Characterization of the γ Subunits of the 7S Nerve Growth Factor Complex†

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ABSTRACT: The γ subunits of the 7S nerve growth factor complex (7S NGF) display arginine esteropeptidase activity. By varying the conditions of electrophoresis in acrylamide gel, it has been demonstrated that the γ-subunit fraction of 7S NGF contains five different proteins, in contrast to the three (γ^1, γ^2, and γ^3) originally described (Smith, A. P., Varon, S. and Shooter, E. M., (1968), *Biochemistry 7*, 3259-3268); the γ^1 and γ^2 subunits, previously thought to be single species, can each be resolved into two components. The two components of the γ^1 subunit have the same isoelectric point, as do the two components of the γ^2 subunit. The distribution of protein among the two components of each of the γ^1 and γ^2 subunits varied from preparation to preparation. Moreover, a shift in this distribution for the γ^1 subunit was accompanied by a parallel shift for the γ^2 subunit. All of the different γ proteins have the same molecular weight. On the basis of the molecular weights of the peptide chains of the γ subunits and of the species which are formed by cross-linking with dimethyl suberimidate, it was concluded that both the γ^1 and γ^2 subunits contain one species with two peptide chains and another with three peptide chains, while the γ^3 subunit is a single species with three peptide chains. The results also suggest that two of the chains in the three-chain species are derived, by proteolytic cleavage, from the larger chain in the two-chain species.

One of the unusual features of the nerve growth factor (NGF[1]) protein is its association, in the 7S NGF complex, with a family of closely related arginine esteropeptidases. The 7S NGF complex, isolated from the male mouse submaxillary gland, contains three types of protein subunits, namely the α, βNGF, and γ subunits (Varon et al., 1968). The latter can be resolved into three components, the γ^1, γ^2, and γ^3 subunits (Smith et al., 1968), each of which is an arginine esteropeptidase showing a relatively high specificity for the hydrolysis of substituted arginine, rather than lysine, esters and amides (Greene et al., 1969). The specific activities of the three isolated enzymes are identical and the level of their activities is regulated by specific subunit interactions in the 7S NGF complex (Greene et al., 1968, 1969). In contrast to the free subunits, whose specific activities toward the synthetic substrates are similar to that of trypsin, 7S NGF has little or no arginine esteropeptidase activity. The three γ subunits are stable after isolation and each one recombines with appropriate amounts of the α and βNGF subunits to reform 7S NGF (Smith et al., 1968). Since 7S NGF isolated from a single lobe of the submaxillary gland also contains the three γ subunits, the appearance of the latter is not related to heterogeneity of the source tissue (Smith et al., 1968).

The role of the γ subunits of 7S NGF has yet to be determined. Enzymes of this type affect cellular growth and differentiation. The mesenchymal growth factor is an esteropeptidase (Attardi et al., 1967) and it has been demonstrated that certain submaxillary gland arginine esteropeptidases affect the growth of cultured rat hepatoma cells (Grossman et al., 1969). More recently, Greene et al. (1971) have shown that the γ subunits release confluent monolayer cultures of chick embryo fibroblasts from contact inhibition. On the other hand, it has been suggested that the γ subunits are responsible for the cleavage of a larger precursor to form the functional NGF peptide chain (Angeletti and Bradshaw, 1971; Moore et al., 1974). Another arginine esteropeptidase, epidermal growth factor (EGF[1]) binding protein, is thought to play a similar role in the production of functional EGF chains (Taylor et al., 1970; Taylor et al., 1974). Since relatively little is known about the γ subunits apart from their enzymatic and fibroblast overgrowth-stimulating properties, their further characterization at the chemical and physical-chemical level was undertaken.

Materials and Methods

Isolation of 7S NGF and its γ Subunits. The method of Varon et al. (1967) was used for the isolation of the 7S NGF complex and the method of Smith et al. (1968) for the isolation of the γ subunits from 7S NGF. The proteins were stored at concentrations of 2 to 7 mg/ml in phosphate buffer, pH 6.7, and ionic strength 0.05 at −20 °C.

Isolation of the Individual γ Subunits. The γ^1, γ^2, and γ^3 subunits in the whole γ-subunit fraction were isolated either by isoelectric focusing in acrylamide gel or by preparative isoelectric focusing. In the first procedure, 120 μg of the γ subunits were loaded onto each of eleven 10% acrylamide gels containing pH 5-8 ampholine prepared by the

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[1] The abbreviations used are: NGF, nerve growth factor; EGF, epidermal growth factor; HMW-EGF, high molecular weight epidermal growth factor; bis-tris, *N,N*-bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane; BAPA, benzoyl-DL-arginine *p*-nitroanilide hydrochloride.

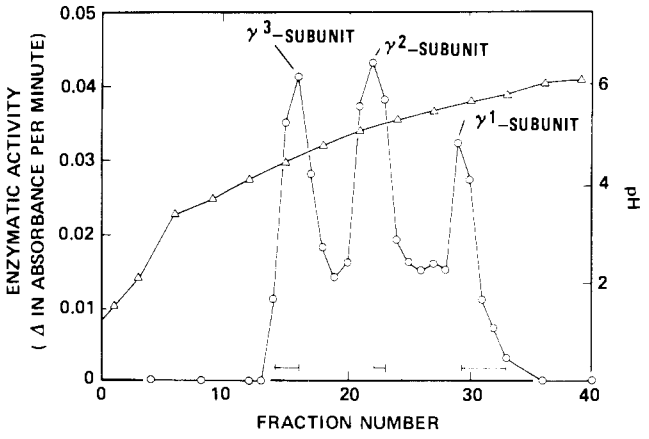

FIGURE 1: Isolation of the γ^1, γ^2, and γ^3 subunits by preparative isoelectric focusing. The procedure is described under Materials and Methods. (○) Enzymatic activity; (△) pH. The fractions were pooled as indicated (—).

method of Server and Shooter (1976). The pH gradient was established and the proteins brought close to equilibrium by applying 100 V for 1 h and 200 V for another 2 h. Following the focusing procedure, one of the gels was soaked in 20% trichloroacetic acid for 10 min and then in the stain fixative of Malik and Berrie (1972) for 15 min to locate the protein zones. Using this as a guide, the other ten gels were sliced up so as to separate the sections of acrylamide which contained the different γ subunits. Slices which contained the same subunit were combined, homogenized gently, and soaked in 2 ml of phosphate buffer, pH 6.7, and ionic strength 0.05 for 30 h at 4 °C. After centrifugation (12 000*g*, 15 min), each supernatant was dialyzed, first against phosphate buffer, pH 6.7 and ionic strength 0.05 containing 0.5 M NaCl to remove ampholine, then against phosphate buffer, pH 6.7 and ionic strength 0.003, lyophilized, and redissolved in 150 μl of deionized water. The yield was 220 μg of the γ^1 subunit, 170 μg of the γ^2 subunit, and 200 μg of the γ^3 subunit.

The preparative isoelectric focusing procedure used a sucrose gradient (0.13–1.3 M) containing 1% ampholine, pH 5–8 in a column of 50-ml capacity. The γ subunits (8.8 mg) were loaded with the light sucrose solution. Isoelectric focusing proceeded for 1 h at 200 V, 4 h at 400 V, and 18 h at 700 V. At the conclusion of focusing, the column outlet was opened and the eluate was collected in fractions of 0.4 ml. The pH of each fraction was determined and the fractions were then assayed for arginine esteropeptidase activity using 1 mM BAPA[1] in 0.05 M Tris-Cl buffer, pH 8.1. In this assay 5 μl of each fraction was added to 500 μl of the substrate and the change in absorbance at 410 nm recorded. Appropriate pools of the fractions (Figure 1) were dialyzed, first against 0.2% acetic acid containing 0.5 M NaCl and then against 0.2% acetic acid, after which the samples were concentrated. The yield was 465 μg of the γ^1 subunit, 510 μg of the γ^2 subunit, and 710 μg of the γ^3 subunit. The γ^1 and γ^2 fractions were not as pure after isolation by this procedure as by the first procedure. In addition, precipitation of protein occurred in a number of the preparative isoelectric focusing experiments preventing adequate separation of the subunits.

Other Proteins and Chemicals. The protein standards for the molecular weight determinations were ovalbumin (Schwarz/Mann), pepsin (Pierce Chemical), lysozyme, myoglobin, cytochrome *c*, and insulin (Sigma Chemical Co.). Ampholine (pH 3.5–10 and pH 5–8) was from LKB-Produktor AB, bis-tris[1] from General Biochemicals, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid from Calbiochem, BAPA from Nutritional Biochemicals Corporation, and dithioerythritol from Cyclo Chemical Corp.

Electrophoresis Using a bis-tris-N-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic Acid System and Isoelectric Focusing. Electrophoresis in 12% acrylamide gels used a bis-tris-*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid system with a resolving pH of 7.05 (Server and Shooter, 1976). Gels were stained with 1% acid fast green in 10% acetic acid and destained in the same solvent. The procedure for the isoelectric focusing in a pH 3.5–10 gradient in 7.5 and 10% acrylamide gels has also been described (Server and Shooter, 1976). Gels were fixed and washed with several changes of 20% trichloroacetic acid and scanned at 280 nm in a Gilford gel scanning attachment to a Beckman DU spectrophotometer. They were then stained with 0.1% napthol blue black in 10% acetic acid and destained in the same solvent.

Sedimentation Equilibrium. Molecular weights were measured with a Beckman Model E ultracentrifuge equipped with interference optics, by the meniscus depletion method of Yphantis (1964). Details of the specific procedures and checking of the optical system by measurement of the molecular weights of standard proteins have been described (Pignatti et al., 1975). Protein solutions were 0.4 to 0.5 mg/ml in concentration and were dialyzed against the appropriate buffer of 0.1 M concentration containing 0.1 M NaCl. The partial specific volume of the γ subunits (0.730 ml g^{-1}) was determined from their amino acid composition (Cohn and Edsall, 1943; McMeekin and Marshall, 1952).

Cross-Linking with Dimethyl Suberimidate. The γ subunits were cross-linked by the method of Davies and Stark (1970). Protein (20 μg) in solution was mixed with an equal volume of 0.4 M borate buffer, pH 8.5, and sufficient 0.2 M borate buffer, pH 8.5, was added to bring protein concentration to 1 mg/ml. Dimethyl suberimidate solution (8 mg of dimethyl suberimidate·2HCl in 940 μl of 0.2 M sodium borate buffer, pH 8.5, neutralized with 60 μl of N NaOH) was added in equal volume to the protein solution and the mixture was incubated at 25 °C for 3 h. Following the incubation period, the cross-linked protein was reduced and carboxamidomethylated as described below. Insulin, cytochrome *c*, myoglobin, and lysozyme were cross-linked by the method of Carpenter and Harrington (1972) as described by Stach and Shooter (1974).

Reduction and Carboxamidomethylation of the γ Subunits for Electrophoresis in the Presence of Sodium Dodecyl Sulfate. Cross-linked protein (20 μg) in 40 μl of the reaction mixture or native protein (18 to 35 μg) in 40 μl of 0.18 to 0.2 M borate buffer, pH 8.5, was mixed with 8 μl of a 2-mercaptoethanol solution (1.5 ml per 10 ml) and 12 μl of a sodium dodecyl sulfate solution (5 g per 100 ml) and incubated for 16 h at 25 °C. Excess iodoacetamide (15 μl of 150 mg/ml solution) was then added to the protein mixture. The latter was allowed to incubate for at least 15 min at 25 °C and was then made 10% in sucrose and 0.005% in bromphenol blue prior to electrophoresis in acrylamide gels in the presence of sodium dodecyl sulfate.

Molecular Weight Determination by Electrophoresis in the Presence of Sodium Dodecyl Sulfate. The method of Weber and Osborn (1969) was used for the electrophoretic analyses in 12% acrylamide gels in the presence of 0.1% so-

dium dodecyl sulfate. The gels were stained with Coomassie brilliant blue (Weber and Osborn, 1969) and scanned at 580 nm. R_f values were determined by the procedure given by Stach and Shooter (1974). Linear calibration plots of migration distance vs. log molecular weight were obtained with cytochrome *c*, pepsin, and ovalbumin after reduction and carboxamidomethylation or with insulin, cytochrome *c*, myoglobin, and lysozyme after cross-linking with dimethyl suberimidate followed by reduction and carboxamidomethylation. The molecular weights of the carboxamidomethylated chains and cross-linked species from the γ subunits were calculated from the regression equation for the appropriate standard curve.

Amino Acid Analyses. These were carried out on a Beckman 116C amino acid analyzer. Proteins were hydrolyzed with 6 N HCl in vacuo (Moore and Stein, 1963). Tryptophan was determined after hydrolysis with *p*-toluenesulfonic acid (Liu and Chang, 1971). The number of half-cystine residues was determined with hydrolysates of reduced and carboxamidomethylated samples. The method of reduction and carboxamidomethylation of proteins for amino acid analyses differed somewhat from the one described above. γ subunits (2.0 mg) in 1.0 ml of 0.05 M Tris-Cl buffer, pH 7.4, were mixed with 1.0 ml of 1.0 M ammonium bicarbonate buffer, pH 8.6, 1.6 g of urea, and 20 μl of a 100 mg/ml of dithioerythritol solution (Zahler and Cleland, 1968). Reduction proceeded for 2 h at 25 °C after which 200 μl of a 30 mg/ml iodoacetamide solution was added. The mixture was allowed to incubate for another 15 min at 25 °C and the reaction was then stopped with the addition of 20 μl of a 100 mg/ml dithioerythritol solution. Excess reagent and buffer were removed by dialysis, although for several preparations lyophilization was substituted for dialysis.

Sulfhydryl Group Determination. The number of free sulfhydryl groups in the protein was determined by reacting 1.5 mg of the γ subunits in 4 ml of 1.0 M Tris-Cl buffer, pH 8.6, 8 M in urea, with 100 mg of iodoacetamide for 30 min at 25 °C. The sample was then dialyzed against distilled water, hydrolyzed and, analyzed for carboxamidomethylcysteine.

Results

The Number of Proteins in the γ Subunit Fraction of 7S NGF. The three major species in the γ-subunit fraction of 7S NGF are readily resolved by electrophoresis in a number of different buffer systems (Smith et al., 1968) or by isoelectric focusing (Figures 1 and 2A,D). In the latter system, the γ^3 subunit, having the lowest isoelectric point (Varon and Shooter, 1970), appeared nearest to the anode and the γ^1 subunit nearest to the cathode. A trace amount of a component, γ′, which appears in increasing amount when the γ subunits are stored at 4 °C (Varon and Shooter, 1970), was visible between the γ^3 and γ^2 subunits. It has now been observed that the γ^1 and γ^2 subunits, which migrate more slowly on electrophoresis than the γ^3 subunit, each resolved into two components when analyzed in the bis-tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid system at pH 7.05 in 12% acrylamide gels (Figure 2B,C). The distribution of protein among the two components of each of the γ^1 and γ^2 subunits varied from preparation to preparation (Figure 2B,C). Moreover, a shift in this distribution for the γ^1 subunit was accompanied by a parallel shift for the γ^2 subunit (Figure 2B,C). Whatever factor was responsible for this shift apparently affected the γ^1 and γ^2 subunits equally. The γ subunits therefore contain five protein species and not three as originally reported (Varon et al., 1968).

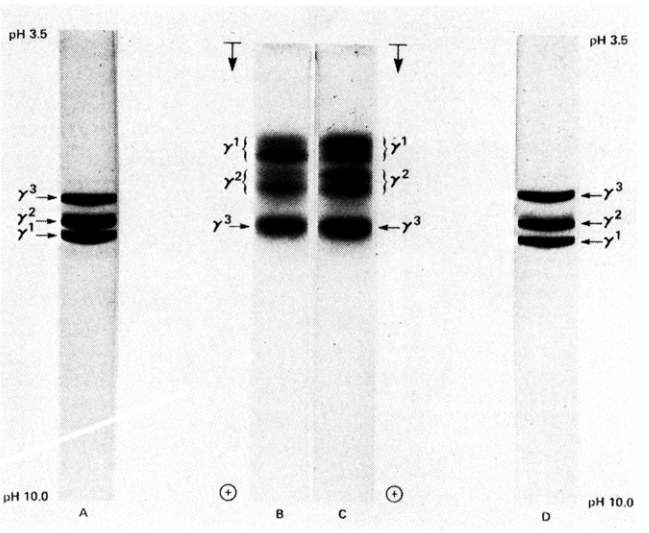

FIGURE 2: Isoelectric focusing and electrophoretic analyses of the whole γ-subunit fraction. Isoelectric focusing was in a pH 3.5–10 gradient in 7.5% acrylamide gels (A, D). Electrophoresis was in 12% acrylamide gels (B, C) in a bis-tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid system, pH 7.05. Details of the procedures are given under Materials and Methods. Two different preparations of the γ subunits were used in these analyses. The γ subunits from one preparation appear on gels A and B, while those from the other preparation appear on gels C and D: (A and D) 100 μg of the whole γ subunit fraction; (B and C) 130 μg of the whole γ-subunit fraction.

The allocation of the pairs of components to the γ^1 and γ^2 subunits, respectively, was based on the analyses of the isolated γ subunits. The three γ subunits separated by isoelectric focusing in acrylamide gel showed only slight cross-contamination (Figure 3A–D). The subsequent electrophoretic analyses showed that both γ^1 and γ^2 subunits contained two species while the γ^3 subunit did not (Figure 3E–H). Moreover, the proportions of the two components in the γ^1 and γ^2 subunits were close to those observed in the original γ-subunit preparation (Figure 3E–G).

Amino Acid Analyses. The amino acid composition of the whole γ-subunit fraction is shown in Table I. Since no free sulfhydryl groups were detected, the data suggest that the γ subunits have six disulfide bridges linking the various chains. Preliminary evidence suggests that the amino acid compositions of the individual γ subunits differ only slightly from that of the whole γ-subunit fraction.

Molecular Weight Determination by Sedimentation Equilibrium. The molecular weight of the γ subunits in acetate buffer at pH 5.4 or in phosphate buffer at pH 6.4 or pH 6.5 was determined by sedimentation equilibrium. The plots of the log of fringe displacement vs. the square of the radial distance were linear at each pH and molecular weights of 25 700, 26 500 and 25 600, respectively, were obtained.

Number and Molecular Weights of the Peptide Chains in the γ Subunits. After reduction and carboxamidomethylation, both γ^1 and γ^2 subunits showed chains of four different sizes on electrophoretic analysis in the presence of sodium dodecyl sulfate (Figure 4A,B). These chains have been classified on the basis of their size as types 1, 2, 3, and 4, and their molecular weights are given in Table II. (The identification of the chains of the different γ subunits by the same numbers is used here as a convenience to stress

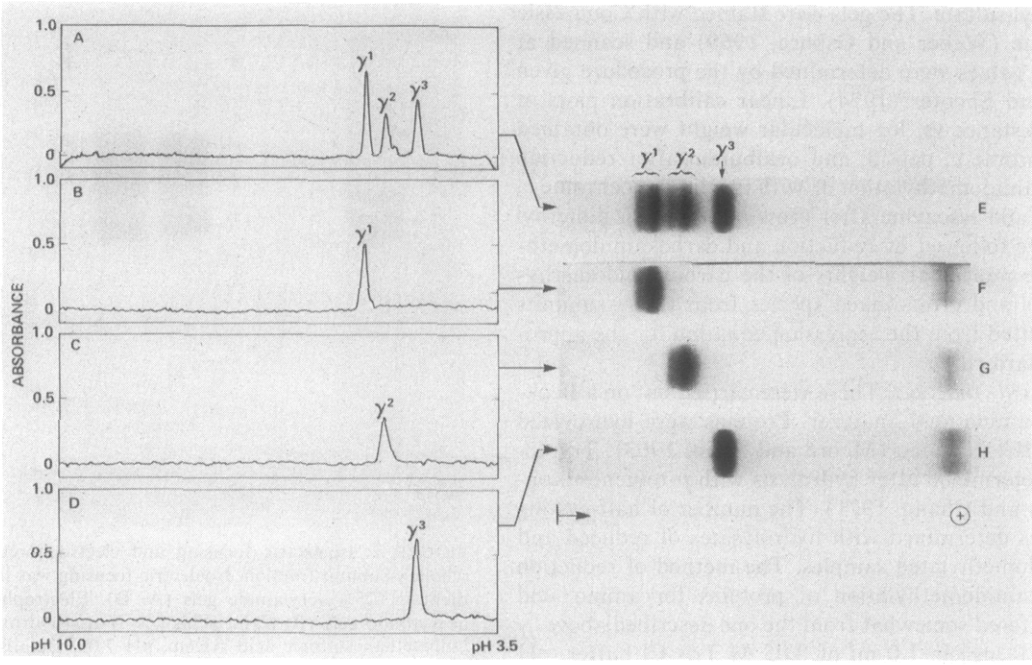

FIGURE 3: Isoelectric focusing and electrophoretic analyses of the individual γ subunits. The γ^1, γ^2, and γ^3 subunits were isolated by isoelectric focusing in acrylamide gels as described under Materials and Methods. The purity of each subunit was determined by isoelectric focusing in a pH 3.5–10 gradient in 10% acrylamide gels. Scans of the gels are presented (B, C, D). A sample of the original whole γ-subunit fraction was also analyzed by the same procedure and the scan (A) presented for comparison: (A) 120 μg of the whole γ subunit; (B) 40 μg of the γ^1 subunit; (C) 30 μg of the γ^2 subunit; (D) 40 μg of the γ^3 subunit. The individual subunits as well as the original γ-subunit fraction were then analyzed by electrophoresis in 12% acrylamide gel (E–H) in a bis-tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid system, pH 7.05, as described under Materials and Methods; (E) 130 μg of the whole γ-subunit fraction; (F) 35 μg of the γ^1-subunit; (G) 26 μg of the γ^2 subunit; (H) 35 μg of the γ^3 subunit. The diffuse band near the anodic end of gels F, G, and H is most likely due to residual ampholine which had been introduced during the isolation of the individual γ subunits and had not been removed during dialysis.

Table I: Amino Acid Analysis of the Whole γ-Subunit Fraction.

Amino Acid	γ-Subunits, residues per 25 900 Daltons[a]
Lysine	18.2
Histidine	5.7
Arginine	3.5
Aspartic acid	31.7
Threonine	17.7
Serine	13.3
Glutamic acid	15.8
Proline	17.4
Glycine	21.8
Alanine	13.3
Half-cystine[b]	11.1
Valine	9.1
Methionine	6.7
Isoleucine	7.4
Leucine	22.3
Tyrosine	8.5
Phenylalanine	8.1
Tryptophan[c]	5.7

[a] Averaged values based on three different analyses. [b] Half-cystine residues were determined as carboxamidomethylcysteine. [c] Values determined after hydrolysis with *p*-toluenesulfonic acid.

similarities of chain composition, but is not intended to denote identity.) In the analysis of the γ^3 subunit, very little of chain 4 was observed (Figure 4C, Table II) and this amount may have resulted from slight contamination by the γ^1 and γ^2 subunits. Moreover, in one preparation of the γ subunits, the γ^1 subunit contained only chains 3 and 4 (data not shown). These observations, taken together, suggest two ways in which the peptide chains can be combined to produce species of the molecular weight of the intact γ subunits, namely by combining chains of types 1, 2, and 3 or by combining chains of types 3 and 4. Although the combined molecular weight of chains of types 2 and 4 also approximates that of an intact γ subunit, such a combination is not possible for the γ^3 subunit at least. On the basis of the above interpretation, then, the γ^1 and γ^2 subunits whose analyses are shown in Figure 4 would each contain two species, one derived from chains 3 and 4 and the other derived from chains 1, 2, and 3, while the γ^3 subunit would be a single species of chains 1, 2, and 3. The finding that the γ^1 and γ^2 subunits, but not the γ^3 subunit, each show two species on disc electrophoresis in the bis-tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid system (Figure 3F–H) supports this interpretation. As expected, the electrophoretic pattern of the whole γ-subunit fraction (Figure 4D), following its reduction and carboxamidomethylation, appears as the sum of the patterns of the individual subunits.

The fact that chains 1 and 2 are not present in every preparation of the γ^1 subunit suggests that, under certain conditions, these peptide chains are derived from one of the larger chains of the γ^1 subunit. On the basis of molecular weight, a reasonable hypothesis is that chain 1 (molecular weight 6600) and chain 2 (molecular weight 8900) are derived by cleavage of chain 4 (molecular weight 15 000); accordingly, for the γ^1 subunit and possibly the γ^2 subunit, the species with chains 1, 2, and 3 would be a proteolytic product of the species with chains 3 and 4. Variations in the distribution of protein among the two peaks of each of the γ^1 and γ^2 subunits on disc electrophoresis in the bis-tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid system (Figure 2) may reflect differences in the extent of

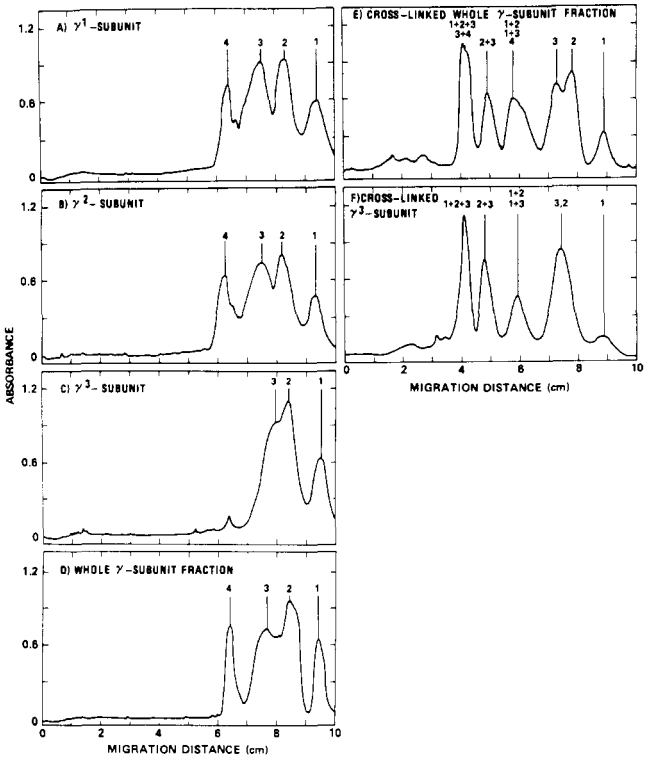

FIGURE 4: Analysis of the reduced and carboxamidomethylated chains and the cross-linked, reduced, and carboxamidomethylated species of the γ subunits. Analyses were carried out by electrophoresis in the presence of 0.1% sodium dodecyl sulfate in 12% acrylamide gels as described in Materials and Methods. Migration was from left to right. Protein samples (18 to 35 μg) were reduced and carboxamidomethylated or cross-linked with dimethyl suberimidate and then reduced and carboxamidomethylated as described under Materials and Methods. The patterns on the left (A-D) are of samples which had not been cross-linked and those on the right (E, F) are of the cross-linked samples: (A) the γ^1 subunit; (B) the γ^2 subunit; (C) the γ^3 subunit; (D) the whole γ-subunit fraction; (E) the whole γ-subunit fraction after cross-linking; (F) the γ^3-subunit after cross-linking. The numbers above the peaks indicate either the chain numbers or the compositions of the cross-linked species. Molecular weights are given in Tables II and III.

Table II: Molecular Weights of Reduced and Carboxamidomethylated Chains of the γ Subunits Determined by Electrophoresis in the Presence of Sodium Dodecyl Sulfate.

	Chains[a]			
Protein Sample	4	3	2	1
γ subunits	16 400	11 400	9400	6800
γ^1 subunit[b]	15 000	11 000	8900	6600
γ^2 subunit	16 300	11 600	9700	6800
γ^3 subunit[c]		1200-9500[d]		6900

[a] Reduced and carboxamidomethylated protein standards were used for molecular weight determinations. [b] The molecular weight values for the chains of the γ^1 subunit are based on a single experiment. All other values provided in the table are the average of results from several experiments. When analyzed in the same experiment, the peptide chains of the different γ subunits migrate similarly (Figure 4). [c] Little of chain 4 was found in the analyses of the γ^3 subunit, and what did appear may have resulted from slight contamination by γ^1 and γ^2 subunits. [d] The peptide chains were insufficiently resolved to permit an accurate estimation of their molecular weights. The values presented define the range of molecular weights for these chains.

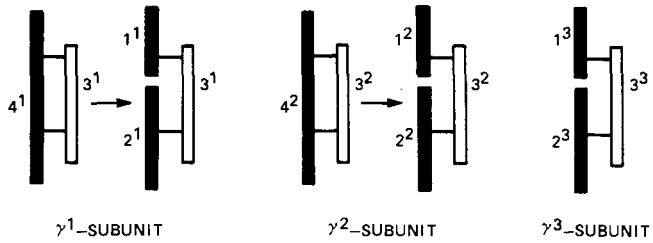

FIGURE 5: Model of the chain compositions of the five proteins in the whole γ-subunit fraction. The γ^1 subunit contains two species, one derived from chains 3^1 and 4^1, and the other derived from chains 1^1, 2^1 and 3^1. The γ^2 subunit also contains two species, one derived from chains 3^2 and 4^2 and the other derived from chains 1^2, 2^2, and 3^2. The γ^3 subunit is a single species of chains 1^3, 2^3, and 3^3. The superscripts identify the different chains on the basis of the isoelectric points of their parent γ molecules. The cross bars indicate that the chains are held together by disulfide bridges. According to the model, the species containing three peptide chains are derived from the species with two chains as indicated by the arrows. Presumably a proteolytic cleavage event (chain 4 → chains 1 and 2) has occurred within the γ^1 and γ^2 subunits and has gone to completion in the γ^3 subunit.

proteolytic cleavage from preparation to preparation.

In the above discussion, the peptide chains have been defined only in terms of their size. Because the three γ subunits have different isoelectric points, some, or perhaps all, of these chains must differ from subunit to subunit in amino acid composition and consequently net charge. The actual chain composition of the subunits can therefore be expressed in the most general terms as $(3^1 + 4^1)$ and $(1^1 + 2^1 + 3^1)$ for the γ^1 subunit, $(3^2 + 4^2)$ and $(1^2 + 2^2 + 3^2)$ for the γ^2 subunit, and $(1^3 + 2^3 + 3^3)$ for the γ^3 subunit, where the superscripts identify the different chains on the basis of the isoelectric points of their parent γ molecules. A model based on the data reported in this section is presented in Figure 5.

Chain Composition and Molecular Weights of the γ Subunits as Determined by Cross-Linking. The results from the cross-linking experiments with dimethyl suberimidate are consistent with the interpretations presented above. The analysis of the cross-linked whole γ-subunit fraction (Figure 4E, Table III) showed the presence of the unreacted chains 1 (molecular weight 6200), 2 (molecular weight 8700), and 3 (molecular weight 10 200). In addition, at least three other bands were observed which resulted from the cross-linking reaction. The slowest migrating band corresponded to a molecular weight of 26 600 and could have resulted from the cross-linking of chains 3 and 4, and the cross-linking of chains 1, 2, and 3. On the basis of their molecular weight, it appears that the species within this slowest migrating band were intact γ-subunit molecules whose peptide chains had been cross-linked to one another. The next band, in order of increasing rate of migration, corresponded to a molecular weight of 21 000 and most likely resulted from the cross-linking of chains 2 and 3. Although the cross-linking of chains 1 and 4 would produce a species with a similar molecular weight, this combination would be unlikely, if, as argued here, these chains occur in separate γ-subunit molecules. Moreover, it was noted that a species of molecular weight 21 300 was formed, in about the same yield as in the example above, when the γ^3 subunit, which contains very little of chain 4, was reacted with dimethyl suberimidate (Figure 4F, Table III). The third band in order of increasing rate of migration was heterogeneous comprising species of molecular weight 14 700 to 15 800. Such heterogeneity would result from the presence of the original chain 4, and from the cross-linking of chains 1 and 2 and

Table III: Molecular Weights of the Chains and Cross-Linked Species of the γ Subunits Determined by Electrophoresis in the Presence of Sodium Dodecyl Sulfate after Cross-Linking with Dimethyl Suberimidate.

Protein Samples	Chains and Cross-Linked Species[a]					
	1 + 2 + 3 3 + 4[b]	2 + 3	1 + 3 1 + 2 4[c]	3	2	1
γ subunits[d]	26 600	21 000	15 800– 14 700[e]	10 200	8700	6200
γ^3 subunits[f]	26 300	21 300	15 600[g]	10 000[g]		6500

[a] Cross-linked, reduced, and carboxamidomethylated protein standards were used for molecular weight determinations. [b] A species derived from those γ subunits (γ^1 and γ^2) which contain chain 4. [c] Present in appreciable amounts in γ^1 and γ^2 only. [d] The values provided are averages based on many experiments. [e] The peptide chain and the two cross-linked species were insufficiently resolved to permit an accurate estimation of their molecular weights. The values presented define the range of molecular weights for the chain and these cross-linked species. [f] The values provided are based on a single experiment. [g] In this analysis, chains 2 and 3 migrated together giving a single, symmetrical peak as did the cross-linked species 1 + 2 and 1 + 3. For each of these peaks, only a single molecular weight value was determined.

the cross-linking of chains 1 and 3. The analysis of the cross-linked γ^3 subunit (Figure 4F, Table III) indicated that these last two cross-linked species were, very likely, formed since a sizeable band corresponding to molecular weight 15 600 was observed in the absence of significant amounts of chain 4. While the amount of the isolated γ^1 subunit was insufficient for cross-linking, analysis of the γ^2 subunit gave results similar to those for the whole γ subunit fraction (data not shown). In summary, the results of the cross-linking experiments can be readily interpreted using the model proposed above on the chain compositions of the various γ species.

Discussion

By varying the conditions of electrophoresis in acrylamide gel, it has been shown that the γ-subunit fraction of 7S NGF contains five different proteins; the γ^1 and γ^2 subunits, previously thought to be single species can each be resolved into two components. In spite of this extra degree of complexity, it is clear that all five proteins have many properties in common. One of these is molecular weight since the sedimentation analysis of the whole γ-subunit fraction showed no evidence of heterogeneity. This has some bearing on attempts to explain the higher resolving power of the present electrophoretic system compared with those of earlier systems. That the extra resolution is achieved because of major differences in size is unlikely, not only because of the demonstrated similarity of molecular weights but also because the same separation was observed at pH 7.0 in acrylamide gels of different pore size. However, the isoelectric points of the two components of the γ^1 subunit are identical (pI = 5.8), as are those of the two components of the γ^2 subunit (pI = 5.6) (Varon and Shooter, 1970). Furthermore, at pH 7.55 in another bis-tris–*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid electrophoretic system, no resolution of the two components in either the γ^1 or the γ^2 subunit was seen (Smith et al., 1968), and they must, therefore, have the same charge-to-mass ratio under these conditions. Thus, the charge differences, which are presumably responsible for the separation of the two components in each of the γ^1 and γ^2 subunits, exist only over a very restricted range of pH. It is unclear how these charge differences arise unless they involve histidine or α-amino residues and their identification, therefore, awaits further knowledge of the primary structure of these proteins.

A model has been presented which predicts the chain compositions of the various γ species and accounts for the five bands observed on electrophoresis of the whole γ-subunit fraction (Figure 5). In summary, the γ^1 and γ^2 subunits each contain two species, one derived from chains 3 and 4 and the other derived from chains 1, 2, and 3, while the γ^3 subunit is a single species of chains 1, 2, and 3. The model also predicts that the species containing three peptide chains are derived from the species with two chains. Presumably a proteolytic cleavage event has occurred within the γ^1 and γ^2 subunits and has gone to completion in the γ^3 subunit. If this interpretation is correct, then the cleavage of chain 4 should produce equal mole quantities of chains 1 and 2 and the proportions of the latter should increase as that of chain 4 decreases. To a rough approximation, these predictions hold for the analyses of the three individual γ subunits (Figure 4A–C), although there is neither sufficient variation in the relative amounts of the various chains in the subunits, nor sufficient resolution of chains 2 and 3 in the γ^3 subunit to allow for more than a qualitative comparison. Substantiation of the model depends on developing methods for separating the five γ species and on at least partial sequence information. It should be noted that this model does not encompass proteolytic events which may be responsible for the differences between the γ^1, γ^2, and γ^3 subunits. Information on the primary sequence of the γ species should also help explain these differences.

The molecular weight data presented here do not agree with those recently reported by Baker (1975). The latter found that protein aggregation occurred in his γ-subunit preparation and estimated a minimal molecular weight of 18 000. Little or no aggregation was noted in the current sedimentation experiments and the molecular weight determined by the equilibrium method (26 000) is in excellent agreement with that obtained in the cross-linking experiments (26 600). Moreover, it is unlikely that the γ subunits can have a molecular weight significantly different from that of either βNGF or the α-subunits since all these proteins have the same sedimentation coefficient (Varon et al., 1968). The molecular weight of βNGF is 26 500 (Angeletti and Bradshaw, 1971; Pignatti et al., 1975) and of the α-subunit, 26 500.

It is of interest that the arginine esteropeptidase subunit in the high molecular weight epidermal growth factor (HMW-EGF) complex, namely the EGF-binding protein (Taylor et al., 1970; Taylor et al., 1974), contains only one major enzyme species. The EGF-binding protein has a chain composition similar to that of the γ^3 subunit in that it contains chains of types 1, 2, and 3 (Server and Shooter, 1976). However, the finding that the EGF-binding protein will not replace the γ subunits in 7S NGF demonstrates that these structurally similar arginine esteropeptidases differ in their binding domains and that subunit interactions in the 7S NGF complex are specific (Server and Shooter, 1976). Such specificity is consistent with the hypothesis that the γ subunits and the EGF-binding protein are involved in the processing of precursors of the growth factor chains with which these enzymes are associated (Taylor et

al., 1970; Angeletti and Bradshaw, 1971). The precursor model would not, however, predict the observed heterogeneity of the γ-subunit fraction from 7S NGF; the significance of there being five species of the γ enzyme remains to be determined.

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