Subunit Structure of High Molecular Weight Mouse Nerve Growth Factor†

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ABSTRACT: Studies from several laboratories have shown that mouse submandibular glands and mouse saliva contain nerve growth factor (NGF) as part of a high molecular weight oligomeric macromolecule composed of three different subunits, termed α, β, and γ. The β-subunit is the nerve growth-promoting protein. The γ-subunit is a serine protease class enzyme of highly restricted substrate specificity. The α-subunit has no known function. This high molecular weight form of nerve growth factor is also a Zn(II)-containing metalloprotein. In the present study, measurements of multiple physicochemical parameters have been used to deduce the subunit structure of high molecular weight NGF. Results demonstrate that it contains two α-, one β- and one γ-subunit together with one tightly bound Zn(II) ion per molecule.

The object of this study is to establish the subunit structure and metal binding stoichiometry of the high molecular weight form of mouse submandibular gland nerve growth factor (HMW-NGF).[1] Before proceeding, it is pertinent to summarize previous studies on this problem—studies that have utilized two different purification schemes for preparation of this high molecular weight form of the growth factor protein.

Varon et al. (1967) first reported that mouse submandibular gland nerve growth factor can exist as a high molecular weight protein, called 7S-NGF. The molecular weight of 7S-NGF has been variously estimated to be between 130 000 (Server & Shooter, 1977) and 140 000 (Varon et al., 1967), and it contains three different kinds of noncovalently bonded subunits termed α, β, and γ (Varon et al., 1968; Greene & Shooter, 1980). The α-subunit, with a molecular weight close to 26 000 (Server & Shooter, 1977), has no known function. The β-subunit, with a molecular weight of 26 518 from sequence information (Angeletti et al., 1973), is the one that stimulates nerve growth (Varon et al., 1968). The γ-subunit is a proteolytic enzyme with highly restricted substrate specificity toward certain argininyl and lysyl peptide bonds (Greene et al., 1968). The γ-subunit has a molecular weight of 27 500 from sequence data (Thomas et al., 1981), and it is a member of the general class of serine proteases (Orenstein et al., 1978). The 7S-NGF oligomer has also been shown to be a Zn(II) metalloprotein, with a Zn(II) content estimated by atomic absorption spectroscopy to be between 1 and 2 mol/mol of protein (Pattison & Dunn, 1975).

The empirical subunit structural formula for 7S-NGF has been stated in several reviews to be $\alpha_2\beta\gamma_2$, and further that 7S-NGF contains two Zn(II) ions per mole [see, e.g., Server and Shooter (1977), Bradshaw (1978), Greene and Shooter (1980), and Nichols and Shooter (1985)]. On the basis of a molecular weight of 130 000–140 000 for 7S-NGF, and the known molecular weights of the three different subunits, such a proposal would account for the total mass of the parent oligomer. However, we have been unable to find any *quantitative* physicochemical study in the literature that establishes an $\alpha_2\beta\gamma_2$–$Zn(II)_2$ structure for this protein.

Several lines of evidence have indicated that the 7S-NGF complex exhibits considerable instability in dilute solution. For example, in their original paper on the isolation of this protein, Varon et al. (1967) observed that during DEAE-cellulose chromatography, extraordinarily high flow rates (350 mL/h) were required to achieve good 7S-NGF recovery. At lower velocities, recovery of 7S-NGF was low, and the protein appeared in many fractions. From studies on purified 7S-NGF, similar conclusions concerning its structural instability were reached by Smith et al. (1969), Baker (1975), and Pantazis et al. (1977).

In the course of studies on the naturally occurring form of NGF as it exists within the mouse submandibular gland, Young et al. (1978) observed that this gland contains multiple molecular forms of NGF. The largest of these species was completely purified by a scheme different from that used for 7S-NGF. Sedimentation equilibrium studies of this protein (HMW-NGF) with simultaneous determination of its partial specific volume (0.688 mL/g) yielded a molecular weight of 116 000 ± 8000 (Young et al., 1978). HMW-NGF contained 1.0 mol of Zn(II) per mole of protein by atomic absorption spectroscopy (Young & Koroly, 1980) as well as the α-, β-, and γ-subunits characteristic of 7S-NGF. Unlike 7S-NGF, however, HMW-NGF retains its oligomeric structure at very low concentrations of protein (Young et al., 1978), and it contained only one γ-subunit per mole (Young & Koroly, 1980).

In light of the above observations concerning the number of γ-subunits and Zn(II) ions per mole of the parent protein—findings that do not agree with the currently accepted view of the structure of salivary gland NGF—it was necessary to measure the number of α- and β-subunits as well. Consequently, we have now utilized multiple physicochemical approaches to determine the complete subunit structural stoichiometry of all of the subunits of HMW-NGF. Results indicate that the empirical subunit formula for this protein

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[1] Abbreviations: HMW-NGF, high molecular weight mouse submandibular gland nerve growth factor, isolated as in Young et al. (1978); 7S-NGF, NGF isolated as described by Varon et al. (1967); EDTA, ethylenediaminetetraacetic acid; pNPGB, *p*-nitrophenyl *p*-guanidinobenzoate; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DFP, diisopropyl phosphorofluoridate; BAPNA, N^{α}-benzoyl-L-arginine *p*-nitroanilide; FPLC, Pharmacia fast protein liquid chromatography system.

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is $\alpha_2\beta\gamma$–Zn(II). The relationship of HMW-NGF to 7S-NGF will be discussed below.

Materials and Methods

Reagents. HMW-NGF was prepared as previously described, and all preparations were shown to be electrophoretically and ultracentrifugally homogeneous (Young et al., 1978). Sequanal-grade guanidine hydrochloride was obtained from Pierce, and pNPGB was from Cal-Biochem. pNPGB was recrystallized once as described by Chase and Shaw (1967). ^{65}Zn (carrier-free) was obtained from New England Nuclear. Crystalline BAPTA was a gift from Dr. Vincent Chau. The individual α-, β-, and γ-subunits were prepared from homogeneous HMW-NGF by (carboxymethyl)cellulose chromatography (Thomas & Bradshaw, 1981) and by FPLC (see below). Sephacryl S-200 was purchased from Pharmacia. Spectral-grade dimethylformamide was obtained from Aldrich.

Specific Subunit Radioimmunoassays. Radioimmunoassays for the purified α-, β-, and γ-subunits of HMW-NGF were developed and performed as described (Young et al., 1979).

Subunit Extinction Coefficients. The interference optical system of a Beckman Model E ultracentrifuge was used to measure the extinction coefficients of the three individual subunits of HMW-NGF at λ 280 and 230 nm (Babul & Stellwagen, 1969). For this purpose, a value of 4.0 Rayleigh fringes was taken to correspond to a protein concentration of 1.0 mg/mL (double-sector cell, 10-mm path length). The following values were obtained for the purified subunits: α, λ 280 nm (1.60 mL mg^{-1} cm^{-1}), λ 230 nm (3.12 mL mg^{-1} cm^{-1}); β, λ 280 nm (1.43 mL mg^{-1} cm^{-1}), λ 230 nm (2.29 mL mg^{-1} cm^{-1}); γ, λ 280 nm (1.59 mL mg^{-1} cm^{-1}), λ 230 nm (3.10 mL mg^{-1} cm^{-1}). These values were all determined with 0.1 M Tris·HCl, pH 7.5, as solvent. Although extinction coefficients determined in dilute aqueous buffer solutions generally decrease in the presence of high concentrations of guanidine hydrochloride (due to unfolding of the secondary and tertiary structure), they would be expected to decrease nearly equally, and by about 10% (Woods et al., 1963). The extinction coefficient of HMW-NGF (λ 280 nm) was taken to be 1.42 mL mg^{-1} cm^{-1} (Young & Koroly, 1980). Spectrophotometric measurements utilized a Varian 2290 split-beam recording spectrophotometer.

Serine Protease Active-Site Measurements. Active-site titrations of HMW-NGF and its γ-subunit with pNPGB were performed as described by Chase and Shaw (1967). The molar extinction coefficient of *p*-nitrophenol (pH 8.30, λ 402 nm) was taken to be 1.69×10^3M^{-1} cm^{-1}. HMW-NGF was dissolved in 0.1 M potassium phosphate, pH 8.3, to a concentration of 0.20 mg/mL (1.72×10^{-6} M). This solution was treated with EDTA (final concentration 6.1×10^{-3} M) for 30 min to activate fully the protease activity (Young, 1979) and then with pNPGB (dissolved in dimethylformamide) to a final concentration of 5.4×10^{-5} M. Values for moles of active-site serine per mole of HMW-NGF were calculated from the burst concentration of *p*-nitrophenol released (λ 402 nm) and a molecular weight of 116 000 for HMW-NGF.

Metal Ion Studies. The Zn(II) content of HMW-NGF was measured both by equilibrium dialysis, utilizing ^{65}Zn(II), and also by spectrophotometric titration of the protein-bound metal with BAPTA at λ 254 and 300 nm. The change in molar absorbance of BAPTA upon Zn(II) chelation was taken to be 1.45×10^4 M^{-1} cm^{-1} (λ 254 nm) and 4.05×10^3 M^{-1} cm^{-1} (λ 300 nm) (Tsien, 1980). For these studies, protein solutions were first dialyzed extensively against Zn(II)-free buffers, prepared by extraction with a 0.01% solution of diphenylthiocarbazone in CCl_4 (Coleman & Vallee, 1960). All labware used for metal binding studies were rinsed with 20% HNO_3 prior to use. Concentrations of HMW-NGF were determined spectrophotometrically and ^{65}Zn(II) concentrations with a Packard γ spectrometer.

Sedimentation Velocity and Active-Enzyme Centrifugation Studies. Sedimentation analyses of HMW-NGF and its subunits were performed with the split-beam photoelectric scanner of a Beckman Model E ultracentrifuge operating at 60 000 rpm and at monochromator wavelengths of λ 280 and 230 nm. The RTIC unit of the instrument was used to control temperature (±0.1 °C), and all values were converted to $s_{20,w}$ based upon solvent relative viscosity and density data.

To measure the sedimentation coefficients of catalytically active species in solutions of HMW-NGF, the active-enzyme centrifugation methods described by Kemper and Everse (1973) were employed. Beckman standard aluminum-filled epon double-sector synthetic boundary centerpieces (12-mm path length) were used as follows. The reference sector was filled with 0.222 mL of a solution containing 0.277 mg/mL protein dissolved in 0.001 M Tris·HCl, pH 7.5. The sample sector contained 0.180 mL of a solution containing 0.1 M Tris·HCl and $(2.4–3.0) \times 10^{-3}$ M BAPNA, pH 7.5. (The above volumes were calculated from microcomparator measurements of the volume of a 2° double-sector centerpiece centrifugal to the capillary channel that connects the two sectors.) On this basis, 0.009 mL of a protein solution (of lower solvent density) is layered over substrate solution (of higher density) during rotor acceleration. Protein concentration was chosen such that 0.009 mL of enzyme solution, dissolved in 1.0 mL of substrate solution, would yield an absorbance change of approximately 0.01 per minute (Kemper & Everse, 1973). A high substrate concentration was selected so that changes in the degree of enzyme–substrate saturation were negligible during the centrifuge run. The centrifuge cell, at a rotor velocity of 60 000 rpm, was scanned automatically at 2-min intervals with light of wavelength 410 nm, and the midpoints of the absorbance vs distance traces were used to compute $s_{20,w}$ for the enzymically active sedimenting species.

FPLC Analyses of the Subunit Composition of HMW-NGF. Cation-exchange chromatographic studies utilized a Pharmacia Mono S HR 5/5 column equilibrated with 0.05 M sodium acetate, pH 4.0. HMW-NGF was dialyzed vs this solution, 0.1–0.8 mg of protein was applied, and the column was developed with a 46-mL linear gradient to 1 M NaCl at a flow rate of 1.0 mL/min. Absorbance was measured by continuous flow-cell monitoring at λ 280 nm. Area analyses of the α, β, and γ peaks were performed both by direct planimetry of the output recorder tracings and by the integrator program of the FPLC instrument. Results are expressed as the means (±SEM) obtained by these two methods. Chromatographic recoveries of the individual purified subunits were also determined.

Results

The close similarity of the molecular weights of the α-, β-, and γ-subunits of HMW-NGF to one another precludes the use of analytical ultracentrifugation methods to determine the complete subunit structure. Similarly, gel electrophoretic analyses suffer from variability of staining of protein bands, with concomitant errors in quantitative determination of subunit concentrations. Likewise, conventional ion-exchange chromatographic methods [such as (carboxymethyl)cellulose] are of little use since the β-subunit of HMW-NGF binds strongly to these resins and cannot be quantitatively recovered. Finally, individual subunit radioimmunoassays designed to

measure the relative ratios of the three different subunits are complicated by the fact that anti-γ and anti-α antisera cross-react with pure α- and γ-subunits, respectively [unpublished results and Anundi et al. (1978)]. Consequently, such assays are quantitatively unreliable for the purpose of determining the subunit composition of HMW-NGF.

The strategy that we have used to deduce the subunit structure is 3-fold as follows: (1) determine the molecular ratio of the γ-subunit to that of HMW-NGF by active-site titration with pNPGB and, using this value, measure the relative amounts of the α- and β-subunits by gel filtration in the presence of a high concentration of guanidine hydrochloride where it can be established that individual subunit recovery is quantitative; (2) measure the relative molar concentration of each subunit by FPLC under solvent conditions where chromatographic recovery of each subunit can be firmly established; (3) finally, measure directly, without resort to active-site titration, the number of moles of γ-subunit released per mole of HMW-NGF in the presence of EDTA by velocity sedimentation.

γ-Subunit Composition of HMW-NGF. Earlier studies from this laboratory suggested that HMW-NGF contains 1 mol of γ-subunit per mole (Young & Koroly, 1980). This conclusion arose from studies which showed that reaction of [^{3}H]DFP with the active-site serine residue of the γ-subunit of HMW-NGF yielded 1 mol of γ-subunit per mole of the parent protein (Young & Koroly, 1980). We have now measured the number of γ-subunits per mole by direct active-site titration with pNPGB and by an ultracentrifuge method which does not rely upon enzyme active sites for its validity.

The serine protease active-site titrant pNPGB was used to determine the content of the γ-subunit within HMW-NGF from spectrophotometric measurements of the burst release of *p*-nitrophenol produced by acylation of the active-site serine (Chase & Shaw, 1967). In the case of the reaction of trypsin with pNPGB, a burst is observed followed by negligible nonspecific hydrolysis of the titrant. In contrast, with HMW-NGF, the immediate burst reaction is followed by slow but distinct enzymic hydrolysis of pNPGB, and the extent of the burst must therefore be determined by extrapolation of the linear hydrolysis rate back to zero time (i.e., the time of reagent addition). In order the ensure that the burst size accurately reflects the true active-site serine concentration, the pNPGB concentration must be significantly larger than its K_m. Following complete activation of the enzyme activity of HMW-NGF by chelation of its bound Zn(II) with EDTA (Young, 1979; Young & Koroly, 1980), the K_m for pNPGB was found from least-squares Lineweaver–Burk plots to be 2.8×10^{-6} M. Thus, concentrations of pNPGB used for active-site titrations were approximately 20 times this value.

Five different HMW-NGF preparations yielded a mean value of 1.0 ± 0.1 (SEM) mol of active-site serine per mole of protein. This finding is in agreement with that determined earlier from [^{3}H]DFP incorporation studies (1.10 mol/mol) (Young & Koroly, 1980).

The γ-subunit content of HMW-NGF has been measured in yet a different way that does not depend upon a knowledge of the number of enzymically active sites. In the course of studies on the effect of EDTA [to chelate protein-bound Zn(II)] upon the macromolecular assembly of HMW-NGF, we observed that EDTA causes dissociation of this protein into two distinct sedimenting boundaries as judged by sedimentation velocity. The following data show that the slower sedimenting boundary is the γ-subunit and that it accounts for all of the γ activity present in the centrifuge cell.

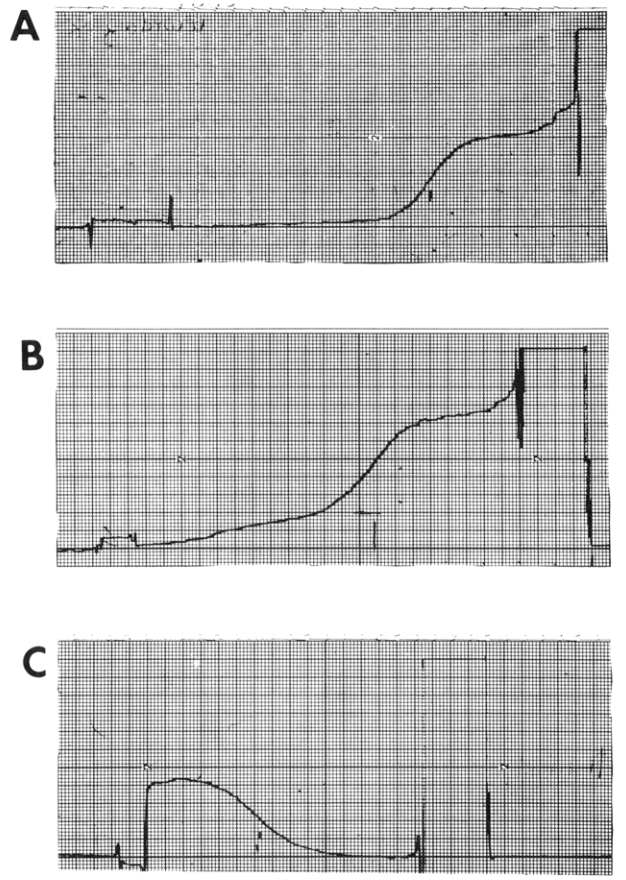

FIGURE 1: Sedimentation velocity and active-enzyme centrifugation of HMW-NGF in the presence and absence of EDTA. Rotor speed, 60 000 rpm. (A) Native HMW-NGF, 462 mg/mL; solvent, 0.1 M Tris·HCl, pH 7.5; temperature, 23.2 °C; λ 280 nm; tracing 30 min after reaching full speed. (B) HMW-NGF, 483 mg/mL, preincubated 30 min with 3.1×10^{-3} M EDTA; solvent, 0.1 M Tris·HCl and 3.1×10^{-3} M EDTA, pH 7.5; temperature, 25 °C; λ 280 nm; tracing 24 min after full speed. (C) Active enzyme centrifugation; HMW-NGF, 277 mg/mL, preincubated 30 min with 3.0×10^{-3} M EDTA; solvent, 0.1 M Tris·HCl and 2.4×10^{-3} M BAPNA, pH 7.5; temperature, 15.8 °C; λ 410 nm; tracing 12 min after reaching full speed.

Table I: Summary of Sedimentation Velocity and Active-Enzyme Centrifugation Studies of Native and EDTA-Activated HMW-NGF[a]

preparation	method	$s_{20,w}$ (S)
native HMW-NGF	SV	7.60 ± 0.06 (n = 4)
native HMW-NGF	AE	7.37 (n = 1)
activated HMW-NGF	SV	2.75 ± 0.14 (n = 4, slow boundary); 6.85 ± 0.09 (n = 4, fast boundary)
activated HMW-NGF	AE	2.65 ± 0.30 (n = 4)

[a] Sedimentation velocity (SV; λ 280 or 230 nm) and active-enzyme (AE; λ 410 nm) centrifugation were performed at 25 °C (±0.1 °C) at 60 000 rpm under conditions described in the text and the legend to Figure 1. For native HMW-NGF, the solvent is 0.1 M Tris·HCl and 1×10^{-6} M Zn(II), pH 7.5; for activated HMW-NGF, the solvent is 0.1 M Tris·HCl and 3.0×10^{-3} M EDTA, pH 7.5, and samples were incubated with this solvent for 30 min prior to the centrifuge run. Sedimentation coefficients represent mean values (±SEM).

Figure 1A depicts a typical scanner trace (λ 280 nm) of HMW-NGF in the absence of EDTA. Only a single sedimenting boundary is seen with $s_{20,w}$ = 7.60 S (Table II). This value is close to that determined earlier ($s_{20,w}$ = 7.37 S) with the more accurate schlieren optical system (Young et al., 1978). Figure 1B shows what happens when the protein is treated with EDTA. Two sedimenting boundaries are seen—a

slower and a faster. The slow boundary has $s_{20,w}$ = 2.75 S and the fast, $s_{20,w}$ = 6.85 S (Table I).

To establish which of these two boundary components exhibits enzyme activity, active-enzyme sedimentation experiments were performed. In this method, a small volume of protein solution is layered over solvent containing a substrate (BAPNA in this case). Scanner traces (λ 410 nm) then reflect hydrolysis of substrate as the enzyme sediments through the substrate solution. To check the accuracy of this method in our system, the sedimentation coefficient of native HMW-NGF was measured in the presence of 1×10^{-6} M Zn(II). A value of $s_{20,w}$ = 7.37 S was obtained (Table I). The sedimentation behavior of HMW-NGF treated with EDTA was then examined. As shown in Figure 1C, only a single component is seen. This component has $s_{20,w}$ = 2.65 S (Table I)—a value to be compared with 2.75 S for the slow component from standard sedimentation velocity monitored at λ 280 nm. No faster component with enzyme activity was detected. Thus, all of the enzyme activity of HMW-NGF dissociates in the presence of EDTA to yield a boundary whose sedimentation coefficient is virtually identical with that of the γ-subunit (Varon et al., 1968).

On the basis of the above information, it is now possible to calculate the weight fraction of γ-subunit that comprises HMW-NGF and that dissociates from it during velocity sedimentation in the presence of EDTA. For this purpose, a series of such runs, like that shown in Figure 1B, were carried out. Solutions of HMW-NGF (480 mg/mL) were incubated 30 min at 25 °C in the presence of 3.1×10^{-3} M EDTA and 0.1 M Tris·HCl, pH 7.5, and then centrifuged at 60 000 rpm at 25 °C. Samples were centrifuged for a time period sufficient to visualize a clear plateau region for the slow (γ) component. (Figure 1B does not yet show a clear plateau region for the slow component. To establish visually this plateau, the faster component must be sedimented to the cell bottom.) The weight fraction of the slow boundary was calculated from its plateau absorbance (λ 280 nm) compared to the total absorbance of all sedimenting components 30 min after reaching full speed. All absorbance values were corrected for radial dilution. Molecular weight values for the slow component were calculated from the weight fraction (slow) and a molecular weight of 116 000 for native HMW-NGF.

From four such experiments, a mean weight fraction of 0.22 ± 0.01 (SEM) was obtained, and this number yields a value of 26 000 ± 1400 for the molecular weight of γ. The molecular weight of γ from sequence information is close to 27 500 (Thomas et al., 1981). Thus, the above analysis also reveals that HMW-NGF contains only one γ-subunit per molecule. It should be pointed out that correction of the above absorbance values for the small differences in extinction coefficients between γ and HMW-NGF makes no difference in the conclusion reached. Further, even if one were to take the molecular weight of HMW-NGF to be as high as 140 000 (Varon et al., 1967), the above data would still be inconsistent with two γ-subunits per molecule.

α- and β-Subunit Stoichiometry. To determine the α- and β-subunit content of HMW-NGF, we have taken advantage of the fact that the β-subunit consists of two identical noncovalently linked polypeptide chains, each of molecular weight 13 259 (Angeletti et al., 1973). Thus, the individual subunit chains of β can be cleanly separated from the 26 000–27 000 molecular weight α- and γ-subunits by gel filtration in the presence of concentrated solutions of guanidine hydrochloride.

Figure 2 depicts a representative elution profile of HMW-NGF following chromatography upon Sephacryl S-200

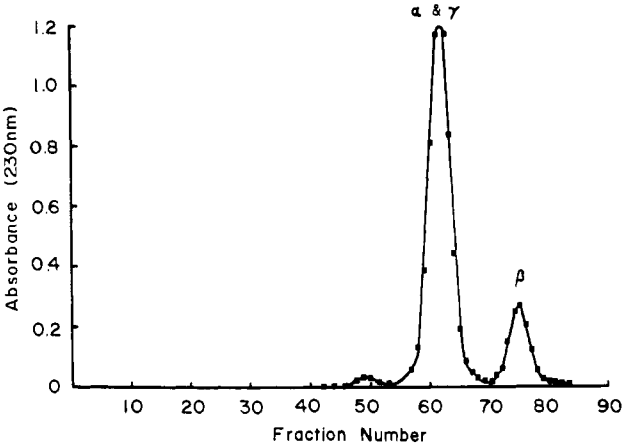

FIGURE 2: Gel filtration of HMW-NGF upon Sephacryl S-200 in 6.1 M guanidine hydrochloride. Column dimensions, 1.0 × 100 cm; flow rate, 13 mL/h; fraction volume, 0.6 mL. HMW-NGF (0.72 mL, 1.37 mg/mL) dialyzed against 6.1 M guanidine hydrochloride was applied to the column. Fractions labeled α + γ and β were identified by specific radioimmunoassays for α, β, and γ. Individual fraction volumes were measured gravimetrically.

equilibrated with 6 M guanidine hydrochloride. Three peaks were visualized. Two major peaks were observed that together accounted for 98% of the total absorbance recovered (Figure 2). The pooled fractions of these two peaks were dialyzed to remove guanidine hydrochloride and then examined for the presence of α-, β-, and γ-subunits by specific radioimmunoassays. The first peak contained only α + γ (no detectable β); the second peak contained only β (no detectable α or γ) (Figure 1). Total absorbance (λ 280 or 230 nm) recovered from column profiles such as that shown in Figure 1 ranged between 95 and 99% of the total absorbance of HMW-NGF initially applied to the column.

Individual fraction volumes (Figure 2) were measured gravimetrically and, together with the extinction coefficients and area analyses of the individual peaks, were used to calculate the total mass of each subunit recovered, as follows. Values for the β-subunit were obtained directly from the mole fraction of this subunit relative to the total chromatographed (and recovered) mass. Values for the α-subunit were computed after first subtracting (from the combined α + γ mass) the weight of the γ-subunit corresponding to 1 mol of γ per mole of HMW-NGF. The molecular weight of HMW-NGF was taken to be 116 000 (Young, 1979), and that of the γ-subunit to be 27 500 (Thomas et al., 1981). Thus, the weight fraction of γ in HMW-NGF is 0.237. Results from three separate determinations yielded the following ratios of moles of subunit per mole of HMW-NGF: α = 2.27 ± 0.03 (SEM) and β = 0.97 ± 0.04. These data indicate that HMW-NGF contains two α- and one β-subunits per molecule.

The α + γ fraction illustrated in Figure 2 contains only the α- and γ-subunits—and in the same proportions in which they exist within HMW-NGF. On the basis of the above data, this ratio should be α:γ = 2:1. To substantiate this inference, the total α + γ fraction from chromatographic analyses such as that shown in Figure 2 was pooled, dialyzed extensively to remove guanidine, concentrated by lyophilization, and redissolved in 0.1 M potassium phosphate, pH 8.3. The concentration of the γ-subunit was then measured by pNPGB titration. The α-subunit concentration was determined by difference from the total protein concentration minus that of the γ-subunit. Results from three different preparations yielded a mean value of 2.1 ± 0.2 (SEM) mol of α/mol of γ. This result is in agreement with that predicted from the results above.

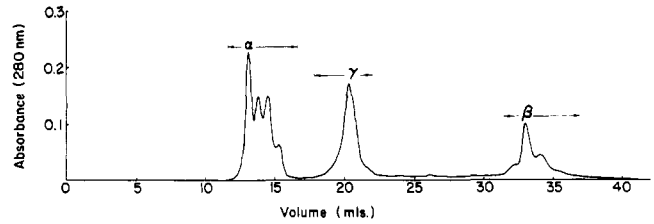

FIGURE 3: FPLC profile of HMW-NGF under subunit-dissociating solvent conditions. Column: Mono S HR 5/5 (Pharmacia); solvent, 0.05 M sodium acetate, pH 4.0, with a 46-mL linear gradient beginning at 4.0 mL to 1 M NaCl at a flow rate of 1.0 mL/min. Individual subunit fractions are marked α, β, and γ.

FPLC Analyses of Dissociated HMW-NGF. Under mildly acidic conditions, HMW-NGF, like 7S-NGF (Varon et al., 1968), dissociates to yield its constituent subunits. While conventional cation-exchange chromatography cannot be used to determine the relative proportions of the individual subunits because of chromatographic losses, FPLC with the Pharmacia cation-exchange resin Mono S does not present this drawback. In initial studies designed to measure chromatographic recoveries of HMW-NGF as well as each of its individual subunits, multiple samples of known total absorbance were applied to Mono-S, and the total eluate absorbance was calculated by integration. For HMW-NGF, protein recoveries following chromatography were always in the range 67–73%. Each of the subunits gave recoveries of between 65% and 70%, regardless of the total protein loaded onto the column.

Figure 3 illustrates a typical chromatogram of HMW-NGF at pH 4.0. At this pH value and below, HMW-NGF is fully dissociated as judged by specific radioimmunoassays of all fractions for α, β, and γ and by enzyme assays for γ. By these criteria, each of the three chromatographic zones shown in Figure 3 contains only one kind of subunit, as marked, and these zones are well separated from one another. Area analyses of each subunit zone from four separate preparations yielded the following subunit ratios: α:γ = 1.78 ± 0.11 (SEM); α:β = 1.99 ± 0.13; and γ:β = 1.18 ± 0.10. These data indicate that HMW-NGF contains two α-, one γ-, and one β-subunits per molecule. The multiple chromatographic peaks shown within the α and β zones in Figure 3 stem from the fact that both subunits are known to contain several (and variable) internal peptide bond cleavages that give rise to multiple electrophoretic isomers (Smith et al., 1968) that can be resolved by FPLC at pH 4.0.

Zn(II) Content of HMW-NGF. Results of an earlier study (Young & Koroly, 1980) indicated that HMW-NGF contains 1 mol of Zn(II)/mol as measured by atomic absorption spectroscopy. As shown below, we have confirmed this stoichiometry by two other independent methods.

Following dithizone extraction of all buffers to remove exogenous Zn(II) ions, and dialysis of HMW-NGF against these buffers, solutions of HMW-NGF were measured spectrophotometrically for Zn(II) content by reaction with the chromophoric Zn(II) chelating reagent BAPTA (Tsien, 1980). Kinetic studies of the reaction of BAPTA with HMW-NGF disclosed a biphasic reaction scheme as follows. The first and most rapid phase was complete within the time required for mixing of reagents (10 s). The second, and slower, phase was studied kinetically, and its half-life was independent both of BAPTA and of protein concentration over a 10-fold range. Kinetic analyses of this reaction according to a first-order rate equation yielded a half-life of 1.3 min. These results are consistent with a scheme whereby BAPTA reacts with HMW-NGF to form a ternary complex, BAPTA–Zn(II)–protein, followed by first-order dissociation to yield the chelator–metal complex (1:1 stoichiometry) and the metal-free protein, according to the following reactions:

$$\text{BAPTA} + \text{Zn(II)–NGF} \xrightarrow{k_1} \text{BAPTA–Zn(II)–NGF} \quad (1)$$

$$\text{BAPTA–Zn(II)–NGF} \xrightarrow{k_2} \text{BAPTA–Zn(II)} + \text{NGF} \quad (2)$$

Similar conclusions have been reached from studies on the reaction of 1,10-phenanthroline with horse liver alcohol dehydrogenase (Vallee & Coombs, 1959) as well as from studies on the reaction of 2′,2″,2‴-terpyridine with 7S-NGF (Pattison & Dunn, 1976). From the overall change in absorbance arising from reactions 1 and 2, the Zn(II) content of four preparations of HMW-NGF was calculated. The solvent was 0.1 M Tris·HCl, pH 7.5 at 25 °C. Total [BAPTA] ranged between 3.1×10^{-5} and 1.8×10^{-4} M, with HMW-NGF concentrations in the range 6.10×10^{-6} to 7.18×10^{-6} M. Results yielded a mean value of 1.25 ± 0.03 mol of Zn(II)/mol of protein.

The Zn(II) content of HMW-NGF was also determined by isotopic exchange with ^{65}Zn(II) by equilibrium dialysis. Standard stock solutions of $ZnSO_4$ were prepared gravimetrically. Carrier-free ^{65}Zn(II) was added, and HMW-NGF (0.4 mL of 0.6–1.0 mg/mL) was dialyzed against 100 mL of a solution of 0.01 M HEPES, pH 7.0, containing 1.03×10^{-5} M Zn(II) and 2.87×10^{-7} M ^{65}Zn(II) for 24 h at 4 °C. After this time period, isotopic exchange was complete. Dialysis was then begun against large volumes of 0.01 M HEPES, pH 7.0, until equilibrium was reached, as judged by the fact that no further change in protein-bound ^{65}Zn(II) with time could be detected. Radioactive analyses of dialysate and dialysand, together with protein concentration, yielded a mean value of 1.03 ± 0.03 (n = 4) mol of bound Zn(II)/mol of HMW-NGF. This value is in agreement with the BAPTA titration results, as well as the atomic absorption spectroscopic data obtained earlier (Young & Koroly, 1980).

Discussion

In this study, several different physicochemical approaches have been used to establish the subunit and metal binding stoichiometries of HMW-NGF. All results indicate that the high molecular weight form of mouse submandibular gland NGF is composed of two α-, one β-, and one γ-subunits, together with 1 mol of Zn(II)/mol of protein (M_r = 116 000). At no time, with five different preparations of this protein, did we obtain values for the γ-subunit approaching 2 mol/mol of HMW-NGF as suggested by Server and Shooter (1977). Nor have we been able to detect values for bound Zn(II) higher than those presented above (Pattison & Dunn, 1975).

One of the requirements for accurate estimation of the above parameters is an accurate value for the molecular weight of the native oligomer. Earlier direct measurements of this value yielded M_r 116 000 ± 8000, together with an apparent partial specific volume, as defined for a three-component system (Casassa & Eisenberg, 1964), of 0.688 mL g^{-1} (Young et al., 1978). Use of this value for $\bar{V}$ has been criticized on the grounds that it differs from that calculated from the amino acid composition of 7S-NGF ($\bar{V}$ = 0.72 mL g^{-1}; Greene & Shooter, 1980). However, there is no priori reason to assume that summation of individual amino acid residue partial volumes should yield a more accurate value for $\bar{V}$ than that directly determined—particularly since equilibrium centrifugation molecular weight data correspond to a value for $\bar{V}$ in a three-component system (Casassa & Eisenberg, 1964), and the experimentally determined value can differ significantly from that calculated from composition. This issue is important since, in dilute aqueous buffer solutions, a 1% error in $\bar{V}$ will

give rise to a 3% error in molecular weight. However, even if a value for $\bar{V}$ = 0.72 mL g^{-1} is used, the molecular weight would rise to about 130 000. Incorporation of this value into the calculations of our data clearly would not significantly change the conclusions drawn. Thus, all of the above information indicates that the subunit structure empirical formula for HMW-NGF is $\alpha_2\beta\gamma$–Zn(II). Use of this stoichiometry, together with the known individual subunit molecular weights, yields a molecular weight for the parent HMW-NGF of 108 000. This value is within 7% of that determined from direct equilibrium sedimentation analyses of this protein (Young et al., 1978).

From studies on reconstitution of 7S-NGF from its three individual subunits, evidence has been presented (Silverman & Bradshaw, 1982) that a complex of subunits with $s_{20,w}$ = 6.1 S can be formed. This result has given rise to the suggestion that perhaps high molecular weight mouse salivary gland NGF actually does contain two γ-subunits per molecule but that somehow our HMW-NGF preparations have lost one of them during purification. Several lines of evidence make this idea unlikely, as follows. First, the sedimentation coefficient of our preparations is $s_{20,w}$ = 7.37 S—a value virtually identical with that reported for 7S-NGF ($s_{20,w}$ = 7.32 S; Silverman & Bradshaw, 1982). Second, with over 50 preparations of native HMW-NGF, we have never detected a species with $s_{20,w}$ = 6.1 S as reported by Silverman and Bradshaw (1982) for 7S-NGF reconstituted from its subunits. Third, the results presented in Figure 1 and Table I reveal that when HMW-NGF is treated with EDTA, the sedimentation coefficient falls from 7.37 S to 6.85 S, one γ-subunit is liberated, and this single subunit accounts for all of the enzyme activity present in the HMW-NGF preparation. In this regard, it is possible to calculate the change in sedimentation coefficient of HMW-NGF that would theoretically be expected if EDTA produced dissociation of either one or two γ-subunits per molecule. In the case of a globular protein such as HMW-NGF, if we make the reasonable assumption that dissociation of HMW-NGF produces no appreciable change in hydrodynamic shape, hydration, or $\bar{V}$, then the Scheraga–Mandelkern equation (Scheraga & Mandelkern, 1953) shows that the ratio of two sedimentation coefficients is given by the two-thirds power of the ratio of respective molecular weights. On this basis, if one γ-subunit (M_r 27 500) dissociated, the sedimentation coefficient of HMW-NGF would decrease to 6.4 S, and this value is similar to the experimental number of 6.85 S obtained for the faster species remaining after γ dissociation (Table I). However, if two γ-subunits dissociated, the sedimentation coefficient would be expected to fall to about 4.8 S—and we have never detected a species with even a similar sedimentation coefficient following treatment of HMW-NGF with EDTA.

From a recent study designed to compare certain molecular properties of HMW-NGF and 7S-NGF, Guerina et al. (1986) concluded that these two proteins are indistinguishable and that they both contain two α-, one β-, and two γ-subunits per molecule. However, this paper presents several problems that make its interpretation difficult, as follows. (1) Estimation of molecular weights of both HMW-NGF and 7S-NGF by gel filtration on molecular weight calibrated columns yielded significantly different results, depending upon the gel filtration medium used. Bio-Gel P-300 gave a mean value of 170 000, and Sephadex G-200 gave 133 000. (2) Gel electrophoretic analyses of the subunits in a dissociating solvent yielded significantly different patterns for HMW-NGF and 7S-NGF [see Figure 5 of Guerina et al. (1986)]. (3) Three different isoelectric species of the γ-subunit from HMW-NGF were detected, whereas five species were observed for 7S-NGF [see Figure 6 of Guerina et al. (1986)]. (4) Analysis of subunit stoichiometry by radioimmunoassays for the individual subunits suffers from two difficulties. First, to determine accurately by radioimmunoassay the number of any given type of subunit that comprises a multisubunit protein, it is necessary that assays be performed under conditions where that subunit is fully dissociated from the parent oligomer. Otherwise, altered immunoreactivity can lead to significant errors. Second, it is known that the α- and γ-subunits display significant immunological cross-reactivity. Anti-α reacts with the γ-subunit, and anti-γ reacts with the α-subunit (Anundi et al., 1978). This feature also makes the use of radioimmunoassays potentially unreliable for quantitative determination of individual subunit stoichiometry. (5) Finally, Guerina et al. (1986) used densitometric scanning of stained, dried polyacrylamide gels of 7S-NGF and HMW-NGF that had been run with acetic acid–urea as solvent. No evidence was presented to show that each subunit is quantitatively dissociated by this solvent. Furthermore, it is well-known that different proteins exhibit widely different staining intensities when treated with Coomassie blue, and no evidence was presented to show that known standards of each subunit could be quantitatively and accurately determined by this method.

We turn now to the question of the relationship of HMW-NGF and 7S-NGF. Present evidence indicates that crude homogenates of mouse submandibular glands contain multiple molecular species with nerve growth factor activity (Young et al., 1978). The largest of these we have called HMW-NGF. In their original paper on the purification of 7S-NGF, Varon et al. (1967) noted that during DEAE-cellulose chromatography, extremely high flow rates (350 mL/h) were required to isolate 7S-NGF. At lower flow rates, NGF (as detected by nerve growth bioassays) was spread over many fractions, and recovery was poor. These results, together with others (Smith et al., 1969; Baker, 1975; Pantazis et al., 1977), led to the idea that perhaps the presence of multiple forms of NGF indicated the operation of processes of proteolytic degradation during isolation of NGF, dissociation of the protein, or both (Pantazis et al., 1977). Such reasoning led to development of a different procedure for purification of the high molecular weight form of salivary gland NGF (Young et al., 1978). HMW-NGF differs from 7S-NGF primarily in the stability of its quaternary structure (to dissociation into subunits) even at very low protein concentrations (Pantazis et al., 1977).

However, at the time all of these studies were performed, it was not recognized that tightly bound Zn(II) plays a role in the structural stability of 7S-NGF [see, e.g., Bothwell and Shooter (1978)]. Thus, although exogenous Zn(II) was not added during purification of either 7S-NGF or HMW-NGF, the stability properties of these two preparations could reflect differences in the endogenous Zn(II) content of the buffer solutions used in their isolation. The HMW-NGF studied here is the predominant and highest molecular weight form of nerve growth factor that we have been able to detect, either in mouse submandibular gland or in saliva (Young et al., 1978; Young & Koroly, 1980; Murphy et al., 1977). Thus, HMW-NGF is probably the naturally synthesized and secreted form of this protein, and it has the structural formula $\alpha_2\beta\gamma$–Zn(II).

Registry No. NGF, 9061-61-4; Zn, 7440-66-6.

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Insulin-Induced Translocation of Glucose Transporters to the Plasma Membrane Precedes Full Stimulation of Hexose Transport†

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ABSTRACT: Insulin stimulation of hexose transport in 3T3-L1 adipocytes was studied at 27 °C. At this temperature, the transport of 2-deoxyglucose was stimulated 8-fold, with a half-time of 9.5 min. Under the same conditions, the increase in cell surface glucose transporters, as measured by labeling in the intact cell with galactose oxidase and tritiated borohydride, was only 2.6-fold. Moreover, the half-times for the increase in cell surface glucose transporters and for the decrease in transporter number in the intracellular pool were both 4 min. Thus, these processes clearly precede the full stimulation of transport. These data are in agreement with immunolocalization studies of the glucose transporter in this cell line and further support the hypothesis that a second mechanism besides translocation is involved in the stimulation of hexose transport by insulin [Blok, J., Gibbs, E. M., Lienhard, G. E., Slot, J. W., & Gueze, H. J. (1988) *J. Cell Biol. 106*, 69–76]. The findings presented here indicate that neither the translocation of glucose transporters to, nor their subsequent insertion into, the plasma membrane is the rate-limiting step in the stimulation of hexose transport by insulin. Rather, there is a second mechanism of activation, which is rate limiting and occurs after the transporter is in the plasma membrane.

Insulin treatment of fat and muscle cells leads to the translocation of glucose transporters from an intracellular site to the plasma membrane; this process results in an increase in the rate of glucose transport (Cushman & Wardzala, 1980; Suzuki & Kono, 1980; Kono et al., 1981, 1982; Karnielli et al., 1981). However, several recent studies have suggested that a second mechanism besides translocation may contribute to the stimulation of glucose transport by insulin. In 3T3-L1 adipocytes, the increase in the glucose transporter content of the plasma membrane upon insulin challenge was determined

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