Reaction of Nerve Growth Factor γ and 7S Nerve Growth Factor Complex with Human and Murine α_2-Macroglobulin†

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ABSTRACT: The kallikrein-like serine proteinase nerve growth factor γ (NGF-γ) reacted with the plasma proteinase inhibitor human α_2-macroglobulin (hα_2M). The hα_2M subunits were cleaved, the electrophoretic mobility of hα_2M in nondenaturing polyacrylamide gels was increased, and the intrinsic fluorescence of hα_2M was increased with a slight blue-shift. These changes are well-characterized components of the α_2M/proteinase reaction mechanism. In N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) hydrolysis experiments, the catalytic efficiency (k_{cat}/K_M) of the hα_2M–NGF-γ complex was decreased by 98.5% compared with free NGF-γ. This decrease is unique since other α_2M–proteinase complexes retain significant amidase activity. For comparison, we determined that the catalytic efficiency of α_2M–trypsin is decreased by 58% compared with free trypsin under equivalent conditions. The rate of NGF-γ inhibition by hα_2M was $(1.0 \pm 0.1) \times 10^4$ M^{-1} s^{-1} as determined by BAPNA hydrolysis. A similar value was determined by monitoring the change in intrinsic fluorescence. NGF-γ, which was bound within the intact 7S NGF complex, also reacted with hα_2M, albeit at a very slow rate. This reaction may have depended exclusively on slow reversible dissociation of NGF-γ from the 7S complex. NGF-γ was rapidly inhibited by murine α_2M (mα_2M). The properties of the NGF-γ/mα_2M reaction and those described for NGF-γ and hα_2M were similar (including the significant decrease in catalytic efficiency after complex formation); however, the NGF-γ inhibition rate constant was $(3.6 \pm 0.4) \times 10^6$ M^{-1} s^{-1}. These studies demonstrate that both hα_2M and mα_2M are significant inhibitors of NGF-γ. Since previous studies have failed to demonstrate inhibition of NGF-γ by other plasma proteinase inhibitors [Au & Dunn (1977) *Biochemistry 16,* 3958–3966], α_2M may be the only major regulator of NGF-γ in plasma.

Nerve growth factor γ (NGF-γ)[1] is a 26-kDa glycoprotein and a member of the kallikrein family of serine proteinases (Greene et al., 1969; Thomas et al., 1981; Evans & Richards, 1985). In the male mouse submaxillary gland and in the exocrine glands of other species, high molecular weight complexes are formed by NGF-γ and two other polypeptides, NGF-α and NGF-β (Varon et al., 1968; Fahnestock, 1991). The largest recoverable complex is termed 7S NGF on the basis of sedimentation velocity; the stoichiometry of 7S is $\alpha_2\beta\gamma_2$ or $\alpha_2\beta\gamma$ (Varon et al., 1967, 1698; Smith et al., 1968; Young et al., 1988). The nerve growth promoting activity of the 7S complex resides exclusively with the β-subunit while the function of the α-subunit is unknown (Isackson et al., 1984; Fahnestock, 1991). NGF-γ is the only subunit with endopeptidase activity; this activity may be important in the processing of the NGF-β precursor (Berger & Shooter, 1977; Isackson et al., 1987; Edwards et al., 1988; Jongstra-Bilen et al., 1989).

In the 7S complex, the activities of the β- and γ-subunits are significantly inhibited. NGF-β does not bind to NGF-specific cellular receptors but is protected from proteolysis (Harris-Warrick et al., 1980). The γ-subunit is enzymatically inactive probably due to association of the active site with the C-terminal Arg residue of NGF-β (Moore et al., 1974; Pattison & Dunn, 1975; Berger & Shooter, 1977; Bothwell & Shooter, 1978). For these reasons, the reversible dissociation of 7S to yield active NGF-β and NGF-γ may represent a key regulatory event for this growth factor complex. Dissociation of 7S is promoted by dilution, changes in pH, and EDTA; the profound destabilization of 7S caused by EDTA reflects the removal of zinc from the complex (Pattison & Dunn, 1976; Bothwell & Shooter, 1978).

The physiologic function of 7S remains unclear. Aggressive behavior in mice results in the massive release of NGF from salivary glands into the blood (Aloe et al., 1986). Once NGF-γ is liberated in the plasma, the proteinase may activate plasminogen and thereby stimulate the fibrinolytic system (Orenstein et al., 1978). Other potential plasma substrates for NGF-γ have not been identified. The regulation of NGF-γ by plasma proteinase inhibitors also remains unclear. Au and Dunn (1977) reacted NGF-γ with a variety of proteinase inhibitors. None of the inhibitors which are normally present in blood demonstrated significant activity; however, α_2-macroglobulin (α_2M) was not studied.

α_2M is a large tetrameric glycoprotein (M_r 718 000) present at high concentration in the plasma (2.0–4.0 μM) and an inhibitor of proteinases from all four major classes (Sottrup-Jensen, 1987). When α_2M reacts with a proteinase, the inhibitor undergoes a major conformational change so that the proteinase is irreversibly "trapped" (Barrett & Starkey, 1973; Gonias et al., 1982). The active site of the bound proteinase is not covalently modified and therefore active against small amide or ester-based substrates (Ganrot, 1966; Rinderknecht et al., 1975). In addition to its role as a proteinase inhibitor, α_2M may also function as a carrier of

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[1] Abbreviations: NGF-γ, nerve growth factor γ; hα_2M, human α_2-macroglobulin; mα_2M, murine α_2-macroglobulin; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; BAPNA, N^{α}-benzoyl-DL-arginine *p*-nitroanilide; PNPGB, *p*-nitrophenyl *p*-guanidinobenzoate.

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various cytokines and growth factors (Gonias, 1992), including NGF-β (Ronne et al., 1979; Koo & Stach, 1989; Koo & Liebl, 1992).

In this investigation, we have demonstrated that NGF-γ is inhibited by both human α_2M (hα_2M) and murine α_2M (mα_2M). In contrast with other α_2M–proteinase complexes, the activity of NGF-γ, when bound to hα_2M or mα_2M, was almost entirely suppressed. Slow reaction occurred between hα_2M and NGF-γ which was introduced as part of intact 7S complex; however, this reaction was probably dependent primarily or exclusively on 7S dissociation.

EXPERIMENTAL PROCEDURES

Materials. D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) was from Chemica Alta (Edmonton, Alberta). Trypsin, N^{α}-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) and *p*-nitrophenyl *p*-guanidinobenzoate (PNPGB) were from Sigma. Na^{125}I was from Amersham Internatonal, and Enzymobeads were from Bio-Rad (Richmond, CA). Mouse submaxillary glands were from Pel Freeze (Rogers, AZ).

Proteins. Human α_2M (hα_2M) was prepared according to the method of Imber and Pizzo (1981). Murine α_2M (mα_2M) was isolated as previously described (Anonick et al., 1989). Absorbance coefficients ($A^{1\%}_{1cm,280nm}$) of 8.93 and 7.8 were utilized to determine the concentrations of hα_2M (Hall & Roberts, 1978) and mα_2M (Gonias et al., 1983), respectively. Hα_2M–methylamine was prepared by reacting hα_2M with 200 mM methylamine hydrochloride at pH 8.2 for 4 h followed by extensive dialysis. After reaction with methylamine, hα_2M adopts a conformation that is nearly equivalent to that of hα_2M which has reacted with proteinase (Gonias et al., 1982).

7S NGF complex and NGF-γ were prepared according to the method of Darling and Shooter (1984) and were homogeneous as determined by SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Trypsin and NGF-γ were active-site-titrated with PNPGB by the method of Chase and Shaw (1967). The concentration of active NGF-γ in the 7S complex was determined by active-site titration with PNPGB after incubation in 5.0 mM EDTA for 30 min to force NGF-γ dissociation (Young et al., 1988). In the present studies, the indicated concentrations of trypsin, free NGF-γ, and NGF-γ in 7S complex are the values determined by active-site titration.

Radioiodination. NGF-γ was radioiodinated with Enzymobeads, as described by the manufacturer. The specific activity was 0.1–0.3 μCi/μg. ^{125}I-NGF-γ retained greater than 95% of the activity of nonradiolabeled NGF-γ, as determined by the velocity of BAPNA hydrolysis.

Inhibition of NGF-γ by PPACK. NGF-γ was incubated with the chromogenic substrate BAPNA (1.0 mM) in the sample cuvette of a Hewlett-Packard 8450 diode-array spectrophotometer at 37 °C. The buffer was 50 mM Tris-HCl, pH 7.4. The absorbance at 410 nm was determined every 5 s. After 300 s, PPACK (final concentration 46 μM) was added, and absorbance monitoring was continued. Absorbance measurements were transformed using the first-derivative function (dA_{410}/dt) to yield substrate hydrolysis velocities which are directly proportional to the concentration of active enzyme at any given time.

Analysis of the Reaction of NGF-γ and 7S Complex with α_2M by PAGE. Reaction of α_2M with proteinases involves a conserved series of well-characterized steps (Sottrup-Jensen et al., 1987). After reversibly associating with α_2M, the proteinase cleaves one or more of the four α_2M subunits near the center of the sequence in an area termed the "bait region" (Barrett & Starkey, 1973; Harpel, 1973; Barrett et al., 1979; Swenson & Howard, 1979; Sottrup-Jensen et al., 1989). For reactions involving hα_2M, the loss of intact subunits (180 kDa) and the generation of subunit cleavage products (90 kDa) may be readily detected by SDS–PAGE under reducing conditions. With mα_2M, loss of intact subunits (primarily 160- and 35-kDa bands by SDS–PAGE) is also accompanied by the generation of 90-kDa cleavage products (Hudson & Koo, 1982; Anonick et al., 1989). Following bait region cleavage, hα_2M and mα_2M undergo a conformational change which may be detected as an increase in α_2M electrophoretic mobility by nondenaturing (native) PAGE; the increase in mobility is referred to as the slow → fast transformation (Barrett et al., 1979; Gonias et al., 1983). Once α_2M has undergone conformational change, the proteinase is irreversibly trapped.

In this investigation, bait region cleavage in hα_2M and mα_2M after reaction with NGF-γ or 7S complex was studied by SDS–PAGE on 5% slabs using the buffer system described by Gonias and Figler (1988). α_2M conformational change was studied by nondenaturing PAGE using the method of Van Leuvan et al. (1981). All incubations involving α_2M and proteinases were conducted at 37 °C. Reactions were terminated prior to electrophoresis by the addition of 0.16 mM PPACK (see Results).

Stoichiometry of NGF-γ Binding to Hα_2M. Hα_2M (1.4 μM) and ^{125}I-NGF-γ (2.9 μM) were incubated in 50 mM Tris-HCl, pH 7.4, for 1 h at 37 °C. The products were then subjected to gel-filtration chromatography on Superose-6. The flow rate was 0.4 mL/min. Binding stoichiometries (moles of NGF-γ per mole of hα_2M) were determined from the radioactivity coeluting in the "high-molecular weight" hα_2M peak (corrected for recovery which was always greater than 80%). When free ^{125}I-NGF-γ was subjected to chromatography in the absence of hα_2M, no radioactivity was recovered at the elution volume of hα_2M. The presented results represents the average (±SD) of seven separate experiments.

Amidase Activity. Hydrolysis of BAPNA (0.025–2.5 mM) by NGF-γ, hα_2M–NGF-γ complex, trypsin, and hα_2M–trypsin complex was studied at 37 °C in 50 mM Tris-HCl, pH 7.4. Hα_2M–NGF-γ was formed by incubating NGF-γ with a 4-fold molar excess of hα_2M for 1 h at 37 °C (conditions that resulted in complete binding of the proteinase to hα_2M). Hα_2M–trypsin complex was formed by reacting trypsin with a 4-fold molar excess of hα_2M. The final concentration of proteinase or the hα_2M–proteinase complex (during BAPNA hydrolysis) was 86 nM. The concentration of *p*-nitroanilide, generated as a product of BAPNA hydrolysis, was determined using an extinction coefficient of 10^4 M^{-1} cm^{-1}.

BAPNA is synthesized as a racemic mixture of D-BAPNA and L-BAPNA. L-BAPNA is cleaved by proteinases while D-BAPNA acts as a competitive inhibitor (Erlanger et al., 1961). Therefore, in the BAPNA hydrolysis experiments, the concentrations of substrate (S) and inhibitor (I) were always identical. Kinetic parameters (k_{cat}, K_M) were determined from double-reciprocal plots according to the equation:

$$1/v = (K_M/V_{max})/[S] + (1 + K_M/K_I)/V_{max}$$

v is the velocity of substrate hydrolysis determined from dA_{410}/dt. K_I is the dissociation equilibrium constant for D-BAPNA. k_{cat} is V_{max} divided by the concentration of active enzyme. For calculation of kinetic parameters, we assumed that K_M and K_I are identical. The y-intercept is then $2/V_{max}$, and the

x-intercept is $-2/K_M$. Erlanger et al. (1961) studied the hydrolysis of BAPNA by trypsin and reported a K_M of 0.9 mM and a K_I of 0.8 mM.

Rate of NGF-γ Inhibition by Hα$_2$M As Determined by BAPNA Hydrolysis. Inhibition of NGF-γ by hα$_2$M was studied under pseudo-first-order conditions. NGF-γ was allowed to hydrolyze BAPNA (1.0 mM) in 50 mM Tris-HCl, pH 7.4, at 37 °C for 300 s with continuous monitoring of the absorbance at 410 nm. Hα$_2$M (0.43–1.3 μM) was then added, and absorbance monitoring was continued for 1000 s. The final concentration of NGF-γ, after addition of hα$_2$M, was 86 nM. Velocities of substrate hydrolysis (v) were determined at 5-s intervals by transforming the data with the first-derivative function (dA_{410}/dt). In separate experiments, we determined that the velocity of BAPNA hydrolysis by α$_2$M–NGF-γ is 5.6% of that demonstrated by free NGF-γ under the conditions specified here. Therefore, in order to determine the fraction of free NGF-γ ($[\text{NGF-}\gamma]_{free}/[\text{NGF-}\gamma]_{total}$) at time = x, the following expression was used:

$$[\text{NGF-}\gamma]_{free}/[\text{NGF-}\gamma]_{total} = 1.06(v_{t=x}/v_{t=0}) - 0.059$$

Plots of $\log([\text{NGF-}\gamma]_{free}/[\text{NGF-}\gamma_{total}])$ against time were linear for more than 3 half-lives. Apparent pseudo-first-order rate constants (k_{app}) were determined from the slopes of the plots and corrected for the presence of substrate as follows:

$$k'_{app} = k_{app}(1 + [S]/K_M)$$

k'_{app} is the corrected value. [S] is the concentration of BAPNA (L-BAPNA + D-BAPNA), and K_m is the Michaelis constant for BAPNA hydrolysis by NGF-γ. k'_{app} was plotted against α$_2$M concentration, and $1/k'_{app}$ was plotted against $1/[\alpha_2M]$ as described by Kitz and Wilson (1962). Scheme A and Scheme B show two mechanisms for the reaction of an enzyme (E) with an irreversible inhibitor (I). In Scheme B, a reversible EI complex is formed (EI*) which then rearranges to form an irreversible complex (EI). Second-order inhibition rate constants (k''_{app}) can be derived from plots of $1/k'_{app}$ against $1/[I]$ irrespective of whether Scheme A or B applies (Kitz & Wilson, 1962).

Scheme A

$$E + I \rightarrow EI$$

Scheme B

$$E + I \leftrightarrow EI^* \rightarrow EI$$

Rate of NGF-γ Inhibition by Mα$_2$M As Determined by BAPNA Hydrolysis. Equimolar concentrations of NGF-γ and mα$_2$M (86 nM) were reacted in the presence of 2.0 mM BAPNA at 37 °C in 50 mM Tris-HCl, pH 7.4. A base-line BAPNA hydrolysis rate was established before addition of mα$_2$M, as described above. The mα$_2$M was then added, and absorbance monitoring was continued. With 2.0 mM BAPNA, the velocity of BAPNA hydrolysis by mα$_2$M was 7.0% of that demonstrated by free NGF-γ. Therefore, the ratio $[\text{NGF-}\gamma]_{free}/[\text{NGF-}\gamma]_{total}$ at different times ($t = x$) was determined as

$$[\text{NGF-}\gamma]_{free}/[\text{NGF-}\gamma]_{total} = 1.07(v_{t=x}/v_{t=0}) - 0.075$$

The reciprocal of $[\text{NGF-}\gamma]_{free}/[\text{NGF-}\gamma]_{total}$ was determined for each time, and the apparent second-order rate constant (k''_{app}) was determined by plotting $[\text{NGF-}\gamma]_{total}/[\text{NGF-}\gamma]_{free}$ against time according to the expression:

$$[\text{NGF-}\gamma]_{total}/[\text{NGF-}\gamma]_{free} = k''_{app}[\text{NGF}]_{total}t/(1 + [S]/K_M) + 1$$

$1 + [S]/K_M$ corrects for the effect of BAPNA on the NGF-γ/mα$_2$M reaction. The plot of $[\text{NGF-}\gamma]_{total}/[\text{NGF-}\gamma]_{free}$ against time should be linear only if the reactants combine in 1:1 stoichiometry to form product. When equimolar concentrations of mα$_2$M and proteinase are reacted, some ternary or 2:1 (proteinase to mα$_2$M) complexes may form together with mostly binary or 1:1 (proteinase to mα$_2$M) complexes (Hudson et al., 1987; Larsson et al., 1989; Sottrup-Jensen, 1987). Formation of 2:1 complexes would be expected to cause slight upward deviation in the plots; however, due to the rapidity of the reaction of mα$_2$M with NGF-γ, it was not possible to determine whether this deviation was present.

Intrinsic Fluorescence Spectroscopy. Fluorescence emission spectra were determined for hα$_2$M, NGF-γ, hα$_2$M–NGF-γ, and hα$_2$M–methylamine in 50 mM Tris-HCl, pH 7.4 at 37 °C, using a Perkin Elmer 650-10s thermostated fluorescence spectrophotometer. The excitation wavelength and slit width were 280 nm and 3 nm, respectively. The concentration of hα$_2$M was 0.1 μM. Spectra of hα$_2$M after reaction with NGF-γ were corrected by subtracting the corresponding free NGF-γ spectra. This spectrum subtraction procedure is legitimate since fluorescence changes resulting from reaction of hα$_2$M with proteinases are due to conformational change in the α$_2$M and not the proteinase (Bjork & Fish, 1982; Straight & McKee, 1982; Christensen & Sottrup-Jensen, 1984).

Rate of Reaction of NGF-γ with Hα$_2$M As Determined by Fluorescence. The kinetics of reaction of equimolar concentrations of NGF-γ with hα$_2$M were studied by measuring the increase in intrinsic fluorescence at 335 nm which accompanies hα$_2$M conformational change (Christensen & Sottrup-Jensen, 1984; Baramova et al., 1990). Excitation was at 280 nm, and the slit width was 3 nm. ΔF_{max} is the increase in intrinsic fluorescence observed when the reaction of hα$_2$M with NGF-γ is complete (measured at 2 h). ΔF_t is the increase in fluorescence at time t. The ratio $\Delta F_t/\Delta F_{max}$ was plotted against time. The results were also plotted according to the expression:

$$1/(1 - \Delta F_t/\Delta F_{max}) = a_0 k''_{app} t + 1$$

a_0 is the initial concentration of NGF-γ or hα$_2$M. As described above, deviation from linearity in this type of plot may be observed if some hα$_2$M–NGF-γ complexes form in 2:1 (proteinase to hα$_2$M) stoichiometry.

RESULTS

Inhibition of NGF-γ by PPACK. Different concentrations NGF-γ (20–120 nM) were allowed to hydrolyze BAPNA at 37 °C. Substrate hydrolysis rates ranged from 7 to 52 nM s^{-1}. When 46 μM PPACK was added, substrate hydrolysis was completely inhibited within the time required for mixing (2–3 s) in each of six different experiments.

Hα$_2$M Bait Region Cleavage by NGF-γ. NGF-γ cleaved the bait regions of hα$_2$M, converting 180-kDa subunits into 90-kDa digestion products (Figure 1). This conversion is typical for proteinases that react with hα$_2$M forming irreversible complexes (Barrett & Starkey, 1973; Harpel, 1973). When 1.4 μM hα$_2$M was reacted with 4.6 μM NGF-γ at 37 °C, subunit cleavage was apparent in less than 10 s; however, the reaction was progressive through at least 30 min. At 60 min, intact 180-kDa subunits still remained. When PPACK

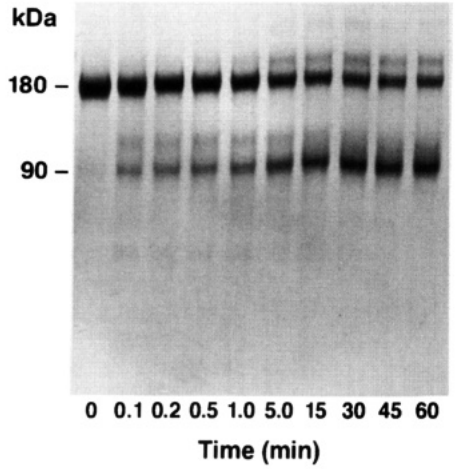

FIGURE 1: Hα_2M bait region cleavage by NGF-γ. Hα_2M (1.4 μM) was reacted with NGF-γ (4.6 μM) at 37 °C for the indicated times. Reactions were terminated with PPACK and the products analyzed by SDS–PAGE. The 180-kDa marker represents the intact hα_2M subunit, and the 90-kDa marker represents the bait region cleavage products.

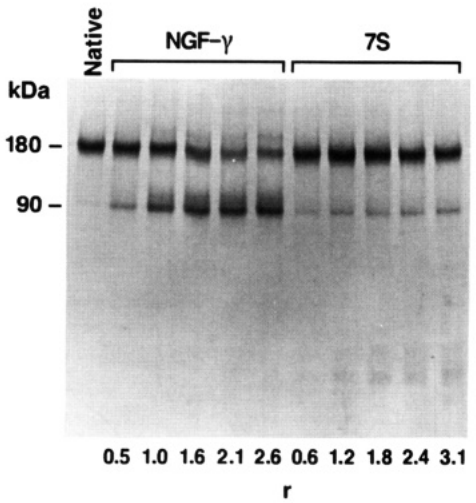

FIGURE 2: Hα_2M bait region cleavage by different concentrations of NGF-γ and 7S complex. NGF-γ and 7S complex were reacted with hα_2M (1.4 μM) for 1 h at 37 °C. The concentration ratio (r) of NGF-γ to hα_2M or NGF-γ within the 7S complex to hα_2M is shown.

(0.16 mM) and hα_2M were reacted simultaneously with NGF-γ, hα_2M subunit cleavage was completely inhibited.

Hα_2M bait region cleavage was studied as a function of NGF-γ concentration (Figure 2). The extent of subunit cleavage, after incubation for 1 h, maximized when the molar ratio (r) of NGF-γ to hα_2M was 2.1:1. At this ratio and at higher ratios, residual uncleaved 180-kDa subunits still remained. Incomplete α_2M subunit cleavage is characteristic of the reaction of α_2M with many proteinases (Sottrup-Jensen, 1987).

Hα_2M bait region cleavage was observed after incubation with 7S complex for 1 h at 37 °C; however, the extent of subunit cleavage was minimal compared with NGF-γ. When higher concentrations of 7S complex were studied or when the reaction time was prolonged, cleavage of the hα_2M bait regions was increased (data not shown).

Hα_2M Conformational Change after Reaction with NGF-γ. Hα_2M that was reacted with NGF-γ unnderwent conformational change as determined by the characteristic increase in mobility by nondenaturing PAGE (Figure 3). Reaction of equimolar concentrations of NGF-γ and hα_2M

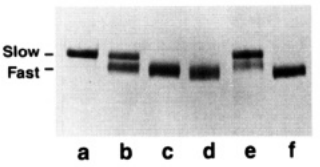

FIGURE 3: Nondenaturing PAGE of hα_2M after reaction with NGF-γ or 7S NGF. Native (unreacted) hα_2M is shown in lane a. Hα_2M (1.4 μM) was reacted with NGF-γ for 1 h at 37 °C and then subjected to native PAGE. The concentration of NGF-γ was 1.4 (lane b), 2.9 (lane c), and 4.4 μM (lane d). Hα_2M was reacted with 6.9 μM 7S complex (based on active-site titration of NGF-γ) in lane e. Hα_2M–trypsin is shown in lane f.

for 1 h converted only about half of the hα_2M into the "fast-form"; all of the hα_2M was converted when the NGF-γ was present in 2.1-fold molar excess to the hα_2M.

In separate experiments, the reaction of NGF-γ (2.8 μM) with hα_2M (0.7 μM) was studied by nondenaturing PAGE as a function of time. A progressive increase in the fraction of "fast-form" was observed; within 30 min, all of the hα_2M was converted (data not shown).

Reaction of hα_2M with a 4.9-fold molar excess of NGF-γ in 7S complex for 1 h caused a small fraction of the hα_2M to undergo conformational change (Figure 3, lane e). Further conversion of the hα_2m into the "fast-form" was observed when the concentration of 7S was increased or the time of reaction extended.

Stoichiometry of the Reaction of NGF-γ with Hα_2M. When 1.4 μM hα_2M was reacted with a 2.1-fold molar excess of NGF-γ, maximal hα_2M subunit cleavage was observed, and all of the hα_2M was converted into the electrophoretic "fast-form" (as shown above). Under identical conditions, the stoichiometry of ^{125}I-NGF-γ binding to hα_2M was 1.6 ± 0.1 mol/mol (n = 7). This result indicates that both ternary (2:1) and binary (1:1) NGF-γ–hα_2M complexes formed. The ability of α_2M to bind 2 mol of proteinase/mol depends on many factors which were outlined in the model presented by Larsson et al. (1989). Importantly, the frequency of ternary complex formation decreases as the incubation ratio (r) of proteinase to α_2M is decreased to values less than 2.0.

BAPNA Hydrolysis by NGF-γ and Hα_2M–NGF-γ. Previous investigators have demonstrated that proteinases which are bound to α_2M retain activity toward small chromogenic substrates; the catalytic efficiency is typically decreased by 50% or less (Rinderknecht et al., 1975; Gonias & Pizzo, 1983a; Cummings & Castellino, 1984). In this study, BAPNA hydrolysis by free NGF-γ, free trypsin, hα_2M–NGF-γ complex, and hα_2M–trypsin complex was examined. The free proteinases cleaved substrate in a manner consistent with simple Michaelis–Menten kinetics; apparently linear reciprocal plots were obtained (Figure 4, panel A). When trypsin was bound to hα_2M, the K_M was slightly increased, and the k_{cat} was slightly decreased compared with free trypsin (Table I). These changes were responsible for a 58% decrease in catalytic efficiency (k_{cat}/K_M).

When NGF-γ was bound to hα_2M, the activity of the proteinase was decreased to an extent which has not been demonstrated with other α_2M–proteinase complexes. An 8.6-fold increase in K_M and a 7-fold decrease in k_{cat} combined to cause a 98.5% decrease in catalytic efficiency compared with free NGF-γ.

Kinetics of NGF-γ Inhibition by Hα_2M. The significantly decreased BAPNA hydrolysis rate of hα_2M–NGF-γ compared with free NGF-γ was exploited to study the kinetics of NGF-γ inhibition. NGF-γ (86 nM) was reacted with different concentrations of hα_2M under pseudo-first-order conditions.

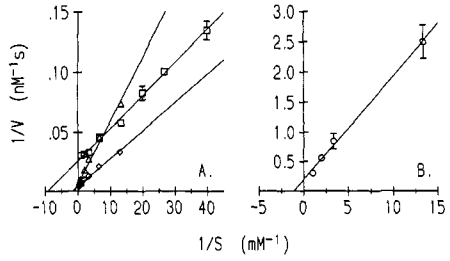

FIGURE 4: Hydrolysis of BAPNA by NGF-γ (□), trypsin (◇), and the hα2M–trypsin complex (△) is shown in panel A. Hydrolysis of BAPNA by the hα2M–NGF-γ complex (○) is shown in panel B (note the change in the scale of the axes). The concentration of active proteinase was 86 nM in all experiments. Missing error bars reflect errors that are within the size of the symbols.

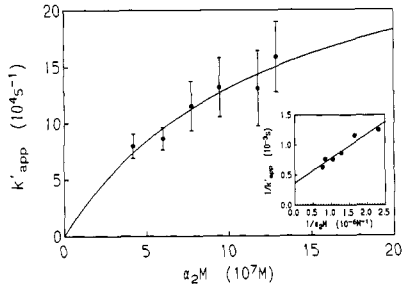

FIGURE 5: Pseudo-first-order rate constants (k'_{app}) for the inhibition of NGF-γ (86 nM) by different concentrations of hα2M. Reactions were conducted at 37 °C. Each value represents the mean ± SEM of at least three independent measurements. The inset shows a double-reciprocal plot of the same data.

Table I: Kinetic Parameters (±SEM) for the Hydrolysis of BAPNA by NGF-γ, Hα2M–NGF-γ Complex, Trypsin, and Hα2M–Trypsin Complex[a]

proteinase	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
NGF-γ	0.22 ± 0.02	0.91 ± 0.04	4.23 × 10³
α2M–NGF-γ	1.9 ± 0.3	0.13 ± 0.02	67.5
trypsin	1.1 ± 0.1	5.5 ± 0.3	5.09 × 10³
α2M–trypsin	2.3 ± 0.3	3.6 ± 0.2	2.15 × 10³

[a] Substrate cleavage was at 37 °C in 50 mM Tris-HCl, pH 7.4.

The k'_{app} increased as a function of hα2M concentration; however, the graph of k'_{app} against [hα2M] was nonlinear (Figure 5). The double-reciprocal plot was linear, and from this plot, a k''_{app} of (1.0 ± 0.1) × 10⁴ M^{-1} s^{-1} was determined for the inhibition of NGF-γ by hα2M.

Intrinsic Fluorescence of Hα2M and Hα2M–NGF-γ Complex. Hα2M conformational change results in an increase in protein intrinsic fluorescence (Bjork & Fish, 1982; Straight & McKee, 1982; Christensen & Storrup-Jensen, 1984; Baramova et al., 1990). As shown in Figure 6, native hα2M exhibited a typical tryptophan emission spectrum with a maximum at 330 nm. Reaction with methylamine caused a 3-nm blue shift in the emission maximum and a 45% enhancement in peak fluorescence. Reaction of hα2M (0.1 μM) with NGF-γ (0.26 μM) also caused the blue shift in the hα2M spectrum; the enhancement in fluorescence was 32%. This change is consistent with that reported for the hα2M–thrombin complex (Bjork & Fish, 1982). NGF-γ concentrations greater than 0.26 μM caused no further increase in fluorescence.

Rate of Hα2M Conformational Change during Reaction with NGF-γ. The intrinsic fluorescence at 335 nm was monitored in order to study the kinetics of hα2M conformational change during reaction with NGF-γ. Figure 7 shows that when equimolar concentrations of hα2M and NGF-γ

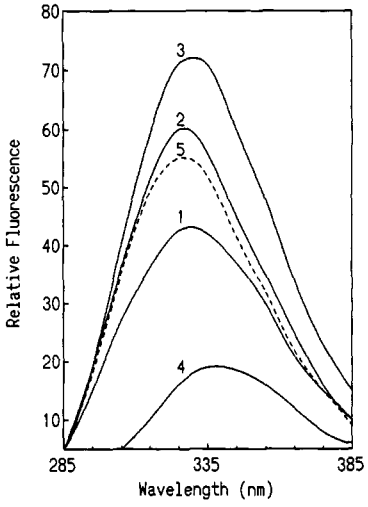

FIGURE 6: Intrinsic fluorescence spectroscopy of hα2M. Emission spectra were determined at 37 °C. The following spectra are shown: 1, native (unreacted) hα2M (0.1 μM); 2, hα2M–methylamine (0.1 μM); 3, hα2M reacted with 0.26 μM NGF-γ; 4, NGF-γ (0.26 μM); 5, spectrum of hα2M–NGF-γ corrected by subtraction for the contribution of NGF-γ.

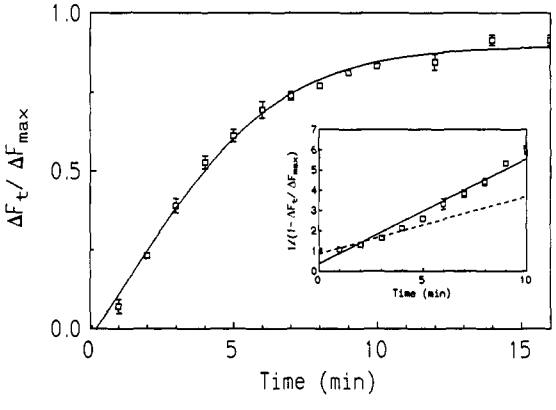

FIGURE 7: Kinetics of the intrinsic fluorescence change when NGF-γ (0.2 μM) reacts with hα2M (0.2 μM). ΔF_t is the increase in fluorescence at time t. ΔF_{max} is the fluorescence increase when reaction is complete. Estimates of k''_{app} were determined from the slopes in the inset. The solid line shows the linear regression of data collected within 10 min. The dashed line shows the linear regression of data collected within 4 min.

(0.2 μM) were reacted at 37 °C, the fluorescence increased progressively, reaching a near-maximum level within 15 min. The graph of $1/(1 - \Delta F_t/\Delta F_{max})$ against time was nonlinear (concave-up), in contrast with comparable plots presented for the reaction of hα2M with plasmin (Christensen & Sottrup-Jensen, 1984) and hemorrhagic metalloproteinases (Baramova et al., 1990). The most likely explanation for the nonlinearity is the formation of 2:1 (NGF-γ–hα2M) complexes in addition to 1:1 complexes. If this is the case, then earlier time points would be expected to yield a more reliable estimate of the rate of hα2M conformational change. Forced linear regression through 10 min in three separate studies yielded a k''_{app} of (4.1 ± 0.1) × 10⁴ M^{-1} s^{-1}. Analysis of the first 4 min yielded a k''_{app} of (2.3 ± 0.1) × 10⁴ M^{-1} s^{-1}. These values are in good agreement with the constants determined by substrate hydrolysis. Other studies performed with 0.1 μM hα2M and 0.1 μM NGF-γ or with 0.1 μM hα2M and 0.15 μM NGF-γ yielded similar constants [(1.0–3.0) × 10⁴ M^{-1} s^{-1}; data not shown]. The similarity in the rate constants determined for NGF-γ inhibition and hα2M conformational change supports models suggesting that α2M bait region cleavage, conforma-

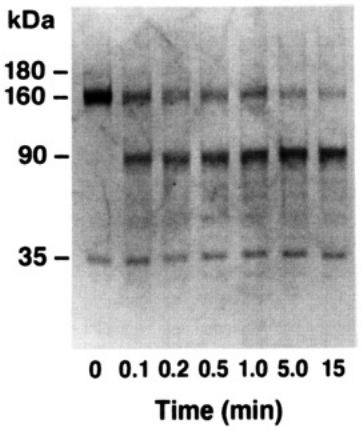

FIGURE 8: Mα_2M bait region cleavage by NGF-γ. Mα_2M (0.7 μM) was reacted with NGF-γ (2.8 μM) at 37 °C for the indicated times. Reactions were terminated with PPACK and the products analyzed by SDS–PAGE. The 180-, 160-, and 35-kDa bands are expected constituents of the mα_2M preparation.

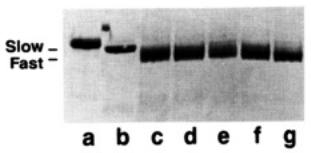

FIGURE 9: Mα_2M conformational change caused by reaction with NGF-γ at 37 °C for different amounts of time. The concentrations of mα_2M and NGF-γ were 0.7 and 2.8 μM, respectively. Reactions were terminated with PPACK and the products analyzed by nondenaturing PAGE. Lane a shows native mα_2M. Lanes b–g shows mα_2M reacted with NGF-γ for 5 s, 15 s, 30 s, 1 min, 5 min, and 15 min.

tional change, and proteinase inhibition occur in rapid succession (Sottrup-Jensen, 1987).

Reaction of NGF-γ with Mα_2M As Determined by PAGE. NGF-γ reacted with mα_2M, cleaving the mα_2M bait regions and generating primarily 90-kDa subunit fragments (Figure 8). Formation of the 90-kDA band is an expected consequence of complex formation between mα_2M and proteinases (Hudson & Koo, 1982; Anonick et al., 1989). A large percentage of the mα_2M subunits were cleaved within 6 s. The remaining 160-kDa subunits seemed to be cleaved at a slower rate.

Reaction of NGF-γ with mα_2M increased the mobility of mα_2M in nondenaturing PAGE (Figure 9). The increase in mobility indicates that mα_2M underwent conformational change (Gonias et al., 1983). The rate of conversion of mα_2M into the "fast-form" by NGF-γ was rapid. The majority of the mα_2M was apparently transformed within 5 s. This result suggests that mα_2M reacts with NGF-γ more rapidly than hα_2M.

Rate of NGF-γ Inhibition by Mα_2M. NGF-γ which was bound to mα_2M did not retain significant amidase activity, similar to hα_2M–NGF-γ complex. It was therefore possible to measure BAPNA hydrolysis in order to determine the rate of NGF-γ inhibition by mα_2M. Initial studies were performed under pseudo-first-order conditions equivalent to those used with hα_2M; however, the NGF-γ was completely inhibited by mα_2M within the time required for mixing. Therefore, experiments were performed using equimolar concentrations (86 nM) of NGF-γ and mα_2M. Under these conditions, reaction still proceeded rapidly to completion; however, it was possible to determine a rate constant. Data obtained before the reaction was 50% complete were utilized to determine the k''_{app} in order to minimize the error that may result from the formation of 2:1 (NGF-γ–mα_2M) complexes. The average value, derived from eight separate experiments with two separate mα_2M preparations, was $(3.6 \pm 0.4) \times 10^6$ M^{-1} s^{-1}.

DISCUSSION

The 7S nerve growth factor complex includes growth regulatory (β-subunit) and endopeptidase (γ-subunit) activity; however, both of these activities are apparently latent within the intact complex (Moore et al., 1974; Pattison & Dunn, 1975; Berger & Shooter, 1977; Bothwell & Shooter, 1978; Harris-Warrick et al., 1980; Fahnestock, 1991). Once secreted into the blood or extracellular spaces, 7S complex dissociation results in the expression of activity. The full range of possible substrates for NGF-γ (in and out of the blood) remains incompletely defined. Au and Dunn (1977) studied the inhibition of NGF-γ by pancreatic trypsin inhibitor, soybean trypsin inhibitor, lima bean trypsin inhibitor, ovomucoid, human α_1-antitrypsin, human antithrombin III, and human C-1 esterase inhibitor. Only pancreatic trypsin inhibitor reacted with NGF-γ. None of the plasma proteinase inhibitors demonstrated activity. Members of the protease nexin family inhibit NGF-γ (Knauer et al., 1982; Van Nostrand & Cunningham, 1987); however, the protease nexins are not typically found in plasma. Therefore, when the present study was initiated, potential regulators of NGF-γ in the blood remained unidentified.

The present investigation demonstrated that NGF-γ is inhibited by both hα_2M and mα_2M. As calculated from the rate constant for the reaction of hα_2M with NGF-γ and the known plasma concentration of hα_2M (2.0–4.0 μM), the half-life of free active NGF-γ in human plasma should be less than 30 s. The half-life of free active NGF-γ in murine plasma is about 0.1 s. These results indicate that α_2M is a major regulator of NGF-γ and the first such regulator identified among physiologically significant plasma proteinase inhibitors. Since α_2M is synthesized by a variety of cells outside the vascular compartment (Sottrup-Jensen, 1987), a role for α_2M in the regulation of NGF-γ in tissues may also be proposed. It should be noted that the existence of NGF-γ in humans remains unresolved. Considerable variability in the NGF system among mammalian species has been reported (Fahnestock, 1991).

The reaction of NGF-γ with hα_2M or mα_2M apparently occurred via a mechanism which is operational for most other proteinases. α_2M subunits were cleaved in the bait region as determined by SDS–PAGE. α_2M then underwent conformational change as determined by nondenaturing PAGE and intrinsic fluorescence. Conformational change is responsible for the irreversible trapping of proteinases within internal binding sites of the α_2M structure (Barrett & Starkey, 1973; Gonias et al., 1982; Sottrup-Jensen, 1987). Since bait region cleavage progresses through the steps of acylation and deacylation, the active site of the "trapped" proteinase is regenerated, and activity against small substrates is retained (Ganrot, 1966; Rinderknecht et al., 1975; Gonias & Pizzo, 1983a; Cummings & Castellino, 1984). The retention of amidase activity by proteinases bound to hα_2M was demonstrated in this study by comparing trypsin with α_2M–trypsin. In contrast, complexes formed by NGF-γ and hα_2M or mα_2M demonstrated unprecedented decreases in amidase activity. The reason why the α_2M–NGF-γ complex differs from other α_2M–proteinase complexes has not been fully determined; however, an interesting analogy may be drawn with the 7S NGF complex. The precursor form of NGF-β is a substrate for NGF-γ (Berger & Shooter, 1977; Isackson et al., 1987; Edwards et al., 1988; Jongstra-Bilen et al., 1989). After

cleavage of an Arg–Arg peptide bond near the C-terminus of the NGF-β precursor, the NGF-γ apparently associates with the new C-terminal Arg residue; this association is responsible for the strong inhibition of NGF-γ activity in the 7S complex (Moore et al., 1974; Berger & Shooter, 1977; Bothwell & Shooter, 1978). There are at least three different Arg residues capable of acting as a P_1 substrate element in the bait region sequence of hα2M (Mortensen et al., 1981). Cleavage of any of these bonds would result in the generation of a new C-terminal Arg residue which could then interact with the active site of NGF-γ similarly to the C-terminal Arg residue in processed NGF-β.

Among the many proteins of the α-macroglobulin family, sequence diversity is greatest in the bait region where proteinases initiate reaction (Sottrup-Jensen et al., 1989; Overbergh et al., 1991). A large number of peptide bonds within the bait region serve as substrates for attacking proteinases; the bait region peptide bond cleaved typically reflects the known specificity of the proteinase (Sottrup-Jensen, 1987; Sottrup-Jensen et al., 1989). Therefore, it is likely that the difference in the rates of inhibition of NGF-γ by mα2M and hα2M reflects differences in bait region sequence. Other examples of Arg-specific proteinases that react more rapidly with mα2M than with hα2M have been reported (Anonick et al., 1989).

Larsson et al. (1989) presented a model to explain variability in the stoichiometry of complex formation between α2M and proteinases. Each mole of α2M is capable of binding 2 mol of proteinase. When α2M reacts with a proteinase, the second proteinase binding site remains available for a finite period of time, after which the inhibitor undergoes a conformational rearrangement to yield a binary α2M–proteinase complex with greatly decreased residual proteinase binding activity. An important factor which determines the frequency of ternary complex formation is the rate of association of the proteinase with α2M binding sites. Our ability to detect a mixture of binary and ternary hα2M–NGF-γ complexes in experiments with ^{125}I-NGF-γ is consistent with the model of Larsson et al. (1989). If we decreased the concentrations of NGF-γ and hα2M (retaining the same ratio of reactants), the percentage of ternary complexes would be expected to decrease. In addition, decreasing the concentration ratio of NGF-γ to α2M during incubation (r) to values less than 2.0 substantially reduces the fraction of ternary complexes formed (Gonias & Pizzo, 1983b).

The slow progressive reaction of hα2M with NGF-γ within 7S NGF complex is consistent with the well-documented lack of activity of the proteinase in the 7S complex. It is highly likely that reaction depends for the most part if not entirely on NGF-γ dissociation. When EDTA was added to promote 7S dissociation, reaction with hα2M was accelerated (data not shown). We cannot at this time rule out a very low level of direct reaction of α2M with NGF-γ in the 7S complex.

Processes which might cause 7S complex dissociation under physiologic conditions are incompletely understood. In our experiments, high concentrations of 7S complex were studied; these conditions favor maintaining the majority of the NGF preparation in the multimeric state (Fahnestock, 1991). In the plasma and tissues, the dilute concentration of 7S complex and local shifts in pH would be expected to promote dissociation.

Dissociation of the 7S NGF complex yields functional polypeptides, all of which may be regulated by α2M. The β-subunit, with growth regulatory activity, binds noncovalently to α2M (Ronne et al., 1979; Koo & Stach, 1989; Koo & Liebl, 1992). The mechanism for this interaction is distinct from that of proteinase trapping (Gonias, 1992). As shown here, the γ-subunit reacts rapidly with α2M, transforming the α2M conformation into one which expresses higher affinity for many cytokines (LaMarre et al., 1991; Gonias, 1992). Whether regulation of NGF-β and NGF-γ may in some manner be coordinated remains to be determined.

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