

Evidence for pro- β -nerve growth factor, a biosynthetic precursor to β -nerve growth factor

(arginyl-esteropeptidase/protein processing/submaxillary gland/gamma subunit/epidermal growth factor)

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ABSTRACT The biosynthesis of β -nerve growth factor (β NGF) was studied in mouse submaxillary glands incubated with L-[³⁵S]cystine. β NGF was isolated from tissue extracts by the addition of antiserum against β NGF and the washed immunoprecipitates were analyzed by sodium dodecyl sulfate gel electrophoresis. With short labeling periods (10 and 25 min) there is a major labeled species with an apparent molecular weight of 22,000 and a smaller peak comigrating with purified β NGF chains (13,260). As time proceeds, the radioactivity in the 22,000 molecular weight peak plateaus, while the label in β NGF continues to increase, until by 4 hr it greatly exceeds the radioactivity of the 22,000 molecular weight species. When glands incubated for 10 min are transferred to medium containing a large excess of unlabeled L-cystine, the 22,000 molecular weight peak gradually declines, and there is a corresponding increase in radioactivity at the β NGF position. The 22,000 molecular weight species isolated from sodium dodecyl sulfate gels possesses all the cystine-containing peptides of β NGF, and possibly two additional ones. When immunoprecipitates from submaxillary glands labeled for 25 min are incubated with the γ subunit (a specific arginyl-esteropeptidase associated with β NGF in the 7S NGF complex), the radioactivity in the 22,000 molecular weight species is converted to the β NGF position. The results suggest that the 22,000 molecular weight species is a biosynthetic precursor to β NGF, and that the γ subunit may function as a specific protease in the processing event.

Nerve growth factor (NGF) is a polypeptide found in nearly all vertebrates which is capable of eliciting profound morphological and biochemical effects from responsive nerve cells, and which is currently thought to play a fundamental role in the development and maintenance of the sympathetic and embryonic sensory ganglia (1, 2). The NGF from male mouse submaxillary glands can be isolated at neutral pH as part of a specific high molecular weight complex called 7S NGF (3, 4). The complex contains three classes of subunits, designated α , β , and γ , which can be purified and recombined to regenerate 7S NGF (4). All the nerve growth-stimulating activity resides in the β subunit (4), which is a dimer of two identical noncovalently associated chains (5, 6), each 118 residues in length (7). The amino acid sequence of these chains has been determined (7), and β NGF has recently been crystallized (8). The γ subunit is a potent arginyl-esteropeptidase, catalyzing the hydrolysis of synthetic arginine esters and amides (9, 10), while no enzymatic activity has yet been found for the α subunit.

One physiological function of 7S NGF may be protection, since β NGF is less susceptible to proteolytic cleavage in submaxillary gland extracts when in the 7S form (11, 12). However, the association of a growth factor with a specific arginyl-esteropeptidase suggests the likelihood of a more active enzymatic role of the complex and its subunits. A clue to what that function might be is provided by the following additional features of 7S

NGF: (i) The COOH-terminal residue (position 118) of each β NGF chain is arginine (7). (ii) The COOH-terminal arginine appears critical for maintaining the 7S complex, since removal of this residue with carboxypeptidase B has little effect on either biological activity (11) or structure (8), yet completely prevents recombination into the 7S complex (11). Furthermore, the arginyl-esteropeptidase activity of the γ subunit is inhibited in 7S NGF (10), suggesting that in the complex the COOH-terminal arginine of β NGF is situated in the active site of the γ subunit. (iii) A strikingly similar situation exists for epidermal growth factor (EGF), another submaxillary gland protein which can likewise be purified in complex with a distinct arginyl-esteropeptidase (EGF binding protein) (13). The arginyl-esteropeptidases from the NGF and EGF complexes have very similar physical, chemical, and immunological properties but EGF binding protein will not substitute for γ in the formation of a 7S-type complex (14). This suggests that the functions of the two enzymes are analogous yet specific for the appropriate growth factor. As with β NGF, the COOH-terminal amino acid of EGF is arginine (13), and this residue is required for complex formation (15) but not for biological activity (16). The esteropeptidase activity of EGF binding protein is similarly inhibited in the complex (15). (iv) β NGF shares some structural and functional features with insulin and proinsulin (17).

These findings, coupled with the observations that many polypeptide hormone precursors are processed by cleavage at specific arginine or lysine residues (18), has led to the prediction (7, 11) that β NGF is initially synthesized as a higher molecular weight precursor (pro- β NGF) with an additional polypeptide length at its COOH-terminal end, and that this precursor is ultimately cleaved on the COOH side of arginine 118 by the action of the γ subunit to yield β NGF. The active site of the arginyl-esteropeptidase then remains complexed with the new COOH-terminal arginine of β NGF and, in conjunction with the α subunit, forms the 7S complex which is the storage form of NGF in the gland. The model thus predicts that the γ subunit is a highly specific protease involved in the biosynthetic processing of the final β NGF molecule from a larger precursor. An analogous proposal has been put forth for EGF and EGF binding protein (13).

In experiments described elsewhere (E. A. Berger and E. M. Shooter, unpublished), we have demonstrated directly by labeling and immunoprecipitation techniques that isolated submaxillary glands synthesize β NGF. Here we present evidence that the initial biosynthetic product is indeed a higher molecular weight precursor (pro- β NGF), and that the γ subunit is capable of cleaving this species to yield β NGF.

Abbreviations: β NGF, β -nerve growth factor; pro- β NGF, precursor to β NGF; EGF, epidermal growth factor; NaDodSO₄, sodium dodecyl sulfate; M_r , molecular weight.

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MATERIALS AND METHODS

β NGF was purified from 7S NGF by the method of Smith *et al.* (19). Purity was confirmed by isoelectric focusing in polyacrylamide gels and by sodium dodecyl sulfate (NaDodSO₄) gel electrophoresis. The γ subunit of 7S NGF was purified as described by Smith *et al.* (19) and had a specific activity of 0.17 μ mol/mg per min when tested at 23° and pH 7.6 against 1 mM α -N-benzoyl-D,L-arginine-*p*-nitroanilide (Sigma). Antiserum was prepared in New Zealand White rabbits by the injection of purified β NGF in complete Freund's adjuvant (20). Antisera were judged to be monospecific by Ouchterlony double diffusion analysis and by immunoelectrophoresis. Antiserum (20–40 μ l) quantitatively precipitated 1 μ g of purified β NGF. ¹²⁵I-labeled β NGF was prepared to a specific activity of 2 to 3×10^7 cpm/ μ g as described (21). L-[³⁵S]Cystine (20–60 Ci/nmol) was purchased from New England Nuclear.

Treatment of Animals. Adult male Swiss Webster mice were castrated. After a period of at least 21 days required for submaxillary gland β NGF levels to fall to a new steady state (20), animals were injected subcutaneously with testosterone propionate (0.2 ml of 5 mg/ml in sesame oil) on days 1 through 4. On day 3, the food was removed and on day 4, animals were killed by cervical dislocation, and the submaxillary glands were removed.

Labeling of Submaxillary Glands with L-[³⁵S]Cystine. Submaxillary glands were cut into small pieces (10–15 mg wet weight) and incubated at 37° on a rotary shaker in an *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes)-buffered medium (pH 7.3) supplemented with 19 unlabeled L-amino acids (all except cystine at concentrations specified by GIBCO for basal Eagle's medium, diploid Earle's powder, and modified Eagle's medium nonessential amino acids), L-[³⁵S]-cystine, Puck's saline G, and Dulbecco's modified Eagle's medium vitamins (GIBCO). The amounts of tissue and isotope, volumes of medium, and times of incubation are indicated for each experiment. After incubation, tissue pieces were rinsed and homogenized in 1 ml of buffer per 75 mg of tissue wet weight, using a glass homogenizer with a medium-fitting Teflon pestle. The homogenization buffer contained 50 mM sodium acetate (pH 4.0), 0.5 M sodium chloride, 1% (vol/vol) Triton X-100, 1 mg of bovine serum albumin per ml, 10 μ g of L-cystine per ml, 200 μ g of sodium azide per ml, 50 μ g of pancreatic trypsin inhibitor per ml (Type I-P, Sigma), and 5 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 40,000 rpm in an A 321 International rotor for 1 hr at 4° and the resulting 105,000 $\times g$ supernatants were analyzed by immunoprecipitation and trichloroacetic acid precipitation.

Isolation of β NGF by Immunoprecipitation. In order to determine the quantity of antiserum required to quantitatively precipitate β NGF from a tissue supernatant, tracer quantities (2–3 ng) of ¹²⁵I-labeled β NGF were added to aliquots of supernatant which were then titrated with various amounts of antiserum. Once the equivalence points were determined, a fresh aliquot of supernatant (0.25–0.3 ml) was diluted in a siliconized conical glass centrifuge tube with 1 ml of wash buffer (homogenization buffer minus pancreatic trypsin inhibitor and phenylmethylsulfonyl fluoride). The appropriate volume of antiserum was added and precipitation was allowed to proceed at 4° for at least 4 hr. The precipitate was collected by centrifugation and washed once by resuspension and centrifugation in 1 ml of wash buffer. The precipitate was dissolved in 0.5 ml of 0.1 M NaOH and then neutralized with 0.5 ml of 0.1 M HCl. Buffer (0.11 ml) containing 0.5 M sodium acetate (pH 4.0), 5 M sodium chloride, 2 mg of sodium azide per ml, and 10 mg of bovine serum albumin per ml was added (as a suspension) followed by a volume of antiserum identical to that used in the

original precipitation, plus an equivalent amount of unlabeled β NGF. After incubation for at least 4 hr at 4°, the immunoprecipitate was collected by centrifugation, suspended in 0.5 ml of wash buffer, and transferred to a fresh siliconized tube. The original tube was rinsed with 0.5 ml of wash buffer and the immunoprecipitate was collected by centrifugation. The overall recovery of β NGF by this procedure, determined by performing the identical isolation with homogenates containing tracer quantities of ¹²⁵I-labeled β NGF, was approximately 75% (E. A. Berger and E. M. Shooter, unpublished).

NaDodSO₄ Gel Electrophoresis. The washed immunoprecipitates were dissolved in 0.2 ml of the NaDodSO₄ sample buffer described by Laemmli (22), modified by the addition of urea to 9 M. After overnight incubation at 37°, samples were applied to discontinuous NaDodSO₄ gels (70 \times 5 cm) (22) containing 15% acrylamide and 0.4% *N,N'*-methylene bisacrylamide. Experiments described elsewhere indicate that the immunoprecipitates were 90% dissociated by this procedure (E. A. Berger and E. M. Shooter, unpublished). Gel slices (2 mm) were incubated overnight at 37° in 10 ml of scintillation fluid containing, per liter of toluene, 4 g of 2,5-diphenyloxazole, 50 mg of 1,4-bis[2-(5-phenyloxazolyl)]-benzene, 54 ml of Protosol (New England Nuclear), and 6 ml of H₂O.

Trichloroacetic Acid Precipitation. Aliquots (10 μ l) of submaxillary gland supernatants were spotted (in triplicate) on Whatman GF-C filter discs. Filters were soaked batchwise (5 ml per filter) twice in 10% trichloroacetic acid (30 min) at 4°, then once in 95% ethanol (5 min), and finally once in anhydrous ether (5 min). They were dried at room temperature and radioactivity was measured.

Isolation of Labeled Peaks from NaDodSO₄ Gels. An immunoprecipitate from an extract of submaxillary glands labeled for 1 hr was washed and electrophoresed as described above. Gel slices (2 mm) were incubated with shaking for 20 hr at 37° in 2 ml of buffer containing 1 mg of NaDodSO₄ plus 200 μ g of sodium azide per ml. Radioactivity was localized by assaying 10- μ l aliquots, and the fractions corresponding to the desired peaks were pooled. Bovine serum albumin (1 mg) was added to each pool. The samples were lyophilized and then dissolved in one-tenth the original volume of buffer containing 8 M urea and 0.2 M Tris-HCl (pH 8.0). Dowex AG 1-X2 (100–200 mesh, chloride form, Bio-Rad) was added (75 μ l of settled resin) (23), and after 10 min at room temperature the suspensions were filtered through glass wool in a 1-ml syringe. The original tubes were washed twice with 0.25 ml of the same buffer. Portions of the resulting protein solutions were reduced and carboxymethylated as described below.

Reduction and Carboxymethylation of Proteins. A portion of the ³⁵S-labeled peak corresponding to the putative β -NGF precursor, isolated as discussed above (containing approximately 0.38 mg of bovine serum albumin in 0.5 ml), was reduced by the addition of 15 μ mol of dithiothreitol. Recrystallized iodoacetic acid (60 μ mol) was added and the solution was incubated at room temperature in the dark for 15 min. After the addition of 2-mercaptoethanol (600 μ mol), the mixture was dialyzed against 0.1 M ammonium bicarbonate (pH 8.2).

Purified β NGF was reduced and carboxymethylated with iodo[³H]acetic acid as described elsewhere (E. A. Berger and E. M. Shooter, unpublished). Tryptic map analysis is described in the legend to Fig. 5.

RESULTS

Kinetics of β NGF Synthesis. Fig. 1 shows the gel profiles of immunoprecipitates from extracts of submaxillary glands labeled for various times. With relatively short incubations (10

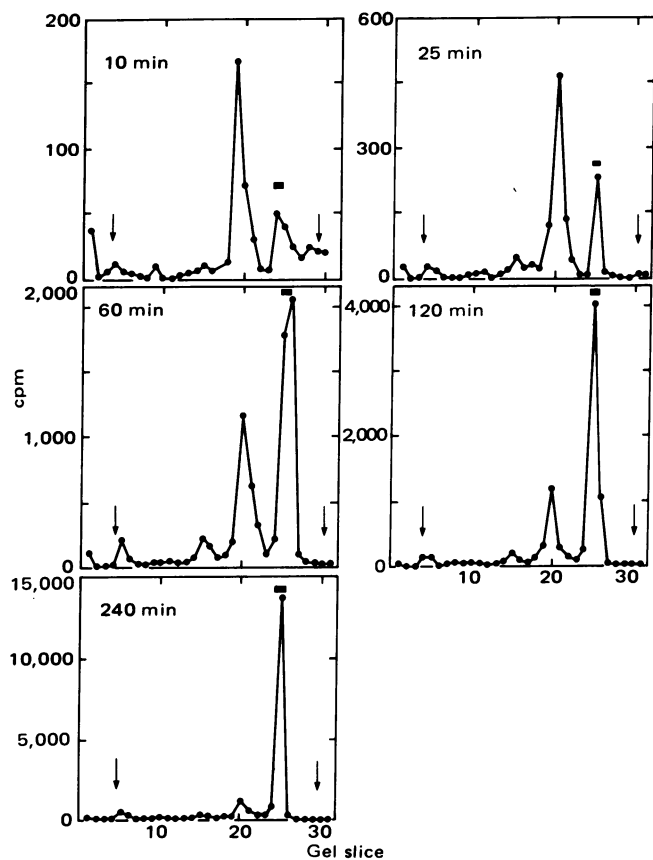


FIG. 1. NaDodSO₄ gel profiles of immunoprecipitates from continuous labeling. Submaxillary gland pieces (270 mg wet weight) were incubated in 0.62 ml of cystine-free medium supplemented with 1.25 mCi of L-[³⁵S]cystine. At the indicated times, 40–60 mg (wet weight) of tissue were removed, rinsed, and homogenized, and the immunoprecipitates from 0.3 ml of supernatant were washed and dissolved in 0.2 ml of NaDodSO₄ sample buffer containing 9 M urea. To each NaDodSO₄ gel 0.16 ml was applied. Note the different scale on each ordinate. In all gels, the left-hand arrow represents the top of the resolving gel, and the right-hand arrow indicates the position of the dye front. Solid bars denote the β NGF position, determined from the mobility of purified β NGF run on a separate gel and stained with Coomassie blue.

and 25 min), the major labeled peak migrates substantially slower than β NGF. The apparent molecular weight (M_r) of this species, determined by its mobility relative to standard proteins on a NaDodSO₄/polyacrylamide slab gel (not shown) is about 22,000. As time proceeds, radioactivity at this position reaches a plateau (Fig. 2). By contrast, radioactivity at the β NGF position dramatically increases after an initial lag, until by 4 hr it far exceeds the label of the 22,000 M_r species (Figs. 1 and 2).

The pulse chase experiment shown in Figs. 3 and 4 further suggests the precursor nature of the 22,000 M_r species. Glands were labeled for 10 min and then either homogenized directly or transferred for various lengths of time to a medium containing a large excess of unlabeled cystine and no additional isotope. As expected after the 10-min pulse, there is a major labeled peak at 22,000 M_r and a smaller peak at the β NGF position (Fig. 3). After an initial increase during the early part of the chase, radioactivity in the 22,000 M_r peak declines and ultimately disappears. By contrast there is a sharp rise in radioactivity at the β NGF position throughout the chase period. These changes occur under conditions where further incorporation of label into trichloroacetic acid-precipitable material is effectively halted (Fig. 4), suggesting that the higher-molecu-

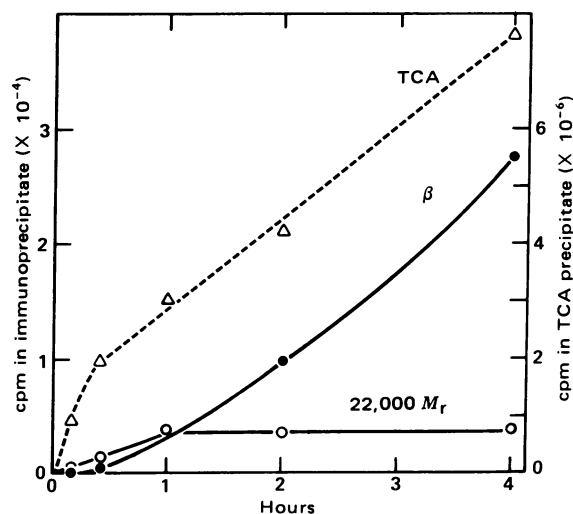


FIG. 2. Kinetics of labeling of the 22,000 M_r species and β NGF. Data were obtained from the experiment shown in Fig. 1 by calculating the total radioactivity in either the 22,000 M_r species or in β NGF at each indicated time, after correcting for average yield of β NGF on immunoprecipitation (75%) and completeness of dissociation of the immunoprecipitates (90%) (left ordinate). Values represent radioactivity from 0.3 ml of supernatant. TCA, trichloroacetic acid.

lar-weight species is indeed being converted to β NGF during the chase period.

Comparison of Tryptic Peptides of β NGF and the 22,000 M_r Species. The 22,000 M_r species was purified from a NaDodSO₄ gel, reduced, and carboxymethylated. Its tryptic peptides were compared on an isoelectric focusing gel with those of purified β NGF, carboxymethylated with iodo[³H]acetic acid (Fig. 5). The 22,000 M_r species apparently possesses all the major cysteine-containing peptides found in β NGF. The

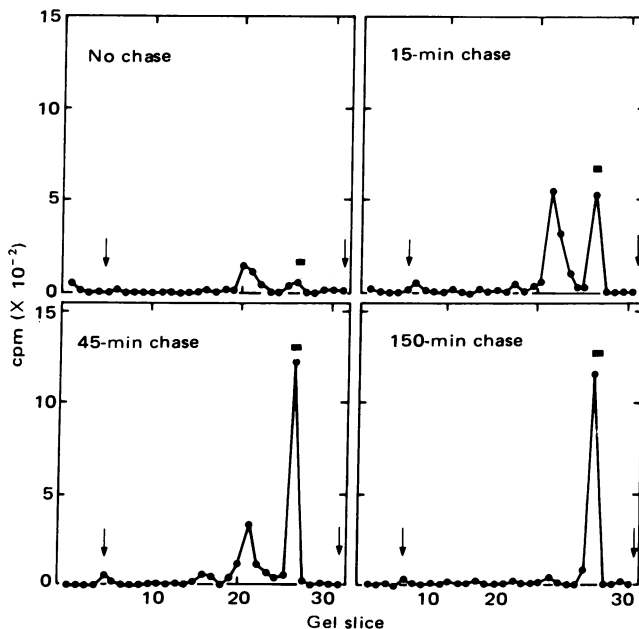


FIG. 3. NaDodSO₄ gel profiles of immunoprecipitates from pulse-chase labeling. Submaxillary gland pieces (240 mg wet weight) were incubated in 0.31 ml of cystine-free medium supplemented with 1.25 mCi of L-[³⁵S]cystine. After 10 min, tissue pieces were rinsed and either homogenized directly (no chase) or transferred to 20 ml of fresh medium containing 115 μ g of unlabeled L-cystine per ml and no additional label. At the indicated times, 40–60 mg (wet weight) of tissue were rinsed and homogenized and the entire washed immunoprecipitates from 0.25 ml of each supernatant were electrophoresed. Solid bars denote the β NGF position.

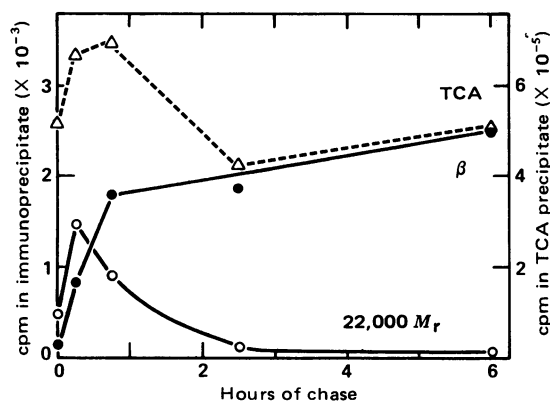


FIG. 4. Pulse-chase kinetics of labeling of the 22,000 M_r species and β NGF. Data were obtained from the experiment shown in Fig. 3 by calculating the total radioactivity in the 22,000 M_r species and in β NGF at each time, after correcting for average yield on immunoprecipitation (75%) and completeness of dissociation (90%) (left ordinate). Values represent radioactivity from 0.25 ml of supernatant. TCA, trichloroacetic acid.

two additional ³⁵S-labeled peaks may represent peptides from the extra polypeptide length. In a similar experiment described elsewhere (E. A. Berger and E. M. Shooter, unpublished), we have shown that the ³⁵S-labeled peptides of the 13,000 M_r species comigrate exactly with the cysteine-containing peptides of purified β NGF.

Effect of γ Subunit on 22,000 M_r Species. Fig. 6 indicates that treatment with the γ subunit of an immunoprecipitate from submaxillary glands labeled for 25 min results in nearly complete conversion of radioactivity in the 22,000 M_r species to the β NGF position. Similar results have been obtained with 22,000 M_r material purified from NaDodSO₄ gels after dissociation of the immunoprecipitate (data not shown).

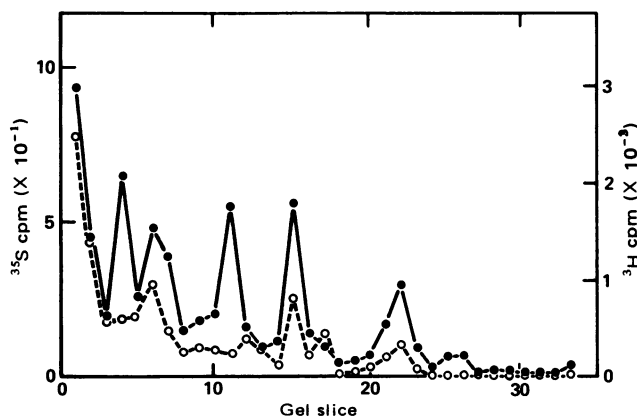


FIG. 5. Isoelectric focusing gel of tryptic peptides of the 22,000 M_r species and β NGF. The ³⁵S-labeled peak corresponding to the 22,000 M_r species was isolated, then reduced and carboxymethylated. A portion of this (1600 cpm containing 125 μ g of bovine serum albumin) was added to lyophilized, purified β NGF that had been reduced and carboxymethylated with iodo[³H]acetic acid (15,000 cpm, 26 μ g). Fifteen micrograms of tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (Worthington) was added and the mixture was incubated for 1 hr at 37°. An additional 15 μ g of trypsin was then added, and after 4 hr at 37° the mixture was lyophilized, then dissolved in 0.1 ml of 1 mM HCl containing 8 M urea and 50 μ g of cytochrome *c*. The sample was applied to a gel (70 × 5 cm) containing 10% acrylamide, 0.2% *N,N'*-methylene bisacrylamide, 2% (wt/vol) ampholines (pH 3.5–10, LKB), and 8 M urea. The anode, at the top, contained 3 mM HCl, and the cathode contained 3 mM NaOH. After focusing, the gel was cut into 2-mm slices. Radioactivity was determined differentially for ³⁵S (●) or ³H (○). The left portion of the graph represents the top of the gel.

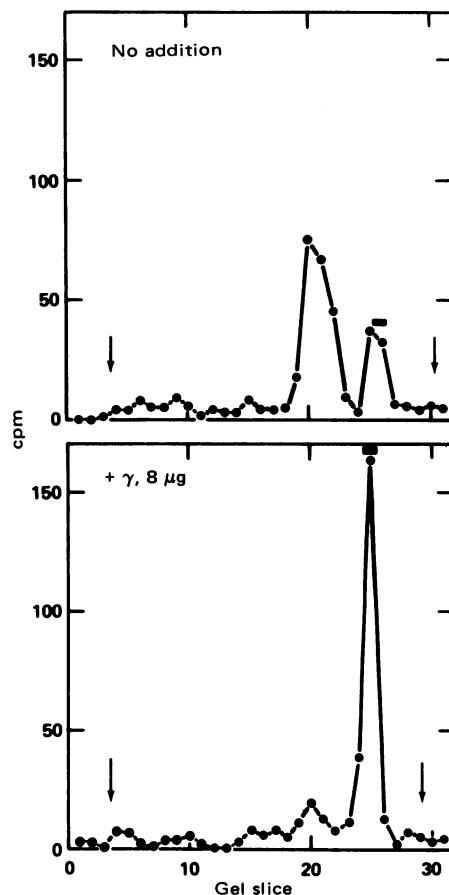


FIG. 6. Effect of the γ subunit on the 22,000 M_r species. Submaxillary gland pieces (510 mg wet weight) were incubated in 0.625 ml of cystine-free medium supplemented with 2.6 mCi of L-[³⁵S]-cystine. After 25 min at 37° the pieces were homogenized, and the washed immunoprecipitate from the 105,000 × *g* supernatant (containing approximately 130 μ g of β NGF) was suspended in 1 ml of 0.1 M Tris-HCl (pH 7.6) containing 0.2 mg of sodium azide per ml. A portion of this suspension (0.01 ml) was incubated either alone or with the γ subunit of 7S NGF (8 μ g). In each case the total volume was adjusted to 0.05 ml with 0.1 M Tris-HCl (pH 7.6). After 2 hr at 37°, the NaDodSO₄ sample buffer plus urea (9 M final concentration) was added, and the samples were applied to NaDodSO₄ gels. Solid bars denote position of β NGF.

DISCUSSION

The data presented herein support the conclusion that the 22,000 M_r peak represents a true biosynthetic precursor to β NGF, and we thus designate this species "pro- β NGF". Its apparent M_r suggests the presence of an additional polypeptide length(s) of approximate size 9000, a rather large segment relative to β NGF (13,260). Our failure to observe such a peak after cleavage with the γ subunit (Fig. 6) may indicate the absence of cysteine residues in the extra segment, although the appearance of two additional peaks in the tryptic digest (Fig. 5) argues against this. Further structural studies will be required to resolve this question, as well as to determine if the additional polypeptide length is at the carboxyl terminus as proposed earlier (7, 11). On the basis of the similarities between β NGF and proinsulin, Boyd *et al.* (24) have predicted a length of 170 residues (M_r 19,000) for the NGF precursor.

The processing of many secretory proteins now appears to involve multiple specific proteolytic events (18, 25, 26), and we have considered this possibility for β NGF. For example, the increase in radioactivity in pro- β NGF during the initial period of the chase (Figs. 3 and 4) is more than can be accounted for

by continued incorporation of label into protein (i.e., the combined radioactivity in pro- β NGF plus β NGF increases approximately 4-fold while trichloroacetic acid-precipitable label rises by only 30–35%). This may reflect the initial production of an earlier precursor form not solubilized by our homogenization conditions or not precipitated by antiserum against β NGF, though the possibility that newly synthesized precursor is cryptic due to compartmentalization remains equally plausible. Second, we have observed that when immunoprecipitates are analyzed with the greater resolution afforded by autoradiography of NaDodSO₄ slab gels (not shown), the 22,000 M_r peak is actually composed of a major band and a minor band of slightly greater mobility which may represent an intermediate in the processing events. Finally, in a number of cases, higher-molecular-weight precursor forms not observed in conventional pulse-labeling experiments can be detected in mRNA-directed cell-free translation systems (25–29). Such a “pre-pro” form may well exist for β NGF.

Despite the growing list of secretory proteins that are synthesized initially as precursors, no specific intracellular cleaving enzymes have yet been identified. We have predicted that the γ subunit is such a specific protease, and indeed its ability to cleave pro- β NGF without having general proteolytic activity (30) supports this idea. However, we have unexpectedly observed that EGF binding protein catalyzes the same conversion with equal potency (unpublished results), and we are therefore not yet able to conclude that the γ subunit is the unique pro- β NGF processing enzyme. The cleavage reactions reported here were performed with precursor molecules that had likely lost some of their native conformation, i.e., precursor bound to antibody (Fig. 6) or purified from a NaDodSO₄ gel after reduction with 2-mercaptoethanol in the presence of urea (not shown). Alternate ways to dissociate pro- β NGF from the antibody without using NaDodSO₄ must be found. In addition, the possibility exists that the α subunit of 7S NGF might confer specificity to the cleavage reaction.

The mouse submaxillary gland contains, in addition to NGF and EGF, a number of other factors capable of affecting growth and differentiation of various target tissues. Thus, two mesenchymal growth factors (31, 32) and a thymocyte transformation factor (33) have been described, and each is reported associated with arginyl-esterpeptidase activity (32). More detailed characterization will be required to determine if these factors exist as distinct high molecular weight complexes with specific arginyl-esterpeptidase enzymes, analogous to NGF and EGF. However, it is intriguing to speculate that the processes discussed here for NGF, namely, cleavage of a biosynthetic precursor in the submaxillary gland by a specific protease and formation of a corresponding high molecular weight complex, may in fact apply to a collection of submaxillary gland factors regulating a variety of growth and developmental functions.

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