Notides, A. C., Hamilton, D. E., & Auer, H. E. (1975) *J. Biol. Chem. 250*, 3945-3950.
Notides, A. C., Hamilton, D. E., & Muechler, E. K. (1976) *J. Steroid Biochem. 7*, 1025-1030.
Notides, A. C., Lerner, N., & Hamilton, D. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4926-4930.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1972) *Biochemistry 11*, 4157-4165.
Puca, G. A., Nola, E., Sica, V., & Bresciani, F. (1977) *J. Biol. Chem. 252*, 1358-1366.
Sakaue, Y., & Thompson, E. B. (1977) *Biochem. Biophys. Res. Commun. 77*, 533-541.
Sherman, M. R., Pickering, L. A., Rollwagen, F. M., & Miller, L. K. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 167-173.
Sherman, M. R., Tuazon, F. B., & Miller, L. K. (1980) *Endocrinology (Baltimore) 106*, 1715-1727.
Stancel, G. M., Leung, K. M. T., & Gorski, J. (1973) *Biochemistry 12*, 2137-2141.
Umezawa, H., & Aoyogi, T. (1977) in *Proteinase in Mammalian Cells and Tissues* (Barrett, A. J., Ed.) pp 637-662, North-Holland Publishing Co., Amsterdam.
Vedeckis, W. V., Freeman, M. R., Schrader, W. T., & O'Malley, B. W. (1980) *Biochemistry 19*, 335-343.
Wachsmuth, E. D., Fritze, I., & Pfleideser (1966) *Biochemistry 5*, 169-174.
Weichman, B. M., & Notides, A. C. (1979) *Biochemistry 18*, 220-225.
Wilson, E. M., & French, F. S. (1979) *J. Biol. Chem. 254*, 6310-6319.
Wrange, O., & Gustafsson, J. Å. (1978) *J. Biol. Chem. 253*, 856-865.

Effects of Limited Tryptic Proteolysis of Bovine Neurophysins on Molecular Properties of Hormone Binding, Self-Association, and Antigenicity†

David M. Abercrombie,‡ Sarojani Angal,§ Reginald P. Sequeira, and Irwin M. Chaiken*

ABSTRACT: Limited tryptic fragmentation of disulfide-intact bovine neurophysins I and II (NP-I and -II, respectively) has been found to cause selective disruption of both hormone binding and neurophysin self-association. Loss of binding interactions, measured as a loss of ability to stimulate retardation of ^{125}I-labeled neurophysin on Met-Tyr-Phe-aminobutylaminoagarose, is complete within 3 h at 37 °C. Reverse-phase high-performance liquid chromatography (HPLC) analysis of tryptic digests of neurophysin I allows detection of two major protein products and the peptide fragment 1-8. Release of the latter N-terminal piece occurs at about the same rate as loss of binding interactions. Reverse-phase HPLC elution behavior before and after performic acid oxidation and amino acid composition of the protein products led to their identification as NP-I-(9-93) (the 9-93 sequence) and [des-19,20]NP-I-(9-93) (the 9-93 sequence with the dipeptide 19-20 missing) for the more rapidly and more slowly formed species, respectively. NP-I-(9-93), unlike intact neurophysin I, is not retarded strongly by either Met-Tyr-Phe-aminobutylaminoagarose or neurophysin II-Sepharose. In contrast, both NP-I-(9-93) and [des-19,20]NP-I-(9-93) are equally as effective as intact NP-I in binding neurophysin I antibodies. The role of amino-terminal residues in promoting hormone binding, self-association, and antigenic recognition interactions is considered.

The neurophysins and their associated neuropeptide hormones oxytocin and vasopressin embody a class of protein-peptide interacting complexes which originate from single-chain, hypothalamic precursors, and, within neurosecretory granules, are transported to and stored in the neurohypophysis before release of proteins and peptides (Livett, 1978; Pickering, 1978; Acher, 1979; Breslow, 1979). As mature proteins, neurophysins exhibit not only hormone binding but also hormone-mediated self-association (Breslow et al., 1971; Chaiken et al., 1975; Nicolas et al., 1976, 1980; Pearlmutter & Dalton, 1980; Tellam & Winzor, 1980; Angal & Chaiken, 1982). Further, given concentrations of neurophysins and hormones in neurosecretory granules that are relatively much higher than K_d values for hormone binding and self-association (Dreifuss, 1975), both the peptide-protein and protein-protein interactions of the mature species likely play a role in stabilizing these molecules during neuronal transport and storage.

The molecular structures responsible for these neurophysin interactions have been defined as yet to only a limited degree. A partially cohesive view of the hormone binding surface has been deduced by a combination of studies such as examination of hormone and peptide ligand binding specificities (Breslow & Weis, 1972; Breslow et al., 1973; Camier et al., 1973; Glasel et al., 1976; Nicolas et al., 1976), spectroscopic changes induced by binding of unlabeled ligands (Balaram et al., 1973; Griffin et al., 1973; Sur et al., 1979) and spin-labeled ligands (Lord & Breslow, 1980), effects of chemical modifications (Walter & Hoffman, 1973; Schlesinger et al., 1979), and photoaffinity labeling (Abercrombie et al., 1982b). The proximity to the hormone binding site of both a carboxylate anion and Tyr-49 has been discussed (Breslow & Garguilo, 1977; Nicolas et al., 1978; Abercrombie et al., 1982b). In

† From the Laboratory of Chemical Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205. *Received June 29, 1982.* Parts of this work were presented at the 66th Annual Meeting of the Federation of American Societies for Experimental Biology, New Orleans, LA, April 1982 (Abercrombie et al., 1982a).

‡ This work is taken in part from a dissertation to be submitted to the Graduate School, University of Maryland, in partial fulfillment of the requirements for the Ph.D. degree in Biochemistry.

§ Present address: Celltech Ltd., Slough SL14DY, United Kingdom.

contrast to data for the hormone binding site, little information is yet available for the neurophysin binding surface participating in self-association. Since hormone binding and self-association are mutually reinforcing, the surfaces involved are not likely to be the same but may be related conformationally.

The present study was undertaken to examine the effects of limited proteolytic degradation on neurophysin interactions and therein to define further the structural bases for these recognition processes. Restricted proteolytic reactions were considered likely for neurophysins in view of the compact structure expected to be produced in these molecules by the presence of seven disulfide bonds per monomer of about 10000 daltons. Indeed, a recent report (Rabbani et al., 1982) has shown that chymotrypsin and thermolysin can effect controlled removal of five and six residues, respectively, from the amino terminus of neurophysin II (NP-II)[1] without altering hormone binding activity. Furthermore, the fact that neurophysins are derived from precursor molecules by limited proteolytic cleavages, including some at basic residues (Land et al., 1982), suggested that the protein is at least fairly resistant to trypsin-like proteases. In the present report, we describe a set of limited proteolytic reactions that can be effected by trypsin. The isolation and chemical identification of major protein products are presented. The restricted tryptic cleavages defined are shown to have no appreciable effect on the antigenicity of the neurophysins. In contrast, these reactions have marked effects on both the interaction of neurophysins with hormone and neurophysin self-association. The results reflect a role of sequence information in the amino-terminal region of the neurophysins in the biologically important interaction properties of these protein carriers of neuropeptide hormones.

Materials and Methods

Neurophysin Preparation and Derivatization. Bovine neurophysins were acid extracted (Hollenberg & Hope, 1968) from either acetone-dried or freeze-dried posterior pituitary tissue (Pelfreez Biologicals, Rogers, AK) and purified by gel filtration and ion-exchange chromatography (Breslow et al., 1971) and then by affinity chromatography on an L-Met-L-Tyr-L-Phe-AH-column (Chaiken, 1979). Affinity-purified neurophysins were routinely freeze-dried and stored at −20 °C. The purity of the samples was determined by amino acid analysis and polyacrylamide gel electrophoresis.

Performic acid oxidation of native and trypsin-digested neurophysins was performed by routine procedures (Chaiken & Hough, 1980; Abercrombie et al., 1982b).

Neurophysins were digested with TPCK–trypsin (Worthington) with or without prior performic acid oxidation. The complete set of tryptic peptides was obtained by digestion after performic acid oxidation (Chaiken & Hough, 1980). The denotation of resultant peptides as "OT-number" follows the convention defined before (Wuu & Crumm, 1976; Chaiken & Hough, 1980). Trypsin digestion of unoxidized neurophysins was performed in 0.1 M ammonium bicarbonate, pH 8.6, at a neurophysin concentration of 1 mg/mL and 13.3% by weight of TPCK-trypsin (except as noted). The samples were incubated at 37 °C for times that ranged up to 21 h. The tryptic cleavage reaction was halted either by adding 11.5 units of aprotinin or by freezing the reaction mixture followed by lyophilization.

Reduction and carboxamidomethylation of NP-I were carried out by using the procedure of Crestfield et al. (1963), with reduction using dithiothreitol in 6 M guanidine hydrochloride and alkylation using iodoacetamide. The reduced–carboxamidomethylated protein was initially dialyzed against water for 24 h with three changes by using no. 16 Nojax dialysis tubing (Union Carbide) prepared as described (Chaiken & Sanchez, 1972) and then purified by affinity chromatography on Met-Tyr-Phe-AH-agarose to remove any underivatized protein. [^{125}I]NP-I and [^{125}I]NP-II were prepared by the Bolton–Hunter procedure (Bolton & Hunter, 1973; Angal & Chaiken, 1982). The specific activity of several fresh preparations was in the range of (0.57–1.4) × 10^6 cpm/μg. These labeled proteins were not used beyond the duration of one half-life of the radioisotope.

Affinity Chromatography. The preparation of affinity matrices used for this study (L-Met-L-Tyr-L-Phe-AH-agarose, L-Met-L-Tyr-L-Phe-AB-agarose, and [NP-II]–Sepharose 4B) has been described before (Angal & Chaiken, 1982). Preparative affinity chromatographic purifications of native and derivatized neurophysins were performed on Met-Tyr-Phe-AH-agarose (0.7 × 7.4 cm). A sample was dissolved in a minimum volume of 0.4 M ammonium acetate, pH 5.7, and applied to the affinity matrix equilibrated with the same buffer. The column was washed with this buffer until the A_{280} of the effluent returned to the base line. Following this, a volume of water that equaled or exceeded the volume of the column was applied to the column to wash out the ammonium acetate. Bound protein was eluted with 0.2 M acetic acid (Chaiken, 1979). For preparation of inactive, derivatized neurophysins, fractions containing the unbound protein in ammonium acetate were collected and dialyzed against water to remove salt and then lyophilized and stored at −20 or −70 °C. Native neurophysins eluted from the affinity column in 0.2 M acetic acid were lyophilized directly without dialysis and stored at −20 °C.

Affinity chromatographic binding analyses of tryptic digests of bovine NP-I and NP-II were obtained with the Met-Tyr-Phe-AB-agarose matrix (0.7 × 6.2 cm) by using procedures analogous to those defined previously (Angal & Chaiken, 1982). Briefly, neurophysin solutions (200 μL total volume) in 0.4 M ammonium acetate, pH 5.7, containing microgram quantities of [^{125}I]NP (20 μL of stock solution defined above) and homologous trypsin-treated neurophysin were applied to the affinity column equilibrated with 0.4 M ammonium acetate, pH 5.7. Fractions of 0.5 mL volume were collected and counted for 0.5 min by using a Packard PGD Prias γ counter.

Direct affinity chromatographic binding analyses of isolated NP-I-(9–93) were performed on the Met-Tyr-Phe-AB-agarose affinity column (see above) and on a [NP-II]–Sepharose matrix (0.9 × 2.8 cm). Details of each elution are described in the legend of Figure 7.

Reverse-Phase HPLC. Elutions of trypsin-digested NP-I samples were carried out on cyanopropylsilyl columns (Zorbax CN, Du Pont). Analytical HPLC peptide mapping was done on a Zorbax CN column (0.46 × 25 cm) as described before for tryptic peptides of oxidized neurophysins (Chaiken &

[1] Abbreviations: NP-I and -II, bovine neurophysins I (oxytocin associated) and II (vasopressin associated); NP-I-(9–93) and [des-19,20]NP-I-(9–93), fragments derived from NP-I by trypsin digestion containing residues 9–93 and 9–18, 21–93, respectively; HPLC, high-performance liquid chromatography; TPCK–trypsin, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone–trypsin; TEAP, triethylammonium phosphate buffer, prepared by adjusting 0.25 N phosphoric acid to pH 3.0 with distilled triethylamine; PBS, phosphate-buffered saline [the pH 7.4 solution was prepared as described previously (Fischer et al., 1977); the pH 7.3 solution was prepared from phosphosaline buffer (Calbiochem), pH 7.6 (containing 0.01% merthiolate), and titrated to pH 7.3 with dilute HCl]; Met-Tyr-Phe-AH-agarose and Met-Tyr-Phe-AB-agarose, Met-Tyr-Phe immobilized through the α-carboxyl to (aminohexylamino)- and (aminobutylamino)agarose, respectively; [^{125}I]NP-I and [^{125}I]NP-II, ^{125}I-labeled neurophysins I and II, respectively.

Hough, 1980). Samples were dissolved in 50 μL of TEAP and injected onto the column equilibrated with TEAP (at room temperature). Elution was effected with a linear gradient, from 100% TEAP to 60% TEAP–40% acetonitrile, starting at sample injection and lasting for 60 min. The flow rate was 0.8 mL/min. Absorbance was monitored at 215 nm. Preparative isolation of NP-I-(9–93) and [des-19,20]NP-I-(9–93) utilized a semipreparative Zorbax CN column (0.94 × 25 cm, Du Pont). Samples injected in 50 μL of TEAP were eluted with a 60-min gradient from 100% TEAP to 70% TEAP–30% acetonitrile, at 3 mL/min. The shallower gradient was useful for increasing the separation of tryptic fragments. Fractions of 0.5 min were collected from each semipreparative run, and those corresponding to equivalent peaks were pooled and dried in vacuo (Savant Speed Vac concentrator).

Amino Acid Analysis. Protein and peptide samples were hydrolyzed for 20–24 h at 110 °C in vacuo with 6 N HCl (constant boiling) supplemented with 10 μL of 5% phenol per mL of acid. Amino acid compositions were determined on a Beckman Model 121 M analyzer. Ratios of moles of residues per mole of protein were computed as described previously (Abercrombie et al., 1982b).

Radioimmunoassay. Antigenicity of neurophysins and derivatives was measured by radioimmunoassay as described previously (Fischer et al., 1977; Chaiken et al., 1982). The fractionated antibodies used for these assays were obtained from antisera raised in rabbits immunized with multi-[poly-(DL-alanyl)]-poly(L-lysine)-conjugated NP-II and unconjugated NP-I (Fischer et al., 1977). Antibodies which specifically recognize NP-I or NP-II were purified from the antisera by chromatography on NP-I– or NP-II–Sepharose 4B, respectively, and stored in PBS, pH 7.4, at −70 °C. For use in a radioimmunoassay, the antibody was thawed and diluted in assay buffer. For assays of the isolated tryptic fragments of NP-I, the assay buffer consisted of 1% bovine serum albumin in PBS, pH 7.3. For assays of other NP-I derivatives, total tryptic digests of NP-I, and -II, and native NP-I, and -II, the assay buffer was composed of 1% bovine serum albumin in PBS, pH 7.4.

The basic procedure for radioimmunoassay consisted of mixing the components together in polyethylene tubes (12 × 75 mm) in a total volume of 100–300 μL: [^{125}I]NP-I or -II, anti-NP-I or anti-NP-II, and unlabeled competitors. *Staphylococcus aureus* cells (Pansorbin, Calbiochem), washed and diluted with the appropriate assay buffer (see above), were added and the samples incubated for 10 min at room temperature to precipitate the antigen–antibody complex. Samples were centrifuged at 2000*g* for 30 min, the supernatant was decanted, and pellets were washed with the assay buffer. The samples were recentrifuged, the supernatant was decanted, and the pellets were counted by using a Packard PGD Prias γ counter. Other details are given under Results.

Results

Time-Dependent Effects of Trypsin on Molecular Properties of Neurophysins I and II. When native NP-I and -II, with all seven disulfides intact, are treated with TPCK–trypsin, a sharp, time-dependent decrease occurs in the ability of either of these proteins to induce formation of self-associated species with high hormone-binding affinity (Figure 1). It has been found previously (Angal & Chaiken, 1982) that native neurophysin, when added to radiolabeled, active neurophysin, will cause an increase in affinity of the latter for the immobilized peptide ligand matrix Met-Tyr-Phe-AB-agarose. This has been shown to be due to self-association of unlabeled with labeled protein and consequent induction of a higher affinity

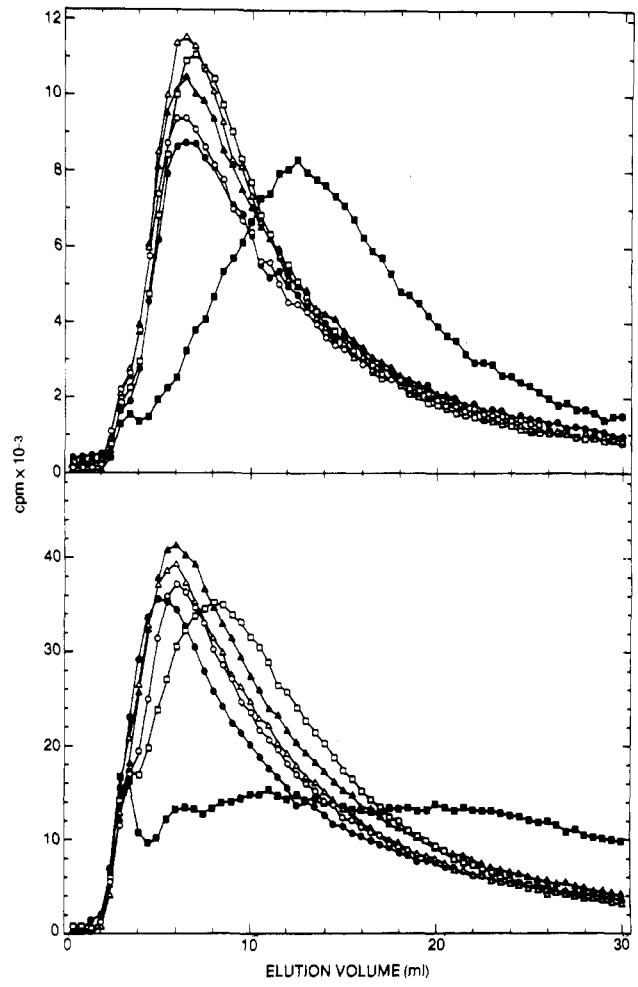

FIGURE 1: Loss of formation of increased-affinity dimers by NP-I and -II upon trypsin digestion as detected by affinity chromatography on Met-Tyr-Phe-AB-agarose. (Top panel) Individual samples of NP-II (300 μg by weight, 202 μg by amino acid analysis) were trypsin digested for (■) 0, (□) 1.5, (△) 3, (○) 7, and (▲) 21 h (Materials and Methods). Each tryptic reaction was quenched by addition of 11.5 units of aprotinin. An aliquot containing 22 μg (by amino acid analysis) of the trypsin-digested protein was mixed with [^{125}I]NP-II (7.0×10^5 cpm) to a total volume of 200 μL, applied to the affinity matrix, and eluted with 0.4 M ammonium acetate, pH 5.7. A control elution was run with [^{125}I]NP-II (7.0×10^5 cpm) alone (●). V_0 (total penetrable volume) for this column is 3.0 mL. (Bottom panel) Individual samples of NP-I (300 μg by weight, 225 μg by amino acid analysis) were trypsin digested as above. A 24-μg portion (by amino acid analysis) of the digested protein at each time was removed, mixed with [^{125}I]NP-I (1.4×10^6 cpm) and chromatographed as above; the control (●) contained [^{125}I]NP-I (1.4×10^6 cpm) only.

of the latter for peptide ligands. It also has been found that this effect is dependent on the presence of a functioning binding site in the unlabeled protein (Abercrombie et al., 1982a). The above chromatographic effect with native protein is shown in Figure 1 by the elutions of ^{125}I-labeled neurophysin without, vs. with, added unlabeled and undigested neurophysin. However, neurophysin treated with 13% by weight TPCK–trypsin at pH 8.6, 37 °C, loses the ability to induce this effect, with the loss being complete by 3 h. Consistent with the above, neurophysin which was trypsin treated for 3 h as in Figure 1 no longer can bind directly to the Met-Tyr-Phe-AB-agarose affinity column (profile not shown).

In contrast to the above, trypsin digestion of the neurophysins leads to no loss of antigenicity. This is shown by the data of Figure 2 for the digested protein samples defined in Figure 1. Here, no significant change is observed in the ability of either trypsin-treated NP-I or trypsin-treated NP-II to

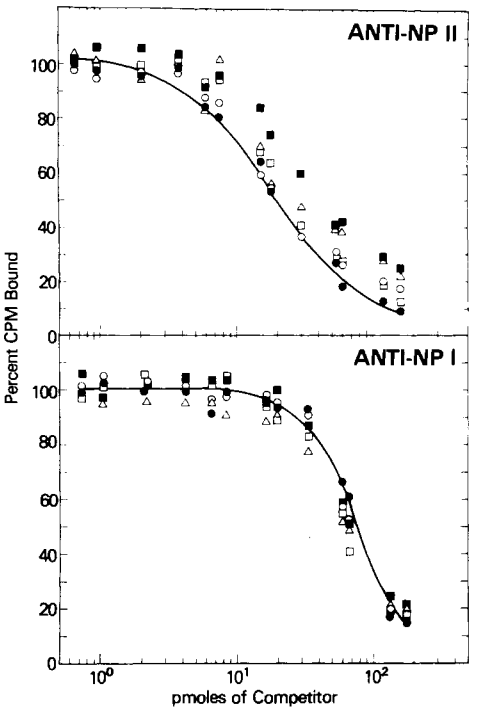

FIGURE 2: Retention of antigenicity of neurophysins upon limited trypsin digestion as detected by radioimmunoassay. From the NP-I and -II samples trypsin digested for (●) 0, (○) 1.5, (□) 3, (△) 7, and (■) 21 h as defined in Figure 1, two 10-μL aliquots were removed. One aliquot was mixed with 190 μL of assay buffer and then diluted serially 1:2 with the same buffer; the other aliquot was mixed with 140 μL of assay buffer and then diluted serially 1:3 with the same buffer. A 100-μL aliquot of anti-NP-I (enough to bind 75% of labeled antigen with no competitor present) or a 120-μL aliquot of anti-NP-II (enough to bind 50% of labeled antigen) was combined with 100 μL of each of the corresponding diluted tryptic digests and 20 μL of [^{125}I]NP-I (3.5×10^4 cpm) or [^{125}I]NP-II (2.3×10^4 cpm). The mixtures were incubated at 4 °C for 1.5 h. The determination of picomoles of competitor present at each dilution was based upon amino acid analysis of initial NP-I and NP-II solutions. Values of "percent cpm bound" were calculated on the basis of 100% for cpm bound when no competitor was added.

compete with the homologous ^{125}I-labeled neurophysin for binding to anti-NP-I or anti-NP-II, respectively. The lack of alteration in competitiveness persists, at least up to 21 h of trypsin treatment, under conditions sufficient to produce functional loss in 3 h (Figure 1). For comparison, the relative antigenicity of NP-I vs. NP-II and the large decrease in antigenicity in disulfide-broken NP-I (performic acid oxidized or reduced–alkylated) are shown by the radioimmunoassay data in Figure 3.

Reverse-Phase HPLC Detection of Time-Dependent Fragmentation of Neurophysin I and Isolation of Major Degraded Forms. The structural changes occurring during the tryptic events which lead to the functional changes seen in Figure 1 were evaluated by reverse-phase HPLC mapping. Previous use of HPLC with cyanopropylsilyl columns has allowed all tryptic fragments of the neurophysins other than dipeptides to be separated and identified (Chaiken & Hough, 1980). For the 93-residue NP-I, the fragments 1–8, 9–18, 21–43, 44–66, and 67–93 all have been identified in tryptic maps of performic acid oxidized NP-I. The dipeptide (19–20) was found to elute, unretarded, in the breakthrough fraction. When essentially the same chromatographic procedure is carried out for native NP-I digested with 13% TPCK–trypsin at pH 8.6, 37 °C, and for varying times, elution profiles were obtained as shown in Figure 4. The appearance of released fragment 1–8, identified by comparison of these profiles with maps of fully digested

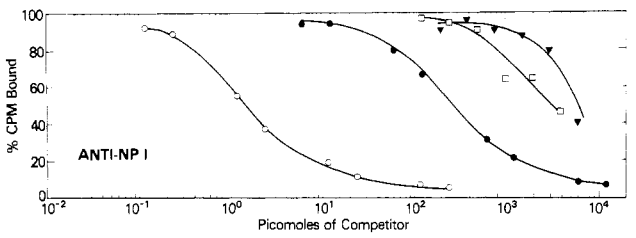

FIGURE 3: Radioimmunoassay of native and chemically modified NP-I. The specified amounts of native NP-I (○) and NP-II (●) as well as reduced–carboxamidomethylated (□) and performic acid oxidized (▼) derivatives of NP-I were mixed with anti-NP-I (enough to bind 14–21% of labeled antigens with no competitor present), [^{125}I]NP-I (9×10^3 cpm), and assay buffer (total volume 100 μL) and incubated at 4 °C for 1.5 h. The quantity of competitor for each assay (in picomoles) is based upon amino acid analysis.

NP-I as well as by amino acid analysis of the eluted peak, was apparent at least as early as 15 min after trypsin addition (the earliest time tested) and was complete by 3 h, as judged by peak size in elution profiles monitored by A_{215}. Comparison of the rate of release of fragment 1–8 in Figure 4 with the rate of functional loss (Figure 1) indicates a strong similarity between the two.

Two relatively large polypeptide products also are produced by trypsin and are denoted in Figure 4 as peaks A and B. Clearly, peak B is transient and appears to convert to peak A with time. Partial insight into the nature of peaks A and B was obtained by mapping performic acid oxidized digests, as well as isolated peaks A and B after performic acid oxidation. As shown in Figure 5, mapping of the 21-h tryptic digest of NP-I, after performic acid oxidation, reveals the presence not only of fragment 1–8 but also of at least one identifiable cysteic acid containing fragment (9–18). The identity of this fragment was confirmed by comparison with the elution position of fragment 9–18 from an exhaustive tryptic digest of the oxidized protein. The amount of fragment 9–18 observed in such maps is quite small at early times of digestion (1.5 and 3 h) and is still increasing (and incomplete) at 21 h. Besides fragments 1–8 and 9–18, three other absorbance peaks are consistently noted after oxidation. From their elution positions relatively late in the gradient, these likely correspond to large polypeptide fragments produced by trypsin. The earliest of these elutes close to the elution position of fragment 21–43. However, this correspondence is not consistent among several maps, making the conclusion that fragment 21–43 is produced in limited digestions only tentative (see below). When peak A (Figure 4) is isolated, oxidized, and reeluted in the Zorbax CN system, fragment 9–18 and the two next most strongly retarded peaks in Figure 5 are observed. In contrast, reelution of peak B after oxidation still shows only one peak, which corresponds to the most retarded peak in Figure 5. These results are consistent with peak A containing at least two or three peptide fragments held together by disulfide bonds and with peak B being a single continuous polypeptide fragment.

The identities of peaks A and B and fragment 1–8 were established more firmly upon isolation of sufficient amounts of these, by HPLC, for subsequent amino acid analysis. For this isolation purpose, a sample of NP-I (5.4 mg) was digested for 1.5 h with 10% by weight TPCK–trypsin by the standard procedure. Three portions containing about 1.5 mg each were dissolved in 50 μL of TEAP and injected onto the semipreparative Zorbax CN column (see Material and Methods). The elution profiles produced were similar to the profiles in Figure 4, except that the peaks eluted later (peaks A and B eluted at 51 and 54 min, respectively, and fragment 1–8 eluted at

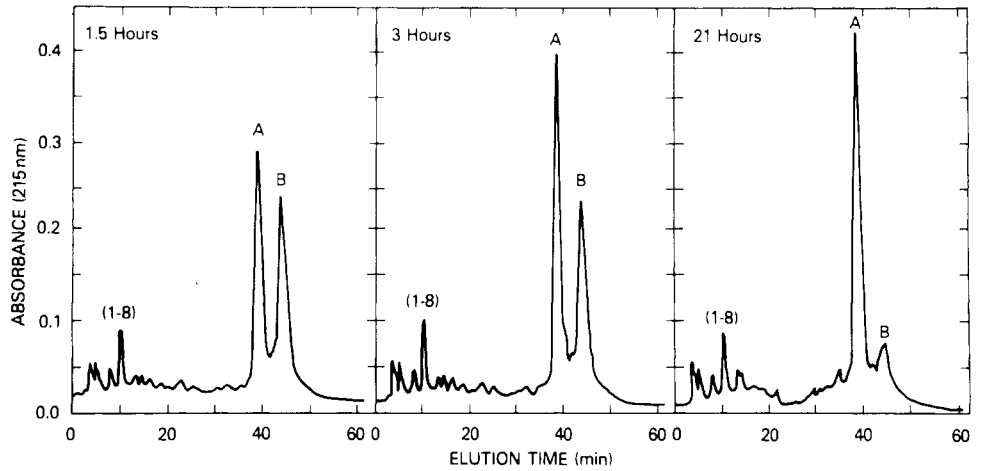

FIGURE 4: Reverse-phase HPLC peptide maps of trypsin-digested NP-I. Samples of 150 μg (by weight) of protein, trypsin digested for the times specified, were injected in TEAP onto the analytical Zorbax CN column (see Materials and Methods). The peak denoted (1–8) corresponds to the tryptic fragment of residues 1–8 of NP-I; peaks A and B are defined in the text.

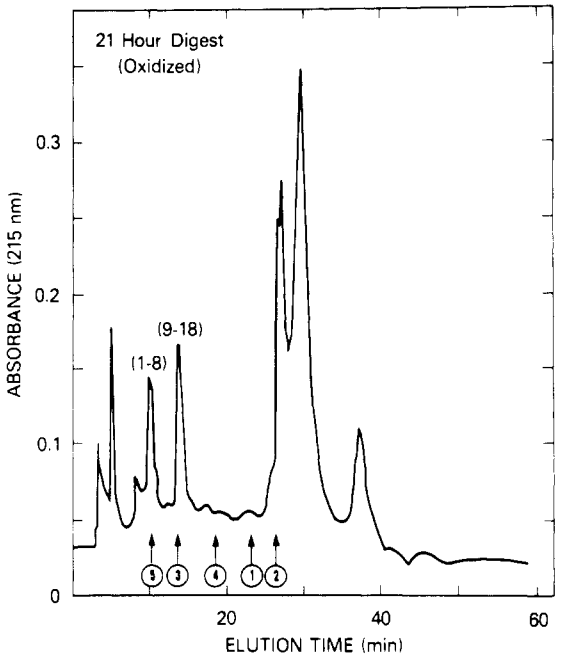

FIGURE 5: Reverse-phase HPLC peptide map of NP-I digested with trypsin for 21 h followed by performic acid oxidation. A 150-μg (by weight) sample was taken from the stock reaction mixture, described in Figure 4, after 21 h and, after lyophilization, subjected to performic acid oxidation. The sample was dried and injected onto the Zorbax CN column. The circled numbers at the bottom of the profile corresponded to the elution position of standard tryptic peptides ("OT peptides") of NP-I obtained by initial performic acid oxidation followed by exhaustive trypsin digestion (Chaiken & Hough, 1980). (OT-5 is residues 1–8, OT-3 is residues 9–18, OT-4 is residues 44–66, OT-1 is residues 67–93, and OT-2 is residues 21–43.) The peak at 38 min corresponds to that obtained for oxidized peak B of Figure 4, while OT-3 and peaks at 27–31 min correspond to those obtained for oxidized peak A of Figure 4.

Table I: Amino Acid Composition of Protein Products Obtained by Semipreparative Zorbax CN Fractionation of Trypsin-Digested NP-I[a]

	calcd residues (mol/mol of protein)[c]		
amino acid residue[b]	peak A	peak B	fragment 1–8
Asp (7)	5.1 (5)	5.2 (5)	2.0 (2)
Thr (2)	2.0 (2)	1.9 (2)	
Ser (6)	5.9 (6)	5.7 (6)	
Glu (10)	10.1 (10)	10.0 (10)	
Pro (9)	8.8 (9)	8.8 (9)	
Gly (14)	12.9 (13)	14.0 (14)	
Ala (9)	7.9 (8)	7.7 (8)	1.1 (1)
$^1/_2$-Cys (14)	10.5 (14)[d]	7.1 (14)[d]	
Val (3)	1.0 (1)	1.0 (1)	1.9 (2)
Ile (2)	1.8 (2)	1.7 (2)	
Leu (6)	3.8 (4)	4.0 (4)	2.1 (2)
Tyr (1)	1.0 (1)	1.0 (1)	
Phe (3)	2.9 (3)	3.1 (3)	
Lys (2)	2.0 (2)	2.0 (2)	
His (1)	0.9 (1)	1.2 (1)	
Arg (4)	1.9 (2)	3.0 (3)	0.9 (1)
(93)	(83)	(85)	(8)

[a] NP-I was digested with TPCK-trypsin and eluted from the semipreparative Zorbax CN column as described in the text. [b] Values in parentheses correspond to numbers of residues expected for native NP-I based upon a sequence of NP-I (Chauvet et al., 1979). A similar sequence for NP-I has been published (Schlesinger et al., 1978) which indicates that the C-terminal residue of NP-I is Leu instead of Gln as in the Chauvet et al. (1979) sequence. The value at the bottom of the column represents total expected residues for native NP-I. [c] Values in parentheses correspond to numbers of residues expected for the following sequences derived from NP-I: peak A, [des-19,20]NP-I-(9–93); peak B, NP-I-(9–93); fragment 1–8, N-terminal eight amino acid residues of NP-I. Values at the bottom of each column represent total expected numbers of amino acids for the tryptic products. [d] Values are uncorrected for partial destruction of half-cystine residues during acid hydrolysis.

23 min) due to the shallower gradient. Amino acid compositions obtained for these peaks are given in Table I.

The composition of peak B indicates that this species contains all residues of NP-I except 1–8. In view of this and the fact that oxidation of peak B leads to only one HPLC peak upon rechromatography, this peak can be identified as the intact fragment, NP-I-(9–93).

Peak A has an amino acid composition consistent with a content of all residues except 1–8, 19, and 20. On this basis, and the observations that oxidation of this peak yields a mixture of fragments including fragment 9–18, peak A can be identified as [des-19,20]NP-I-(9–93). However, the extent to which the 21–93 sequence in this latter derivative may be internally cleaved cannot be deduced completely from the present data. In view of the slow apparent appearance of a peak tentatively identified as fragment 21–43 in oxidized tryptic digests (Figure 5), it is concluded provisionally that some of [des-19,20]NP-I-(9–93) has a discontinuity between residues 43 and 44. The cleavages at the 18–19, 20–21, and 43–44 bonds all occur without fragment separation (except for the dipeptide 19–20) as long as the protein product remains disulfide intact. This suggests that the disulfide bonds, as yet unassigned with certainty for either of the major bovine

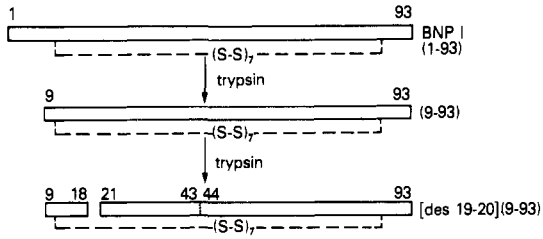

FIGURE 6: Diagram of proposed scheme for trypsin digestion of NP-I. The presence of seven disulfide bonds in the intact (BNP I) and trypsin-digested NP-I is indicated by "$(S\text{-}S)_7$". The trypsin cleavage sites in disulfide-broken NP-I are between residues 8–9, 18–19, 20–21, 43–44, and 66–67 (Chauvet et al., 1979; Chaiken & Hough, 1980). The dotted line between residues numbered 43 and 44 in [des-19,20]NP-I-(9–93) indicates a proposed slow tryptic cleavage site in native (disulfide intact) NP-I (lower line).

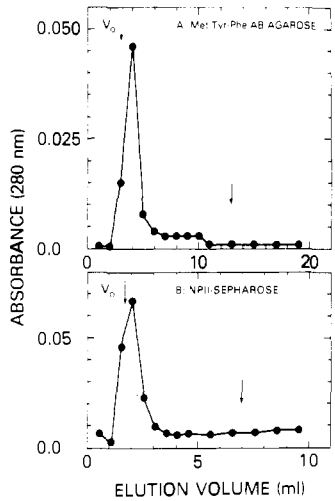

FIGURE 7: Assay of binding properties of NP-I-(9–93) by affinity chromatography. (A) Isolated NP-I-(9–93) (75 μg) in 300 μL of ammonium acetate (containing about 0.1 M phosphate from HPLC purification and having a pH of 5.2) was applied to the Met-Tyr-Phe-AB-agarose matrix equilibrated with 0.4 M ammonium acetate. Fractions were 1 mL. Initial elution was with the ammonium acetate buffer, pH 5.7, through 10 mL, after which the column was washed with 5 mL of water followed by 5 mL of 0.2 M acetic acid. The unmarked arrow indicates the peak position for the elution of 75 μg of native NP-I. (B) NP-I-(9–93) [75 μg, as in (A)] was applied to [NP-II]–Sepharose equilibrated with 0.4 M ammonium acetate, pH 5.7, and eluted with the same buffer. Fractions were 0.5 mL. The unmarked arrow indicates the elution position of 75 μg of native NP-I. The arrow marked V_0 indicates the elution position of noninteracting protein on each column.

neurophysins, are arranged in such a way that the native NP-I sequences 9–18, 21–43, and 44–93 are all cross-linked concomitantly by disulfides. On the basis of the above data, a time course of limited trypsin digestion of NP-I can be deduced. This is shown schematically in Figure 6.

Molecular Properties of NP-I-(9–93) and [des-19,20]NP-I-(9–93). That the disruption of neurophysin binding properties upon trypsin digestion is due to fragment 1–8 release is shown by characterization of peak B, NP-I-(9–93), by affinity chromatographic analysis. The profile of Figure 7A shows that NP-I-(9–93) does not bind strongly to Met-Tyr-Phe-AB-agarose, indicating the loss of effective peptide binding in the hormone binding site. Further, Figure 7B shows that the NP-I-(9–93) species is not retarded significantly on [NP-II]–Sepharose, a phenomenon which does occur, as shown, for unmodified NP-I. Thus, both ligand binding and self-association are reduced in NP-I-(9–93). This dual reduction would lead to the loss of the affinity chromatographic effect shown in Figure 1.

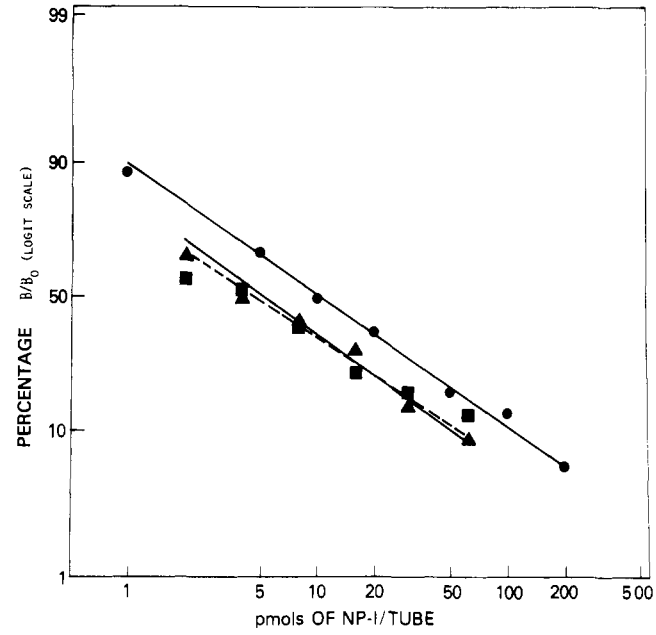

FIGURE 8: Radioimmunoassay for NP-I tryptic products, (▲) NP-I-(9–93) and (■) [des-19,20]NP-I-(9–93). Specified amounts of tryptic cleavage products were combined with anti-NP-I (enough to bind 25% of the labeled antigen with no competitor present), [^{125}I]NP-I (3×10^3 cpm), and enough assay buffer to make a total of 300 μL and incubated at 4 °C for 18 h. The competition data are plotted as percentage antigen bound [ratio of cpm bound with competitor present (B) to cpm bound without competitor (B_0)] vs. the amount of competitor in the log–logit transformation format (Midgley et al., 1969). The latter emphasizes the parallelism of competition curves for native NP-I and isolated tryptic products. The standard curve (●) shows competition by native NP-I.

Radioimmunoassay of isolated NP-I-(9–93) confirms that this species, while inactive, is fully antigenic (as expected from the data of Figure 2). Competition profiles for NP-I-(9–93) as well as [des-19,20]NP-I-(9–93) are shown in Figure 8. Both of these species are essentially as effective as intact neurophysin I in competing with [^{125}I]NP-I for binding to anti-NP-I. The data show that substantial internal peptide bond cleavage can occur without loss of the antigenic character of NP-I.

Discussion

The results presented here show that limited proteolytic cleavage of NP-I and -II can be effected by trypsin, leading to inactivated products. The cleavage events with NP-I have been particularly straightforward to evaluate, due in large part to the more restricted set of tryptic peptides possible and the ability to detect all of the latter, save one dipeptide (Gly-19-Arg-20), by reverse-phase HPLC on cyanopropylsilyl columns. The cleavage at residue 8 of NP-I is the most rapid tryptic event. This leads to release of the fragment 1–8 peptide and the large protein fragment, NP-I-(9–93). Affinity chromatographic characterization of the latter (Figure 7) shows that this product (as predicted from analysis of initially obtained total digests in Figure 1) has a greatly reduced ability to bind peptide ligand in the hormone binding site. Further, the product has reduced ability to self-associate with intact neurophysin, a property previously shown to be interdependent with peptide binding (Nicolas et al., 1978, 1980; Angal & Chaiken, 1982). Thus, residues in the N-terminal octapeptide must contribute in some obligatory way to the functional state of NP-I.

The mechanisms by which removal of N-terminal residues leads to loss of hormone binding and self-association of NP-I

are not completely definable from the present results. The data of Figure 1, taken with the known structural relatedness of NP-I and -II (Chauvet et al., 1979; Breslow, 1979), suggest the likelihood that inactivation also occurs in NP-II upon N-terminal tryptic cleavage at the 8–9 bond. In contrast, a previous study of NP-II proteolysis has shown that removal from this protein species of residues 1–5 by chymotrypsin and residues 1–6 by thermolysin does not lead to inactivation (Rabbani et al., 1982). Thus, perhaps it is removal of only those residues closest to the 8–9 bond, namely, residues 7 and 8, which leads to inactivation. This sequence is Leu-Arg for NP-II and Val-Arg for NP-I. Among several neurophysins sequenced so far, residue 7 varies among Leu, Val, and Met, while residue 8 is always Arg. In spite of the above, delineation of a particular role for N-terminal residues in two distinct interaction sites, one for hormone binding and a second for self-association, is subject only to conjecture. That these residues, particularly 7 and 8, might contribute indirectly to the overall conformational stabilization of binding surfaces is one possibility.

In contrast to the disruption of hormone binding and self-association, limited tryptic cleavage in the N-terminal region causes no decrease in neurophysin interaction with affinity-fractionated antibodies. Since NP-I and -II are distinguishable antigenically with these antibodies (Figure 3 and similar results with anti-NP-II), and since they have identical interior sequences (from half-cystine-10 to half-cystine-74), the trypsin results suggest that antigenic specificity resides in the C-terminal region. Furthermore, antigenicity of inactive trypsin products makes it likely that the determinants are dependent upon local structural order but not overall conformational intactness. Related to this, antigenicity is greatly decreased by disulfide elimination (oxidized and reduced–alkylated derivatives, Figure 3). These results suggest that local structural order required for antibody interactions may reside in disulfide-stabilized loop regions, areas whose antigenic structure could be stabilized even with some sequences of neurophysin missing but not when all disulfides are broken and the polypeptide chain is less constrained structurally. For NP-I, the C-terminal region of residues 75–84 is bounded by half-cystines-74 and -85 and is of different sequence from the corresponding region in NP-II (which also is bounded by half-cystines at positions 74 and 85). This region may well contain one or more of the determinants responsible for antigenicity differences between NP-I and -II.

The pattern of tryptic digestion of neurophysin I gives some insight into the conformational compactness of these proteins. The rapid appearance of fragment 1–8 as observed by HPLC argues that the 8–9 bond is the first hydrolyzed. This would suggest that the N-terminal segment is in a much less ordered state than that of segments containing other potential tryptic cleavage sites and therefore more accessible to the protease. It would seem that all subsequent tryptic cleavages, at bonds 18–19 and 20–21, and perhaps 43–44, occur only after the 8–9 cleavage. This view is consistent with the proposal that cleavage at residue 8 may lead to significant conformational destabilization of the main body of the neurophysin molecule and resultant disruption of the two binding surfaces, one for peptide ligands and the other for another neurophysin molecule.

That the main portion of the intact neurophysin molecule is conformationally compact and therefore relatively resistant to proteolysis could be related to a need for proteolytic resiliency of neurophysin–hormone complexes during their storage, after in vivo processing of precursors. The latter reactions apparently take place in neurosecretory granules (Sachs et al., 1969; Gainer et al., 1977; Brownstein et al., 1980), making the environment of the mature neurophysin–hormone complexes one in which proteases create a potentially hostile environment. While processing proteases likely have defined specificities which would preclude rapid hydrolysis at sequences within the mature, native neurophysins, it may well be conformational compactness which suppresses slower hydrolysis by the proteases at less favored sites at least in the 9–93 region. Formation of multimeric neurophysin–hormone complexes induced by hormone-mediated self-association also may help protect neurophysin against proteolyic inactivation in neurosecretory granules.

The present study defines the use of limited tryptic digestion in establishing a role for residues in the amino-terminal region in neurophysin–hormone binding and self-association. That these residues may act to stabilize the conformation of neurophysin, and therein the proper organization of binding surfaces, is implied from the pattern of tryptic digestion. While the precise role of amino-terminal residues cannot be defined with certainty as yet, NP-I-(9–93) could provide a basis for further delineating the function of these residues, by reconstitution of NP-I-(9–93) with synthetic N-terminal peptides of native or analogue sequence using methods of semisynthesis (Chaiken, 1981).

Added in Proof

After completion of this work and manuscript, a paper appeared (Breslow et al., 1982) which reported data on the tryptic cleavage of the amino-terminal neurophysin octapeptide and the chemical modification of arginine-8. Their results and proposal of a role for arginine-8 in neurophysin function are consistent with the data of the present paper.

Acknowledgments

We thank Dr. Paul A. Hargrave for his help and advice in the early stages of this project and Jonathan Seeman for his help with high-performance liquid chromatography and amino acid analyses.

References

Abercrombie, D. M., Angal, S., & Chaiken, I. M. (1982a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 5337.

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

Acher, R. (1979) *Angew. Chem., Int. Ed. Engl. 18*, 846–860.

Angal, S., & Chaiken, I. M. (1982) *Biochemistry 21*, 1574–1580.

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

Bolton, A. E., & Hunter, W. M. (1973) *Biochem. J. 133*, 529–539.

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

Breslow, E., & Weis, J. (1972) *Biochemistry 11*, 3474–3482.

Breslow, E., & Gargiulo, P. (1977) *Biochemistry 16*, 3397–3406.

Breslow, E., Aanning, H. L., Abrash, L., & Schmir, M. (1971) *J. Biol. Chem. 246*, 5179–5188.

Breslow, E., Weis, J., & Menendez-Botet, C. J. (1973) *Biochemistry 12*, 4664–4653.

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

Brownstein, M. J., Russell, J. T., & Gainer, H. (1980) *Science (Washington, D.C.) 207*, 373–378.

Camier, M., Alazard, R., Cohen, P., Pradelles, P., Morgat, J., & Fromageot, P. (1973) *Eur. J. Biochem. 32*, 207–214.

Chaiken, I. M. (1979) *Anal. Biochem. 97*, 302–308.
Chaiken, I. M. (1981) *CRC Crit. Rev. Biochem. 11*, 255–301.
Chaiken, I. M., & Sanchez, G. R. (1972) *J. Biol. Chem. 247*, 6743–6747.
Chaiken, I. M., & Hough, C. J. (1980) *Anal. Biochem. 107*, 11–16.
Chaiken, I. M., Randolph, R. E., & Taylor, H. C. (1975) *Ann. N.Y. Acad. Sci. 248*, 422–450.
Chaiken, I. M., Fischer, E. A., Giudice, L. C., & Hough, C. B. (1982) in *Hormonally Active Brain Peptides: Structure and Function* (McKerns, K. W., & Pantič, V., Eds.) pp 327–347, Plenum Press, New York.
Chauvet, M. T., Codogno, P., Chauvet, J., & Acher, R. (1979) *FEBS Lett. 98*, 37–40.
Crestfield, A. M., Moore, S., & Stein, W. H. (1963) *J. Biol. Chem. 238*, 622–627.
Dreifuss, J. J. (1975) *Ann. N.Y. Acad. Sci. 248*, 184–201.
Fischer, E. A., Curd, J. G., & Chaiken, I. M. (1977) *Immunochemistry 14*, 595–602.
Gainer, H., Sarne, Y., & Brownstein, M. J. (1977) *J. Cell Biol. 73*, 366–381.
Glasel, J. A., McKelvy, J. F., Hruby, V. J., & Spatola, A. F. (1976) *J. Biol. Chem. 251*, 2929–2937.
Griffin, J. H., Alazard, R., & Cohen, P. (1973) *J. Biol. Chem. 248*, 7975–7978.
Hollenberg, M. D., & Hope, D. B. (1968) *Biochem. J. 106*, 557–564.
Land, H., Schütz, G., Schmale, H., & Richter, D. (1982) *Nature (London) 295*, 299–303.
Livett, B. G. (1978) *Int. Rev. Cytol., Suppl. No. 7*, 53–237.
Lord, S. T., & Breslow, E. (1980) *Biochemistry 19*, 5593–5602.
Midgley, A. R., Niswender, G. D., & Rebar, R. W. (1969) *Acta Endocrinol. (Copenhagen), Suppl. No. 142*, 163–184.
Nicolas, P., Camier, M., Dessen, P., & Cohen, P. (1976) *J. Biol. Chem. 251*, 3965–3971.
Nicolas, P., Wolff, J., Camier, M., DiBello, C., & Cohen, P. (1978) *J. Biol. Chem. 253*, 2633–2639.
Nicolas, P., Batelier, G., Rholam, M., & Cohen, P. (1980) *Biochemistry 19*, 3565–3573.
Pearlmutter, A. F., & Dalton, E. J. (1980) *Biochemistry 19*, 3550–3556.
Pickering, B. (1978) *Essays Biochem. 14*, 45–81.
Rabbani, L. D., Pagnozzi, M., Chang, P., & Breslow, E. (1982) *Biochemistry 21*, 817–826.
Sachs, H., Fawcett, Y., Takabatake, Y., & Portanova, R. (1969) *Recent Prog. Horm. Res. 25*, 447–491.
Schlesinger, D. H., Audhya, T. K., & Walter, R. (1978) *J. Biol. Chem. 253*, 5019–5024.
Schlesinger, D. H., Audhya, T. K., Hoffman, P. L., & Walter, R. (1979) *Proc. Int. Congr. Biochem., 11th, Abstr. 603* (Abstr. 11-3-S5).
Sur, S. S., Rabbani, L. D., Libman, L., & Breslow, E. (1979) *Biochemistry 18*, 1026–1036.
Tellam, R., & Winzor, D. J. (1980) *Arch. Biochem. Biophys. 201*, 20–24.
Walter, R., & Hoffman, P. L. (1973) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 32*, 1974.
Wuu, T., & Crumm, S. E. (1976) *Biochem. Biophys. Res. Commun. 68*, 634–639.

Conformational States of Tubulin Liganded to Colchicine, Tropolone Methyl Ether, and Podophyllotoxin†

Jose Manuel Andreu and Serge N. Timasheff*

ABSTRACT: The conformational effects of colchicine, podophyllotoxin, and tropolone methyl ether binding to tubulin have been studied. Conditions for the stability of the purified calf brain protein were established, and the effects of binding were examined by means of difference absorption spectroscopy, circular dichroism, fluorescence, activation of tubulin GTPase, and tubulin self-association reactions. The tubulin–colchicine complex was isolated and characterized. It displays quenched intrinsic protein fluorescence, ligand fluorescence, and GTPase activity, probably accompanied by minor perturbations in the secondary structure. The conformation of the tubulin–colchicine complex appears to be nonidentical with that of the unliganded protein. Podophyllotoxin was not found to induce any of the mentioned changes. This ligand seems to bind through a hydrophobic interaction of its trimethoxybenzene ring with tubulin, as does colchicine. Binding of tropolone methyl ether, which is the analogue of the other part of the colchicine molecule that binds to tubulin, produced effects consistent with a ligand-linked conformational change. The small perturbation by tropolone methyl ether of the circular dichroism spectrum of tubulin resembles changes induced by colchicine.

The antimitotic drugs colchicine and podophyllotoxin are known to bind strongly to soluble tubulin (Cortese et al., 1977; Garland, 1978). The liganded protein can be incorporated into microtubules with the consequence that the formation of these tubulin polymers is inhibited by substoichiometric amounts of the drugs (Olmsted & Borisy, 1973; Margolis & Wilson, 1977; Sternlicht & Ringel, 1979; Margolis et al., 1980; Lambeir & Engelborghs, 1980). The binding of colchicine to tubulin is slow, but the high apparent affinity is difficult to measure quantitatively by equilibrium techniques due to the instability of the protein (Wilson & Bryan, 1974). Garland (1978) carried out a kinetic study of the binding and fitted the results by a binding scheme consisting of a fast reversible association, followed by a slow ligand-induced conformational

† From the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachussetts 02254. *Received November 20, 1981; revised manuscript received April 8, 1982.* Publication No. 1419. This work was supported by Grants CA 16707 and GM 14603 from the National Institutes of Health and Fogarty International Fellowship F05TW02983 (J.M.A.).

0006-2960/82/0421-6465$01.25/0