*ENZYMATIC ACTIVITIES OF MOUSE NERVE GROWTH FACTOR AND ITS SUBUNITS**

By Lloyd A. Greene,† E. M. Shooter,‡ and Silvio Varon†

UNIVERSITY OF CALIFORNIA (SAN DIEGO) AND STANFORD UNIVERSITY SCHOOL OF MEDICINE, PALO ALTO

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The nerve growth factor (NGF), discovered by Levi-Montalcini and her coworkers,[1] has traditionally been described as a protein that selectively stimulates the growth of sympathetic and embryonic sensory ganglia. NGF is present in snake venoms[2] and, even more abundantly, in the submaxillary gland of the adult male mouse.[3] Purified products from these two sources were reported to have molecular weights of the order of 20,000 and 44,000, respectively. More recent investigations[4–6] have shown that in crude extracts of the mouse gland, the NGF activity is associated exclusively with a protein that has a sedimentation coefficient of about 7*S*, indicative of a molecular weight of about 140,000. This 7*S* NGF protein has been isolated in a purified form.[4]

The 7*S* NGF protein is stable only within a narrow pH range between 5 and 8, outside which it dissociates reversibly, ultimately into subunits of molecular weights of around 25,000–30,000 with a parallel, and also reversible, three- to fourfold loss of total biological activity.[5, 6] These subunits, while grossly uniform in size, can be resolved by electrophoresis or ion-exchange chromatography into three groups. At neutral pH, one group (α) carries a relatively high negative charge, another group (γ) is less negatively charged, and the third (β) is positively charged. The low NGF activity still measurable after dissociation is found exclusively with the β subunits. No reassociation at neutral pH is observed among subunits of the same group or between α and γ subunits. On the other hand, β subunits will combine with either α or γ subunits to form complexes of approximate molecular weights 60,000 and 120,000, respectively, that do not show an increase in biological activity. All three types of subunits are needed to reconstitute the original, highly active 7*S* NGF protein. Both the γ and the α subunits are heterogenous, and each group comprises three major components that can be isolated by ion-exchange chromatography or electrophoresis.[7] These individual α or γ subunits arise from multiple forms of the 7*S* NGF protein that have the same general subunit composition but differ in the types of α or γ subunits they contain. These multiple forms appear to be already present in the crude extract of even a single mouse submaxillary gland. The roles that α and γ subunits play in respect to the biologically active β subunit on the one hand and the responsive tissues on the other remain to be determined.

Various aspects of the NGF activity are being reinvestigated by using the parent 7*S* NGF protein and its derivatives. One such aspect was the possible occurrence of enzymatic activities. This paper reports a potent enzymatic activity of the 7*S* NGF protein and identifies the γ subunits as the active portion of the molecule in this regard.

Materials and Methods.—The 7*S* NGF protein was isolated from mouse submaxillary glands as previously described.[4] The methods for the separation of the α, β, and γ sub-

unit groups on *O*-(carboxymethyl)cellulose (CM-cellulose) and the resolution on *O*-(diethylaminoethyl)cellulose (DEAE-cellulose) of the γ group into its individual subunits are given elsewhere.[7] All materials were prepared and stored at 0–4°C. The trypsin (crystallized three times) used for comparative enzymatic studies was purchased from Worthington Biochemical Corporation, and its chymotrypsin activity was inhibited by use of L-1-tosylamido-2-phenylethyl chloromethyl ketone.[8]

Proteolytic activity, as measured by the hydrolysis of casein (Hammersten, purchased from Mann Research Laboratories), was assayed according to the method of Kunitz[9] and expressed in trypsin units (TU^{cas}). Enzymatic activity on α,N-benzoyl-L-arginine ethyl ester (BAEE)[10] was measured at pH 7 and 25° by using 2.9 ml of 1 mM BAEE (Calbiochem) in 66.7 mM phosphate buffer and 0.1 ml of the sample to be tested. Hydrolysis of the substrate was followed by the absorbance changes at 253 mμ against an enzyme-free substrate blank. Activities are reported in μmoles of BAEE hydrolyzed per minute for the linear portion of the reaction. Specific activities on either substrate were calculated on the basis of protein values determined by the method of Lowry *et al.*[11]

Results.—Enzymatic activities of NGF and its subunits: The results of the various enzymatic assays are summarized in Table 1. When tested on casein,

TABLE 1. *Specific enzymatic activities of NGF, NGF subunits, and trypsin.*

	Substrate	
	Casein*	BAEE†
Trypsin	3.5	27
NGF	0.01	32
α	0	0
γ	0.02	180
β	0	0.2–1.6

Assay procedures are described in detail in *Materials and Methods.*

* Proteolytic activity is expressed in TU^{cas}. Incubation was for 20 min (trypsin) or 24 hr (NGF, α, γ, β).

† Activity is expressed in μmoles BAEE hydrolyzed/min/mg enzyme. Enzyme used per assay was 1 μg (trypsin, NGF, γ) or 10 μg (α, β).

7*S* NGF showed a slight but definite proteolytic activity of 0.01 TU^{cas}, a level that required 24 hours' incubation for reliable readings. In contrast, trypsin assayed at 3.5 TU^{cas} after 20 minutes' incubation. When tested on the BAEE substrate, however, NGF showed a specific activity comparable to that of trpysin (32 and 27 μmoles/min/mg, respectively). The hydrolysis of BAEE by NGF did not proceed initially at a linear rate as did the hydrolysis by trypsin, but exhibited a lag period after which a maximal linear rate was maintained until about 85 per cent of the substrate was consumed (see Fig. 4). The activity reported in Table 1 was computed on the basis of this maximal linear rate. The same behavior was observed over a range of 0.6 to 6 μg per assay.

These results prompted an examination of the activity of the NGF subunits on the two substrates. The α subunits were inactive both on casein and on BAEE in concentrations up to 15 μg per ml of assay mixture. The β subunits exhibited no detectable activity on casein over a similar concentration range, but did show some activity on BAEE, the level of which varied from preparation to preparation between 0.16 and 1.6 μmoles/min/mg. The γ subunits were active on both substrates. On casein, their specific activity was 0.02 TU^{cas}, which, like the activity of NGF, amounted to less than 1 per cent of that of trypsin. In contrast, the activity of the γ material on BAEE (180 μmoles/min/mg) was about

sixfold higher than that of trypsin. As with trypsin, but not with NGF, the hydrolysis proceeded linearly from the start of the reaction until about 80 per cent of the substrate was consumed (see Fig. 4).

Identification of the enzyme with the γ subunits: To determine whether the observed BAEE activity of the β and the γ subunits was a property of these species themselves or of accompanying contaminants, the following experiments were carried out:

(*a*) 7*S* NGF was separated into its constituent subunits by chromatography on a CM-cellulose column,[7] and the fractions were assayed for both protein and enzymatic activity (Fig. 1). Enzymatic activity measured on BAEE and ab-

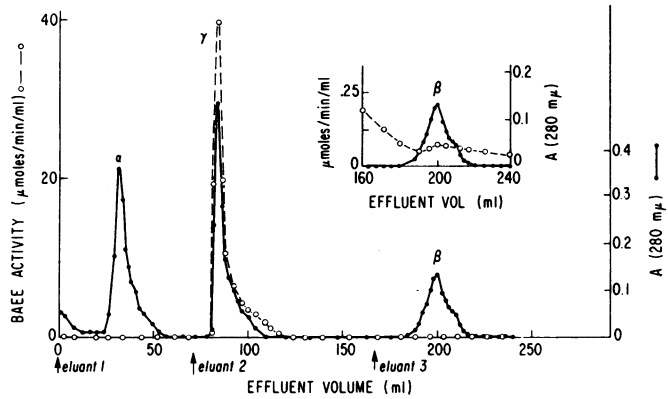

FIG. 1.—Enzymatic activity of the NGF subunits. 15 mg of 7*S* NGF protein were dissociated by dialysis against sodium acetate buffer (I = 0.1), pH 4.0, and loaded on a microgranular CM-cellulose column (0.9 × 20 cm) equilibrated with the same buffer containing 0.15 *M* NaCl.

Eluants 1 and 2: 0.15 and 0.35 *M* NaCl, respectively, in the acetate buffer. *Eluant 3:* 1.0 *M* NaCl in sodium glycinate buffer (I = 0.1), pH 9.4. Enzymatic activity was assayed with BAEE as the substrate on fractions directly or after 10-fold dilution.

Inset: assays on fractions obtained during elution of β subunits but with a 20-fold expanded scale for activity on BAEE.

sorbance at 280 mμ paralleled each other in the eluates of the γ but not of the β subunits. The slight enzymatic activity of the β fractions (Fig. 1, *inset*) arose from a prolonged trail of the γ material eluted before it, the carry-over into the β fraction being less than 0.5 per cent of the activity collected with the γ fraction.

(*b*) The sedimentation properties of the enzyme were examined on sucrose gradients with γ protein alone or after concentration in the presence of excess α and β subunits at neutral pH, a condition known to result in the recombination of the three types of subunits into 7*S* NGF.[5, 6] These experiments revealed that the enzymatic activity sedimented with the γ protein in the expected 2.5*S* region when only γ subunits were present (Fig. 2*a*) but shifted completely into the 7*S* NGF region of the gradient after recombination (Fig. 2*b*). Thus these results demonstrate that the enzymatic activity is an inherent property of the γ subunits of the 7*S* NGF protein.

Multiple forms of the γ enzyme: The γ subunits comprise three major com-

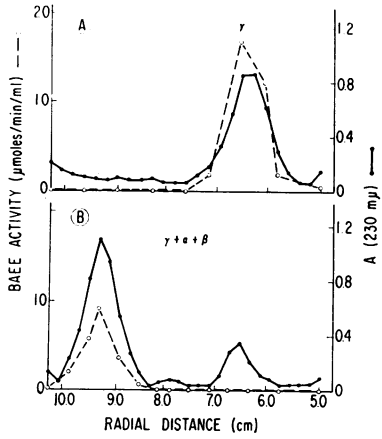

FIG. 2.—Sucrose gradient sedimentation of γ subunits alone and after recombination with excess α and β subunits.

(*A*) 90 μg of γ subunits were loaded in 150 μl of 0.05 *M* tris(hydroxymethyl)aminomethane (Tris)-chloride buffer, pH 7.4.

(*B*) 50 μg of γ subunits were mixed with 110 μg of α and 96 μg of β subunits and the volume was reduced to 200 μl by pressure dialysis against Tris buffer.

Sedimentation was for 13 hr at 60,000 rpm at 5° in a 5–20% sucrose gradient in the same buffer. Fractions were diluted fivefold prior to enzymatic assay.

ponents of similar size and ability to recombine with α and β subunits, but with different net charge.[7] To examine the enzymatic activity of these individual components, γ material was resolved by DEAE-cellulose chromatography,[7] the column fractions were assayed both for protein and for activity on BAEE, and the identity of the three components was verified by acrylamide gel electrophoresis. As shown in Figure 3, the enzymatic activity and protein patterns were closely correlated, indicating that the γ^1, γ^2, and γ^3 subunits of NGF each possess enzymatic activity. When assayed under identical conditions, all three γ species proved to have the same specific activity (Fig. 4), equal to that of the unfractionated γ material (Table 1). Like the latter, the individual enzyme species hydrolyzed BAEE at a constant rate from the beginning of the reaction. For comparison, Figure 4 also shows the characteristic time course of the hydrolysis of BAEE by the 7*S* NGF protein.

Discussion.—The high levels of protease activity of the submaxillary gland of the mouse have been known for a long time.[12] The activity resides in the tubular portion of the gland, a structure that depends on testosterone for its full development.[12–14] Like the protease activity, NGF activity resides in the tubular structure and is high in adult male, but not female, mouse glands.[15] Recently, enzymes with both esterase and peptidase activities have been reported in a number of mouse submaxillary preparations. In one investigation,[16] two differ-

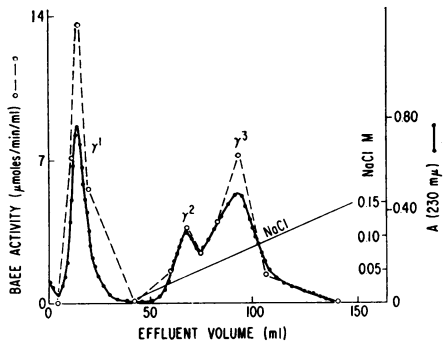

FIG. 3.—Enzymatic activity of the individual γ subunits. Isolated γ protein (7 mg) was concentrated to 0.5 ml in 0.05 *M* Tris-chloride buffer, pH 7.4, and loaded on a DEAE-cellulose column (0.9 × 12 cm) equilibrated with the same buffer. γ^1 subunits were eluted directly with Tris buffer; γ^2 and γ^3 subunits were resolved with a linear NaCl gradient between 0 and 0.15 *M* in the same buffer. Fractions were assayed on BAEE after fivefold dilution.

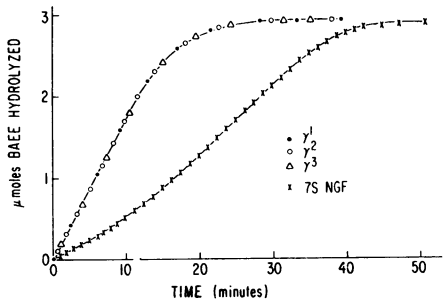

FIG. 4.—The hydrolysis of BAEE by γ^1, γ^2, and γ^3 subunits and by 7S NGF. Assays were performed on 1 μg of each γ subunit and 3 μg of 7S NGF. Details are given in *Materials and Methods*.

ent molecular species having these enzymatic activities were recognized in a partially purified preparation of mesenchymal growth factor. For one of these species, the enzymatic and biological activities appeared to be associated. In another case,[17] it was reported that an estero-peptidase and had been purified and adversely affected NGF activity. It was not clear, however, whether this was attributed to an enzymatic degradation of NGF or to some interference with its action at the target-cell level. A third study[18, 19] reported combined estero-peptidase activities in two chromatographic fractions from mouse gland extracts. Two distinct enzyme forms were purified and their synthesis was shown to increase in female glands after testosterone treatment. Their possible relationship to NGF or other growth factors was not examined.

The enzymes reported here have shown, like those just reviewed, a potent activity on BAEE. They also have shown a low, measurable, proteolytic activity as measured on casein. The remarkable feature of these enzymes, however, is that they belong to the molecular system of NGF and have actually been isolated as, or from, purified 7S NGF protein itself. As with the NGF activity,[4–7] the enzymatic activity occurs in a number of molecular forms. At the subunit level (γ species), the three isomers γ^1, γ^2, and γ^3 have the same specific activity (Fig. 4), the same molecular weight of about 25,000–30,000, the same reactivity toward the α and β subunits, and only differ, as far as is known, in their net charge. A fourth isomer, γ', which appears to derive from γ^1 upon aging,[7] remains to be investigated in terms of enzymatic activity. At the parent-complex level, the 7S NGF protein (or $\alpha\beta\gamma$ species), with a molecular weight of about 140,000, also exists in multiple forms. These depend not only on which γ isomers but also on which α isomers participate in their makeup.[7]

The present findings have shown that the 7S NGF protein, whose growth-promoting activity is well established, is also endowed with enzymatic activity. The two activities reside in different portions of the molecule, the β and the γ subunits respectively. Other proteins in which the different subunits serve different functions are known, e.g., tryptophan synthetase.[20] There are as yet no clues as to the possible activities of the remaining subunit, α. It is clear, however, that the activities of the β and γ subunits are affected by the presence of the other two. Thus, a given amount of β protein has a higher and more stable growth-promoting activity when combined with α and γ subunits than in the isolated form.[5, 6] For the enzymatic activity, both the kinetics and the specific activity of the γ subunits differ from those of the $\alpha\beta\gamma$ complexes. The time

course of the enzymatic hydrolysis of BAEE by 7*S* NGF shows a lag phase (Fig. 4), while the difference in specific activity between the the two molecular forms (Table 1 and Fig. 4) is larger than can be accounted for by the fact that γ subunits contribute some 30–40 per cent of the total protein of the $\alpha\beta\gamma$ species.[21] Whether the enzymatic activity of 7*S* NGF is related to its growth-promoting activity is a question that remains for further investigation.

Summary.—The high-molecular-weight (7*S*) form of NGF has been shown to possess a potent esterase activity on the substrate BAEE. Upon dissociation of the parent molecule, this enzymatic activity is exhibited by the γ subunits alone. The γ^1, γ^2, and γ^3 species, which make up the γ fraction, have identical specific activities. The high-molecular-weight form differs from the isolated subunit in both its specific activity and kinetic pattern of hydrolysis.

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† Departments of Chemistry and Biology and School of Medicine, University of California, San Diego. One of us (L. A. G.) was a National Science Foundation Graduate Trainee, 1967–68.

‡ Department of Genetics and Lt. Joseph P. Kennedy, Jr., Laboratories for Molecular Medicine, Stanford University School of Medicine, Palo Alto, California.

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