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Proteolytic Activity of Nerve Growth Factor: A Case of Autocatalytic Activation†

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ABSTRACT: Nerve growth factor is a highly specific protease that can convert plasminogen to plasmin and that can hydrolyze certain synthetic N-substituted arginine esters (e.g., N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester (TAME); N. S. Orenstein et al. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5497). Hydrolysis of TAME is characterized by a lag phase of lower velocity which precedes development of the steady-state maximal velocity. Kinetic analyses indicate that this behavior stems from autocatalytic activation of a nerve growth factor (NGF)-zymogen by NGF. As isolated from the mouse submandibular gland at high concentration, NGF is largely enzymically inactive. Upon high dilution, the protein undergoes autocatalytic activation with concomitant generation of full enzymic activity. The biologic significance of this unusual property of NGF is not clear, but it may serve to prevent expression of enzymic activity until the protein reaches its target cell(s) or until it recognizes its physiological substrate.

Recent studies have shown that crude extracts of male mouse submandibular glands contain multiple molecular forms of nerve growth factor (NGF)[1] (Young et al., 1978). In fact, by using a variety of chromatographic and electrophoretic procedures, at least six different forms of NGF can be observed. Of these six species, only one is stable in highly dilute solution; i.e., it does not dissociate into its constituent polypeptide chains. In contrast, all other forms of mouse salivary gland NGF are appreciably unstable in dilute solution. These observations led us to propose that the several unstable forms of the protein arise by proteolytic degradation of the single stable form—either in vivo or during the process of isolation in vitro (Young et al., 1978).

The single stable form of gland NGF (HMW-NGF) has been purified to homogeneity by chromatographic, ultracentrifugal, and electrophoretic criteria (Young et al., 1978). It has a molecular weight of 116000, and it contains as part of its quaternary structure a noncovalently linked subunit(s) which is immunochemically and electrophoretically indistinguishable from that of the smaller 2.5S form of NGF, first described by Bocchini & Angeletti (1969). Furthermore, the 116000 molecular weight stable form of the protein is the predominant species of NGF which is secreted in extraordinarily high concentrations in mouse saliva (Murphy et al., 1977a,b). Thus, it would appear that this is the principal form of NGF which is destined for export by the mouse submandibular gland.

One other form of high molecular weight salivary gland NGF has been described. In 1967, Varon et al. (1967) reported the isolation of an NGF species with a sedimentation coefficient of 7 S, from which a molecular weight of 140000 was estimated. These authors also reported that all of the NGF activity in gland homogenates is associated with the 7S species. We have been unable to confirm this result (Young et al., 1978). Furthermore, it is now known that, like the multiple unstable species of NGF present in gland extracts, 7S-NGF also is unstable and that it dissociates into its subunits at relatively high protein concentrations (Baker, 1975; Pantazis et al., 1977).

In another study, Greene et al. (1969) observed that 7S-NGF possessed esterase activity toward several N^{α}-substituted arginine and lysine ester substrates (e.g., TAME). This observation led us to ask whether the stable 116000 molecular form of gland NGF referred to above also exhibits esterase activity—and further, what protein substrates it might hydrolyze. In a recent study, we have shown that the stable form of NGF also hydrolyzes arginine and lysine esters and furthermore that it can convert plasminogen to plasmin with subsequent lysis of a fibrin clot (Orenstein et al., 1978). NGF-mediated fibrinolysis is strictly plasminogen dependent: no fibrin hydrolysis occurs in the absence of plasminogen. DFP inhibits the plasminogen activation reaction, and thus HMW-NGF appears to be a member of the general class of serine proteases. At the present time, plasminogen activation is the only known enzymic action of HMW-NGF upon a substrate of physiologic importance and of nonneural origin. Whether the reaction has any physiologic significance is not known.

In their study on the hydrolysis of arginine esters by 7S-NGF, Greene et al. (1969) observed that, when the protein was diluted from high to low concentration, the maximum catalytic velocity of the reaction was achieved only after a lag phase of lower velocity. Furthermore, predilution of the

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[1] Abbreviations used: NGF, nerve growth factor; HMW-NGF, high molecular weight form of NGF prepared as described by Young et al. (1978); EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl phosphofluoridate; TAME, N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester.

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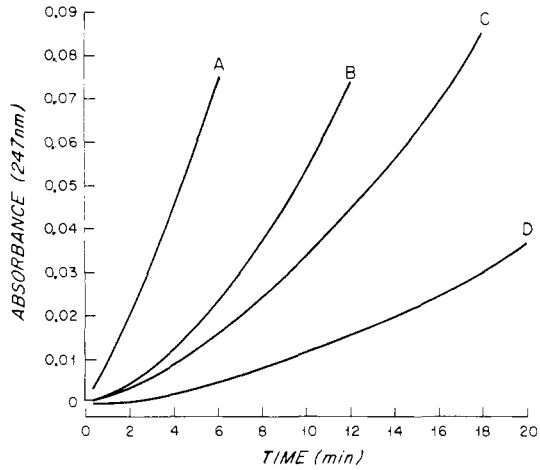

FIGURE 1: Enzymic activity of HMW-NGF as a function of enzyme concentration and time. A solution of HMW-NGF (420 μg/mL) was diluted with 0.1 M potassium phosphate, pH 7.0, containing 2 mM TAME at 25 °C, and the increase in absorbance (247 nm) was recorded vs. time: (A) 10.4 μg/mL HMW-NGF; (B) 5.2 μg/mL; (C) 2.6 μg/mL; (D) 0.52 μg/mL.

enzyme for a period of time (followed by addition of substrate) served to abolish the lag phase. These authors interpreted their results on the basis of dilution-induced progressive dissociation of 7S-NGF into its subunits, with concomitant appearance of enzymic activity (see also Smith et al. (1969) for a similar interpretation).

In the study presented below, we have examined the kinetic behavior of the stable form of salivary gland HMW-NGF with respect to the time course of its hydrolysis of a synthetic ester substrate. A lag phase is observed, and all available evidence indicates that HMW-NGF comprises an autocatalytic self-activating enzyme system. At high concentrations, the protein is largely inactive; but, as the protein concentration is progressively reduced, an NGF-zymogen is autocatalytically activated with ultimate generation of full enzyme activity. This behavior, not dissociation, is responsible for the lag phase. Other than the autocatalytic activation of trypsinogen and pepsinogen (Kunitz & Northrup, 1936; Herriott, 1938a,b), we know of no precedent for this type of reaction.

Experimental Procedures

Reagents. Double-glass-distilled H_2O was used for all solutions, and buffer salts were reagent grade. DFP was obtained from Pfaltz and Bauer, Stamford, CT, and TAME from Sigma. HMW-NGF was prepared as previously described (Young et al., 1978). All preparations were shown to be electrophoretically homogeneous, and four different preparations were used in this study. Protein solutions were stored at −20 °C in 200-μL aliquots with 0.10 M potassium phosphate, pH 7.0, as solvent.

Physical Measurements. Protein concentrations were measured by absorbance at λ 280 nm with use of an extinction coefficient of 1.92 mL mg^{-1} cm^{-1} (Young et al., 1978). Enzyme reaction rates were measured with TAME as substrate (1–2 mM) and with a Cary Model 15 split-beam recording spectrophotometer. The molar absorbance of N^{α}-*p*-toluenesulfonyl-L-arginine was taken to be 540 at λ 247 nm (Hummel, 1959). All spectrophotometric measurements were recorded at 25 °C with 0.1 M potassium phosphate, pH 7.0, as solvent. For determination of reaction rates in the presence of higher concentrations of TAME (~5 mM), a Radiometer autotitrator (TTTI), autoburette, and titrigraph were used. For these studies, the solvent was 0.1 M KCl, and 1 mM NaOH was used as titrant at a constant pH 7.0 at 25 °C.

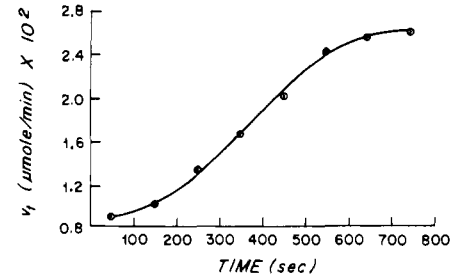

FIGURE 2: Rate of appearance of enzyme activity as a function of time. A solution of HMW-NGF (420 μg/mL) was diluted with 0.1 M potassium phosphate, pH 7.0, containing 2 mM TAME at 25 °C (final HMW-NGF concentration, 2.14 μg/mL), and the increase in absorbance (247 nm) was recorded vs. time. Values of the instantaneous velocity (v_i) of TAME hydrolysis were obtained from the slope of this curve as a function of time.

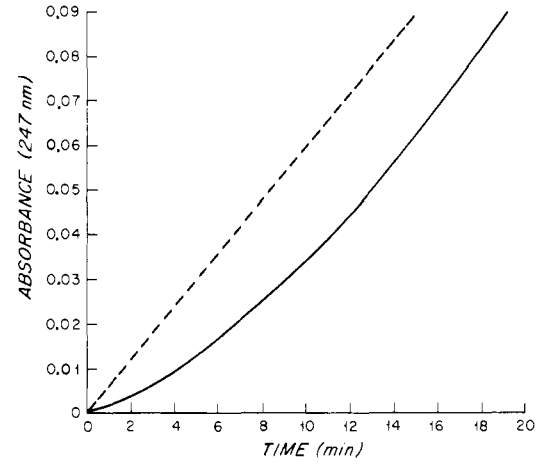

FIGURE 3: Enzymic activity of HMW-NGF preincubated in buffer (---) and not preincubated (—). HMW-NGF, 104 μg/mL, was diluted with 0.1 M potassium phosphate, pH 7.0, to give a final concentration of 2.6 μg/mL. This solution was incubated at 25 °C for 1 h, at which time 10 μL of 0.2 M TAME was added to 1.0 mL of protein. An identical solution of HMW-NGF was diluted directly from 104 μg/mL with buffer containing TAME and recording was begun immediately.

To ensure that all measured reaction velocities were zero order with respect to TAME concentration, K_m for TAME hydrolysis was estimated from Lineweaver–Burk plots by using fully activated HMW-NGF at 25 °C. Over the substrate concentration range 0.27×10^{-4} M to 2.2×10^{-4} M, these plots were strictly linear and yielded a value for $K_m = 6.5 \times 10^{-5}$ M. Consequently, TAME concentrations of 1×10^{-3} M or higher were employed for all enzyme assays.

Results

Figure 1 illustrates the kinetic behavior of HMW-NGF (TAME as substrate) when the protein is diluted from a high concentration (420 μg/mL) to progressively lower concentrations. It will be seen that a lag phase in the reaction occurs at all protein concentrations and that the duration of this phase increases as the protein concentration decreases. Furthermore, when the instantaneous velocity of the TAME hydrolytic reaction is plotted as a function of time (Figure 2), the *rate of appearance* of enzymic activity initially *increases* as time passes. Figure 3 demonstrates that the lag phase can be abolished by predilution of the enzyme. For these experiments, HMW-NGF (initially at a concentration of 420 μg/mL) was diluted to 2.6 μg/mL and allowed to remain at 25 °C for 1 h. After this time period, TAME was added and the reaction velocity was measured. As a control, NGF was diluted directly into the TAME solution at zero time. In the latter case, a lag

Table I: Maximal Specific Activities of HMW-NGF as a Function of Protein Concentration

protein concn (μg/mL)[a]	v_{act} (μmol min^{-1} mg^{-1})[a]
0.05	5.13
0.20	4.30
0.93	5.73
3.50	6.85
14.6	6.24
72.5	2.61
833[b]	0.43

[a] HMW-NGF was diluted from an initial concentration of 420 μg/mL to the concentrations indicated. These solutions were incubated at 25 °C for 3 h and then analyzed for TAME hydrolysis by adding 10 μL of 0.1 M TAME to 1.0 mL of NGF solution. Solvent: 0.1 M potassium phosphate, pH 7.0. No lag phase was observed at any protein concentration. v_{act} is the velocity of the fully activated enzyme. [b] The activity of this solution was measured directly by adding 10 μL of NGF solution.

phase occurred as expected; with the prediluted enzyme, the lag phase was absent (Figure 3).

As noted earlier, Greene et al. (1969) observed a lag phase in their studies on the enzymic properties of 7S-NGF, and they attributed this feature to dissociation of the protein, with a progressive increase in activity as the enzymically active species was liberated from the parent protein. Furthermore, they reported that the maximum specific enzyme activity (in μmol min^{-1} mg^{-1}) increased greatly as the 7S-NGF concentration was lowered (from about 2 μg/mL to about 0.02 μg/mL). We have been unable to confirm this observation. Table I presents values for the maximum velocity of TAME hydrolysis by fully active HMW-NGF (v_{act}) as a function of protein concentration. (The lag phase was eliminated here by prior dilution of the protein for 3 h to the indicated concentrations, followed by addition of TAME.) As shown in Table I, the specific enzyme activity remains essentially constant over a 300-fold range of protein concentration (0.05–14.6 μg/mL). At higher concentrations of HMW-NGF, the specific activity does fall, and this phenomenon will be explained.

With regard to the lag phase, this feature cannot be explained by a protein dissociation mechanism for several reasons. First, on kinetic grounds alone, such behavior is inconsistent with a dissociation reaction such as, e.g., AB → A + B (with AB enzymically inactive and A or B active) since the rate of appearance of A and B is first order in [AB] and this rate cannot increase as a function of time. Yet as shown in Figure 2, the rate of appearance of activity does increase with time in the initial stage of the reaction. Second, as shown in Figure 1, the lag phase becomes progressively longer as the enzyme concentration is reduced. Yet the half-life of a dissociation reaction is independent of the initial protein concentration. Third, HMW-NGF, purified as previously described (Young et al., 1978), is a stable protein that does not dissociate at concentrations comparable to those used for Figure 1.

One possibility which could serve to explain the results shown in Figure 1 is activation of the reaction by the products of TAME hydrolysis. However, this mechanism is eliminated by the observation that predilution alone is sufficient to abolish the lag phase (Figure 3). Furthermore, we have studied the reaction in the presence of 1 mM N^{α}-*p*-tosyl-L-arginine plus methanol (spectrophotometrically), and in the presence of 5 mM products (with the pH-stat). Under these conditions, the lag phase remained unchanged.

Autocatalytic Activation of NGF. We now present evidence to show that the lag phases depicted in Figures 1 and 2 can

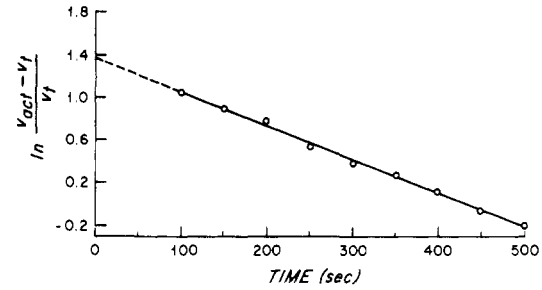

FIGURE 4: Representative plot of ln $(v_{act} - v_t)/v_t$ vs. t, according to an autocatalytic activation scheme. Data like those presented in Figure 1 were analyzed according to eq 4. HMW-NGF was diluted from a concentration of 420 to 4.7 μg/mL at zero time in the presence of 1 mM TAME. Solvent: 0.1 M potassium phosphate, pH 7.0, at 25 °C.

Table II: Kinetic Constants for Autocatalytic Activation[a]

protein concn (μg/mL)	k (L mol^{-1} min^{-1})	v_0 (μmol min^{-1} mg^{-1})	$t_{1/2}$ (min)
10.4	13.0×10^6	1.30	5.5
5.2	13.0×10^6	1.28	7.9
2.6	14.1×10^6	1.40	8.7
1.0	14.4×10^6	1.50	12.6

[a] HMW-NGF, initially at a concentration of 420 μg/mL, was diluted directly into the TAME solution to yield the indicated final protein concentrations. Solvent: 0.1 M potassium phosphate, pH 7.0; temperature, 25 °C; [TAME] = 2 mM. Data were analyzed according to eq 4 and 5.

be fully explained by a kinetic mechanism which involves autocatalytic self-activation of the enzyme as it is diluted from high protein concentrations (~0.5 mg/mL) to much lower concentrations (1–10 μg/mL).

For a reaction of the kind shown by eq 1, where P is enzymically inactive protein and P′ is active enzyme and where the reaction is catalyzed by P′, the rate of activation is given by eq 2, where k is the second-order rate constant. If A is the total protein concentration and x is the concentration of active enzyme, then eq 3 results. Integrating this equation within the limits $x = x_t$ at time $= t$ and x_0 = concentration of active enzyme at $t = 0$ yields eq 4. A value for the half-life ($t_{1/2}$) of an autocatalytic reaction can also be obtained, and we have chosen to define it as the time required to reduce $A - x_0$ by one-half. At this time, $x_{t_{1/2}} = x_0 + (A - x_0)/2$. Substituting in eq 4 yields eq 5. To determine values for x_t and x_0, we have taken advantage of the observation (Table I) that the maximum (steady state) turnover number for TAME hydrolysis is independent of HMW-NGF concentration below 10–20 μg/mL. Thus $v_t = ax_t$, $v_{act} = aA$, and $v_0 = ax_0$, where a is a constant, v_0 is the velocity at $t = 0$, v_t is the velocity at

$$P \rightarrow P' \tag{1}$$

$$\frac{d[P']}{dt} = k[P][P'] \tag{2}$$

$$\frac{dx}{x(A - x)} = k\,dt \tag{3}$$

$$kAt = -\ln\left(\frac{A - x_t}{x_t}\right) + \ln\left(\frac{A - x_0}{x_0}\right) \tag{4}$$

$$t_{1/2} = \frac{1}{kA} \ln\left(\frac{A + x_0}{x_0}\right) \tag{5}$$

Table III: Autocatalytic Activation of HMW-NGF at High Protein Concentrations

protein concn (μg/mL)[a]	time (h)	k (L mol^{-1} min^{-1})	v_0 (μmol min^{-1} mg^{-1})	$t_{1/2}$ (min)
298 → 2.89	0	4.8×10^6	1.7	13.6
298 → 2.89	2	5.5×10^6	1.7	11.8
875 → 8.50	0	2.0×10^6	1.0	13.1
875 → 8.50	5	3.3×10^6	1.2	7.3

[a] HMW-NGF was allowed to remain at high concentrations at 25 °C for the indicated times and then diluted to the concentration given after the arrow. Solvent: 0.1 M potassium phosphate, pH 7.0, at 25 °C; [TAME] = 2 mM. Data were analyzed according to eq 4 and 5.

$t = t$, and v_{act} is the velocity obtained with the fully activated protein.
is the velocity obtained with the fully activated protein.

Figure 4 presents a representative plot of ln $(v_{act} - v_t)/v_t$ vs. t according to eq 4. At all protein concentrations used in this study, such plots were always strictly linear. Table II summarizes values for k, v_0, and $t_{1/2}$ over a tenfold range of protein concentrations. For these experiments, a solution of HMW-NGF initially at the relatively high concentration of 420 μg/mL was diluted to yield the concentrations shown in the table, and the velocity of TAME hydrolysis was recorded over time. To obtain values for v_{act} for use with eq 4, the protein was prediluted for 1 h in the absence of TAME to the concentration shown in Table II. This procedure serves to activate the enzyme fully. Values for v_{act} cannot be obtained from the kinds of curves shown in Figure 1 since v_{act} is not reached until after the concentration of TAME becomes limiting and the enzyme is no longer saturated. It will be seen that the initial velocities (in μmol min^{-1} mg^{-1}) and second-order rate constants do not change significantly as a function of protein concentration, whereas (as expected for an autocatalytic reaction) the half-time increases appreciably as the protein concentration is lowered.

The results of Figures 1, 2, and 3 and Table II indicate that dilution of HMW-NGF from an initially high concentration promotes activation of the enzyme and that the kinetics of the reaction are consistent with autocatalysis. These observations led to the prediction that solutions of HMW-NGF preincubated at very high concentrations should become autocatalytically activated very slowly, if at all. The results of Table III indicate that this is the case. Two solutions of the protein, one at a concentration of 298 μg/mL and the other at 875 μg/mL, were incubated at 25 °C for 2 and 5 h, respectively, and then the kinetics of TAME hydrolysis were measured following 100-fold dilution of the protein. Control solutions (not preincubated) were used for comparison. As shown in Table III, preincubation of 298 μg/mL NGF for 2 h results in little, if any, activation. The half-times and the rate constants are similar. After 5 h, a small degree of activation has occurred. Thus, we infer that high dilution of the enzyme is required for any appreciable autocatalytic activation to occur; i.e., the activation reaction is strongly inhibited if the protein concentration is kept high.

Another prediction of an autocatalytic activation scheme is that the reaction should be irreversible. Accordingly, a 420 μg/mL solution of HMW-NGF was diluted 100-fold with 0.1 M potassium phosphate, pH 7.0, and incubated 1 h at 25 °C. Under these conditions, as shown above (Figure 3), the lag phase is eliminated. The dilute solution of protein was then lyophilized, reconstituted with buffer to its original concentration, and again diluted 100-fold (to 4.20 μg/mL) directly into a solution of 1 mM TAME. The lag phase was completely

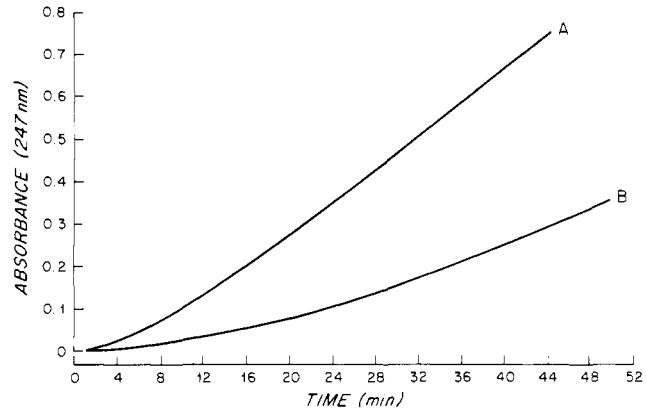

FIGURE 5: Effect of DFP upon the kinetics of activation. A solution of HMW-NGF, 38.5 μg/mL, was treated with DFP (0.011 M) for 10 min at 25 °C. An identical solution of HMW-NGF served as control. These solutions were then further diluted with 0.1 M potassium phosphate, pH 7.0, plus 2 mM TAME, and the velocity of TAME hydrolysis was measured as a function of time. The final [HMW-NGF] in the substrate solution was 3.6 μg/mL. (A) Control: $v_0 = 3.8$ μmol min^{-1} mg^{-1}, $v_{act} = 11.9$ μmol min^{-1} mg^{-1}, $k = 6.8 \times 10^6$ L mol^{-1} min^{-1}, $t_{1/2} = 3.7$ min. (B) DFP treated: $v_0 = 1.6$, $v_{act} = 7.1$, $k = 3.1 \times 10^6$, $t_{1/2} = 12.5$.

by dilution of the protein is irreversible. Once the lag phase has been eliminated by dilution, it cannot be restored by reconcentration.

Effects of Activation and of Inhibition of Enzyme Activity upon the Activation Reaction. If autocatalytic activation is responsible for the results presented above, then it follows that any reagent which inhibits the enzyme should decrease k (increase $t_{1/2}$). Conversely, any reagent which activates the enzyme should increase k (decrease $t_{1/2}$).

(a) Inhibition Studies. In an earlier study, we showed that DFP inhibits the catalytic activity of HMW-NGF in converting plasminogen to plasmin as well as the hydrolysis of TAME. With TAME as substrate, the DFP-inhibition reaction is second order (as expected) with a rate constant = 14.4 L mol^{-1} min^{-1} and a half-life = 53 min (at a DFP concentration of 9.0×10^{-4} M) (Orenstein et al., 1978).

Based upon these results, experiments were designed to study the kinetics of activation of an enzyme preparation which had been partially inhibited by DFP. For this purpose, HMW-NGF at a concentration of 38.5 μg/mL was treated for 10 min with 0.011 M DFP. (Under these conditions, the activity of the enzyme with TAME as substrate is inhibited about 40%.) Following DFP treatment, the enzyme was diluted to a final concentration of 3.6 μg/mL in the presence of 2 mM TAME. A solution of HMW-NGF without DFP was used as a control. Figure 5 illustrates the two kinetic profiles and the legend summarizes the kinetic constants which were analyzed according to eq 4 and 5. As shown in Figure 5, the lag phase is greatly prolonged by DFP treatment of the enzyme. Accordingly, the rate constant, initial velocity, and maximum velocity are reduced. The half-life of the reaction is increased more than threefold.

(b) Activation Studies. In an earlier study, Pattison & Dunn (1975, 1976) reported that 7S-NGF contains 1–2 g-atoms of Zn^{2+} per mol of protein and that removal of this ion by chelation with EDTA greatly (ca. tenfold) increases the arginine esterase activity of this form of NGF. Thus, they concluded that the enzymic activity of 7S-NGF is normally inhibited by Zn^{2+}. We do not yet know whether Zn^{2+} is involved in the structure of our HMW-NGF, but as shown below, EDTA will activate this enzyme also. However, our interpretation of the results of this activation differs from that

Table IV: Effect of EDTA upon the Steady-State Velocity of HMW-NGF[a]

protein concn (μg/mL)	v_{act} (μmol min^{-1} mg^{-1})	
	−EDTA	+EDTA
11.2	7.3	8.5
0.84	7.0	7.0

[a] A solution of HMW-NGF (420 μg/mL) was diluted to the given concentration with either 0.1 M potassium phosphate, pH 7.0, or potassium phosphate plus 5 mM EDTA. After 1 h (to activate fully the enzyme), 10 μL of 0.1 M TAME was added to 1.0 mL of protein solution, and the reaction velocity was measured at 25 °C. No lag phases were observed in any of these reactions.

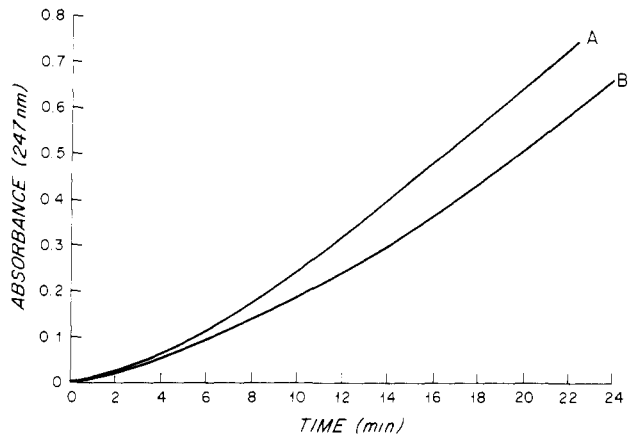

FIGURE 6: Effect of EDTA upon the kinetics of activation. HMW-NGF, 420 μg/mL, was diluted to a final concentration of 11.2 μg/mL with: (A) 0.1 M potassium phosphate, 5 mM EDTA, pH 7.0; (B) the same buffer without EDTA. Reaction velocities were measured in the presence of 2 mM TAME at 25 °C. EDTA: v_0 = 1.8 μmol min^{-1} mg^{-1}, $k = 4.0 \times 10^6$ L mol^{-1} min^{-1}, $t_{1/2}$ = 4.6 min. Control: v_0 = 1.9, $k = 2.7 \times 10^6$, $t_{1/2}$ = 6.1.

of Pattison & Dunn (1976) as follows. (1) Treatment of our preparations of NGF with EDTA does not change the activity of the enzyme. Rather, EDTA increases only *the rate of the autocatalytic activation reaction.* (2) EDTA increases neither v_0 nor v_{max} above those values which can be achieved simply by diluting the enzyme.

Table IV illustrates the effect of a high concentration of EDTA (5 mM) upon the maximal velocity of TAME hydrolysis. For these experiments, HMW-NGF (initially at a concentration of 420 μg/mL) was diluted to the indicated concentrations and incubated for 1 h at 25 °C to eliminate the lag phase; then, TAME was added to obtain values for v_{act}. As shown in Table IV, EDTA has no significant effect upon the maximum velocity achieved. Simple dilution of the protein without EDTA produces the same result.

However, as shown in Figure 6, EDTA does have a significant effect upon the kinetics of the activation reaction. In the presence of EDTA, the length of the lag phase is shortened when compared with the control solution without EDTA. The legend to Figure 6 presents kinetic constants obtained from analyses of the data according to eq 4 and 5. Within experimental error, values for v_0 and v_{max} are unchanged by EDTA. In contrast, EDTA increases the rate constant of the activation reaction, and it also decreases $t_{1/2}$.

Discussion

Taken together, the results presented above indicate that HMW-NGF comprises an autocatalytic self-activating enzyme system. This conclusion is based upon the following observations. (1) The length of the lag phase increases as the protein concentration decreases. (2) Once the lag phase has been eliminated (by high dilution of the protein), it cannot be restored. The reaction is irreversible. (3) The rate of appearance of enzyme activity increases with time. (4) Partial inhibition of the enzyme with DFP significantly prolongs the lag phase, whereas treatment of HMW-NGF with EDTA produces the opposite effect. (5) Over a wide range of protein concentrations, the kinetics of TAME hydrolysis obey the autocatalytic activation scheme described by eq 4.

In their study on the arginine esterase activity of 7S-NGF, Greene et al. (1969) observed a lag phase which they attributed to progressive dissociation of the protein as it was diluted, with concomitant appearance of enzyme activity. Consequently, we have carefully examined the possibility that dissociation of HMW-NGF might also be responsible for the observed appearance of activity. There are three main reasons which indicate that a dissociation reaction cannot explain the results presented above. First, a reaction of the kind NGF (inactive) → dissociation products (active) cannot explain the kinetic results summarized in the preceding paragraph. The half-life of a dissociation reaction does not vary with changes in initial protein concentration. Second, the *rate of dissociation* of a dissociation reaction does not increase with time; yet, Figure 2 shows that the rate of appearance of enzyme activity does increase with time. Finally, earlier studies have shown that HMW-NGF, at concentrations considerably below those used in the present study, is stable and that it does not dissociate (see Figure 7 in Young et al. (1978)). Thus, although 7S-NGF and the HMW-NGF used in this present study are prepared by quite different methods (Varon et al., 1967; Young et al., 1978, respectively), and although 7S-NGF is known to dissociate in dilute solution (Baker, 1975; Young et al., 1978), it is possible that Greene et al. (1969) were also observing an autocatalytic reaction with 7S-NGF.

From studies on the enzymic properties of 7S-NGF, Pattison & Dunn (1975, 1976) found that a variety of metal ion chelating reagents activate this form of the protein. Although these authors also observed a lag phase, they did not study this property as a function of protein concentration, and the mathematical treatment which they used to analyze their data did not include a protein concentration-dependent term. Yet as shown in Figure 1 and Table II, the length of the lag phase and the calculated half-lives of the activation reaction are clearly dependent upon protein concentration. Furthermore, simple dilution of the enzyme serves to activate it; EDTA is not required (see Table IV). However, as shown in Figure 6, EDTA does increase the second-order rate constant of activation, and it decreases the half-life. A plausible explanation for these results is that EDTA changes neither v_0 nor v_{act} (see Table IV and the legend to Figure 6) but that it releases the enzyme from inhibition and thereby enhances the autocatalytic reaction rate. Presumably this is accomplished by removal of a metal ion.

The results shown in Table I reveal that, at high protein concentrations, the specific enzymic activity of HMW-NGF is low (about 5–10% of that which can be observed at high dilution following complete activation). As shown in an earlier study (Orenstein et al., 1978), this low level of activity is a property of the intact HMW-NGF molecule; it does not stem from a contaminant of the preparation. Furthermore, the results of Table III reveal that not only is the specific activity very low but also that the activation reaction proceeds very slowly at high protein concentrations. Thus, both the catalytic activity with TAME as substrate as well as the autocatalytic activation reaction are strongly inhibited at high protein concentrations. Dilution of the enzyme, as well as addition

of EDTA, releases it from inhibition. Taken together, these observations suggest that a metal ion is responsible for inhibiting autocatalytic activation and that, at high dilution, this ion dissociates from the protein.

A question now arises: how many kinds of active sites does the enzyme possess? That is, is the autocatalytic site the same as the one that hydrolyzes the synthetic arginine substrate? While this question cannot be precisely answered at present, the available evidence is consistent with the existence of one kind of site and, further, that this site contains a serine residue. For example, the autocatalytic reaction progressively produces new sites which also can hydrolyze TAME, and both the autocatalytic reaction as well as TAME hydrolysis are inhibited by DFP.

The biologic significance of an autocatalytic activation reaction for HMW-NGF is not clear. Yet we have shown that extremely high concentrations of this protein are secreted in mouse saliva (Murphy et al., 1977a,b), and it could be that the enzyme remains largely inactive until it becomes diluted during passage through the alimentary tract. (Such a mechanism implies that NGF plays a heretofore unsuspected role in the gastrointestinal tract, and no such role has yet been discovered.) Another possibility is that the zymogen remains inactive until it comes in contact with its naturally occurring substrate. In this regard, the results of Figure 3 demonstrate that preincubation of the enzyme at low concentration in the absence of any exogenous substrate will promote full activation. But it must be remembered that the HMW-NGF used in this study is a pure protein and that it has been purified from its in vivo milieu with reagent grade buffers containing no added metal ions. Consequently, it could be that, in vivo, the enzyme is normally inhibited and that it becomes activated only when it recognizes its substrate.

From results arising from studies on 7S-NGF, several authors have suggested that the enzymic activity associated with this protein is responsible for converting one of its newly synthesized subunits (a pro-β-NGF) to β-NGF. (This is the subunit of 7S-NGF which is responsible for promoting neurite outgrowth in the sensory ganglion assay system.) According to this scheme, the neurite outgrowth promoting factor is initially synthesized as a larger (presumably inactive) molecule which is then posttranslationally converted to β-NGF (Angeletti & Bradshaw, 1971; Moore et al., 1974; Berger & Shooter, 1977). An additional feature of this postulated mechanism is that the enzyme which is responsible for the pro-β-NGF $\rightarrow$ β-NGF conversion remains attached to β-NGF in the 7S-NGF complex and that its enzymic activity is consequently inhibited, only to be fully expressed when 7S-NGF dissociates (see Berger & Shooter, 1977, for a summary of these arguments). Based upon the results of the present study, we believe this mechanism to be unlikely. First of all, dissociation of HMW-NGF cannot account for the observed enzyme kinetic behavior. Second, the stable form of NGF exists largely as a zymogen after it has been purified from submandibular gland extracts. Between 90 and 95% of its potential enzymic activity still remains to be realized (following autocatalytic activation). Consequently, it is difficult to see why a zymogen, whose full enzymic activity remains to be expressed, would be responsible for converting a precursor (pro-β-NGF) to β-NGF since very little autocatalytic activation of the enzyme has occurred, even when the postulated posttranslational cleavage event is finished.

The foregoing arguments are not intended to imply that a larger precursor form of the nerve growth promoting subunit of NGF does not exist. In fact, evidence has been presented that such a precursor of β-NGF may be initially synthesized by the mouse submandibular gland (Berger & Shooter, 1977). What we are implying is that, if a precursor does exist, then it seems unlikely that a largely inactive zymogen is responsible for processing it.

To our knowledge, other than the zymogen-NGF $\rightarrow$ NGF reaction, only the activations of pepsinogen $\rightarrow$ pepsin and of trypsinogen $\rightarrow$ trypsin comprise autocatalytic systems. But we suspect that other similar reactions will be discovered that may play an important role in expression of the biologic activity of other growth factors.

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