Proteolytic Modification of the β Nerve Growth Factor Protein†

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ABSTRACT: At least part of the difference in structure between the two commonly used preparations of nerve growth factor (NGF) proteins, β and 2.5S NGF, is explained by proteolytic modification of the C termini of their two peptide chains. In βNGF isolated from the purified 7S NGF complex, the major species, β^1NGF, has C-terminal arginine residues on both its chains while the minor species, β^2NGF, has one C-terminal arginine chain and one C-terminal threonine chain, threonine being the penultimate residue of the chains. Both C-terminal arginine residues of β^1NGF are removed by carboxypeptidase B producing β^3NGF or bisdesarginine β^1NGF. β^2NGF is an intermediate in this conversion. The submaxillary gland extract also contains a carboxypeptidase B like enzyme(s) which causes partial loss of the C-terminal arginine from βNGF. The isolation of the NGF protein from 7S NGF before the latter has been purified therefore leads to mixtures of β^1, β^2, and β^3NGF whose proportions depend on the extent of proteolysis. Such preparations have been designated 2.5S NGF. Cleavage of the C-terminal arginine does not occur with either enzyme when βNGF is present in the complex 7S NGF rather than free, suggesting that either or both the α and γ subunits protect the C terminus of βNGF from proteolysis. Removal of both C-terminal arginine residues has no effect on the size or specific NGF activity of βNGF but does completely prevent the re-formation of a 7S complex. The role of the arginine residues in dictating the stability of the 7S NGF complex is consistent with the hypothesis that the arginine esteropeptidase γ subunit is the enzyme responsible for cleaving longer pro-βNGF to the functional βNGF chains at these particular residues.

Significant advances have been made in the characterization of the nerve growth factor (NGF)[1] proteins which enhance the growth and differentiation of sympathetic and embryonic sensory nerve cells (Levi-Montalcini, 1966) since the early reports of their isolation from snake venom (Cohen, 1959) and from the adult mouse submaxillary gland (Cohen, 1960). The description by Varon *et al.* (1967) of a new method for isolating NGF activity from the mouse submaxillary gland emphasized that the activity is associated with a relatively large subunit containing protein, 7S NGF. Varon *et al.* (1968) showed further that the 7S NGF complex dissociates reversibly at mild extremes of pH and that it contains three different types of subunits. Only one of these three subunit types in 7S NGF, the basic subunit or βNGF, elicits the NGF response (Varon *et al.*, 1968). One of the other subunits, the γ subunit, displays arginine esteropeptidase activity (Greene *et al.*, 1969) while the third type, the acidic α subunit, inhibits the activity of the γ subunit in the 7S NGF complex (Greene *et al.*, 1969). Recent work has therefore been aimed at understanding the significance of the 7S NGF complex, and, as described here, one possible clue comes out of a detailed comparison of the structure of the two most commonly used NGF preparations, βNGF and 2.5S NGF.

The βNGF subunit of 7S NGF comprises two identical peptide chains, each with three intrachain disulfide bridges, whose molecular weights are approximately 12,000 as determined by sodium dodecyl sulfate electrophoresis (Greene *et al.*, 1971) or 12,800 by sedimentation equilibrium (P. Pignatti, unpublished data). The βNGF dimer dissociates into its component peptide chains in sodium dodecyl sulfate, 8 M urea, or 2 M guanidine hydrochloride (Greene *et al.*, 1971). The other NGF preparation which has been extensively used and characterized is the one isolated by Bocchini and Angeletti (1969) and now called 2.5S NGF (Angeletti and Bradshaw, 1971). Originally thought to have a mol wt close to 30,000 (Bocchini and Angeletti, 1969; Angeletti *et al.*, 1971) it is now known, on the basis of the complete amino acid sequence, to have a mol wt of 26,500 (Angeletti and Bradshaw, 1971; Angeletti *et al.*, 1973a). The two peptide chains, A and B, in 2.5S NGF are identical, except that B is shorter than A by the first eight amino acid residues from the N terminus, and each chain has three intrachain disulfide bridges (Angeletti *et al.*, 1971, 1973a,b).

It seemed likely from the procedure by which it was isolated that 2.5S NGF was also derived from 7S NGF and was therefore closely related to βNGF. This was substantiated by the similarity of their sedimentation coefficients (Varon *et al.*, 1968; Bocchini and Angeletti, 1969), amino acid composition (Varon and Shooter, 1970; Bocchini, 1970; Angeletti *et al.*, 1971), isoelectric points (Greene *et al.*, 1971; Bocchini, 1970) and, as noted above, by their molecular weights and the number of peptide chains. The amino acid sequence data on βNGF also support this close relationship and suggest that βNGF contains two of the longer A chains of 2.5S NGF (Perez-Polo *et al.*, 1972).

In the course of the sequence work on βNGF, heterogeneity at the C terminus of the peptide chains was observed, which led, as described in this report, to a study of the role of the C-terminal arginine residues in determining the stability of the 7S NGF complex. This work suggests some possible functions

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[1] Abbreviations used are: bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane; NGF, nerve growth factor; EGF, epidermal growth factor.

for 7S NGF over and above its role as the parent complex for βNGF.

Materials

Ampholine, pH 3–10, was obtained from LKB-Produkter AB, bis-tris[1] from General Biochemicals, and *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid from Calbiochem. Dowex AG2-X10 came from Bio-Rad.

Carboxypeptidase B (sp act. 185 units/mg) and carboxypeptidase A (sp act. 60 units/mg) were acquired from Sigma Chemical Co. Both enzymes were prepared for use as described by Potts *et al.* (1972) and stored in 2 M NH_4HCO_3 at −20°.

7S NGF was isolated from adult male mouse submaxillary glands as previously described (Varon *et al.*, 1967). The subunits were isolated from 7S NGF according to the method of Smith *et al.* (1968), except that the elution of the β subunit (βNGF) from the carboxymethylcellulose (CM-cellulose) employed 0.24 M ethanolamine buffer (pH 10.6), rather than the 0.05 M sodium glycinate buffer (pH 9.4). The 2.5S NGF protein was isolated according to the method of Bocchini and Angeletti (1969). An aliquot of the supernatant of the sodium acetate (pH 5.0) dialysis step of this procedure was removed and kept frozen and is referred to here as "dialysate." All proteins were prepared and stored at 4°. All other chemicals were of reagent grade and were used without further purification.

Methods

Carboxypeptidase Digestion. Digestion of βNGF with carboxypeptidase A or B was carried out in 0.2 M NH_4HCO_3 at pH 8.5 unless otherwise specified. For the removal of carboxypeptidase B from the final product the digestion was stopped by the addition of 10% acetic acid to pH 3.6. After dialysis against 0.2% acetic acid (pH 3.6), overnight, the mixture was placed on a CM-cellulose column (2.5 cm × 2.5 cm), prepared in 0.05 M sodium acetate buffer (pH 4.0). Elution of the carboxypeptidase B was accomplished with 0.05 M sodium acetate buffer (pH 4.0) containing 0.65 M NaCl. The product, β^3, was eluted with 0.24 M ethanolamine buffer (pH 10.7).

Carboxyamidomethylation and Succinylation. Carboxyamidomethylation of βNGF was performed according to the method described by Greene *et al.* (1971) and succinylation was performed by the procedure of Klotz (1967).

Amino Acid Analyses. These were carried out on a Beckman 121 amino acid analyzer. Deproteinization of all samples was done essentially by the method of Stein and Moore (1954) except that a small column of Dowex AG2-X10 (1 × 0.5 cm) was used to remove the picric acid and precipitated protein. Norleucine was used as an internal standard.

NGF Activity. The biological activities of 7S and βNGF and of the modified βNGF proteins were measured by the standard *in vitro* bioassay using 9-day-old embryonic chick sensory ganglia (Levi-Montalcini *et al.*, 1954; Varon *et al.*, 1972).

Electrophoresis and Isoelectric Focusing. Electrophoresis in acrylamide gel used the discontinuous bis-tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer system described by Smith *et al.* (1968). The gels were stained with 0.1% Naphthol Blue Black in 10% acetic acid, destained in the same solution, and scanned in a Gilford 2000 spectrophotometer at 580 nm. Isoelectric focusing in acrylamide gel followed the procedure outlined by Greene *et al.* (1971). The gels were run for 3 hr at 100 V, excess ampholine was removed by repeated washing with 20% Cl_3CCOOH, and the gels were then stained and scanned as described above. Isoelectric focusing was also carried out in the presence of urea by incorporating 8 M urea into both the acrylamide gel and sample. Such gels were run for 1 hr at 100 V and 2.5 hr at 200 V and then treated and stained in the same way as the urea-free gels.

Buffers. Buffers were prepared according to Datta and Gryzbowski (1961) and pH values were determined at 25°.

Results

C-Terminal Sequence of βNGF. The three C-terminal amino acid residues of 2.5S NGF have been shown to be . . . Ala-Thr-Arg-COOH (Angeletti and Bradshaw, 1971; Angeletti *et al.*, 1973b). The results given in Table I show that the C termini of the β subunit of 7S NGF and of 2.5S NGF are identical. Digestion of carboxyamidomethylated and succinylated βNGF with B alone released arginine, threonine, and alanine in that order (Table I). The threonine and alanine residues appeared presumably because of contamination of the carboxypeptidase B with carboxypeptidase A. Digestion with both carboxypeptidase B and carboxypeptidase A released stoichiometric amounts of all three residues (Table I). Using both exopeptidases in this manner, the recovery of alanine and threonine approached 100% (96 and 97%, respectively) while that of arginine was only 88%. This significant difference suggested that a small proportion (12%) of the peptide chains of βNGF did not have C-terminal arginine residues, an observation that was confirmed in the digestion of βNGF with carboxypeptidase A alone (Table I). Since the specificity of carboxypeptidase A is such that it does not cleave C-terminal arginine residues, the release of threonine and alanine with carboxypeptidase A alone indicated again that approximately 10% of the βNGF chains have C-terminal threonine rather than arginine residues.

The analysis of βNGF by isoelectric focusing is in keeping with these results. Typical βNGF preparations showed one major species (designated β^1) and a second minor species (designated β^2) of lower isoelectric point in the approximate ratio of 9:1 (Figure 1a). It seemed likely therefore that in β^1

TABLE I: C Terminus of βNGF.

Amino Acid	nmol of Amino Acid Released by: Carboxypeptidase B, 15 min	Carboxypeptidase B, 30 min	Carboxypeptidase A + Carboxypeptidase B, 30 min	Carboxypeptidase A, 30 min
Arg	7.2	9.3	9.3	0.0
Thr	3.5	5.9	10.3	0.6
Ala	1.4	2.9	10.1	0.3

[a] βNGF (10.6 nmol, 137 μg, carboxyamidomethylated and succinylated as described under Methods) was digested with exopeptidase(s) in 0.2 M NH_4HCO_3 (pH 8.5), at a weight ratio of βNGF:enzyme of 40:1. At the indicated times, the samples were frozen, lyophilized, and treated for amino acid analysis as described under Methods. The results indicate that βNGF contains 88% of C-terminal arginine chains and 12% of C-terminal threonine chains.

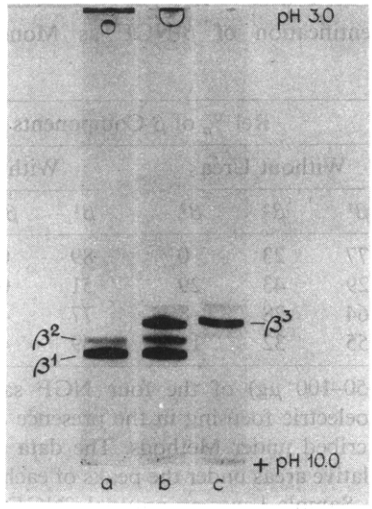

FIGURE 1: Characterization of β^3NGF by isoelectric focusing. βNGF (390 μg) was converted to β^3 by cleavage with carboxypeptidase B (1.0 μg) for 2 min at 25° in 0.2 M NH_4HCO_3 (pH 8.5). This sample was dialyzed against 0.05 M sodium glycinate buffer (pH 3.0) to remove excess NH_4HCO_3, and the carboxypeptidase B removed as described under Methods. The β- and β^3NGF samples were finally dialyzed against 0.05 M sodium acetate buffer (pH 4.0). Analyses were carried out by isoelectric focusing in pH 3–10 gradients in acrylamide gels as described under Methods: (a) 25 μg of βNGF; (b) 25 μg of βNGF and 18 μg of β^3; (c) 18 μg of β^3.

both peptide chains had C-terminal arginine residues while in β^2 only one of the two chains ended in arginine. The results presented in the following section confirmed this prediction.

Formation and Characterization of Bisdesarginine βNGF. Treatment of native βNGF with carboxypeptidase B in various ratios of carboxypeptidase B:βNGF caused a pronounced shift in the isoelectric point of β^1 toward a more acidic value (Figure 1c). The product of this reaction, β^3, had an even lower isoelectric point than β^2, the minor component in βNGF (Figure 1b). Even at very low ratios of carboxypeptidase B:βNGF (1:300), the conversion of β^1 to β^3 was complete within 3 min at 25°. The minor species, β^2, also disappeared during the course of the incubation.

The enzymatic modification of βNGF occurred with a concomitant release of stoichiometric amounts of C-terminal arginine residues from both β chains (Table II). Under these conditions no other amino acids were released by carboxypeptidase B from native βNGF. To show conclusively that the arginines released were the C-terminal residues, the new C-terminal amino acid of β^3 was determined by carboxypeptidase A digestion (Table III). As anticipated from the sequence data, threonine, the penultimate residue of the β chains, was released in 95% yield. Near stoichiometric amounts of alanine were also released but at a slower rate (Table III) in confirmation of the data presented in Table I. These results show that treatment of the native β^1NGF with carboxypeptidase B selectively and rapidly releases arginine from the C termini of both chains causing a marked change in isoelectric point. The new protein which is formed, β^3, is therefore bisdesarginine β^1. The rapid conversion of β^1 to β^3 demonstrates that the C-terminal arginine residues are extremely susceptible to proteolytic cleavage. The removal of the C-terminal arginine residues did not alter the molecular weight and therefore dimeric character of β^3 (P. Pignatti and J. B. Moore, Jr., unpublished data). In addition, the NGF activity of βNGF was unaltered by carboxypeptidase B treatment as shown in Figure 2. Both βNGF (containing 90% of the β^1 species) and β^3 gave a maximum biological response at 10 ng/ml.

TABLE II: Identification of the Amino Acid Released by Carboxypeptidase B Digestion of Native βNGF.[a]

Sample	Arginine Released (nmol)	
	Exptl	Calcd
1	11.4	11.6
2	9.3	9.8

[a] The two βNGF samples were digested with carboxypeptidase B for 3 min at 25° at a weight ratio of βNGF:carboxypeptidase B equal to 300:1. The experimental values were corrected for loss during deproteinization using norleucine as an internal standard. The theoretical values refer to the amount of C-terminal arginine residues present in the βNGF samples determined from the proportions of β^1- and β^2NGF.

TABLE III: Determination of the C Terminus of β^3NGF.[a]

Amino Acid Released	nmol of Amino Acid Released at (min)				
	5	30	120	240	360
Threonine	0.7	2.1	4.4	5.5	5.9
Alanine	0.0	0.7	2.0	3.4	5.2

[a] Reduced and carboxyamidomethylated β^3NGF (70 nmol) was digested with carboxypeptidase A at a weight ratio of β^3:carboxypeptidase A equal to 15:1. Aliquots (6.2 nmol of β^3) were removed at the indicated times, frozen, and lyophilized. The samples were treated for amino acid analysis as described under Methods.

Identification of β^2 as a Dimer Containing C-Terminal Arginine and C-Terminal Threonine Chains. The minor component, β^2, present in preparations of βNGF has an isoelectric point midway between those of β^1 and β^3 (Figure 1b). This suggested that β^2 was an NGF dimer in which only one

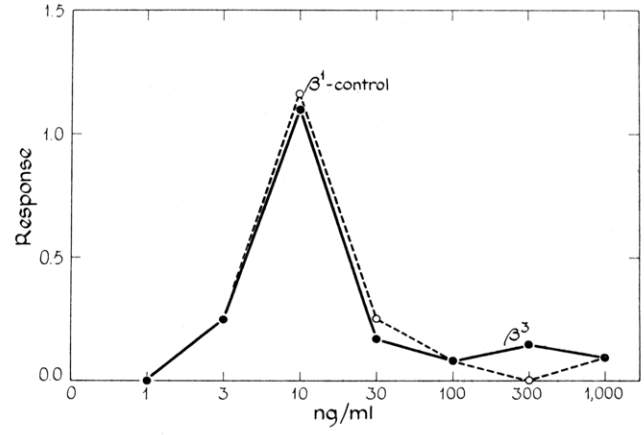

FIGURE 2: The biological activity of β^3NGF. The β^1NGF dimer (50 μg) was incubated with 0.170 μg of carboxypeptidase B for 3 min at 25°. The reaction was terminated by placing a small aliquot (0.2 ml) into 0.8 ml of rooster plasma, and the biological activity was determined by the method of Levi-Montalcini *et al.* (1954).

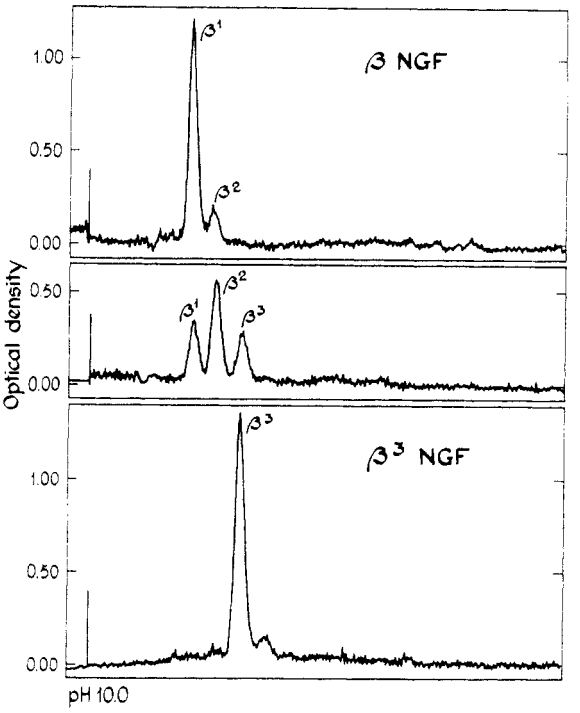

FIGURE 3: The intermediate (β^2NGF) in the conversion of β^1- to β^3NGF. βNGF (50 μg) was incubated (a, top) alone, (b, middle) with 40 ng of carboxypeptidase B, and (c, bottom) with 0.4 μg of carboxypeptidase B for 10 min at 25° in 0.2 M NH_4HCO_3 (pH 8.5). The reaction was terminated by the addition of 0.4 ml of 0.05 M sodium acetate buffer (pH 3.4) and the sample was analyzed by isoelectric focusing in pH 3–10 gradients in acrylamide gel as described under Methods.

of the two chains had a C-terminal arginine residue. Two methods were used to confirm this point.

In the first method, the proteolytic cleavage of β^1NGF by carboxypeptidase B was slowed down sufficiently to demonstrate that β^2 was an intermediate in the conversion of β^1 to β^3. The isoelectric focusing analyses of a reaction in which very small amounts of carboxypeptidase B were used are shown in Figure 3. Under these conditions a significant amount of the original β^1 species remained and only 20% of β^3 was formed. The major component, comprising 50% of the total protein, was β^2 (Figure 3b). Continued digestion of this mixture with higher concentrations of carboxypeptidase B converted all the β^1 and β^2 to the β^3 species (Figure 3c). β^2 is therefore an intermediate in the formation of β^3.

The second method made use of the observation that the βNGF dimer dissociates into single chains in 8 M urea (Greene *et al.*, 1971). The types of chains in a given βNGF dimer could therefore be determined by isoelectric focusing in the presence of 8 M urea. Both the β^1 and β^3 species gave single major bands (chains) in such analyses with the products of the β^3 dissociation having, as expected, the lower isoelectric point (results not shown). Dissociation of β^2 should, if it is a dimer composed of one C-terminal arginine chain and one C-terminal threonine chain, produce equal amounts of the β^1 and β^3 chains. In the absence of purified β^2NGF this hypothesis was tested by comparing the composition of mixtures of the three β species in the presence and absence of 8 M urea (Table IV). The dissociation of β^2 in 8 M urea was complete for the first two mixtures shown in Table IV and it is clear in both instances that the chains in β^2 distributed themselves equally among the β^1 and β^3 chains. Upon dissociation the 43% of β^2

TABLE IV: Identification of β^2NGF as Monodesarginine β^1NGF.[a]

NGF Sample	Rel % of β Components					
	Without Urea			With Urea		
	β^1	β^2	β^3	β^1	β^2	β^3
1	77	23	0	89	0	11
2	29	43	29	51	0	49
3	64	28	8	77	4	19
4	55	32	13	69	4	27

[a] Aliquots (50–100 μg) of the four NGF samples were analyzed by isoelectric focusing in the presence and absence of urea as described under Methods. The data are given in terms of the relative areas under the peaks of each component in the gel scan. Sample 1 was an unusual βNGF preparation with a high proportion of β^2NGF, sample 2 was obtained by the partial cleavage of βNGF with carboxypeptidase B as shown in Figure 3b, and samples 3 and 4 were 2.5S NGF preparations made by the procedure of Bocchini and Angeletti (1969). For the urea-containing gels, the same weight of NGF sample as used in the urea-free analyses was added to 10 M urea to give a final concentration of 8 M and the solutions stood for 4 hr at 25° before analysis.

in the second sample, for example, enhanced the β^1 and β^3 chains by an additional 22 and 20%, respectively. Although the dissociation of β^2 in the last two samples reported in Table IV was not quite complete the same pattern of equal distribution of the chains of β^2 among β^1 and β^3 was noted. Both the evidence from dissociation and from the partial carboxypeptidase B digestion support the conclusion that β^2 is the monodesarginine β^1 dimer.

Protection of βNGF in 7S NGF from C-Terminal Cleavage. The last two NGF mixtures used in the dissociation experiments described in Table IV were 2.5S NGF preparations and it is clear that they contain greater amounts of mono- and bis-desarginine β^1, ~30 and ~10% respectively, than a typical βNGF preparation. Since 2.5S NGF is isolated from 7S NGF before it is completely purified these results suggested that the cleavage of the C-terminal residues was caused by a carboxypeptidase B enzyme(s) in the submaxillary gland extract. Furthermore, βNGF in 7S NGF appeared to be protected from this proteolysis. Experiments to explore this aspect of 7S NGF function are reported in Tables V and VI. The data in Table V show the proportions of β^1-, β^2-, and β^3NGF in β- and 7S NGF preparations before and after digestion with carboxypeptidase B at various pH values. These data are expressed in the last two columns of Table V as the per cent of the C-terminal arginine residues released compared to those originally present in the controls. At pH 6.6, where 7S NGF is a stable complex, very little C-terminal arginine was released from βNGF in the 7S complex compared to free βNGF. In contrast, at pH 10.0 where 7S NGF is completely dissociated (Varon *et al.*, 1968) both βNGF in the complex and free βNGF showed almost complete loss of the C-terminal arginine residues. At intermediate pH values of 5.0 and 8.6 where 7S NGF is only partially dissociated, the proportion of arginine residues released from the βNGF in 7S NGF was less than from the free subunit. The result at pH 7.4, where approximately one-half of the arginine residues was

TABLE V: Protection of the C-Terminal Arginine Residue of βNGF in 7S NGF from Cleavage by Carboxypeptidase B.[a]

	Rel % of β components in						% of C-terminal Arginine Lost from β Chains in	
	βNGF			7S NGF				
Digestion pH	β^1	β^2	β^3	β^1	β^2	β^3	βNGF	7S NGF
Control	91	9	0	93	7	0	0	0
5.0	0	6	87	12	41	41	98	76
6.6	0	8	86	86	14	0	98	3
7.4	0	0	96	46	24	30	100	41
8.6	0	0	96	4	20	62	100	88
10.0	0	9	91	0	0	90	98	100

[a] βNGF (50 μg) and 7S NGF (300 μg) were incubated with carboxypeptidase B (0.2 and 1.2 μg, respectively) for 5 min at 25° at the appropriate pH in a total volume of 250 μl. The samples were analyzed by isoelectric focusing in a pH 3–10 gradient in acrylamide gel as described under Methods.

TABLE VI: Protection of C-Terminal Arginine Residues of βNGF in 7S NGF from Cleavage by Submaxillary Gland Enzyme(s).[a]

	Rel % of β Components in						% of C-Terminal Arginine Lost from β Chains in	
	βNGF			7S NGF				
Digestion pH	β^1	β^2	β^3	β^1	β^2	β^3	βNGF	7S NGF
Control	86	14	0	90	10	0	0	0
5.0	64	34	2	73	23	4	13	11
6.6	41	43	16	88	12	0	33	1
7.4	58	36	8	91	9	0	18	0
8.6	62	32	6	87	10	3	16	3
10.0	62	33	5	74	19	7	15	14

[a] βNGF (50 μg) or 7S NGF (300 μg) was incubated with 50 μl of dialysate (see Methods; OD_{280} = 1.10) for 36 hr at 4° at the appropriate pH in a total volume of 300 μl. The samples were analyzed by isoelectric focusing in a pH 3–10 gradient in acrylamide gel as described under Methods.

released from the subunit in the complex, is of interest because this pH falls within the originally defined pH stability limits of 7S NGF (Varon *et al.*, 1968). The conclusion from these studies using carboxypeptidase B was that over a narrow but well-defined range of pH the β subunit in 7S NGF is well protected from the action of this enzyme. A similar conclusion was reached using the carboxypeptidase B like activity in the submaxillary gland extracts as shown in Table VI. A suitable source of the gland enzyme(s) was the dialysate as defined under Methods and aliquots of this material were incubated with β- and 7S NGF at various pH values for 36 hr at 4°. Again, at pH 6.6 cleavage of the C-terminal arginine of the βNGF in 7S NGF was completely inhibited compared to cleavage in the free subunit, while at pH 10.0 with 7S NGF dissociated, there was essentially no difference. Under the particular conditions used here, the extent of cleavage with the

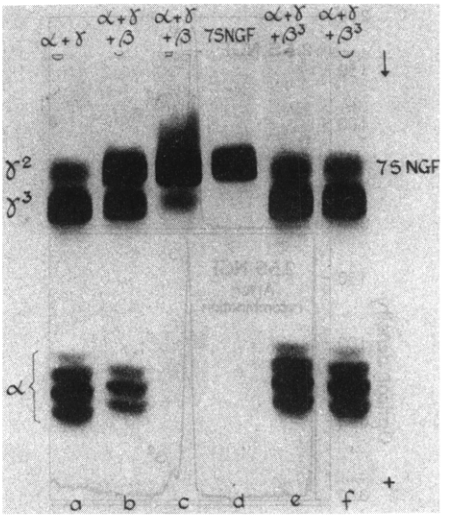

FIGURE 4: Inability of β^3NGF to recombine with the α and γ subunits of 7S NGF to form a 7S complex. Aliquots of α (77 μg) and γ (66 μg) subunits were added to β- or β^3NGF and dialyzed against 0.05 M phosphate buffer (pH 6.8) for 16 hr at 4°. The samples were analyzed by acrylamide gel electrophoresis in the bis-tris–*N*-tris-(hydroxymethyl)methyl-2-aminoethanesulfonic acid system as described under Methods. The analyses shown are (a) α + γ subunits, (b) α + γ + 16 μg of βNGF, (c) α + γ + 32 μg of βNGF, (d) 100 μg of 7S NGF, (e) α + γ + 16 μg of β^3NGF, and (f) α + γ + 32 μg of β^3NGF.

dialysate enzymes was less than with carboxypeptidase B. The range of pH over which the C termini of the βNGF in 7S NGF was protected was greater with the dialysate enzymes than with carboxypeptidase B as shown by the results at pH 7.4 and 8.6 (Table VI), the latter pH being within the alkaline dissociation range for 7S NGF. On the acid side of the pH stability region for 7S NGF, *i.e.*, at pH 5.0, the dialysate enzymes behaved much like carboxypeptidase B. It is clear from these results that the carboxypeptidase B like enzyme(s) in the dialysate, like carboxypeptidase B itself, acts more effectively on the free rather than the complexed βNGF. While the loss of these arginine residues has, as already noted, no effect on the specific NGF activity or molecular weight of βNGF it does affect one other property of this subunit, namely its ability to associate with the α and γ subunits.

Inability of β^3NGF to Re-Form 7S NGF. Two different methods were used to determine if the removal of the C-terminal residue of the βNGF altered the stability of the 7S NGF complex. The first was based on the known ability of the α and γ subunits to recombine with β^1NGF to re-form 7S NGF (Varon *et al.*, 1968; Shooter and Varon, 1970). The electrophoretic analysis of mixtures of the α and γ subunits with β^1NGF compared to that of α and γ subunits (Figure 4a–c) showed the formation of a significant amount of 7S NGF as identified by its characteristic mobility (Figure 4d). In contrast, the addition of β^3NGF to the same α–γ subunit mixture had no effect and no 7S NGF was formed (Figure 4e,f).

The second method involved the characterization of the βNGF species which did associate with the α and γ subunits when the latter were added to 2.5S NGF. 2.5S NGF was chosen because it contained significant amounts of each of the three βNGF species. The experiment involved adding an excess of α and γ subunits to 2.5S NGF at the appropriate pH for recombination, subsequent isolation of the re-formed 7S NGF by gel filtration on Sephadex G-150, and finally the

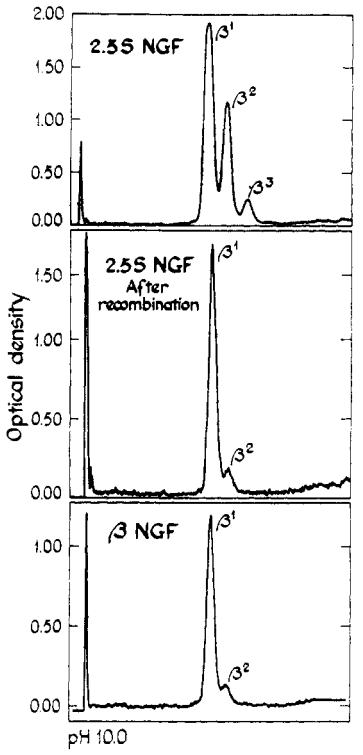

FIGURE 5: Analysis of the β species in 2.5S NGF which recombine with the α and γ subunits of 7S NGF. 2.5S NGF (1.45 mg in 1.0 ml), α subunit (5.4 mg in 1.5 ml), and γ subunit (5.8 mg in 0.5 ml) were combined and dialyzed against 0.05 M phosphate buffer (pH 6.8) for 16 hr at 4°. The sample was then filtered through a Sephadex G-150 column (100 × 2.5 cm) equilibrated in the same buffer and the 7S NGF peak isolated as described by Varon *et al.* (1972). The composition of the β species in the 7S NGF made from 2.5S NGF was compared to that in the original 2.5S NGF by isoelectric focusing in a pH 3–10 gradient in acrylamide gel as described under Methods. The analyses shown are: (a) 70 μg of 2.5S NGF, (b) 240 μg of the 7S NGF made from 2.5S NGF showing only the β species, and (c) 160 μg of βNGF.

analysis of the βNGF species in the purified complex by isoelectric focusing. These analyses (Figure 5) showed that whereas 2.5S NGF contained 10% of β^3NGF, the re-formed 7S NGF had none of this species. Furthermore, while the ratio of β^2:β^1 NGF in the original 2.5S was 33:67, the ratio in the re-formed 7S NGF was only 10:90. This experiment not only supports the earlier conclusion that β^3NGF is unable to recombine with the α and γ subunits but also demonstrates that these two subunits have a significantly higher affinity for β^1- than for β^2NGF. The C-terminal arginine residues of βNGF are therefore critically involved in the association of the subunits in the 7S NGF complex.

Discussion

A Comparison of β- and 2.5S NGF. The main difference between the isolation procedures for β- and 2.5S NGF is the stage at which 7S NGF is dissociated prior to the isolation of its basic, NGF-active subunit. In the isolation of βNGF, the 7S NGF complex is dissociated only after it has been extensively purified (Varon *et al.*, 1968), whereas in the isolation of 2.5S NGF, 7S NGF is dissociated after only one step in the 7S NGF purification procedure (Figure 6). In both methods, 7S NGF is dissociated at mildly acidic pH and the subsequent separation of the basic NGF proteins is achieved in both procedures by chromatography on CM-cellulose at this acidic pH

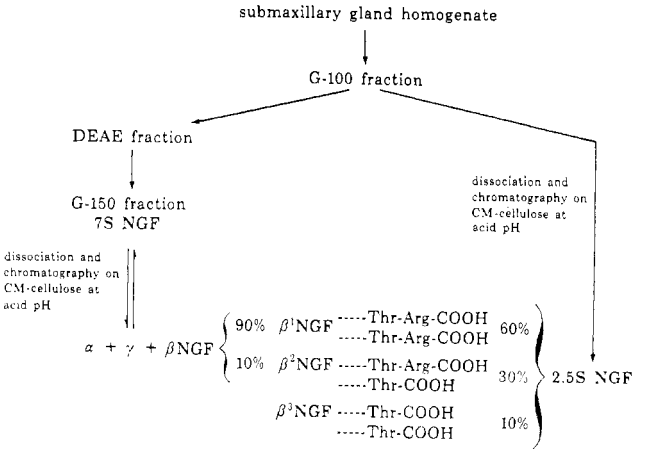

FIGURE 6: The relationship between 7S, β-, and 2.5S NGF. The isolation procedure for 7S NGF is abbreviated from Varon *et al.* (1967), for 2.5S NGF from Bocchini and Angeletti (1969), and for the individual α, γ, and βNGF subunits of 7S NGF from Smith *et al.* (1968). The β^1, β^2, and β^3NGF species are defined by the C-terminal amino acid residues of their two peptide chains. The figures opposite these species indicate the proportion of each present in typical β- or 2.5S NGF preparations.

(Figure 6). The NGF protein isolated from purified 7S NGF (βNGF) is not exposed to the action of certain of the proteolytic enzymes of the submaxillary gland, whereas the NGF protein isolated by the alternate procedure (2.5S NGF) is so exposed. The results presented here now explain, in part, the differences between β- and 2.5S NGF.

The C-terminal residues of the native βNGF chains are rapidly and selectively released by either carboxypeptidase B or the carboxypeptidase B like enzyme(s) in the submaxillary gland. The first species which is formed is β^2NGF or monodesarginine β^1NGF and the final product is β^3NGF or bisdesarginine β^1NGF. β^3NGF retains the specific NGF activity and dimeric character of β^1NGF. The disappearance of β^2NGF on incubation with 8 M urea and the concomitant production of equal amounts of β^1 and β^3 chains confirm that β^2NGF also contains two chains. Furthermore, incubation of βNGF with the carboxypeptidase B like enzymes of the gland produces a mixture of the three species which approximate in composition to that of 2.5S NGF, *i.e.* β^1:β^2:β^3 = 60:30:10 (Table IV). This type of proteolysis evidently occurs during the isolation of 2.5S NGF[2] when 7S NGF is dissociated into its subunits early in the procedure and before the carboxypeptidase B like enzymes have been removed. In contrast, the C-terminal arginine residue of βNGF is protected from cleavage in the 7S NGF complex and the chains of the purified βNGF retain most of their C-terminal arginine residues. The interrelationship of these various NGF species is illustrated in Figure 6.

There are a number of possible reasons why even βNGF preparations contain a small proportion of monodesarginine β^2NGF. Traces of the carboxypeptidase B like enzymes might remain in purified 7S NGF, sufficient to cause proteolysis during the isolation of the βNGF, or alternatively some cleavage of βNGF in 7S NGF could occur during purification of the latter. With regard to this last point, it was noted here

[2] The presence in 2.5S NGF of peptide chains lacking C-terminal arginine residues was also determined during the amino acid sequence work by R. A. Angeletti and R. A. Bradshaw, unpublished data.

that carboxypeptidase B (but not the carboxypeptidase B like enzymes in the dialysate) did cause extensive cleavage at the C terminus of βNGF in 7S NGF in Tris-HCl buffer at pH 7.4 (Table V), one of the buffers used during the isolation of 7S NGF. Moreover, it has been recently observed that some dissociation of 7S NGF occurs in this particular buffer even though the pH is within the general pH stability range of 7S NGF (Varon *et al.*, 1972). These observations could account for the small amount of cleavage found in βNGF preparations particularly in view of the fact that no attempt was made to identify all the possible carboxypeptidase B like enzymes in the gland. A third possibility is that the β^2 and β^3 species as well as β^1 occur in significant amounts in the gland. If this is so, then only those species which have an affinity for the α and γ subunits, *i.e.* β^1 and to a considerably less extent β^2, will be isolated as 7S NGF. This situation is illustrated by the composition of the 7S product of the recombination of 2.5S NGF with the α and γ subunits (Figure 5). Its βNGF profile resembles that of a typical βNGF preparation even though the initial 2.5S NGF used in the recombination contained ~30% of β^2 and 10% of β^3NGF (Figure 5b). If this explanation is correct, neither the 7S nor the 2.5S NGF isolation methods would recover the full NGF activity in the gland extract because the free β^2- and β^3NGF would be excluded in the first gel filtration step.

The 2.5S NGF preparation also differs from βNGF at the N termini of the chains where in 2.5S but not in βNGF one of the two chains lacks the eight N-terminal residues (Angeletti and Bradshaw, 1971; Perez-Polo *et al.*, 1972). The gland extract contains the enzyme responsible for this specific cleavage and again βNGF in the 7S NGF complex suffers significantly less cleavage from this activity than does free βNGF (W. C. Mobley, unpublished data). It appears, therefore, that one general function of the 7S NGF complex is to protect the peptide chains of βNGF from proteolysis by a variety of submaxillary gland enzymes.

A Possible Role for the γ Subunit of 7S NGF. The identification of the C-terminal residue of the β- or 2.5S NGF chains as arginine has given rise to speculation about the possible origin of these chains and prompted the present study of the properties of β^3, bisdesarginine β^1NGF. The submaxillary gland contains another growth factor, the epidermal growth factor (EGF),[1] which consists of two EGF subunits and two arginine esteropeptidase subunits, the latter being similar to the γ subunit of 7S NGF (Taylor *et al.*, 1970). The EGF subunit is relatively small and acidic and also has arginine as its C-terminal amino acid. Noting the existence in the submaxillary gland of a number of arginine esteropeptidases (Angeletti and Angeletti, 1967), Taylor *et al.* (1970) suggested that the EGF protein might be derived from a longer precursor by the selective action of an arginine esteropeptidase at this particular arginine residue. The finding that 2.5S NGF also had C-terminal arginine residues led to a similar suggestion for the NGF chains (Angeletti and Bradshaw, 1971). The subsequent demonstration of homology between the amino acid sequences of the A chain of 2.5S NGF and of proinsulin strengthened this hypothesis (Frazier *et al.*, 1972).

The present study has shown that the β^3NGF species which lacks the C-terminal arginine residues differs from β^1NGF in its inability to recombine with the α and γ subunits. The C-terminal arginine residues of βNGF are therefore critical for the stability of 7S NGF. One possible interpretation of this result is that the C-terminal arginine residue is recognized by and binds specifically to the active site of the γ arginine esteropeptidase subunit. This interpretation identifies the γ enzyme as the cleaving enzyme which processes a longer pro-βNGF chain to the functional βNGF chain. In the absence of the terminal arginine enzyme, the γ enzyme no longer recognizes the remaining βNGF chain as the product of its cleavage.

An unusual feature of such a postulate is that the γ enzyme remains bound to its product after the hydrolysis of the peptide bond and indeed is joined by the third subunit, the α subunit. The reasons for this are not entirely clear although there are perhaps some analogies with the trypsin–trypsin inhibitor system (Laskowski and Sealock, 1971). The α subunit is responsible for inhibiting the activity of the γ enzyme in the complex (Greene *et al.*, 1969) and the complex itself, as noted above, provides an environment where the βNGF chains are protected from further proteolytic attack. In addition, the combination of a carboxypeptidase B like enzyme in the gland and an association–dissociation reaction which is sensitive to the presence or absence of a C-terminal arginine residue provides a possible regulatory pathway for the control of the dissociation of 7S NGF. Other possible explanations for the lack of interaction between β^3NGF and the other subunits, such as conformational changes on removal of the arginine residue, remain to be explored.

The existence of a precursor for the NGF chains remains to be proven. However, the analogies with the proinsulin–insulin system noted above and the fact that this latter conversion also involves a carboxypeptidase B like activity in removing a C-terminal arginine residue from an intermediate in the conversion (Nolan *et al.*, 1971) suggest further studies of the 7S NGF system along these lines are warranted. Such studies may help also to distinguish between a number of functional models for 7S NGF recently presented by Varon and Raiborn (1972).

Acknowledgment

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CORRECTIONS

"Effect of Phenobarbital and Naphthalene on Some of the Components of the Electron Transport System and the Hydroxylating Activity of House Fly Microsomes," by Jorge Capdevila, Antonio Morello, Albert S. Perry, and Moises Agosin,* Volume 12, No. 7, March 27, 1973, page 1445.

In the legend to Figure 1, 3rd and 4th lines: "Results expressed per 10 g of flies" should read "Results expressed per 2 g of flies."

In the legend to Figure 2, 3rd line: "Results expressed per 10 g of flies" should read "Results expressed per 2 g of flies."

In Table II, column 4, line 3, the value 4.56 should read 45.6.

"Specific Interaction of Peptides with Nucleic Acids. Evidence for a "Selective Bookmark" Recognition Hypothesis," by Edmond J. Gabbay,* Karl Sanford, C. S. Baxter, and Lou Kapicak, Volume 12, Number 21, October 9, 1973, page 4021.

In Table IV Lys-TrpNH_2 (**37**) should be Lys-TyrNH_2 (**37**).

In the last paragraph, p 4027, (1) L-Lysyl-L-tyrosinamide (**44**) ... should read instead L-Lysyl-L-tryptophanamide (**44**)...