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Reaction of the Basic Trypsin Inhibitor from Bovine Pancreas with the Chelator-Activated 7S Nerve Growth Factor Esteropeptidase†

Alice M.-J. Au‡ and Michael F. Dunn*

ABSTRACT: The native 140 000 molecular weight nerve growth factor protein from the mouse submaxillary gland (7S NGF_n) is a multisubunit zinc metalloprotein which regulates the differentiation of sensory and sympathetic ganglia in vivo. The 7S NGF_n oligomer contains a masked trypsin-like proteolytic activity which is activated by the sequestering and removal of the 7S NGF_n-bound zinc ions by divalent metal-ion chelators. The proteolytic activity of the oligomer is associated with the γ subunit, while growth activity resides with the β subunit. In this study, the susceptibility of the proteolytic activity to inhibition by seven protein protease inhibitors, the basic trypsin inhibitor from bovine pancreas (PTI), soybean trypsin inhibitor, lima bean trypsin inhibitor, ovomucoid, human α_1-anti-trypsin, human antithrombin III, and human C-1 esterase inhibitor, has been investigated. Of these inhibitors, only PTI is an inhibitor for the proteolytic activity. By the use of sucrose density gradient sedimentation, isoelectric focusing gel electrophoresis, gel filtration, equilibrium sedimentation, and protease activity studies we have established that PTI does not react with 7S NGF_n; however, PTI undergoes rapid, stoichiometric reactions with both the EDTA-activated 7S NGF species (7S NGF_a) and with the isolated γ subunit. Reaction of PTI with 7S NGF_a results in the inhibition of the proteolytic activity and the dissociation of the 7S oligomer to a mixture of the α and β subunits and the γ subunit–PTI complex. In contrast to the reaction of NGF_a with PTI, the reaction of a low-molecular-weight substrate, α-*N*-benzoyl-L-argininamide, does not alter the state of aggregation of the 7S oligomer.

The 140 000 molecular weight nerve growth factor protein (7S NGF)[1] from the mouse submaxillary gland is a zinc metalloprotein (Pattison and Dunn, 1975, 1976a,b) composed of three types of subunits (designated α, β, and γ), each of molecular weight 20 000 to 30 000 (Varon et al., 1967a,b, 1968; Baker, 1975a,b). This protein is involved in the regulation of growth and differentiation for the sensory and sympathetic chains (Levi-Montalcini, 1965; Schenkein, 1972; Varon et al., 1967a,b). The 7S NGF subunits carry separate biological activities; neuronal outgrowth activity is associated with the β subunit, a potent trypsin-like protease activity is associated with the γ subunit, and the α subunit has been reported to enhance the survival of sensory neurons under conditions of mechanical stress (Varon et al., 1967a; Greene et al., 1968, 1969; Varon and Raiburn, 1972).

Previous work from this laboratory (Pattison and Dunn, 1975, 1976a,b) has shown that the reaction of divalent metal-ion chelators with native 7S NGF (hereafter designated

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[1] Abbreviations used are: NGF, nerve growth factor; PTI, pancreatic trypsin inhibitor; IAI, β-*trans*-indolacryloylimidazole; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, (ethylenedinitrilo)tetraacetic acid; BAPNA, α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide; BAA, α-*N*-benzoyl-L-argininamide; BU, biological unit (Levi-Montalcini and Hamburger, 1953).

7S NGF_n) activates the proteolytic activity of the 7S NGF-bound γ subunits at least sevenfold. The activation process involves the sequestering and removal of the tightly bound 7S NGF zinc ions ($K_D \simeq 10^{-11}$ M) by the chelator. In this work, we have investigated the reaction of activated 7S NGF (hereafter designated 7S NGF_a) with the Kunitz pancreatic trypsin inhibitor (PTI). Greene (1969) has reported that PTI is a potent inhibitor of both the proteolytic activity of the γ subunit and of the residual activity associated with 7S NGF_n. As will be shown, PTI reacts rapidly and specifically with both 7S NGF_a and the free γ subunit, but PTI does not react with 7S NGF_n. The effects of the reaction of a small artificial substrate (α-*N*-benzoyl-L-argininamide) and the effects of the reaction of PTI on the integrity of the 7S NGF_a oligomer are also described.

Experimental Section

Materials. The γ subunit and 7S NGF_n were isolated according to the procedures of Smith et al. (1968) and Varon et al. (1967a), respectively. The 7S NGF_n exhibited an in vitro growth-promoting activity of ~1 BU, in good agreement with previous reports (Varon et al., 1967a; Smith et al., 1968) when assayed in the organ culture procedure of Levi-Montalcini and Hamburger (1953). The 7S NGF_n preparation was further purified by gel filtration over Bio-Gel P-150 to remove lower molecular weight species for the sedimentation equilibrium studies (see below, and Results). The basic pancreatic trypsin inhibitor (Kunitz), soybean trypsin inhibitor, lima bean trypsin inhibitor, and ovomucoid were purchased from Sigma. α_1-Antitrypsin, antithrombin III, and C-1 esterase inhibitor from human plasma were provided by the National Fractionation Center of the American Red Cross.[2] Rabbit muscle phosphoglucose isomerase was kindly provided by Professor E. A. Noltmann. The sucrose used in the density gradient centrifugation studies was purchased from Schwarz/Mann. All other reagents and materials were purchased from commonly available sources.

Methods. Protease activities were determined at 25 ± 0.2 °C according to the procedure of Pattison and Dunn (1975). PTI titrations were carried out as follows: *β-trans*-indolacryloylimidazole (IAI) reacts stoichiometrically at the active site of α-chymotrypsin, forming the relatively stable indolacryloyl-acyl-enzyme (IA-enzyme) (Bernhard et al., 1965). The difference spectrum of IAI vs. IA-enzyme exhibits a maximum at 390 nm. By monitoring the change in absorbance at this wavelength a titration of the catalytically functional enzyme is achieved, and the titration end point corresponds to the normality of the α-chymotrypsin solution. An α-chymotrypsin stock solution standardized via this procedure was then used to determine the normality of a PTI stock solution by the following procedure: 1 mL of standardized α-chymotrypsin was added to both the reference and sample cuvettes. An aliquot of the unknown PTI solution was added to the sample cuvette and an equal volume of buffer was added to the reference cuvette. After incubation for 1 h at 25 °C, IAI was added to both cuvettes and the OD_{390} was recorded immediately. These last two steps were repeated with increasing concentrations of PTI using fresh α-chymotrypsin aliquots until the titration end point, as indicated by no further optical density change at 390 nm, was reached. The standardized PTI solution then was used in the titrations of the γ subunit and 7S NGF_a as described in the caption to Figure 1.

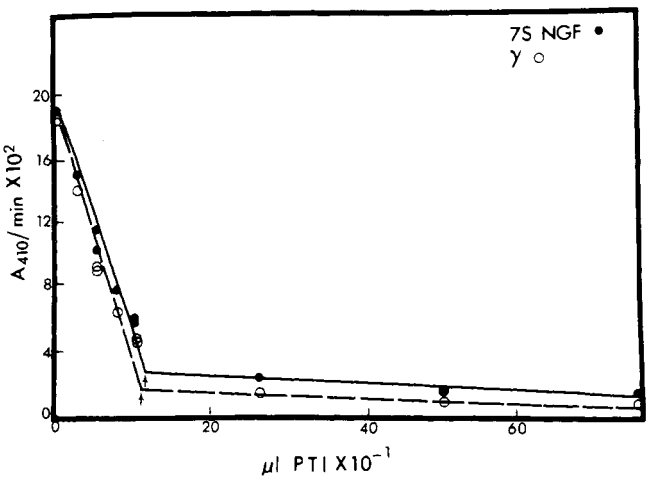

FIGURE 1: Titration of the proteolytic activities of 7S NGF_a (●) and γ (○) by reaction with PTI. Solutions of 7S NGF_a and γ were adjusted by dilution to give nearly identical protease activities (measured as the rate of BAPNA hydrolysis, A_{410}/min). Then microliter amounts of PTI were added to 1-mL aliquots of each solution and incubated for 1 h before measuring the remaining proteolytic activity via the rate of BAPNA hydrolysis. Concentrations: PTI stock solution, 5.90 μM (see Methods); γ, ~0.70 μM; 7S NGF_a, ~0.3 μM.

Sedimentation in 5 to 20% linear sucrose gradients was carried out at 5 °C using the procedure of Martin and Ames (1961) in 4.4-mL capacity polyallomer tubes. The following proteins were run in parallel experiments with the 7S NGF samples to calibrate the gradients: PTI (1.0 S), bovine ribonuclease A (2.0 S), hen egg white lysozyme (1.9 S), myoglobin (2.0 S), bovine α-chymotrypsin (2.4 S), bovine trypsin (2.5 S), bovine trypsin-PTI complex (2.9 S), hemoglobin (4.4 S), bovine carbonic anhydrase (3.0 S), ovalbumin (3.6 S), bovine serum albumin (4.3 S), rabbit muscle phosphoglucose isomerase (7.0 S).

Equilibrium centrifugation studies using the Beckman Model E ultracentrifuge equipped with an AN-J rotor were carried out on samples of 7S NGF_n, 7S NGF_a, and on samples of 7S NGF_n plus PTI and 7S NGF_a plus PTI to compare molecular weights via the long-column method (Chervenka, 1970). The runs were carried out at 10 °C with 7S NGF protein concentrations of approximately 0.8 mg/mL and using capillary-type 12-mm Epon synthetic boundry double-sector cells. The optical density across the cell was monitored with the photoelectric scanner attachment. All runs were made at 10 589 rpm in either 50 mM Tris-HCl buffer (pH 7.40 at 25 °C), or in sodium phosphate buffer (pH 7.87 at 25 °C). Both buffers contained sufficient KCl to give ionic strengths of 0.15.

Isoelectric focusing gels containing pH 3.5 to 10 ampholine (LKB products) were prepared by Wrigley's procedure (Wrigley, 1968). Horse heart cytochrome *c*, 10 μL of 2 mg/mL (pI 9.8), was used in each run as a visual indicator to establish when the gels were focused. All focusing experiments were carried out at 5 °C for 3 h at 100 V. The gels were fixed and stained in the quick Coomassie blue stain.

The gel-filtration studies were performed as described in the figure captions (see Results). Acid-urea and $NaDodSO_4$-urea gel electrophoresis experiments were carried out with 7S NGF_n and 7S NGF_a according to the procedure of Panyim and Chalkey (1969) and Bruening et al. (1967), respectively. Dansylation of N-terminal residues was carried out by a modification of the procedure of Hartley (1970).

[2] α_1-Antitrypsin (lyophilized powder, lot No. 1986), C-1 esterase inhibitor (lyophilized powder, lot No. 9B), and antithrombin III (concentrate, lot 51-IIA) were kindly provided by the American Red Cross National Fractionation Center, Blood Research Laboratory, Bethesda, Md. 20014, with partial support of NIH Grant HE 1388 (HEM).

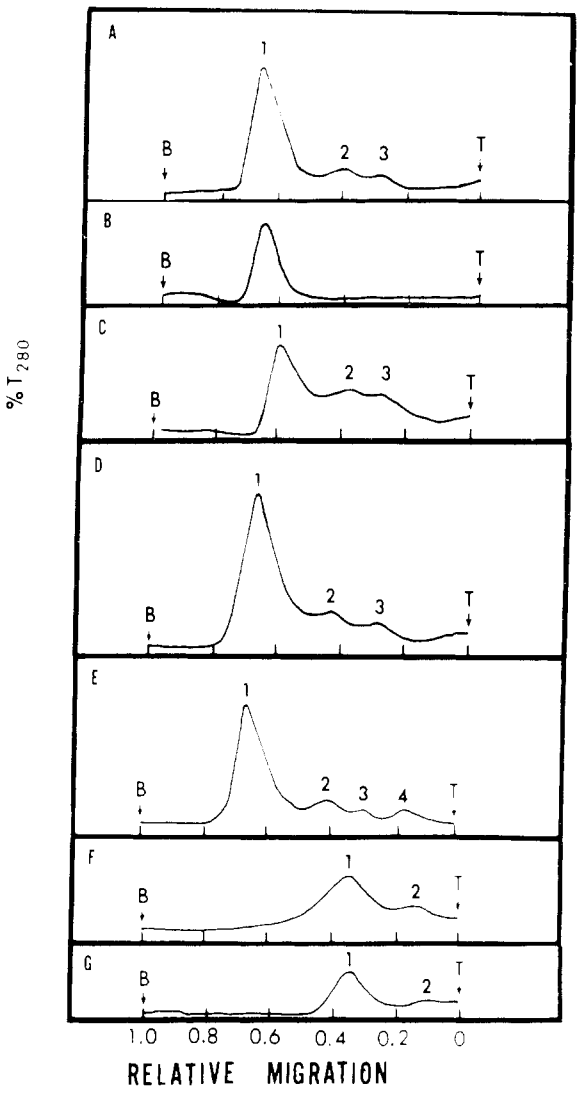

FIGURE 2: Comparison of the effects of buffer, EDTA, and PTI on the sedimentation of 7S NGF in 5 to 20% linear sucrose gradients at 5 °C. Buffer effects: (A) 7S NGF_n in 50 mM, pH 7.97, Tris-HCl buffer; (B) 7S NGF_n in pH 7.97 sodium phosphate buffer, $I = 0.05$. EDTA effects in 50 mM, pH 7.97, Tris-HCl buffer; (C) 7S NGF_a in the presence of 0.1 mM EDTA; (D) 7S NGF_a in the presence of 0.1 mM EDTA and 1 mM $ZnSO_4$; PTI effects in 50 mM, pH 7.97, Tris-HCl buffer; (E) 7S NGF_n (0.35 mg) preincubated for 1 h with excess PTI (0.156 mg); (F) 7S NGF_a (0.35 mg) preincubated for 1 h with excess PTI (0.156 mg); (G) the γ subunit (0.08 mg) preincubated for 1 h with excess PTI (0.156 mg). A total sample volume of 100 μL was applied to each gradient. B and T refer to the bottom and to the top of the gradient respectively.

Results

The basic Kunitz trypsin inhibitor from bovine pancreas (PTI) has been reported to inhibit the trypsin-like proteolytic activities of both the γ subunit and 7S NGF_n (Greene, 1969). The titration isotherms presented in Figure 1 demonstrate that the reaction of PTI with either activated 7S NGF (7S NGF_a) or the γ subunit results in a nearly stoichiometric loss of proteolytic activity for each addition of PTI. Note in Figure 1 that solutions of 7S NGF_a and the γ subunit which have been adjusted by dilution to give the same steady-state turnover rate for the hydrolysis of α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) yield nearly identical titration end points. (The titration end point here is taken as the sharp break point in the isotherms indicated by the vertical arrows). Hence, within the limits of experimental error (±20%), these data establish that 7S NGF_a and the γ subunit exhibit the same specific activities. Note also that the stoichiometric equivalence points occur at a residual level of activity of ~10%. This residual activity is attenuated by further additions of PTI, but the dependence of the decrease in activity on the concentration of PTI beyond the equivalence point suggests the occurence of a much weaker interaction between the γ species involved and PTI. Based on the reported extinction coefficients for the γ subunit and for 7S NGF_n (Server and Shooter, 1976), the titration end points are consistent with a γ:PTI stoichiometry ratio of 1:1 and a γ:7S NGF subunit stoichiometry ratio of 2:1 (see Experimental Section).

Rapid kinetic studies using the proflavin-displacement technique (Bernhard et al., 1966; Brandt et al., 1967; Feinstein and Feeney, 1966) indicate that the reactions of the initially formed (weak) complex to give the stable PTI complexes occur on similar time scales for both proteins, with $t_{1/2} \simeq 5$ s under conditions similar to those used in Figure 1 (A. M.-J. Au and M. F. Dunn, unpublished results).

Sucrose gradient centrifugation provides a convenient means for resolving the 7S NGF species (mol wt 140 000) from lower molecular weight aggregates and the individual subunits (mol wt 20 000 to 30 000) (Varon et al., 1967a,b; Smith et al., 1969). A typical sucrose gradient profile for 7S NGF_n in a 5–20% sucrose gradient at 5 °C (50 mM Tris-HCl buffer, pH 7.97) is shown in Figure 2A. Note that in addition to the 7S NGF_n band (peak 1) near the bottom of the gradient there are present two other bands (peaks 2 and 3) which correspond to $s_{20,w}$ values of ~5, and 2.5–3 S (as determined by comparison with $s_{20,w}$ standards) in agreement with the studies of Varon et al. (1967). The relative amounts of the three peaks vary with the age of the preparation; the amounts of the 5S and 2.5–3S peaks increase with age at the expense of the 7S peak. Isoelectric-focusing studies (data not shown) indicate that all three subunit classes (i.e., α, β, and γ) are present in each peak from the profile.

In contrast, the amounts of 5S and 2.5–3S material relative to the 7S peak are greatly reduced in gradients buffered with pH 7.97 sodium phosphate (ionic strength 0.05), as is apparent in Figure 2B. Our investigations (A. M.-J. Au and M. F. Dunn, unpublished results) indicate that the difference in the amounts of 5S and 2.5–3S material in Tris-HCl buffered and sodium phosphate buffered gradients has its origins in a specific salt effect which destabilizes the 7S NGF oligomer.

The gradient profile in Figure 2C shows that EDTA-activated 7S NGF in Tris-HCl buffer gives a profile that is similar, but not identical, to the profile for 7S NGF_n (Figure 2A). Note that, under identical centrifugation conditions, the major band (peak 1) of 7S NGF_a does not migrate as far into the gradient as does 7S NGF_n. Although the difference in relative migration distance is small, as documented in Table I, the difference is reproducible, and according to the standard statistical Student's *t* test (Campbell, 1974) the difference is significant. Furthermore, the difference was found in both the Tris-HCl buffered gradient and in the sodium phosphate buffered gradient. The relative migration distances of the 5S and 2.5–3S bands in comparison to the native sample do not show a significant difference. However, the relative amounts of the 5S and 2.5–3S bands are increased in the EDTA-treated sample (compare Figure 2A,C). Note also that the decrease in mobility of the ~7S peak is reversed when 7S NGF_a is placed in a gradient containing zinc ion in a concentration which is in excess of the EDTA concentration present in the gradient (Figure 2D).

Sedimentation equilibration centrifugation studies at 10 °C (data not shown) using the analytical ultracentrifuge and the long-column method (Chervenka, 1970) were carried out to

TABLE I: Sucrose Density Gradient Centrifugation of Native and EDTA-Activated 7S NGF in 5 to 20% Linear Gradients and 50 mM Tris-HCl Buffer, pH 7.97, at 5 °C.

Sample No.	Conditions	Relative Mobilities[a] 7 S	5 S	2.5 S
1	7S NGF_n	0.670	0.443	0.292
2	7S NGF_n	0.666	0.428	0.298
3	7S NGF_n	0.673	0.435	0.282
4	7S NGF_n	0.660	0.427	0.317
5	7S NGF_n	0.675	0.425	0.306
6	7S NGF_n	0.670	0.440	0.305
7	7S NGF_n	0.680	0.440	0.296
		Av 0.671 ± 0.016	0.430 ± 0.021	0.299 ± 0.027
8	7S NGF_a[b]	0.600	0.412	0.282
9	7S NGF_a[b]	0.630	0.422	0.321
10	7S NGF_a[b]	0.617	0.432	0.308
11	7S NGF_a[b]	0.630	0.437	0.296
12	7S NGF_a[b]	0.600	0.403	0.317
		Av 0.615 ± 0.026	0.421 ± 0.028	0.305 ± 0.010
		t value[c] 3.488 (sig)[d]	2.082 (ns)[e]	−0.689 (ns)[e]

[a] Relative mobility refers to the ratio of the distance migrated (measured from the top of the gradient) to the total length of the gradient. [b] 7S NGF preincubated with 0.1 mM EDTA and sedimented in gradients containing 0.1 mM EDTA. [c] Standard *t* value is 2.764, $P < 0.01$. [d] sig, significant. [e] ns, nonsignificant.

determine whether or not the apparent change in $s_{20,w}$ value on EDTA activation is due to a change in molecular weight. Analysis of the sedimentation profiles via plots of ln ΔOD vs. r^2, where ΔOD refers to the protein OD at 280 nm and r refers to the distance from the center of the rotor, gave linear plots with identical slopes for samples of 7S NGF_n and 7S NGF_a that had been subjected to an additional purification step involving gel filtration of 7S NGF_n over Bio-Gel P-150 (see materials under Experimental Section). Since the slope of this plot is proportional to the weight-average molecular weight, the apparent change in $s_{20,w}$ value accompanying activation does not appear to be due to a change in molecular weight.

The gradient profile in Figure 2E shows that preincubation of 7S NGF_n with excess PTI does not influence either the positions or the relative amounts of the 7S, 5S, or 2.5–3S bands (peaks 1, 2, and 3). The fourth peak at the top of the gradient consists of PTI and is located at a relative migration distance consistent with the $s_{20,w}$ value for PTI (~1 S). However, preincubation of 7S NGF_a with excess PTI drastically alters the gradient profile (Figure 2F). The resulting profile consists of a relatively broad band (peak 1) corresponding to 2.5–3S and a band (peak 2) consisting of PTI. Isoelectric focusing studies (see below) show that the 2.5–3S peak consists of the α and β subunits and the γ subunit–PTI complex. The sucrose gradient sedimentation profile of the γ subunit preincubated with excess PTI is shown in Figure 2G for comparison. Peak 1 was identified as the γ subunit–PTI complex while peak 2 consists of excess PTI.

Analytical ultracentrifuge sedimentation studies confirm these findings. A plot of ln ΔOD vs. r^2 for a solution containing 7S NGF_n plus excess PTI yielded a linear plot with a slope identical to the slopes obtained for 7S NGF_n and 7S NGF_a (as described above), while solutions containing 7S NGF_a and excess PTI gave linear plots with considerably reduced slopes, indicating a drastic change in molecular weight (i.e., a change from ~140 000 to ~30 000 mol wt).

Since the degree of resolution achieved in these sucrose gradients precludes the possibility of detecting whether or not PTI forms a complex with 7S NGF_n (viz., Figure 2E) due to the low molecular weight of PTI (6500), fractions from the gradients were assayed along the profile for proteolytic activity with the chromogenic substrate BAPNA, assayed both in the presence and in the absence of added EDTA. Figure 3 compares the intrinsic protein fluorescence profile with the proteolytic activity profile along the gradient. The data in Figure 3A compare protein fluorescence and proteolytic activities for the gradient shown in Figure 2A. Note that in the absence of EDTA the activity levels are relatively low and that the 7S band (fractions 4–8) and 2.5–3S band (fractions 20–25) exhibit comparable activities, whereas EDTA treatment enhances the activity of the 7S band 10- to 15-fold while the 2.5–3S band is virtually unaffected by EDTA treatment. The degree of EDTA activation achieved for fractions taken from the 5S region (fractions 10–15) of the gradient is not more than threefold.

The fluorescence and the activity profiles in Figure 3B for the gradient in which 7S NGF_n and PTI were preincubated (Figure 2E) are identical, within the limits of reproducibility, to those in Figure 3A except near the top of the gradient (fractions 26–35). This region of the gradient (as previously described) consists of PTI. The close similarities of the profiles in Figure 3A,B demonstrate that the preincubated mixture of 7S NGF_n and PTI cleanly separates on sedimentation, and, since the activity profiles are not altered, the 7S band contains negligible amounts of PTI.

Similar comparisons of protein fluorescence and proteolytic activity with 7S NGF_a (data not shown) demonstrate that the ~7S band (peak 1 in Figure 2C) exhibits a proteolytic activity which is comparable to that of the corresponding peak from the 7S NGF_n gradient after EDTA treatment.

To further characterize the reaction of PTI with 7S NGF, mixtures of PTI with both 7S NGF_n and 7S NGF_a were examined via isoelectric focusing gel electrophoresis. Previous studies (Varon and Shooter, 1970) have shown that the 7S NGF_n oligomer dissociates to subunits when subjected to isoelectric focusing conditions. Therefore, isoelectric focusing using polyacrylamide gels containing pH 3.5–10 ampholine results in the focusing of the individual subunits at pH values which correspond to their apparent pI values (Varon and Shooter, 1970). Under the conditions used it is possible to resolve the γ fraction into at least three electrophoretically distinct species, and a fourth species can be detected under conditions of higher resolution (Varon and Shooter, 1970; Stach et al., 1976). Although both the α and β fractions also consist

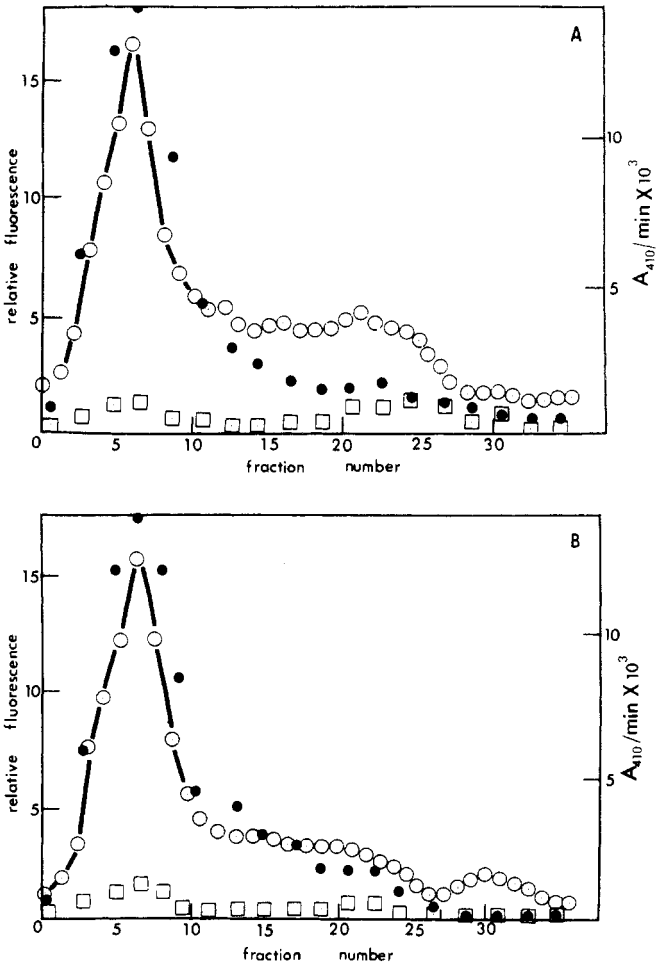

FIGURE 3: Comparison of proteolytic activity profiles in 5 to 20% linear sucrose gradients for 7S NGF_n (A) and 7S NGF_n preincubated with excess PTI for 1 h (B). The activity profile in A corresponds to the sucrose gradient shown in Figure 2A, the profile in B corresponds to the gradient shown in Figure 2E. (○) Relative protein fluorescence, (□) proteolytic activity before treatment with EDTA, (●) proteolytic activity after treatment with EDTA. Fractions 5-7, 11-13, 21-24, and 29-31 correspond to $s_{20,w}$ values of approximately 7, 5, 2.5-3, and 1 S, respectively.

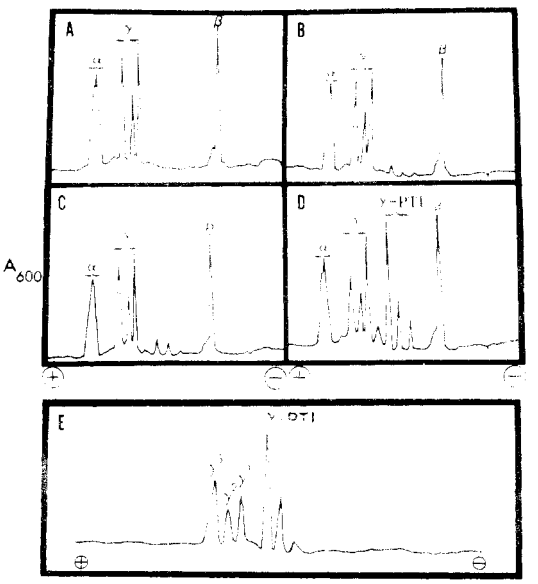

FIGURE 4: Comparison of the effects of PTI on the isolelectric focusing behavior of 7S NGF_n and 7S NGF_a in pH 3.5-10 ampholine. The protein samples (in sucrose) were layered at the anodic end of the gel (+) prior to focusing. In A, 25 μL of 30 μM 7S NGF_n was run as a control. The same amount of 7S NGF_n preincubated with excess PTI (80 μM) for various time periods before focusing was used in the experiments. Preincubation times: B, <1 min; C, 10 h. In D, after 10 h of incubation with PTI, a sample identical to the one employed in C was incubated for an additional 1 h with 0.1 mM EDTA prior to focusing. In E, 10 μM γ was incubated with ~20 μM PTI for 1 h prior to focusing. The gels were stained with quick Coomassie blue.

of electrophoretically distinct species, the steepness of the pH gradient in the pH 3.5-10 ampholine system at the pH extremes prevents a clean separation of the individual α and β species into distinct bands in these gels. Hence, the α and β subunits appear as slightly broadened bands.

The gel scan presented in Figure 4A is illustrative of a typical isoelectric focusing gel for 7S NGF_n. The gel scans shown in Figure 4B,C show that the preincubation of 7S NGF_n with PTI for time periods up to 10 h (Figure 4C) does not alter the pattern of species detected by isoelectric focusing. Note that PTI, due to its high p*I* value (≥10), runs off of the end of the gel. In marked contrast, preincubation of 7S NGF_a with PTI for 1 h before focusing yields a substantially altered pattern of species (viz., Figure 4D). The resulting gel (Figure 4D) contains considerable amounts of three new species exhibiting p*I* values which are more basic than the various γ species, and the total amount of the γ species detected relative to the amounts of α and β species is decreased. The same pattern is obtained when 7S NGF_a and PTI are subjected to focusing immediately after mixing. Traces of the new species appear to be present in Figure 4B,C. Isoelectric focusing gels (Figure 4E) run on mixtures of γ and PTI clearly identify these new species as γ subunit-PTI complexes. Note in Figure 4D,E that the relative amounts of the γ subunit-PTI species do not appear to occur in the same ratio as do the individual γ species. This fact suggests that the various γ species bind PTI with differing affinities. Such a finding is consistent with the previously noted residual activity level which persists at the titration end point in the titration shown in Figure 1.

Since the results presented in Figure 1 demonstrate that 7S NGF_a and γ exhibit comparable specific activities toward BAPNA, and since the reaction of PTI with 7S NGF_a brings about the dissociation of the 7S oligomer yielding a mixture of α and β subunits and the formation of the γ subunit-PTI complex (viz., Figures 3 and 4), it was of interest to determine whether or not the proteolytically active species derived from EDTA activation is the high-molecular-weight (~7 S) oligomer or a lower molecular weight species. Therefore, gel-filtration studies using Bio-Gel P-100 (Figure 5A-C) were undertaken to determine the effects of the presence of a saturating concentration of a low-molecular-weight substrate on the aggregation state of 7S NGF_a. The artificial substrate, α-*N*-benzoyl-L-argininamide (BAA), was chosen, since (a) this compound is hydrolyzed at a rate which is ~100-fold slower than the rate of BAPNA hydrolysis, and (b) the UV spectrum of BAA and the hydrolysis products do not interfere with the measurement of the BAPNA optical density changes which occur on hydrolysis. Thus, relative proteolytic activities using BAPNA as substrate can be measured easily in the presence of high concentrations of BAA.

The experiment shown in Figure 5 involves the comparison of elution patterns for the passage of 7S NGF_n and 7S NGF_a through the same P-100 Bio-Gel column in the presence and in the absence of BAA. The choice of P-100 Bio-Gel was dictated by the following constraints: (a) the column must be capable of resolving a 140 000 mol wt species from 25 000 mol wt species, and (b) the elution time must be fast in comparison to the rate of depletion of substrate in order to assure that the

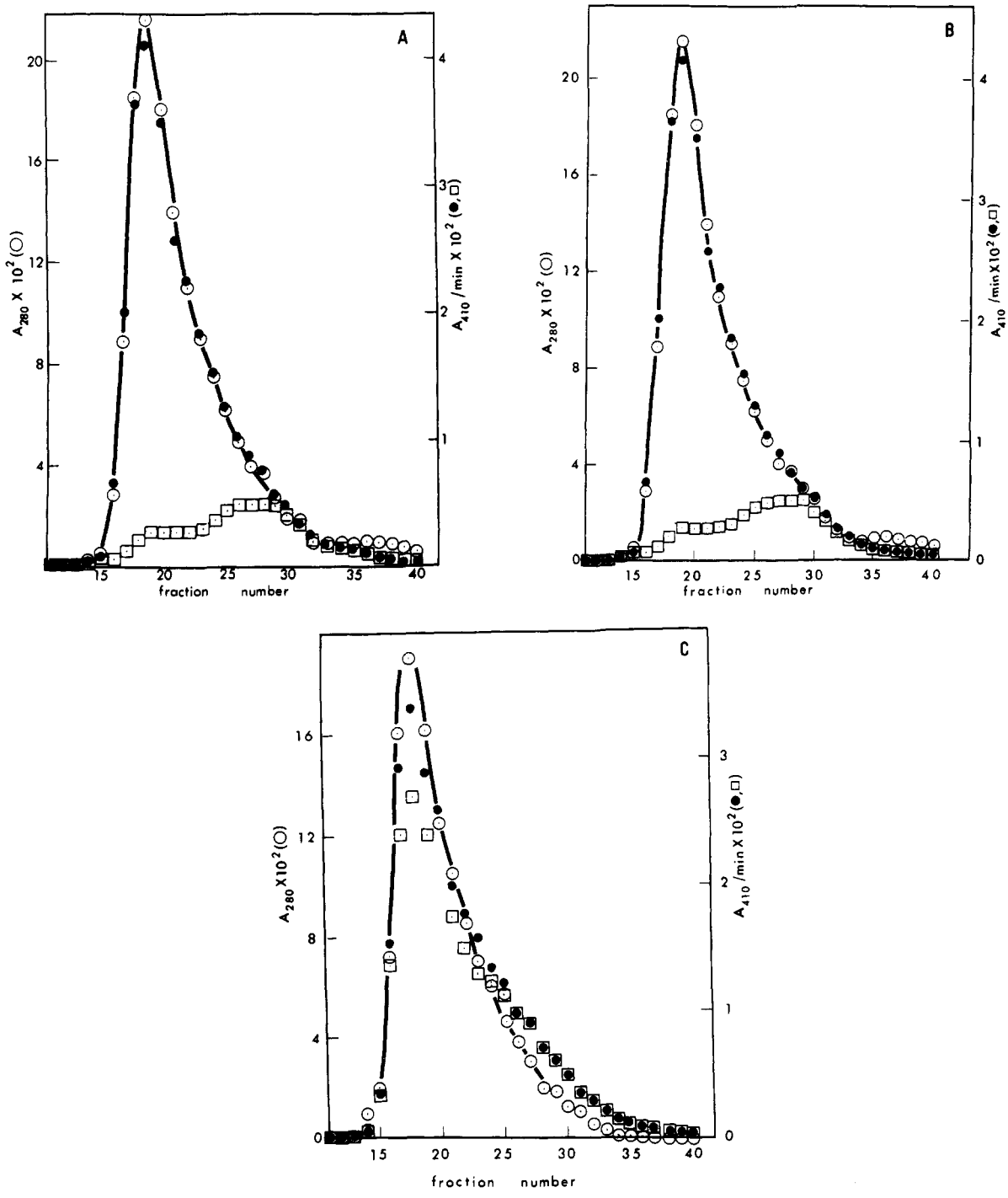

FIGURE 5: Comparison of Bio-Gel P-100 elution profiles for 7S NGF_n and 7S NGF_a in the presence and absence of α-*N*-benzoyl-L-argininamide (BAA) at pH 7.97 and 5 °C. A, 7S NGF_n; B, 7S NGF_n in the presence of 1 mM BAA; C, 7S NGF_a in the presence of both 1 mM EDTA and 1 mM BAA. (○) A_{280}; (□) proteolytic activity assayed as the rate of hydrolysis of α-*N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) measured at 410 nm in the absence of 0.1 mM EDTA; (●) proteolytic activity measured in the presence of 0.1 mM EDTA after incubation for 1 h at room temperature. The molecular weight standards Blue Dextran (>2 000 000 mol wt), rabbit muscle aldolase (160 000 mol wt), bovine chymotrypsinogen (25 500 mol wt), and horse heart cytochrome *c* (13 000 mol wt) eluted in fractions 16, 17, 28, and 34, respectively.

enzyme is continuously presented with saturating levels of substrate during passage through the column.

In Figure 5A–C, most of the 7S NGF_n or 7S NGF_a applied to the column elutes just behind the column void volume and corresponds to an ~140 000 mol wt species. However, the shape of the trailing edge of the OD_{280} profile suggests the presence of small amounts of lower molecular weight species. The activity profile measured in the absence of EDTA more clearly indicates sample heterogeneity: note in Figure 5A,B that the activity peak, fraction 27 (~25 000 mol wt), does not coincide with the protein peak (fraction 19), although there is a shoulder on the activity profile in the vicinity of the protein peak. In contrast, the activity profile obtained in the presence of EDTA closely corresponds with the protein OD_{280} profile, and the EDTA-activated 7S NGF_a species exhibits an activity which is 10- to 15-fold greater than that of the native protein.

Note that the protein OD_{280} profile in 5C is nearly identical to the OD_{280} profiles observed in Figure 5A,B. Note also that the peak in the activity coincides with the peak in the OD_{280}

profile, and that the levels of activity found correspond to fully activated 7S NGF_a. Since the elution of 7S NGF_a in comparison to 7S NGF_n is unperturbed by the presence of a saturating excess of BAA, this experiment strongly indicates that the interaction of a small artificial substrate such as BAA with the 7S NGF_a-bound γ subunits does not influence the aggregation state of the activated oligomer.

Greene (1969) has reported that γ is not inhibited by the trypsin inhibitors from soybean or lima bean, or by ovomucoid. Our experiments confirm these findings. We also have extended the survey of macromolecular protease inhibitors to include human α_1-antitrypsin, human antithrombin III, and human C-1 esterase inhibitor.[3] Whereas these three proteins are potent trypsin inhibitors, 10- to 50-fold molar excesses of these proteins did not inhibit either the γ subunit or 7S NGF_a.

In an attempt to determine whether or not EDTA activation of the 7S NGF-proteolytic activity results in the proteolytic modification of the 7S NGF subunits, freshly prepared samples of 7S NGF_n and 7S NGF_a were subjected to both acid–urea and $NaDodSO_4$–urea electrophoresis. These gel electrophoresis experiments failed to show any differences in the number or intensities of the various NGF polypeptide chains of 7S NGF_n and 7S NGF_a resolved in these gels.

The dansylation of 7S NGF_n and 7S NGF_a N-terminal residues was also carried out in an attempt to determine whether or not EDTA activation results in the proteolytic modification of the NGF subunits. Again, these experiments failed to detect any difference in the number or intensities of the various dansylated N-terminal residues derived from the chains making up the 7S NGF_n and 7S NGF_a subunits.

Discussion and Conclusions

Greene (1969) in a preliminary account has reported that the esteropeptidase activities associated with both the γ subunit and 7S NGF_n are inhibited by PTI. The experiments reported in this study demonstrate that the 7S NGF_n species does not undergo a detectable reaction with PTI (viz., Figures 2, 3 and 4), whereas the EDTA-activated species undergoes a relatively rapid reaction ($t_{1/2}$ = 5 s) which results in the loss of esteropeptidase (Figure 1) and ultimately the dissociation of the oligomer to a mixture of the α and β subunits and the formation of the γ subunit–PTI complex (Figures 2 and 4). Hence, the sequestering and removal of zinc ion from 7S NGF_n by EDTA renders the active sites of the 7S NGF_a-bound γ subunits accessible both to artificial small-molecule substrates (e.g., BAPNA and BAA) and to the large polypeptide serine protease inhibitor, PTI.

The loci for zinc-ion binding to 7S NGF are not known. The α and β subunits exhibit little or no affinity for zinc ion (Pattison and Dunn, 1975). The γ subunit exhibits an affinity for zinc ion ($K_i \simeq 10^{-6}$ M; Pattison and Dunn, 1975) which is several orders of magnitude lower than that of 7S NGF_n ($K_D \simeq 10^{-11}$ M; Pattison and Dunn, 1976b). Although removal of zinc ion brings about a 10- to 15-fold change in the proteolytic activity of 7S NGF, it cannot be concluded that the zinc-ion sites on 7S NGF_n and the inhibiting zinc-ion site on the free γ subunit are one and the same.

Kinetic studies (Pattison and Dunn, 1976a) show that the 7S NGF-bound zinc ions each bind 1 mol of the chromophoric chelator 2,2′,2″-terpyridine rapidly; the specific second-order rate constant for this step, 1×10^6 M^{-1} s^{-1}, is identical to the rate at which this chelator forms an inner sphere complex with the aquo-zinc ion (Holyer et al., 1966). Following this rapid step, there occurs a second, slower step in which the bisterpyridine–zinc ion complex and 7S NGF_a are formed concomitantly. The kinetics and stoichiometries of this sequence suggest that three of six putative zinc-ion coordination positions are occupied by easily replaced ligands (perhaps water molecules) and that the remaining three positons are occupied by tightly bound ligands from the protein. Note that the x-ray structure of insulin (Blundell et al., 1972) shows just such a ligand field about the two zinc ions in the crystalline zinc–insulin hexamer. These inferences suggest a possible structural similarity between the zinc–insulin hexamer and 7S NGF_n. Although the sequence homology between the β chain and guinea pig proinsulin (Angeletti and Bradshaw, 1971; Frazier et al., 1972; Angeletti et al., 1973) is modest (~20%), it is interesting to note that there is a histidyl residue in the sequence of the β chain (residue 8) at a position which corresponds to the insulin histidyl residue (residue B10) involved in coordination of the zinc ions of the zinc–insulin hexamer. Thus, His-8 of the β chain is a good candidate for one of the zinc ligands in 7S NGF_n.

Titration of the γ subunit by PTI (Figure 1) yields a sharp end point which corresponds in stoichiometry to the formation of a 1:1 complex between the γ subunit and PTI. Since solutions of 7S NGF_a and solutions of γ adjusted by dilution to give identical turnover rates yield nearly identical titration end points, 7S NGF_a and the γ subunit exhibit the same specific activities (on a per-site basis). The residual (~10%) activity remaining at the titration end point (Figure 1) appears to be associated with a γ species which binds PTI only weakly. Greene et al. (1968, 1969) have reported that the various γ species detected by isoelectric focusing exhibit closely similar (if not identical) specific activities and specificities. Hence, the residual activity probably is contributed by a γ species which accounts for approximately 10% of the total γ enzyme present. Using the 280-nm extinction coefficient for 7S NGF_n reported by Server and Shooter (1976), the titration end point obtained in the PTI titration of 7S NGF_a indicates a subunit stoichiometry of 2 mol of γ subunit per mol of 7S NGF. This stoichiometry is consistent with the proposals of others (Berger and Shooter, 1976; Server and Shooter, 1976; Baker, 1975a; Jeng et al., 1976).

The fact that 7S NGF_n preparations exhibit a residual proteolytic activity may indicate that the native zinc metalloprotein possesses an intrinsic protease activity that differs in specific activity and/or specificity from that of the free γ subunit or 7S NGF_a. However, the sucrose density gradient centrifugation studies (Figures 2 and 3) and the gel-filtration studies (Figure 5) argue against this hypothesis. These experiments clearly show that as much as 75% of the residual proteolytic activity associated with 7S NGF_n (isolated according to the procedure of Varon et al., 1967a) is due to the presence of the free γ subunit. The fact that the high-molecular-weight fractions from both the sucrose gradient and the Bio-Gel P-100 column exhibit measurable levels of activity (~6% relative to 7S NGF_a) could be due (a) to the dissociation of the oligomer under the conditions of the activity assay, (b) to the loss of zinc ion from the protein, or (c) to the selective irreversible loss of α or β subunits.

Since previous studies from this laboratory (Pattison and Dunn, 1975, 1976a,b) strongly indicate that the chelator-induced activation of the 7S NGF esteropeptidase yields an activated 7S oligomer, the finding that the PTI reaction with

[3] This latter possibility seems unlikely, since the acid–urea and $NaDodSO_4$–urea gel electrophoresis experiments and the N-terminal dansylation studies comparing freshly prepared 7S NGF_n and 7S NGF_a (see Results) strongly suggest that EDTA activation of the 7S NGF proteolytic activity does not result in the proteolytic modification of α, β, or γ.

7S NGF_a results in dissociation of the oligomer was unexpected. A priori, one possible explanation for this unexpected reaction is that the actual proteolytically active species is a lower molecular weight species, such as the free γ subunit, which arises via a dissociation process induced by substrate binding to the 7S NGF_a-bound γ subunits. The gel-filtration studies presented in Figure 5 strongly indicate that the binding of small artificial substrates (i.e., BAA) does not exert a gross influence on the molecular weight of 7S NGF_a. Therefore, we conclude that the reaction of PTI with the 7S NGF-bound γ subunits triggers the dissociation of the oligomer, as shown in eq 1 and 2

$$7S\ NGF_n + 2EDTA \rightleftharpoons 7S\ NGF_a + 2Zn(EDTA) \quad (1)$$

$$7S\ NGF_a + 2PTI \rightleftharpoons 7S\ NGF_a(PTI)_2 \rightarrow 2\gamma PTI + x\alpha + y\beta \quad (2)$$

(where x and y are the stoichiometry coefficients for the α and β subunits within the 7S NGF_a oligomer). It is possible that the γ subunit–PTI protein–protein interface sterically interferes (via van der Waals contacts) with interactions between the 7S NGF_a subunits that are critical to the stability of the 7S species. Alternatively, the binding of PTI to the active site of γ may stabilize a conformation state of the γ subunit which does not bind the other NGF subunits with sufficient affinity to yield a stable 7S NGF–PTI oligomer.

Since the reaction of PTI with 7S NGF_a also brings about dissociation of the oligomer, whereas the artificial substrate BAA appears to undergo reaction without altering the state of aggregation of 7S NGF_a, implies that the protein topologies adjacent to the active sites of 7S NGF_a and γ are different. Therefore, the specificities of 7S NGF_a and γ toward macromolecular substrates may be different in instances where the interactions between enzyme and macromolecular substrate extend significantly beyond the scissile residue. Indeed, both x-ray structure studies and kinetic studies have shown that for proteases such as trypsin, α-chymotrypsin, elastase, and subtilisin there occur specific interactions between enzyme and polypeptide substrates which extend considerably beyond the region of the catalytic site (Rühlmann et al., 1973; Segal et al., 1971; Shotten et al., 1971; Kraut et al., 1971).

The high resolution x-ray structures and the thermodynamic studies of the trypsin- and the anhydrotrypsin–PTI complexes (Huber et al., 1974, 1975; Rühlmann et al., 1973; Laskowski and Sealock, 1971; Vincent et al., 1974; Vincent and Lazdunski, 1972; Janin and Chothia, 1976) clearly show that the stability of the trypsin–PTI complex resides (a) primarily in hydrophobic forces (solute aggregation driver by water cohesiveness), and (b) secondarily in the large number of weak bonding interactions (van der Waals forces and Coulombic interactions) which make up the protein–protein interface. Similar interactions almost certainly must account for the stability of the γ subunit–PTI complex.

Furthermore, it is apparent that the more restricted specificities (in comparison to trypsin) exhibited by γ and other trypsin-like enzymes (i.e., thrombin, enterokinase, kallikaren, plasmin, acrosin, Reich et al., 1975) must be due to steric strictures imposed by the topography of subsites adjacent to the active site which define the interface between enzyme and macromolecular substrate. The finding that PTI is a potent inhibitor of both γ and 7S NGF_a while the soybean trypsin inhibitor shows no inhibiting activity is particularly striking, since comparison of the high-resolution three-dimensional x-ray structures of the trypsin–PTI complex (Huber et al., 1974, 1975; Rühlmann et al., 1973) and the trypsin–soybean inhibitor complex (Sweet et al., 1974) show the van der Waals contacts and Coulombic interactions between trypsin and the two inhibitors to be extremely similar.

The in vivo biological function of the 7S NGF_n protein is open to speculation. The studies of Perez-Polo et al. (1972), Moore et al. (1974), and Pattison and Dunn (1975, 1976a,b) as well as this work demonstrate that the binding domains between the 7S NGF subunits are highly specific, and that 7S NGF_n appears to be the predominant molecular form of NGF in the mouse submaxillary gland homogenate. Hence, if the only function of the γ subunit is to convert a "pro-β" to β (Moore et al., 1974; Angeletti et al., 1973), then, since only catalytic amounts of the activating enzyme are required for the conversion of a putative "pro-β" to β, the existence of a stable complex (7S NGF_n) containing α, β, and γ in fixed stoichiometry ratios would appear to serve no useful biological purpose.

The fact that the proteolytic activity of the 7S NGF-bound γ subunits is virtually completely inhibited in 7S NGF_n suggests that the native oligomer is a precursor form of the active growth factor. Indeed, it is reasonable to speculate that 7S NGF_n is a "packaged" form of β which may play one or more of the following physiological roles: (a) regulation of the storage and/or release of the active form of the growth factor following synthesis, (b) transport to the target cells via the serum and/or the nervous system, (c) proteolytic modification of a receptor or surface element of the neuronal target cell, (d) proteolytic modification of α and/or β by γ during transport or at the target cell.[3]

These studies have shown that, of the macromolecular PTI-like protease inhibitors thus far examined (PTI, soybean trypsin inhibitor, lima bean trypsin inhibitor, ovomucoid, human α_1-antitrypsin, human anti-thrombin III, human C-1 esterase inhibitor), only PTI inhibits γ and 7S NGF_a. Nevertheless, there is a component present in mouse serum which is a potent inhibitor of both the γ and the 7S NGF_a proteolytic activities (A. M.-J. Au and M. F. Dunn, unpublished results). Perhaps activation of 7S NGF_n by an indogenous zinc-ion chelator followed by reaction of 7S NGF_a with a specific PTI-like inhibitor provides a mechanism by which the proteolytic activity of the oligomer and the release of α and β from the oligomer both are regulated in vivo?

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