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Nerve growth factor: A protease that can activate plasminogen

(mouse submandibular gland/fibrinolysis/urokinase/streptokinase/serine proteases)

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ABSTRACT **The single, highly stable form of mouse submandibular gland nerve growth factor (NGF), prepared as described by Young *et al.* [(1978) *Biochemistry* 17, 1490–1498] is a protease of restricted specificity that can convert plasminogen to plasmin. In the absence of plasminogen, NGF is not fibrinolytic, nor does it hydrolyze casein at a measurable rate. Treatment of NGF with diisopropyl fluorophosphate inhibits its ability to activate plasminogen as well as its capacity to hydrolyze certain synthetic arginine esters. These results indicate that NGF is a member of the class of serine proteases. Since NGF is known to be secreted at high concentrations in mouse saliva, it may serve to activate plasminogen (with subsequent fibrinolysis) somewhere in the alimentary tract. Plasminogen activation is the only known action of NGF upon a biologically important non-neural substrate.**

Recent studies from one of our laboratories (1, 2) have shown that extracts of the male mouse submandibular gland contain multiple molecular forms of nerve growth factor (NGF). By using several chromatographic procedures, we have resolved at least six distinct species (2). Of these six, only one (of molecular weight 116,000) remains intact at high dilution. All of the other five forms of NGF are unstable in dilute solution, and at relatively high concentrations they dissociate into their constituent polypeptide chains (2). Moreover, the stable 116,000-molecular weight NGF is the predominant form of the protein that is secreted in mouse saliva (1, 2). The origin of the several unstable forms of NGF is not clear, although it has been suggested that they probably arise from proteolytic degradation of the single stable form, either within the submandibular gland *in vivo* or during the process of isolation *in vitro* (2).

Until recently, two different molecular forms of mouse submandibular gland NGF have received extensive study. These two forms arise because two different schemes are used in their purification from gland extracts. One of these, called 2.5S NGF (3), has a molecular weight of 26,000, and the other, termed 7S NGF (4), has a molecular weight of approximately 140,000. These two proteins are related in that 7S NGF contains as part of its subunit structure a component that is closely similar to 2.5S NGF (4). In solution at relatively high protein concentrations, 7S NGF is unstable, and it dissociates to yield a mixture of its component polypeptide chains (5, 6). Both 2.5S and 7S NGF are biologically active in stimulating neurite outgrowth from embryonic sensory and sympathetic ganglia in culture (3, 4).

In 1968, Greene *et al.* (7) first observed that 7S NGF possessed enzymic activity with certain synthetic arginine-containing substrates. We now show that the completely purified, stable form of NGF described above (2) is also an arginine esterase as well as a highly specific protease that can promote fibrinolysis by converting plasminogen to plasmin. Moreover, release of soluble peptides from fibrin is strictly plasminogen dependent—no detectable hydrolysis of fibrin occurs in the absence of plasminogen. To our knowledge, this is the only known biochemical effect of NGF upon a physiologically important, non-neural substrate.§

MATERIALS AND METHODS

Reagents. All buffer salts were reagent grade. $Na^{125}I$ was obtained from New England Nuclear; Sephadex and blue dextran 2000, from Pharmacia; and bovine serum albumin (three times recrystallized), from Sigma. Plasminogen-free human fibrinogen was a generous gift from Yu-Lee Hao, American Red Cross, Washington, DC. It was iodinated with ^{125}I by the method of Helmkamp *et al.* (9). Plasminogen was isolated from fresh human plasma by the method of Deutsch and Mertz (10). Human urokinase was made available through the courtesy of William Blackmore (Sterling–Winthrop Laboratories) and J. C. Fratantoni (National Heart, Lung, and Blood Institute). Fetal calf serum was obtained from GIBCO, and chloramine-T was the Eastman product. Tosyl-L-arginine methyl ester (Tos-Arg-OMe), tosyl-L-arginine amide (Tos-Arg-NH_2), tosyl-L-lysine methyl ester (Tos-Lys-OMe), benzoyl-L-tyrosine ethyl ester (Bz-Tyr-OEt), and 2-mercaptoethanol were obtained from Sigma. Bovine pancreatic trypsin and chymotrypsin were obtained from Worthington, and azocasein, from Pentex, Kankakee, IL. Diisopropyl fluorophosphate (iPr_2-*P*-F) was the Pfaltz–Bauer product.

Plasminogen Activator Assay. Conversion of plasminogen to plasmin was determined by measurement of the release of soluble ^{125}I-labeled fibrin (^{125}I-fibrin) peptides from ^{125}I-fibrin as described earlier (11). One hundred percent activity was defined as the amount of ^{125}I-fibrin peptides solubilized by an excess of urokinase.

Preparation of NGF. The stable 116,000-molecular weight form of NGF from male mouse submandibular glands was completely purified as described (2). 2.5S NGF was purified by the procedure of Bocchini and Angeletti (3).

Physical Methods. Protein concentrations were measured by absorbance at 280 nm (2) and by the Lowry procedure (12). Hydrolysis of the several synthetic protease substrates was followed with a Cary model 15 double beam recording spectrophotometer (λ = 247 nm: Tos-Lys-OMe, Tos-Arg-NH_2, Tos-Arg-OMe; λ = 256 nm: Bz-Tyr-OEt). Polyacrylamide gel

Abbreviations: NGF, nerve growth factor; 2.5S NGF, NGF isolated as in ref. 3; 7S NGF, NGF isolated as in ref. 4; Tos-Lys-OMe, α-*N*-*p*-tosyl-L-lysine methyl ester; Tos-Arg-OMe, α-*N*-*p*-tosyl-L-arginine methyl ester; Tos-Arg-NH_2, α-*N*-*p*-tosyl-L-arginine amide; Bz-Tyr-OEt, α-*N*-benzoyl-L-tyrosine ethyl ester; iPr_2P-F, diisopropyl fluorophosphate.

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§ It will be shown elsewhere that the enzymic properties of the stable salivary gland NGF used in the present study (2) are strikingly different from those that have been reported for 7S NGF (7, 8)—differences which may stem from the instability of 7S NGF. Whether 7S NGF can convert plasminogen to plasmin is not known, but it seems reasonable to suspect that it would.

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Table 1. Hydrolysis of synthetic ester and amide substrates by NGF*

Substrate	NGF, μg/ml	Specific activity, μmol/min·mol
Tos-Arg-OMe	1.0	6.96×10^5 (7.90×10^5)†
Tos-Lys-OMe	8.8	4.64×10^4 (9.12×10^5)†
Tos-Arg-NH_2	8.8	None detected
Bz-Tyr-OEt	8.8	None detected (2.93×10^6)‡

* Solvent = 0.1 M potassium phosphate, pH 7.0; temperature = 25°C. All substrates were tested at a concentration of 1 μmol/ml.

† Numbers in parentheses are specific activities of trypsin expressed on a molar basis.

‡ Number in parentheses is specific activity of chymotrypsin expressed on a molar basis.

electrophoresis of NGF was performed as follows at 4°C (2). Samples were electrophoresed with 7.5% polyacrylamide gels at neutral pH. Both upper and lower reservoir buffers contained 0.05 M sodium phosphate, pH 7.0. Protein was dissolved in 0.01 M sodium phosphate, pH 7.0, containing 0.5 M sucrose and a trace of bromphenol blue. Electrophoresis was performed at a constant current of 8 mA/gel. Under these conditions NGF migrates toward the anode (2).

Radioimmunoassay of NGF. NGF, purified as previously described (2), was iodinated as follows: NGF (5.0 μg in a total volume of 10 μl) was added to 2 mCi of $Na^{125}I$ dissolved in 300 μl of 0.40 M potassium phosphate, pH 7.0. Chloramine-T (1 mg/ml dissolved in H_2O), 10 μl, was added at 25°C, followed 30 sec later by 10 μl of a 36 mM solution of 2-mercaptoethanol in H_2O. ^{125}I-labeled NGF (^{125}I-NGF) was separated from the iodination reagents by Dowex-1 chromatography (13).

Antisera to NGF were prepared in rabbits as follows: NGF (200 μg) in complete Freund's adjuvant was injected subcutaneously in multiple sites. The animals were given booster shots 26 days later with 100 μg of NGF injected in the same fashion; this was repeated after an additional 36 days and the animals were bled 6 days later. Antisera prepared in this way quantitatively bind ^{125}I-NGF at a final dilution of $1:10^4$. The radioimmunoassay procedure is exactly as described for 2.5S NGF (13, 14).

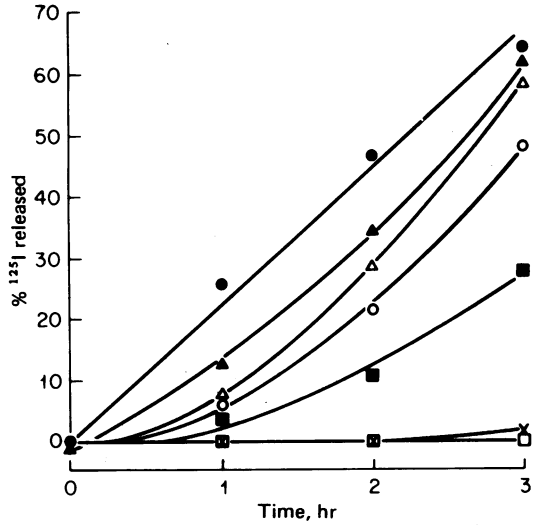

FIG. 1. Conversion of plasminogen to plasmin by NGF. Plasminogen activation assays were performed as described in the text as a function of NGF concentration. The total volume of each reaction mixture was 0.5 ml. ●, 0.083 ng of urokinase; ▲, 880 ng of NGF; △, 440 ng of NGF; ○, 220 ng of NGF; ■, 88 ng of NGF; □, 600 ng of NGF in the absence of plasminogen; ×, 1100 ng of 2.5S NGF.

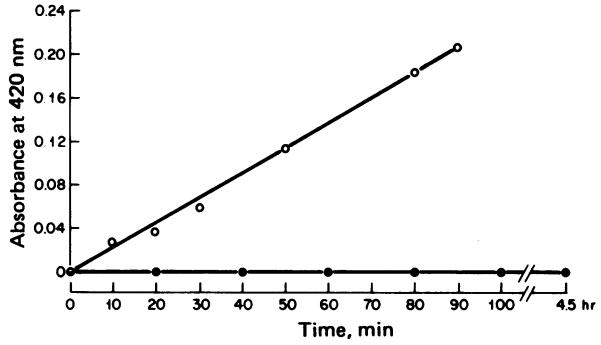

FIG. 2. Enzymatic activity of NGF and trypsin upon azocasein. Solutions of azocasein (4 mg/ml dissolved in 0.1 M potassium phosphate, pH 7.0) were treated with trypsin at 1 μg/ml (○) or NGF at 4.3 μg/ml (●) at 25°C. At the indicated time periods, protein was precipitated by addition of 10% perchloric acid at 0°C, and the concentrations of soluble peptides were measured by absorbance at 420 nm.

RESULTS

By using NGF preparations isolated and purified as described (2), we have measured enzymic activity upon several synthetic ester substrates. Table 1 reveals that this structurally stable form of NGF hydrolyzes both Tos-Arg-OMe and Tos-Lys-OMe. The rate of hydrolysis of Tos-Arg-OMe is nearly ten times that of Tos-Lys-OMe, and on a molar basis NGF is as active as trypsin with Tos-Arg-OMe as substrate. No hydrolysis of Tos-Arg-NH_2 or Bz-Tyr-OEt was detected. Moreover, 2.5S NGF has no detectable activity with any of the substrates listed in Table 1.

These observations suggested that the 116,000-molecular weight form of NGF is a protease of restricted specificity. The general class of plasminogen activators [e.g., urokinase (15) and the tissue activator secreted by cells in culture (16)] all have a high degree of specificity for an arginine residue on the

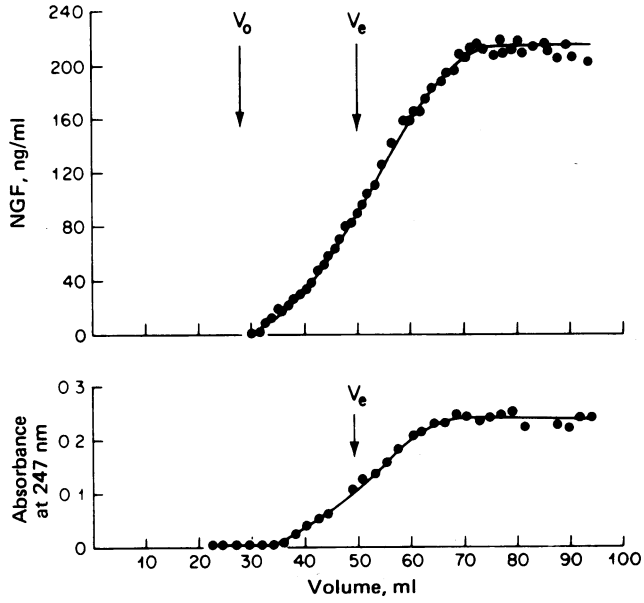

FIG. 3. Sephadex G-200 frontal elution profile of NGF. A large volume of NGF (100 ml) at 210 ng/ml was applied to a 1.6 × 46-cm column of Sephadex G-200 to establish a plateau region of concentration. Individual fractions were measured by radioimmunoassay (*Upper*) and by Tos-Arg-OMe hydrolysis (*Lower*). Tos-Arg-OMe concentration was 1 μmol/ml at 25°C. Absorbance (247 nm) was measured 5.5 hr after addition of substrate at 25°C. V_e and V_0 represent the elution volumes of NGF and dextran blue 2000, respectively. Column solvent, 0.1 M potassium phosphate (pH 7.0) containing 1 mg of bovine serum albumin per ml; temperature, 4°C.

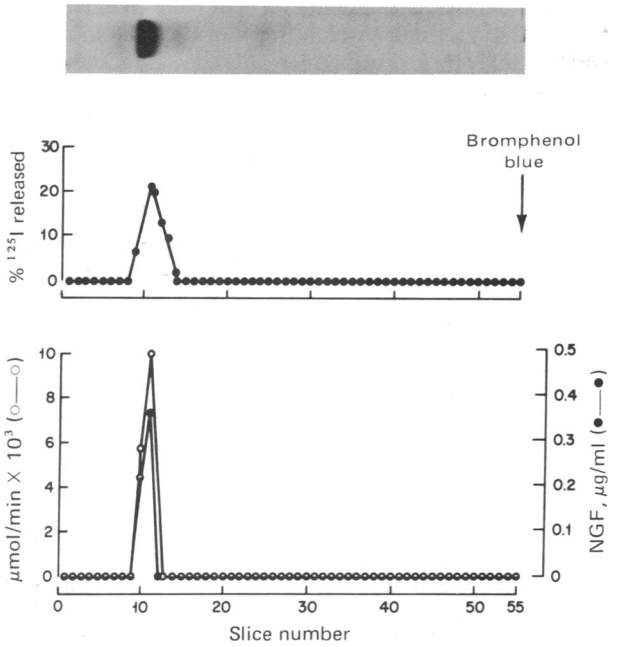

FIG. 4. Gel electrophoretic mobility of NGF as measured by protein staining, radioimmunoassay, Tos-Arg-OMe hydrolysis, and plasminogen activation. NGF (10 μg in 20 μl) was applied to each of three gels as described (2) at 4°C. (*Top*) Gel stained with Coomassie blue. (*Middle*) Gel was divided into 1-mm slices, which were then eluted with 0.1 M potassium phosphate (pH 7.0) and measured for plasminogen activator activity. (*Bottom*) Gel was sliced and NGF was measured by hydrolysis of Tos-Arg-OMe (○) and by radioimmunoassay (●).

COOH-terminal side of the bond to be split. We therefore sought to determine whether NGF had plasminogen activator activity.

Fig. 1 reveals that NGF is indeed capable of converting plasminogen to plasmin. Several concentrations of NGF were examined in the ^{125}I-fibrin plate assay for comparison with urokinase. A lag phase occurred in the release of ^{125}I-soluble peptides and the duration of this lag phase increased with decreasing concentrations of NGF. No lag phase was detected with urokinase. [A kinetic lag phase in the cleavage of fibrin has also been observed in assays of the plasminogen activator secreted by cells in culture (16).] Fig. 1 also reveals that the release of soluble ^{125}I-fibrin peptides by NGF is strictly plasminogen dependent. No cleavage of fibrin was detected in the absence of plasminogen. In contrast, 2.5S NGF has little, if any, activity in this assay system.

The results of Fig. 2 indicate that, whereas trypsin readily hydrolyzes azocasein, NGF does not, at least under the conditions of these experiments (4.5 hr at 25°C). Thus, NGF possesses a considerable degree of proteolytic specificity, because neither fibrin (see Fig. 1) nor azocasein is hydrolyzed.

To minimize the possibility that the conversion of plasminogen to plasmin depicted in Fig. 1 arose from a contaminant of the NGF preparations, gel filtration chromatography as well as gel electrophoretic studies were carried out with parallel measurements of (*i*) Tos-Arg-OMe hydrolytic activity, (*ii*) plasminogen activator activity, and (*iii*) radioimmunoassay.

Fig. 3 (*upper*) illustrates a G-200 Sephadex frontal elution profile of NGF at a protein concentration of 210 ng/ml as measured by radioimmunoassay. Fig. 3 (*lower*) shows the elution profile of NGF as measured by hydrolysis of Tos-Arg-OMe. Within experimental error, the elution volumes (V_e = 51.0 ml, immunoassay; and V_e = 49.5 ml, Tos-Arg-OMe hydrolysis) are indistinguishable.

To compare the mobility of enzymic activity with protein

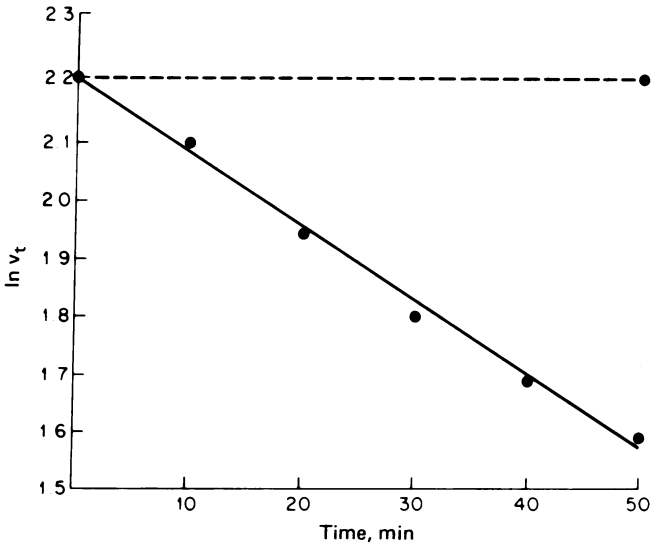

FIG. 5. Kinetics of inhibition of NGF by iPr_2P-F with Tos-Arg-OMe as substrate. A solution of NGF (1.89 μg/ml) dissolved in 0.1 M potassium phosphate, pH 7.0, was treated with iPr_2P-F (in isopropanol) at a final concentration of 0.9 mM at 25°C. At the indicated times, the reaction velocity of Tos-Arg-OMe hydrolysis was measured in the presence of 1 μmol of Tos-Arg-OMe per ml. Solutions of NGF containing the same final concentration of isopropanol were used as controls. —, iPr_2P-F-treated NGF; - - -, control.

mobility in yet another system, we carried out the studies illustrated by Fig. 4. NGF was subjected to polyacrylamide gel electrophoresis upon three identical gels. One gel was stained for protein; the second was sliced into 55 1-mm slices for measurement of Tos-Arg-OMe hydrolysis and for determination of NGF by radioimmunoassay; and the third gel was sliced for determination of plasminogen activator activity (see the legend to Fig. 4 for details). As shown in Fig. 4, Tos-Arg-OMe hydrolysis, NGF by radioimmunoassay, the stained band, and plasminogen activator activity all had identical electrophoretic mobilities in this system. Taken together, the results of Figs. 3 and 4 indicate that the activation of plasminogen by NGF is a specific property of this protein and that it is not due to a contaminating enzyme.

The results illustrated by Figs. 5 and 6 demonstrate that the enzymic activity of NGF is inhibited by iPr_2P-F. Fig. 5 presents a plot of ln v_t versus time after treatment of NGF with iPr_2P-F

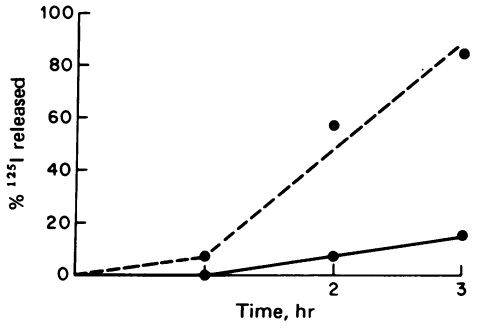

FIG. 6. Inhibition of plasminogen activator activity of NGF by iPr_2P-F. A solution of NGF (3.5 μg/ml dissolved in 0.1 M potassium phosphate, pH 7.0) was treated for 1 hr with iPr_2P-F at a final concentration of 16 mM. An identical control solution of NGF was treated for the same time period with the same volume of isopropanol used for the iPr_2P-F reaction. Both samples were then dialyzed overnight at 4°C against a large volume of 0.1 M Tris·HCl, pH 8.0, to remove iPr_2P-F. Aliquots (250 μl) of these solutions were then assayed for plasminogen activation as described in the text. —, iPr_2P-F-treated NGF; - - -, control.

at a final concentration of 0.9 mM. Here, v_t represents the velocity of Tos-Arg-OMe hydrolysis at time t after addition of iPr_2P-F. It will be seen that iPr_2P-F inhibits NGF according to second-order kinetics, as it does in its inhibition of trypsin, urokinase, and the tissue-derived plasminogen activator secreted by cells in culture (16). The second-order rate constant for the NGF/iPr_2P-F reaction in the presence of 0.9 mM iPr_2P-F is 14.4 liter mol^{-1} min^{-1}, and the half-time is 53 min. Fig. 6 shows that iPr_2P-F also inhibits activation of plasminogen by NGF. Taken together, the results of Figs. 5 and 6 indicate that NGF is a iPr_2P-F-inhibitable enzyme and that, as such, it is a member of the general class of serine proteases.

DISCUSSION

The results presented above indicate that the single stable form of mouse submandibular gland NGF (2) is a protease and that it is capable of converting plasminogen to plasmin. The results of Figs. 3 and 4 strongly indicate that this activity is not due to a contaminating enzyme, and the results of Figs. 1 and 2 show that NGF displays a considerable degree of substrate specificity.

Furthermore, the results of Figs. 5 and 6 indicate that hydrolysis of Tos-Arg-OMe as well as the activation of plasminogen by NGF is inhibited by iPr_2P-F. Consequently, we infer that NGF, like tissue activator (16), is a member of the general class of serine proteases.

From examination of the legend to Fig. 1, it will be appreciated that the plasminogen-to-plasmin converting activity of mouse NGF is much less than that of human urokinase. On the other hand, it is not at all clear that urokinase should be used as the standard for comparison. For example, plasminogen activators are known to be secreted by several types of neoplastic cells in culture (16), and the specific activities of these enzymes (on a molar basis) remain to be compared directly with that of NGF. In this regard, it should be noted that NGF has also been shown to be secreted by a wide variety of both primary as well as transformed cells in culture (17). Whether cell-secreted plasminogen activator and cell-secreted NGF are structurally similar remains to be determined.

The biologic significance of the plasminogen activator activity of NGF is not clear. For example, plasminogen activator is only one of many proteases with restricted substrate specificity toward certain basic amino acid-containing peptide bonds, including some enzymes of the blood-clotting system (18), the complement system (19), and the kallikrein system (20). Thus, it could be that plasminogen is not the physiologic substrate for NGF *in vivo*. Alternatively, it has been shown that very high concentrations of NGF are secreted in mouse saliva (1, 21) and that the predominant form of NGF in saliva is the stable 116,000-molecular weight form of the protein used in the present study (1, 2). Furthermore, the concentration of NGF that is continuously secreted into mouse saliva is nearly 1000 times the maximal concentration depicted in the plasminogen activator assay shown in Fig. 1 (1). Consequently, mouse saliva NGF may play a role in the activation of plasminogen somewhere in the alimentary tract.

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