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Characterization and Isolation of Proteolytically Modified Nerve Growth Factor†

William C. Mobley, Anton Schenker,‡ and Eric M. Shooter*

ABSTRACT: The peptide chains of β nerve growth factor (βNGF) were shortened at their NH_2 termini by cleavage with a specific submaxillary gland endopeptidase(s) after histidine in position number 8 and the modified proteins characterized by sequence analysis, electrophoresis in the presence of sodium dodecyl sulfate, and by nonequilibrium isoelectric focusing. The NH_2-terminal octapeptide released by the endopeptidase was recovered in 11% yield. Removal of >97% of the NH_2-terminal octapeptide sequences from βNGF had no effect on its dimeric structure, its biological activity, or its ability to reform the 7S NGF complex with stoichiometric amounts of the α and γ subunits. Bisdes(1-8)-βNGF proteins lacking from 15 to 90% of their COOH-terminal arginine residues were prepared by altering the pH, temperature, or salt concentration at which βNGF was incubated with various fractions of the submaxillary gland homogenate. A rapid procedure for the isolation of NGF was devised which involved a two-column fractionation of crude extracts of adult male mouse submaxillary glands, taking advantage of the differing isoelectric points of the parent 7S NGF complex and its βNGF subunit. During isolation by this procedure the NGF dimer lost 17-20% of its COOH-terminal arginine residues and 35% of its NH_2-terminal octapeptide sequences. A method for determining the proportion of NGF chains which have lost COOH-terminal arginine residues or NH_2-terminal octapeptide sequences, or both, was developed. The method depended on the observation that intact chains, which contain two extra histidine residues compared with chains lacking the NH_2-terminal octapeptide, migrated more rapidly than the cleaved chains in the acidic region of the pH gradient established for isoelectric focusing.

Several different methods of isolating the nerve growth factor (NGF)[1] protein from the submaxillary glands of adult male mice have been described (Varon et al., 1968; Bocchini and Angeletti, 1969; Perez-Polo and Shooter, 1975; Jeng and Bradshaw, 1976). In one of them (Varon et al., 1968) the protein, βNGF, is isolated as one of the subunits of the purified 7S complex (Varon et al., 1967). In the other methods the NGF protein is isolated directly from the gland homogenate without prior separation of the complex. These proteins do not have exactly the same structure because the βNGF dimer undergoes two specific proteolytic cleavages when exposed to enzymes in the submaxillary gland extract. One of these cleavages results in the removal of either or both the COOH-terminal arginine residues from the two identical peptide chains in the NGF dimer (Angeletti et al., 1973; Moore et al., 1974). The second results in the production of chains which are shorter by eight amino acid residues at their NH_2 termini than the βNGF chains (Angeletti and Bradshaw, 1971; Angeletti et al., 1973; Mobley et al., 1974).

The properties of the βNGF dimers which lack one or both COOH-terminal arginine residues have been studied in detail (Moore et al., 1974). While complete removal of these residues does not affect the biological activity of βNGF (Moore et al., 1974), it does prevent the protein from reassociating with the other α and γ subunits of 7S NGF to reform the high-molecular-weight complex, a finding which argues in favor of the existence of a longer precursor to the βNGF chain (Angeletti and Bradshaw, 1971; Moore et al., 1974; Berger and Shooter, 1976). This report now describes a method for the complete

† From the Departments of Genetics, Biochemistry, and Neurobiology and the Lt. Joseph P. Kennedy Jr. Laboratories for Molecular Medicine, Stanford University School of Medicine, Stanford, California 94305. *Received March 29, 1976.* This research was supported by grants from the National Institute of Neurological and Communicative Disorders and Stroke (NS 04270), the National Science Foundation (GB 31982), and by a Medical Scientist Training Program grant from the National Institutes of Health (GM 01922). Part of this work was carried out while one of the authors (E.M.S.) was a Faculty Scholar of the Josiah Macy Jr. Foundation in the Department of Pathology, University of Geneva, Switzerland.

‡ Fellow of the Swiss National Science Foundation. Present address: Institute fur Hirnforschung der Universitat Zurich, 8008 Zurich, Switzerland.

[1] Abbreviations used are: $NaDodSO_4$, sodium dodecyl sulfate; NGF, nerve growth factor; βNGF, the preparation made according to the procedure of Varon et al. (1968); βNGF dimer and βNGF chains, the intact βNGF dimer and βNGF chains, respectively; mono- and bisdes(1-8)-βNGF, the βNGF dimers lacking one and both, respectively, of the NH_2-terminal octapeptide sequences; mono- and bisdes-$Arg^{1\,8}$-βNGF, the βNGF dimers lacking one and both, respectively, of the COOH-terminal arginine residues; Bistris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Bis, *N,N'*-methylenebisacrylamide; Tris, tris(hydroxymethyl)aminomethane; CPB, carboxypeptidase B; endopeptidase, the endopeptidase which specifically cleaves the histidine-methionine peptide bond—the eighth peptide bond from the NH_2 terminus of the βNGF chain; CM, carboxymethyl.

removal of the octapeptide sequences from the NH_2 termini of the βNGF dimer and discusses some of the properties of the modified protein. Based on the properties of the proteins modified by proteolytic cleavage, it has now been possible to derive simplified procedures for the isolation of NGF and for the determination of its chain composition. Jeng and Bradshaw (1976) have recently described another rapid procedure for isolating NGF which results in the almost complete cleavage of the NH_2-terminal octapeptide sequences and have also described the properties of this protein in detail.

Materials and Methods

NGF Proteins. The 7S NGF complex was isolated by the method of Varon et al. (1967) with minor modifications (Varon et al., 1972). The βNGF subunit of 7S NGF was isolated as described by Smith et al. (1968), except that βNGF was usually eluted from the CM-cellulose column with 0.05 M sodium borate buffer, pH 10.3, containing 1.5 M NaCl. The procedure of Bocchini and Angeletti (1969) was used for preparing 2.5S NGF. The α and γ subunits of 7S NGF were obtained by the procedure of Smith et al. (1968).

Chemicals. Cyanogen bromide was purchased from Baker Chemical Co., guanidine hydrochloride (Sequanal grade) and sodium dodecyl sulfate (Sequanal grade) were from Pierce Chemical Co., dansyl chloride was from Sigma, polyamide layers were from Gallard-Schlesinger, urea (ultrapure) was from Schwarz/Mann, Bis from Bio-Rad Laboratories, and ampholine (pH 3 to 10) from LKB Produktor AB. CM-cellulose (CM-32) was the product of Whatman and Sephadex G-50 (fine) of Pharmacia.

Cyanogen Bromide Cleavage of βNGF. The procedure described by Gross (1967) was used with a molar ratio of cyanogen bromide to βNGF of 200:1. The βNGF sample (13.2 mg of βNGF in 25 ml of 0.1 N HCl) was dialyzed against 0.1 N HCl for 16 h at 4 °C. To this solution was added 21 mg of cyanogen bromide in 9.7 ml of 0.1 N HCl to bring the concentration of βNGF to 0.38 mg/ml. The mixture was incubated in the dark at 23 °C for 4 days with stirring, was frozen, lyophilized, and finally dissolved in 5 ml of 0.2% acetic acid and frozen at −20 °C for storage. A control sample of βNGF (1.28 mg) was carried through the same treatment in the absence of cyanogen bromide.

The Preparation of Bisdes(1–8)-βNGF Species. A number of different fractions of the mouse submaxillary gland extract were used to achieve the limited proteolysis of βNGF by the specific endopeptidase. The most efficient removal of the NH_2-terminal octapeptide was achieved with a fraction prepared as follows. A homogenate of the gland (5 g of gland homogenized with 15 ml of ice cold water in a Waring blender for 1 min was a convenient scale, although smaller amounts can be prepared) was centrifuged at 25 000*g* for 1 h and then adjusted to pH 4.9 with 1% acetic acid. The precipitate was removed by centrifugation at 15 000*g* for 30 min. The supernatant, identified as the "acidified supernatant", was used for the limited proteolysis of βNGF. The acidified supernatant (0.1 ml) was mixed with a 1 mg/ml solution of βNGF (5.0 ml) previously dialyzed against 0.05 M sodium acetate buffer, pH 4.9, to give a ratio of βNGF protein to acidified supernatant protein of 5:1, and the mixture was incubated at 37 °C for 24 h. The mixture was then dialyzed for 12 h against 0.05 M sodium acetate buffer, pH 4.0, at 4 °C and then loaded onto a CM-cellulose column (2.0 × 10 cm, Whatman CM-32) equilibrated in the same buffer. After washing the sample onto the column with 40 ml of buffer, the column was eluted stepwise with: 40 ml of 0.05 M sodium acetate buffer, pH 4.9, and 0.4 M in NaCl; 30 ml of 0.05 M acetate buffer, pH 4.9; 100 ml of 0.05 M Tris-Cl buffer (pH 9.0); finally with the pH 9.0 buffer containing 0.4 M NaCl. The flow rate was 40 ml per h. The modified βNGF protein comprised the last fraction. Analysis (vide infra) showed that 97% of the NH_2-terminal octapeptide sequences were removed as well as 60% of the COOH-terminal arginine residues.

Another fraction called "dialysate" was used when more extensive (90%) cleavage of the COOH-terminal arginine residues was required in addition to removal of the NH_2-terminal octapeptide sequences. The dialysate was prepared by the method described by Moore et al. (1974) from the 7S NGF-containing pool obtained in the Sephadex G-100 gel filtration of the clarified gland supernatant (Varon et al., 1967; Moore et al., 1974). This pool was dialyzed against 0.05 M sodium acetate buffer, pH 4.9, the precipitate removed by centrifugation (15 000*g* for 30 min), and the clear solution called the dialysate stored frozen at −20 °C until needed. For proteolysis a mixture of βNGF in 0.05 M sodium acetate buffer, pH 4.9, and the dialysate (5.5 mg βNGF with 1.8 ml of dialysate containing 8.9 mg of protein) was incubated at 23 °C for 7 days and the modified βNGF protein isolated by chromatography on CM-cellulose as described above. It lacked >90% of both NH_2-terminal octapeptide sequences and COOH-terminal arginine residues (vide infra).

For the preparation of bisdes(1–8)-βNGF which retained most of the COOH-terminal arginine residues, a method involving the proteolysis of endogeneous βNGF in the gland extract was employed. It was based on the finding that the CPB-like activity in the gland extract was largely inhibited at pH 4.0 in the presence of 0.4 M NaCl. The centrifuged homogenate of the submaxillary glands was prepared as described above but using 20 g of glands and then dialyzed for 24 h against several changes of 1 mM phosphate buffer, pH 6.8 at 4 °C. An appropriate volume of glacial acetic acid was added with stirring to the dialyzed solution to bring its pH to 4.0 and solid NaCl also added to give a final concentration of 0.4 M. The precipitate was removed (15 000*g* for 15 min) and the clear solution incubated for 3 days at 4 °C. After incubation it was loaded onto a CM-cellulose column (2.5 × 15 cm) equilibrated with 0.05 M sodium acetate buffer, pH 4.0, containing 0.4 M NaCl. The column was washed with 200 ml of the same buffer and then with 100 ml of 0.05 M sodium acetate buffer, pH 4.0, and eluted first with 100 ml of 0.05 M Tris-Cl buffer, pH 9.0, and secondly with this latter buffer containing 0.4 M NaCl, all at a flow rate of 50 ml per h. The last protein fraction again contained the modified βNGF protein. It lacked 85% of the NH_2-terminal octapeptide sequence but only 15% of the COOH-terminal arginine residues (vide infra).

A Modified Procedure for Isolating NGF.[2] Frozen submaxillary glands (20 g) obtained from about 100 mice were thawed and homogenized with 100 ml of cold water in a Waring blender for 1 min. The homogenate was then centrifuged at 25 000*g* for 1 h and the clear supernatant (ca. 100 ml) dialyzed against two 4-l. lots of 0.02 M phosphate buffer, pH 6.8, for 16 h at 4 °C. All further steps were carried out at the same temperature.

[2] The two common, low-molecular-weight preparations have been designated as βNGF (Varon et al., 1968) and 2.5S NGF (Bocchini and Angeletti, 1969). In order to avoid using another trivial abbreviation, the present preparation is referred to only as NGF. This preparation may be distinguished from the earlier ones or from any other NGF preparation by stating the percentage of the NGF chains which lack the COOH-terminal arginine residues (17–20%) and the NH_2-terminal octapeptide sequences (35%).

The dialyzed supernatant was passed through a CM-cellulose column (25 × 12 cm) (flow rate 50 ml per h) equilibrated in 0.02 M phosphate buffer, pH 6.8, and the column washed with the same buffer until the absorbance of the eluate at 280 nm fell below 0.5.

The eluate (ca. 160 ml) was then dialyzed against two 4-l. lots of 0.25 mM phosphate buffer over a 24-h period to reduce the buffering capacity of the solution. To the dialyzed solution was added one-ninth of its volume of 0.5 M sodium acetate buffer, pH 4.0, to rapidly reduce the pH and dissociate 7S NGF and establish a final buffer concentration of 0.05 M. Sufficient solid NaCl was then added to bring the final concentration of NaCl to 0.4 M. After standing for 5 min to allow the precipitate to form, the solution was centrifuged at 25 000*g* for 30 min and the pellet discarded.

The acidified solution was immediately loaded onto a second CM-cellulose column (2.5 × 15 cm) equilibrated with 0.05 M sodium acetate buffer, pH 4.0, containing 0.4 M NaCl and the nonadsorbing material eluted from the column with ~150 ml of the same buffer. After washing the column with 50 ml of 0.05 M sodium acetate buffer, pH 4.0, the remaining protein was eluted in two steps. Elution with 0.05 M Tris-Cl buffer, pH 9.0 (ca. 100 ml), produced a faint red-colored fraction containing half the remaining protein which eluted as soon as the pH of the column rose above 8.0. The purified NGF fraction was eluted last with 0.05 M Tris-Cl buffer, pH 9.0, containing 0.4 M NaCl. The NGF peak usually displayed asymmetry on its trailing edge.

Molecular Weight Determination. The sedimentation equilibrium meniscus depletion method of Yphantis (1964) was used. Samples at 0.4 to 0.5 mg/ml protein concentration were dialyzed against 0.10 M sodium acetate buffer (pH 4.0) containing 0.1 M NaCl. Sedimentation was carried out in a 12-mm double sector cell using 0.13 ml of protein solution at 36 000 rev/min at 25 °C for 24 h. The partial specific volume of the modified βNGF proteins was taken to be 0.725.

Biological Assay. The biological activity was determined by the standard procedure (Varon et al., 1972; Levi-Montalcini et al., 1954) using explanted sensory ganglia from 8-day-old chick embryos.

Electrophoresis and Isoelectric Focusing. Electrophoresis in a Bistris-Tes system of pH 7.55 in 7.5% acrylamide gels was as previously described (Moore et al., 1974). Electrophoresis in sodium dodecyl sulfate and urea-containing acrylamide gels was performed according to Swank and Munkres (1971) using 5 × 120 mm, 12.5% gels with a 1:10 ratio of Bis to acrylamide. The concentration of Tris-phosphate buffer, pH 6.8, was increased from 0.1 to 0.17 M in both the gel solution and the buffer reservoirs. Protein samples (10–30 μg, except where specified) were incubated prior to electrophoresis in 0.17 M Tris-phosphate buffer containing 1% sodium dodecyl sulfate for 5 min at 100 or at 37 °C for 4 h plus 65 °C for 1 h. When these gels were used in the presence of a reducing agent, the upper buffer contained 0.03 M mercaptoacetic acid and the sample incubation medium contained 1% 2-mercaptoethanol. Electrophoresis was carried out for 60 to 70 mA h with currents no greater than 4 mA per tube. Gels were fixed and stained in 1% acid fast green in methanol-acetic acid-water (45:10:45 by volume) for 2 to 3 h at 37 °C and destained with the same solvent.

Isoelectric focusing in a pH 3–10 gradient in 7.5% acrylamide gels in the absence or presence of 8 M urea followed the procedure given earlier (Greene et al., 1971; Server and Shooter, 1976). For the interrupted procedure protein samples were made up in freshly prepared 9 M urea. Optimum resolution of the four different NGF chains was achieved after 400 V h in gels 70 mm in length and 675 V h in gels 100 mm in length. Gels were scanned at 620 nm after staining with 0.1% Naphthol Blue Black or at 560 nm after staining by the procedure of Malik and Berrie (1972).

Amino Acid and Peptide Analysis. Samples for amino acid analysis were treated according to the method of Moore and Stein (1963). Hydrolysis was carried out at 110 °C for 16 h and amino acid analysis was performed with a Beckman Spinco Model 120 B amino acid analyzer. Eluates from the gel filtration on Sephadex G-50 were analyzed for peptide material by the method of Hirs (1967). Protein concentrations were determined by the method of Lowry et al. (1951).

NH_2-Terminal Amino Acid Determination. The procedures for both peptide and protein analysis were those of Gray (1972) and for the thin-layer chromatography on polyamide sheets those of Weiner et al. (1972). Approximately 5 to 10 nmol of material was taken for dansylation. For the thin-layer chromatography 10 to 30 pmol of standard dansyl amino acids or of the unknown in 10% formic acid was separated on 5 × 5 cm polyamide sheets (Weiner et al., 1972).

Sequence Analysis. Amino acid sequence analyses were carried out on a Beckman automated sequenator. Successive cycles of the Edman degradation were carried out following the general technique of Edman and Begg (1967). Before analysis the proteins were carbamoylmethylated by the method of Morris et al. (1971).

The carbamoylmethylated des(1-8)-βNGF (612 nmol) was dissolved in 50% acetic acid and successive 0.5-ml volumes were applied to the cup and dried. The analysis used a modified slow protein program (Beckman 04270) with 0.5 M Quadrol buffer. The amino acid >PhNCS derivatives were identified by gas-liquid chromatography by a method similar to that described by Pisano and Bronzert (1969).

High-Voltage Electrophoresis. Electrophoresis was carried out in a Gilson Model DW high voltage electrophorator in a pyridine-acetic acid-water (5:5:390 by volume) mixture at pH 4.7 using Whatman 3 MM paper (46 × 57 cm). The conditions for electrophoresis were 2000 V for 60 min at 25 °C after sample application in a 2-cm strip 10 cm from the anode edge of the paper. Peptides were eluted (after marking with 0.02% ninhydrin) with pyridine-acetic acid-water (1:1:1 by volume).

Results

The Detection of Native and Modified NGF Chains. NGF chains shortened by removal of their NH_2-terminal octapeptide sequences were sufficiently different in molecular weight from native chains (the molecular weight difference is ca. 1000) to be resolved from the latter by electrophoresis in the presence of sodium dodecyl sulfate and urea (Figure 1). These analyses confirmed what was already known from sequence data (Angeletti and Bradshaw, 1971; Angeletti et al., 1973; Móbley et al., 1974) that 2.5S NGF has a higher proportion of shorter (faster migrating) chains than βNGF. Whereas βNGF had only 8% of the shorter chain (Figure 1E), 2.5S NGF contained between 40 and 50% (Figure 1F). The identity of the faster migrating chain was confirmed by showing that it had a similar mobility to NGF chains shortened by nine residues at their NH_2 termini by cleavage with cyanogen bromide at the single methionine residue in position 9 (Angeletti et al., 1973). (The separation of the des(1-9)-βNGF chain from the NH_2-terminal nonapeptide is shown in Figure 3a). Loss of one or both COOH-terminal arginine residues from the βNGF dimer produces species of lower isoelectric points than βNGF (Moore

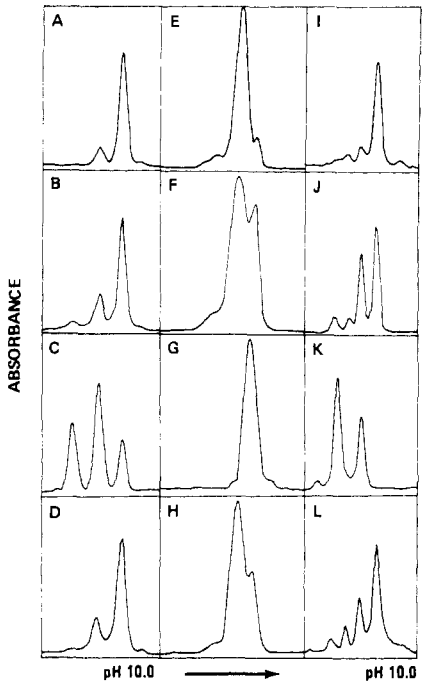

FIGURE 1: Comparison of βNGF, 2.5S NGF, bisdes(1-8)-βNGF, and the NGF isolated by the new procedure, by isoelectric focusing, and electrophoresis in acrylamide gel. The analyses in the first column (A to D) were obtained by isoelectric focusing in 7.5% acrylamide gels in a pH 3 to 10 gradient; in the second column (E to H) by electrophoresis in 12.5% acrylamide gels in the presence of sodium dodecyl sulfate and urea; and in the third column (I to L) by isoelectric focusing in 7.5% acrylamide gels in the presence of 8 M urea stopping the analysis after 400 V h. The procedures were as outlined in Materials and Methods. The analyses of βNGF are shown in the top line, of 2.5S NGF in the second line, of bisdes(1-8)-βNGF in the third line, and of NGF isolated by the new procedure in the bottom line. Protein loads were between 25 and 50 μg. Only those sections of each gel scan containing the protein peaks are reproduced. The remaining sections were equivalent to background scores. In the first column the peaks correspond to (from left to right) bisdes-Arg^{118}-βNGF, monodes-Arg^{118}-βNGF, and βNGF; in the second column, the two peaks are the βNGF and the des(1-8)-βNGF chains, respectively, and in the third column the four chains are (left to right) des-Arg^{118}, des(1-8)-βNGF; des-Arg^{118}-βNGF; des(1-8)-βNGF; and βNGF.

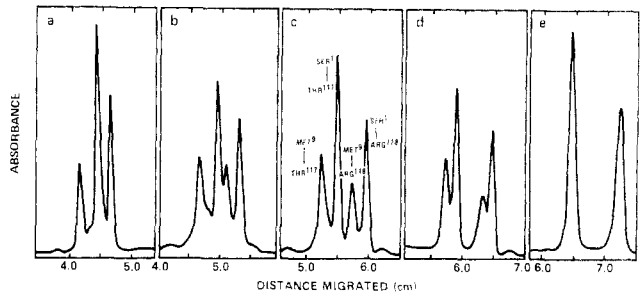

FIGURE 2: The separation of the four different NGF chains during isoelectric focusing in the presence of 8 M urea. Isoelectric focusing was in a pH 3-10 gradient in 8 M urea with gels 100 mm in length as described in Materials and Methods. A sample of 30 μg of NGF isolated as described in footnote *i* of Table I was loaded onto each of five gels and electrofocusing carried out for (a) 450, (b) 575, (c) 675, (d) 770, and (e) 975 V h. Gels were scanned at 560 nm after staining by the Malik and Berrie (1972) procedure.

et al., 1974). Equilibrium isoelectric focusing of βNGF and 2.5S NGF showed that the latter contained slightly more of the dimer lacking one COOH-terminal arginine residue than βNGF as well as small amounts of the dimer lacking both such residues (Figure 1A,B).

A single analytical system for distinguishing complete NGF chains from the three different chains which result from the specific proteolytic cleavages at either or both NH_2 or COOH termini has now been developed. The octapeptide which is released from the NH_2 termini of the βNGF chains contains two histidine residues (Angeletti and Bradshaw, 1971; Angeletti et al., 1973). At acid pH, therefore, the intact βNGF chain has two extra positive charges compared with the shorter, des(1-8)-βNGF chain. Provided the sieving effect of the acrylamide gel is insufficient to distinguish between intact and short chains, the former should migrate through the acidic regions of a pH 3-10 gradient more rapidly than the latter. This has been found to be true in isoelectric focusing analyses carried out in the presence of urea to dissociate NGF dimers into chains. The separation of the different chains in an NGF preparation as the protein moved toward equilibrium in the pH gradient is shown in Figure 2. In the early stages three peaks were resolved (Figure 2a). Somewhat later the center component resolved into two peaks (Figure 2b), and at an appropriate time (Figure 2c) the four components were optimally resolved. With continued migration the two minor components moved closer to the major ones (Figure 2d) until each pair finally fused and banded at the same equilibrium pH (Figure 2e). The final equilibrium positions of these two components corresponded to those of NGF chains with and without their COOH-arginine residues, respectively (Moore et al., 1974). If the argument given above is correct, then the slower (minor partner) of each pair of components appearing at the intermediate stage of focusing should be the chain which also lacks the NH_2-terminal octapeptide. The four different types of NGF chains can, therefore, be identified with the four components as shown in Figure 2c. To confirm these identifications, a number of different NGF preparations were analyzed by this new procedure and the results compared with those obtained by isoelectric focusing in the absence of urea (which gives the extent of COOH-terminal arginine cleavage) and by electrophoresis in the presence of sodium dodecyl sulfate and urea (which gives the extent of NH_2-terminal cleavage). The data are shown in Table I. Over a wide range of both NH_2- and COOH-terminal cleavage, the new procedure gave values comparable to those obtained with the two separate analytical systems. This procedure of interrupted isoelectric focusing, therefore, permits a rapid determination of the chain composition of a given NGF preparation in a single analysis. The analyses of βNGF and 2.5S NGF by this procedure are shown in Figure 1I,J.

The Endopeptidase Cleavage of the βNGF Chains. In analyses of 2.5S NGF or other partially degraded NGF preparations by either electrophoresis under denaturing conditions or by nonequilibrium isoelectric focusing, no evidence was found for chains intermediate in size between βNGF and des(1-8)-βNGF chains such as might result from sequential proteolysis from the NH_2 terminus. This has now been confirmed by isolation of the NH_2-terminal octapeptide itself. After incubation of βNGF with the dialysate, the mixture was lyophilized, dissolved in 50% acetic acid, and subjected to gel filtration on Sephadex G-50 in the same solvent (Figure 3b). A single major peak of protein was eluted at the exclusion volume of the column as in the chromatography of the cyanogen bromide treated βNGF (Figure 3a); this component presumably contained bisdes(1-8)-βNGF as well as proteins of the dialysate. Of the lower molecular weight fractions, pool 1 contained most peptide material and was eluted at approximately the same volume as the NH_2-terminal nonapeptide

TABLE I: Comparison of the Chain Composition of Several NGF Preparations.

NGF Prep or Method of Isolation	% Des-Arg^{118}-βNGF Chain IEF[a]	% Des-Arg^{118}-βNGF Chain IEF 8 M Urea[c]	% Des(1-8)-βNGF Chain NaDodSO$_4$[b]	% Des(1-8)-βNGF Chain IEF 8 M Urea
βNGF[d]	7	8	8	9
2.5S NGF[e]	17	15	45	44
NGF[f]	17	20	35	34
NGF[g]	24	25	64	59
1 column[h]	33	33	45	38
2 column[i]	60	58	35	33
Bisdes(1-8)-βNGF[j]	57	56	>97	100

[a] Analysis by the method given by Greene et al. (1971) and Server and Shooter (1976); see also Materials and Methods. [b] Analysis by the method of Swank and Munkres (1971). [c] Analysis by the method described in the text. Isoelectric focusing (70 mm gels) was stopped after 400 V h. [d] Isolated by the procedure of Varon et al. (1968). [e] Isolated by the procedure of Bocchini and Angeletti (1969). [f] Isolated by the procedure described in this paper. [g] Isolated by the procedure described in this paper, except that the eluate from the first CM-cellulose column was dialyzed for 24 h against 0.05 M acetate buffer (pH 4.0). [h] NGF isolated by the one-column procedure as follows: supernatant was prepared as described in Materials and Methods and rapidly adjusted to pH 4 by the addition of one-ninth of its volume of 0.5 M acetate buffer, pH 4.0. It was then dialyzed against 0.5 M acetate buffer (pH 4.0) for 24 h before NaCl was added to a final concentration of 0.4 M and fractionation on the second CM-cellulose column equilibrated in this solvent. [i] NGF isolated by a procedure to allow extensive cleavage of the COOH-terminal arginine residues. Homogenate was prepared as described in Materials and Methods and dialyzed against 0.25 mM phosphate buffer (pH 6.8) for 24 h. The pH was adjusted to 4.0 by addition of one-ninth volume of 0.5 M acetate buffer, pH 4.0, NaCl added to 0.4 M, and the sample applied to a CM-cellulose column (2.5 × 15 cm) equilibrated in 0.05 M acetate buffer (pH 5.5) containing 0.1 M NaCl. Nonadsorbed proteins were washed from the column with 200 ml of the same buffer and an NGF fraction eluted by raising the NaCl concentration to 0.5 M. This fraction (40 ml) was applied to a CM-cellulose column equilibrated in 0.05 M Tris-Cl buffer, pH 9.0, the column washed with 100 ml of the same buffer, and NGF eluted by raising the NaCl concentration to 0.4 M. [j] Prepared as described in this paper.

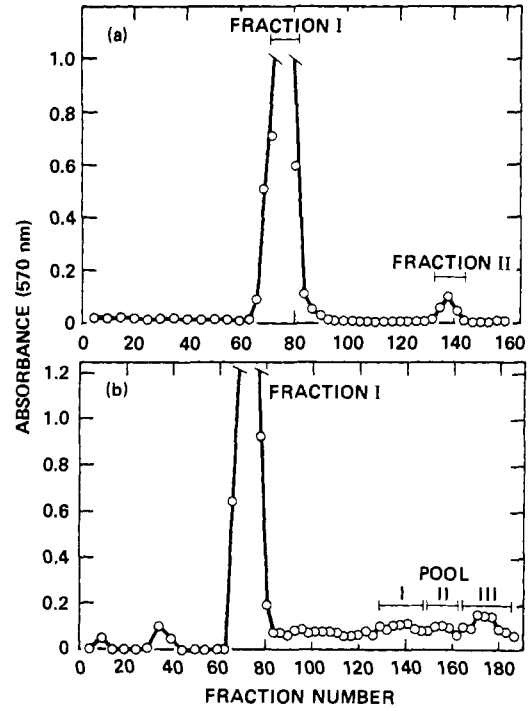

FIGURE 3: (a) Gel filtration of the products of the reaction of βNGF with cyanogen bromide. The βNGF (0.9 nmol) was treated with cyanogen bromide as described in Materials and Methods. After lyophilization, the powder was dissolved in 2.0 ml of 50% acetic acid and loaded on a 1.5 × 150 cm column of Sephadex G-50 fine equilibrated with the same solvent. The flow rate was 15 ml per h and 1.4-ml fractions were collected. A 150-μl portion of each fraction was used for peptide analysis by the ninhydrin procedure as described in Materials and Methods. The inclusion volume of the column was reached at fraction 90. (b) Gel filtration of the products of the incubation of βNGF with dialysate. The βNGF (0.7 nmol) was incubated with 3.24 ml of dialysate (containing 16.2 mg of protein) as described in Materials and Methods and lyophilized. The powder was dissolved in 2 ml of 50% acetic acid and the mixture analyzed by gel filtration under the conditions described in a.

(Figure 3a, fraction II). Pool 1 was further purified by high-voltage electrophoresis. Two peptides were identified by ninhydrin staining at 18.6 and 6.5 cm from the origin toward the cathode, and the more basic major peptide had the amino acid composition shown in Table II. This analysis together with the finding that its NH_2-terminal amino acid was serine by the dansyl method identified it as the NH_2-terminal octapeptide. It was obtained in 11% yield.

The Preparation and Characterization of Bisdes(1-8)-βNGF. The bisdes(1-8)-βNGF was prepared by proteolysis of βNGF with the acidified supernatant as described in Materials and Methods and the primary identification made by amino acid and sequence analyses. The amino acid analysis of the protein (Table II) was close to that anticipated for the chains of bisdes(1-8)-βNGF. Most residue numbers were identical or within one residue except for valine which was low by two residues. The NH_2-terminal amino acid of the modified protein was identified as methionine and the sequence of the following 20 residues was identical with that of residues 10 through 29 of the βNGF chain. Since serine, the NH_2-terminal amino acid residue of the βNGF chain, was not detected in the first cycle, it appeared that the removal of the NH_2-terminal octapeptide sequences from both βNGF chains was complete. On electrophoresis in the presence of sodium dodecyl sulfate a single major band appeared which migrated with the faster of the two bands in β or 2.5S NGF (Figure 1G). By this criteria less than 2% of the original chain remained. The same result was obtained when the protein sample was treated with 1% 2-mercaptoethanol to reduce the three internal disulfide bridges in the peptide chains and the electrophoresis conducted in gels which also contained mercaptoacetic acid (data not shown). A small amount of a species with molecular weight lower than that of the des(1-8)-βNGF chain was also seen in these analyses (Figure 1G). Its composition has not been examined.

Analysis of the protein by the nonequilibrium isoelectric focusing procedure revealed the presence of only two types of chains (Figure 1K). One of these migrated in the same position as the βNGF chain lacking the NH_2-terminal octapeptide and the other in the position of the βNGF chain lacking both the NH_2-terminal octapeptide and the COOH-terminal arginine residue. These identifications were made by comparison with the βNGF and 2.5S NGF preparations of known chain composition (Figure 1I,J) and by the analysis of a mixture of the bisdes(1-8)-βNGF preparation and βNGF (data not shown). These analyses also demonstrated that the removal of the NH_2-terminal octapeptide sequences was complete. The chain present in largest amount in bisdes(1-8)-βNGF lacked the COOH-terminal arginine residue and accounted for 60% of

TABLE II: Amino Acid Composition of Fractions Obtained from Endopeptidase Cleavage of βNGF.

Amino Acid	Number of Residues			
	Des(1-8)-βNGF[a]	Des(1-8)-βNGF[b]	Pool I Peptide[c]	βNGF (1-8)[b]
Lys	8.2	8		
His	2.1	2	2.4	2
Arg	6.8	7		
Asp	12.4	11		
Thr	13.6	13	1.2	1
Ser	9.8	9	1.5	2
Glu	8.5	8		
Pro	1.3	1	1.0	1
Gly	5.6	5		
Ala	8.5	8		
Cys	4.9	6		
Val	10.1	12	1.1	1
Met	0.53	1		
Ile	4.5	5		
Leu	2.8	3		
Tyr	2.2	2		
Phe	6.1	6	1.0	1
Trp	3[d]	3		

[a] Prepared by incubation of βNGF with acidified supernatant as described in Materials and Methods. Data expressed as residues per 12 400 g. [b] Values taken from the data of Angeletti et al. (1971). [c] Calculated by assigning phenylalanine the value of 1.0. [d] Assumed value.

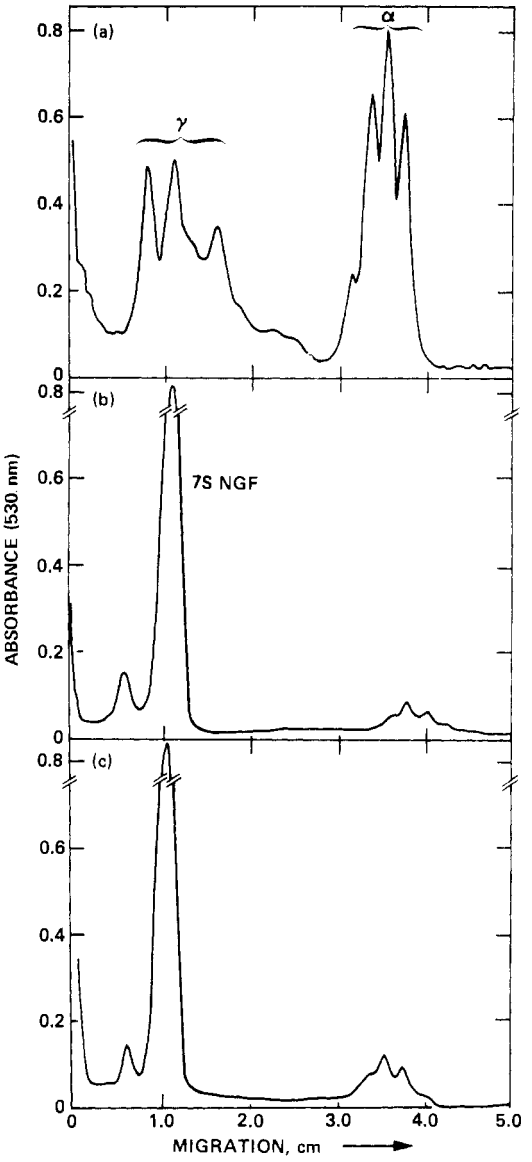

FIGURE 4: The ability of bisdes(1-8)-βNGF to recombine with the α and γ subunits. Mixtures of 50 μg of α subunit and 50 μg of γ subunit were incubated in 0.05 M phosphate buffer, pH 6.8, with 30 μg of βNGF or 30 μg of the bisdes(1-8)-βNGF which retained 85% of its COOH-terminal arginine residues for 16 h at 4 °C. The mixtures were then analyzed by electrophoresis in 7.5% acrylamide gels in the Bistris-Tes system at pH 7.55 as described in Materials and Methods. (a) Mixture of the α and γ subunits; (b) recombination of α and γ subunits with βNGF; (c) recombination of α and γ subunits using bisdes(1-8)-βNGF. The position of control 7S NGF was determined in separate analyses and by addition of 7S NGF to the mixtures.

the total. The same result was obtained by analyzing the protein by isoelectric focusing in the absence of urea which showed that 50% of the species lacked one COOH-terminal arginine residue and 32% both such residues (Figure 1C). Other preparations of bisdes(1-8)-βNGF more and less modified at their COOH-termini were obtained by altering the incubation conditions.

A bisdes(1-8)-βNGF preparation which retained most of its COOH-terminal arginine residues was prepared by proteolysis of endogenous βNGF in the gland supernatant as described in Materials and Methods. The method depended on the fact that the activity of the CPB-like enzyme(s) was largely inhibited at pH 4.0 by low temperature and relatively high (0.4 M) salt concentration. In such preparations, 85% of the NH_2-terminal octapeptide sequences were removed while about 85% of the COOH-terminal arginine residues remained intact. Conversely, prolonged incubation of βNGF with the dialysate (see Materials and Methods) resulted in the release of more than 90% of both NH_2-terminal octapeptide sequences and COOH-terminal arginine residues.

The Properties of Bisdes(1-8)-βNGF. The appearance in these preparations (as shown in Figure 1C) of hybrid species which contain one chain ending in COOH-terminal arginine and one chain lacking this residue suggested that bisdes(1-8)-βNGF retained the dimer character of βNGF. This was confirmed by measuring the molecular weight of the modified protein. The value of 25 400 obtained from the linear plot of the ln fringe displacement vs. the square of the radial distance is in excellent agreement with that (24 680) calculated for a βNGF dimer lacking its NH_2-terminal octapeptide sequences. The same result was obtained with bisdes(1-8)-βNGF species lacking either 15% or >90% of the COOH-terminal arginine residues.

The ability of the bisdes(1-8)-βNGF to reform a 7S NGF complex was also tested. Because the COOH-terminal arginine residues are critical to the recombination process, the bisdes(1-8)-βNGF preparation which retained 85% of these residues was used in this experiment. The addition of a given amount of bisdes(1-8)-βNGF to an equimolar mixture of the α and γ subunits produced the same amount of 7S NGF as did the addition of an equivalent amount of βNGF (Figure 4). The major recombined product was identified as 7S NGF by comparison with analyses of standard 7S NGF and by addition of the latter to the recombination mixture (data not shown). This experiment therefore shows that the bisdes(1-8)-βNGF reforms 7S equally as well as the βNGF dimer. The identity of the minor component migrating more slowly than 7S NGF is not known, although it may be an αβ complex resulting from a slight excess of α subunit in the recombination mixture

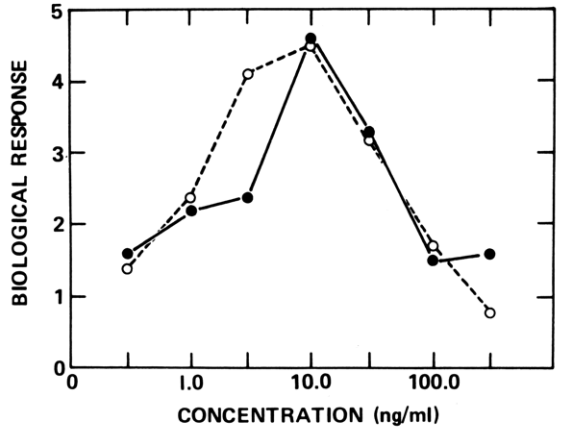

FIGURE 5: The biological activity of bisdes(1-8)-βNGF. The bisdes(1-8)-βNGF was prepared using the acidified supernatant as outlined in Materials and Methods. Biological activity is expressed on an arbitrary scale from 0 (no fiber outgrowth) to 5 (maximum fiber outgrowth). (- - - O - - -) βNGF; (—● —) bisdes(1-8)-βNGF.

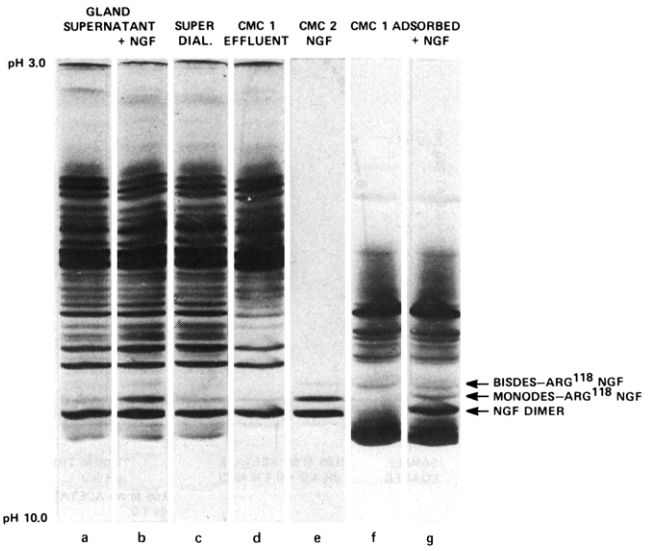

FIGURE 6: The protein composition of various fractions obtained during the isolation of NGF. Analyses were made by isoelectric focusing in 7.5% acrylamide gels as described in Materials and Methods. Gels were stained by the Malik and Berrie (1972) procedure. (a) Supernatant (2 mg of protein); (b) supernatant (2 mg of protein) plus 25 μg of NGF; (c) supernatant dialyzed against 0.02 M phosphate buffer, pH 6.8 (2 mg of protein); (d) eluate from first CM-cellulose column (2 mg of protein); (e) final fraction (NGF) from second CM-cellulose column (25 μg of protein); (f) eluate from first CM-cellulose column obtained with 0.05 M Tris-Cl buffer, pH 9.0, containing 0.4 M NaCl (500 μg of protein); and (g) the sample described in f plus 25 μg of NGF. NGF or NGF dimer refers to βNGF or βNGF dimer, respectively.

(Server and Shooter, 1976).

Because of the relative inaccuracy of the bioassay, biological activities obtained with 2.5S NGF (lacking only 40 to 50% of the octapeptide) cannot reliably assess the activity of chains shortened at their NH_2 terminus. The biological activity of bisdes(1-8)-βNGF was therefore compared with that of βNGF using the standard bioassay procedure with explanted sensory ganglia from 8-day-old chick embryos. The maximum neurite outgrowth was obtained with similar concentrations of the two proteins and the extent of neurite outgrowth was also about the same over a wide range of concentrations (Figure 5). Thus, removal of the NH_2-terminal octapeptide sequences has no effect on the biological activity of the protein.

A Rapid Procedure for the Isolation of NGF. Given that complete removal of the COOH-terminal arginine residues (Moore, et al., 1974) of the NH_2-terminal octapeptide sequences or of both has no effect on the biological activity of the NGF protein, it is possible to simplify the procedures for its isolation. The one described here utilizes the differences in the isoelectric points of 7S (5.1) and βNGF (9.3) (Varon and Shooter, 1970) to remove basic proteins from the gland homogenate on one ion-exchange column before the basic NGF protein is itself isolated from the complex on a second column. The procedure is given in detail in Materials and Methods and has been applied to from 1 g to in excess of 200 g of glands.

The major fraction of the NGF activity as well as about 80% of the total protein was not adsorbed to the first CM-cellulose column at pH 6.8 (Table III). Isoelectric focusing analyses showed that the protein adsorbed to the column comprised most of the proteins more basic than the βNGF dimer as well as a number of proteins with lower isoelectric points than NGF (Figure 6c,d,f). The position at which the NGF proteins in the supernatant electrofocused under these conditions was established by addition of NGF to the supernatant or to the adsorbed protein (Figure 6b,g). Practically all the purification was achieved on the second CM-cellulose column (Table III and Figure 7). While at least 99% of the protein passed through the column, more than 95% of the NGF activity was adsorbed. The first fraction eluted with the buffer at pH 9.0 contained only 1-2% of the adsorbed NGF activity while the major part of the activity eluted in the final NGF protein fraction (Table III). The yield was of the order of 7 mg NGF from 20 g of glands and the overall recovery of NGF activity about 40% of that in the gland supernatant. The yield is comparable to that obtained in the isolation of 2.5S NGF and significantly greater than for βNGF isolated from 7S NGF. The biological activity of this NGF fraction was indistinguishable from that of β or 2.5S NGF.

The loss of COOH-terminal arginine during isolation was similar to that seen in 2.5S NGF; about 25% of the NGF dimers lacked one and 5% both such residues (Figure 1D). About 35% of the NGF chains lacked the NH_2-terminal octapeptide

TABLE III: Protein and NGF Content of Fractions Obtained in the Isolation of NGF.

Fraction[a]	Vol (ml)	Protein (mg)	Biol. Act.[b] (BU × 10^{-6})	Recovery (%)	Spec Act. (ng/BU)
1. Supernatant	105	2000	1.6	(100)	1280
2. Dialyzed supernatant	117	1950	1.5	95	1300
3. Eluate from first CM-cellulose column	164	1500	1.3	80	1170
4. Eluate adjusted to pH 4.0 and 0.4 M NaCl	212	1250	0.95	61	1310
5. NGF fraction from second CM-cellulose column	32	7	0.63	40	11

[a] The preparation of the fractions is described in Materials and Methods. [b] BU refers to the biological unit, as previously described (Levi-Montalcini and Angeletti, 1968).

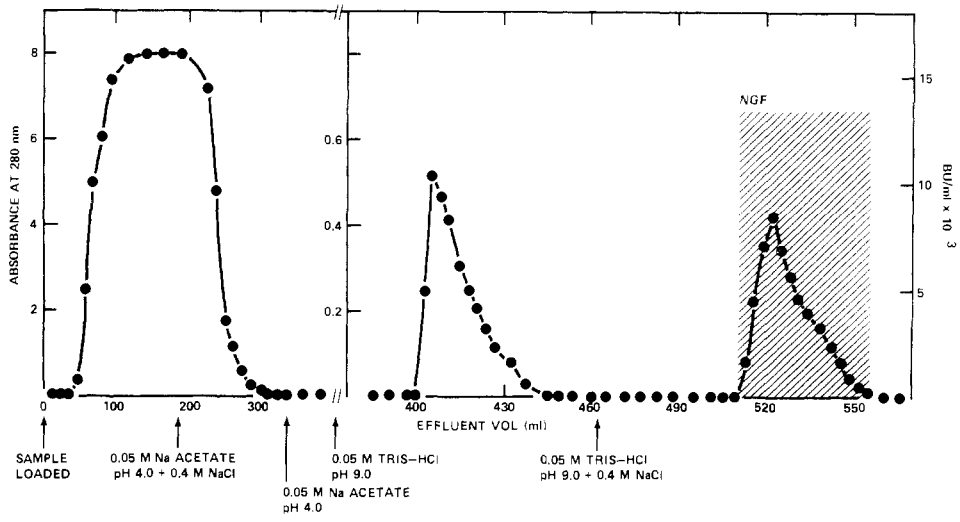

FIGURE 7: CM-cellulose chromatography of acidified eluate from the first CM-cellulose column. See Materials and Methods for column procedures. The protein load was 1250 mg and the fraction size was reduced from 12 to 3 ml after starting elution with 0.05 M Tris-Cl buffer, pH 9.0. The hatched area indicated the presence of NGF as detected by bioassay. BU refers to the biological unit, as previously described (Levi-Montalcini and Angeletti, 1968).

as judged by electrophoresis in sodium dodecyl sulfate (Figure 1H) or by nonequilibrium isoelectric focusing (Figure 1L). The molecular weight was determined to be 26 600. The identity of the small amount of material(s) of higher isoelectric point and higher molecular weight than the NGF proteins has not yet been determined.

Discussion

The Specific Proteolysis of βNGF. The βNGF protein acts as a substrate for at least two enzymes in the submaxillary gland and they in turn can be used as probes to explore certain structure–function relationships in NGF. By varying the conditions for incubation of βNGF with the gland homogenate or fraction derived from it, it is possible to obtain NGF proteins lacking the COOH-terminal arginine residue or the NH_2-terminal octapeptide sequences or both. Neither the complete removal of the COOH-terminal arginine residues by the carboxypeptidase-B-like enzyme (Moore et al., 1974) nor of the NH_2-terminal octapeptide sequences by the endopeptidase has any effect on the biological activity of NGF, and the terminal sections of its chains are not, therefore, involved in its interaction with the specific NGF receptors in the responsive ganglia (Bannerjee et al., 1973; Herrup and Shooter, 1973; Frazier et al., 1974). Hogue-Angeletti et al. (1974) also determined that the octapeptide itself has no biological activity. Furthermore, neither of these proteolytic modifications sufficiently affects the interchain binding in the NGF dimer to alter its sedimentation properties. However, the absence of the COOH-terminal arginine residues does affect interaction between the subunits of the 7S NGF complex (Moore et al., 1974) while the absence of both NH_2-terminal octapeptide sequences does not. Bisdes-Arg^{118}-βNGF completely fails to interact with the α and γ subunits to reform 7S NGF (Moore et al., 1974), while monodes-Arg^{118}-βNGF will do so but much less efficiently than βNGF (Perez-Polo and Shooter, 1975). A comparable situation exists with the EGF protein where removal of its COOH-terminal arginine residues also prevents its interaction with the specific esteropeptidase (EGF-binding protein) with which it normally forms a high-molecular-weight complex (Server et al., 1976). These findings have been used to argue in favor of the idea that both NGF and EGF are synthesized as longer precursor peptide chains which are processed to the biologically active chains by the arginine esteropeptidase enzyme in each complex (Angeletti and Bradshaw, 1971; Moore et al., 1974; Server et al., 1976). Data have recently been obtained for the NGF system which supports this contention (Berger and Shooter, 1976). The removal of the COOH-terminal arginine residue from βNGF may also affect the binding of zinc ions which are known to be critically involved in the stability of 7S NGF (Pattison and Dunn, 1976). A comparable situation exists for the NH_2-terminal octapeptide and the COOH-terminal arginine residues with respect to their enzymatic cleavage from the parent molecule. It is known that the proteolytic release of the octapeptide is prevented if βNGF is in the 7S NGF complex rather than free[3] and the same holds true for release of the COOH-terminal arginine residues (Moore et al., 1974). The protection afforded βNGF at its NH_2 termini is not directed at preserving the receptor binding site of βNGF, its binding domains for the α and γ subunits, or the binding domains of the NGF chains themselves. It may be directed at controlling the release of the NH_2-terminal octapeptide itself, a process which would be dictated by the stability of the 7S NGF complex under any given set of conditions.

The Significance of the Endopeptidase Activity. The proteolytic cleavage of the eighth peptide bond in the βNGF peptide chains by a soluble endopeptidase(s) in the submaxillary gland extract is an interesting phenomenon whose physiological significance is not yet understood. The isolation of the NH_2-terminal octapeptide, even if in less than quantitative yield, suggests that the proteolysis occurs only at this single peptide bond and not by sequential removal of the first eight NH_2-terminal amino acid residues. This point is further substantiated by the fact that only one specific, shorter peptide chain was detected by electrophoresis under denaturing conditions with βNGF samples which had been cleaved to widely differing extents. The amino acid sequence data also lead to the same conclusion since only chains with NH_2-terminal serine (the original βNGF chains) or NH_2-terminal methionine (the des(1–8)-βNGF chains) residues were detected in

[3] W. C. Mobley, A. Schenker, and E. M. Shooter, unpublished observations.

preparations like 2.5S NGF where the cleavage is incomplete (Angeletti et al., 1973).

The peptide bond which is hydrolyzed is a histidine–methionine bond. This is apparently a rare peptide bond in proteins since only a few examples are found in known protein sequences (Dayhoff, 1972). It is of interest to know whether the endopeptidase is specific for this peptide bond. This cannot be determined from the present data. Experiments aimed at determining specificity await the purification of the enzyme which is now being attempted. It will also be of interest as one approach in attempting to understand the physiological role of the octapeptide to see if the endopeptidase is present either in plasma where it could act on circulating NGF, or in sympathetically innervated tissues other than the submaxillary gland where it could act on NGF prior to its uptake at the nerve terminal (Hendry et al., 1974).

The Form of NGF in Extracts of the Submaxillary Gland. Although it is recognized that the 7S NGF complex is a major form of NGF in extracts of the gland (Perez-Polo and Shooter, 1974; Varon and Shooter, 1970), it is not clear that it is the only form. The question as to whether significant amounts of the low-molecular-weight NGF are also present has not been previously answered. The new procedure for isolating NGF speaks to this issue and suggests that this is not so. If low-molecular-weight NGF was present in the gland extract, it would be adsorbed onto the first CM-cellulose column at pH 6.8. Not only was the recovery of NGF activity high after this column procedure (Table III), suggesting that little NGF was adsorbed, but very little NGF activity (ca. 0.3% of the NGF activity loaded) was desorbed from this column when it was eluted with the buffer which desorbs NGF from CM-cellulose. Similarly, very little (if any) NGF protein was detected in the analysis of the protein eluted from the first column (Figure 6f,g). Even if the recovery of NGF under these conditions was low (i.e., 50%), the amounts of low-molecular-weight NGF in the extract would still be extremely small.

The 7S NGF complex is dissociated under the conditions of the isoelectric focusing analysis and the βNGF dimer which is produced is recognizable by its characteristic equilibrium position on the gel (Figure 6a,b). This identification is aided by the presence of monodes-Arg^{118}-βNGF which bands at a slightly lower isoelectric point than the intact βNGF dimer. The ratio of the amount of these two NGF proteins to the total protein was increased somewhat in the eluate from the first column as compared with that in the supernatant (Figure 6a,c,d). If the supernatant had contained significant amounts of βNGF, the proportion would have decreased.

Furthermore, any βNGF in the supernatant would have been subject to proteolytic cleavage during its extended dialysis at neutral pH, producing increasing quantities of mono- and bisdes-Arg^{118}-βNGF. No increase in the amount of these two modified NGF species was seen in the dialyzed supernatant (Figure 6c) compared with the original supernatant (Figure 6a). Therefore, the NGF which is present in the supernatant is protected from cleavage and is present as 7S NGF. The current data suggest that 7S NGF is probably the exclusive form of NGF in the gland extract.

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^{13}C Nuclear Magnetic Resonance Study of Molecular Motions and Conformational Transitions in Muscle Calcium Binding Parvalbumins†

Donald J. Nelson,‡ Stanley J. Opella,*,§ and Oleg Jardetzky

ABSTRACT: ^{13}C nuclear magnetic resonance is used to detect the Ca^{2+} ion controlled conformational transition in muscle calcium binding parvalbumin and to study its intramolecular motions. Nuclear relaxation parameters are used to evaluate the reorientation rates of the protein and some of the amino acid side chains. While peripheral residues exhibit greater motional freedom than the protein interior, an interesting finding is that significant rapid internal motion is present in the phenylalanine rings comprising the hydrophobic core of the protein.

Understanding the function of proteins requires not only knowledge of their structure, but also of the degrees of motional freedom allowed within the structure. In some cases of simple enzymes, knowledge of the static configuration alone may suffice to deduce the catalytic mechanism. However in regulatory and transport proteins, or proteins involved in generating mechanical work, where function is inextricably linked to structural change, knowledge of the available conformational options and segmental mobility is essential.

Many physical, especially spectroscopic methods, can and have been used to detect molecular motions in proteins. Few are suited to describe them in detail. The basic requirement for such a description is that motions of different portions of the molecule be monitored simultaneously. For this reason, high resolution nuclear magnetic resonance is particularly well suited to the study of this problem.

Since the early days of the development of the method, internal motions in polymers have been detected (Bovey, 1959; Jardetzky, 1964a,b) and the particular usefulness of relaxation methods for their investigation had been recognized (Jardetzky and Jardetzky, 1962; Jardetzky, 1964b). In recent years several groups have embarked on a more detailed study of intramolecular motions in proteins (Roberts, 1975). ^1H NMR[1] studies of protein mobility have relied on qualitative features of averaging spectra to place lower limits on the effective rates of internal reorientation (Snyder et al., 1975; Campbell et al., 1975; Wüthrich and Wagner, 1975; Cave et al., 1976). Quantitative interpretations based on relaxation data have been carried out for ^{19}F-labeled proteins (Hull and Sykes, 1975) and for proteins studied by ^{13}C NMR in natural abundance (Allerhand et al., 1971a,b; Opella et al., 1974; Oldfield and Allerhand, 1975a,b; Oldfield et al., 1975) or with site enrichment (Browne et al., 1973; Hunkapiller et al., 1973). These studies have made it apparent that to obtain quantitative information on the motion of individual amino acid side chains, and on changes of these motions with changes in protein conformation, it is necessary to use multiple relaxation parameters to reduce the number of physical models which can be compatible with the data.

^{13}C magnetic resonance provides a particularly informative approach to the problem of internal motions. First, it offers the opportunity of measuring two relaxation parameters (the longitudinal relaxation time T_1 and the nuclear Overhauser enhancement, NOE) with a significantly different functional dependence on the correlation times. The transverse relaxation time T_2 is in principle also useful for this purpose, but is experimentally less accessible for the spectra of large molecules, or generally spectra with limited resolution and signal-to-noise ratios and especially for noise-decoupled ^{13}C spectra. Second, theoretical considerations are greatly simplified for the large class of carbons with directly bonded hydrogens since the dominant relaxation mechanism is dipolar. Third, the distance between the dominant dipoles is given by the C–H bond length, which is constant and known, allowing accurate correlation time calculations at least for isotropic motion. The determination of the rates of internal motion, which are generally anisotropic, is a more complex problem, discussed in more detail below.

Muscle calcium binding proteins (MCBP) have been chosen for this study for several reasons. The MCBPs are of relatively low molecular weight (mol wt ~12 000), highly soluble, and acidic (Pechere et al., 1971). Multiple isotypes are usually isolated from a given species (e.g., there are three major isotypes obtained from common carp (*Cyprinus carpio*)). These isotypes are convenient for comparative studies and greatly aid

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‡ Present address: Department of Chemistry, Clark University, Worcester, Massachusetts 01610.

§ Present address: Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19174. This work is taken in part from the doctoral dissertation of S.J.O., Stanford University, 1974.

[1] Abbreviations used: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; MCBP, muscle calcium binding proteins; TN-C, troponin trimer complex; ALC, myosin alkaline-extractable light chain protein; cAMP, cyclic adenosine 3′,5′-monophosphate; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; Me_4Si, tetramethylsilane.