.5 M HOAc but being precipitated at 2.0 M NaCl-0.5 M HOAc. The relationship between HMW and LMW is at present uncertain, and it is not known if LMW is derived from HMW by further proteolytic cleavage (Reese

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