Drummond, G. I., & Yamamoto, M. (1971) *Enzymes, 3rd Ed. 4*, 337–354.
Eckstein, F. (1975) *Angew. Chem., Int. Ed. Engl. 14*, 160–166.
Eckstein, F. (1977) *Biochim. Biophys. Acta 483*, 1–5.
Eckstein, F. (1978) *Acc. Chem. Res. 12*, 204–210.
Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685–1691.
Hackney, D. D., & Boyer, P. D. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3133–3137.
Hasselbach, W. (1974) *Enzymes, 3rd Ed. 10*, 431–467.
Hollander, V. P. (1971) *Enzymes, 3rd Ed. 4*, 449–498.
Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839–10845.
Janson, C. A., Degani, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 3743–3749.
Jarvest, R. L., & Lowe, G (1979) *J. Chem. Soc., Chem. Commun.*, 364–366.
Jones, S. R., Kindman, L. A., & Knowles, J. R. (1978) *Nature (London) 275*, 564–565.
Kanazawa, T., & Boyer, P. D. (1973) *J. Biol. Chem. 248*, 3163–3172.
Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.
Koshland, D. E., Jr., & Springhorn, S. S. (1956) *J. Biol. Chem. 221*, 469–476.
Levine, D., Reid, T. W., & Wilson, I. B. (1969) *Biochemistry 8*, 2374–2380.
Lowe, G., Potter, B. V. L., Sproat, B. S., & Hull, W. E. (1979) *J. Chem. Soc., Chem. Commun.*, 733–735.
Morton, R. K. (1953) *Nature (London) 172*, 65.
Murray, W. A., & Atkinson, M. R. (1968) *Biochemistry 7*, 4023–4029.
Reid, T. W., & Wilson, I. B. (1971) *Enzymes, 3rd Ed. 4*, 373–415.
Richard, J. P., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7757–7758.
Richard, J. P., Ho, H.-T., & Frey, P. A. (1978) *J. Am. Chem. Soc. 100*, 7756–7757.
Richard, J. P., Douglas, C. P., Ives, D. H., & Frey, P. A. (1979) *J. Biol. Chem 254*, 4339–4341.
Sheu, K.-F. R., & Frey, P. A. (1977) *J. Biol. Chem. 252*, 4445–4448.
Tsai, M.-D. (1979) *Biochemistry 18*, 1468–1472.
Tsai, M.-D., & Chang, T. T. (1980) *J. Am. Chem. Soc. 102*, 5416–5418.
Tsai, M.-D., Huang, S. L., Kozlowski, J. F., & Chang, C. C. (1980) *Biochemistry 19*, 3531–3536.
Webb, M. R., & Trentham, D. R. (1980) *J. Biol. Chem. 255*, 1775–1779.

Nerve Growth Factor Zymogen. Stoichiometry of the Active-Site Serine and Role of Zinc(II) in Controlling Autocatalytic Self-Activation†

Michael Young* and Mary Jo Koroly

ABSTRACT: Mouse submandibular gland nerve growth factor (NGF) is a 116000 molecular weight protease with a high degree of specificity for certain lysyl and arginyl bonds. This protein can activate plasminogen and it is also a member of the general class of serine proteases [Orenstein, N. S., Dvorak, H. A., Blanchard, M. H., & Young, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5497]. As isolated, NGF is an enzymically inactive zymogen. Upon dilution from high to very low protein concentrations or upon treatment with EDTA, the zymogen undergoes autocatalytic activation. Atomic absorption spectroscopy measurements reveal that NGF contains 1 g-atom of tightly bound Zn(II) per mol. Reaction of the fully autoactivated protease with [^{3}H]DFP yields 1 mol of labeled serine per mol of enzyme. All results indicate that as long as Zn(II) remains bound to the zymogen, autocatalytic activation is inhibited. Removal of this ion, by dilution of the protein or by chelation, initiates autoactivation. The physiologic purpose of this unusual reaction is not known but it may be that Zn(II) serves to act as a control ion which keeps the protein in an inactive form (the zymogen) until it recognizes its naturally occurring substrate.

Recent studies from this laboratory have shown that the predominant form of mouse submandibular gland nerve growth factor (NGF)[1] is a protein of molecular weight 116000 (Young et al., 1978) which is secreted at very high concentrations into mouse saliva (Murphy et al., 1977a,b). Other lower molecular weight forms of NGF are also present in both salivary gland extracts and saliva, and evidence has been presented which indicates that these smaller species are degradation products of the larger 116000 molecular weight form (Young et al., 1978; Murphy et al., 1977b).

The observation that the mouse submandibular gland contains a high molecular weight form of NGF is not a new one. In 1967, Varon et al. (1967) isolated a protein which they called 7S-NGF which was estimated to have a molecular weight of 140000. Evidence from several sources indicates that 7S-NGF is an unstable protein and that it dissociates into its subunits at relatively high protein concentrations (Varon et al., 1967; Greene et al., 1969; Smith et al., 1969; Baker, 1975; Pantazis et al., 1977). When the individual subunits of 7S-NGF are recombined, the resulting protein exhibits the

† From the Department of Biochemistry and Molecular Biology, University of Florida, College of Medicine, Gainesville, Florida 32610. *Received February 19, 1980.* This work was supported by National Institutes of Health Grants CA 17137 and NS 13674.

[1] Abbreviations used: NGF zymogen, 116000 molecular weight nerve growth factor zymogen prepared as described by Young et al. (1978); 7S-NGF, nerve growth factor prepared as described by Varon et al. (1967); EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl phosphorofluoridate; Tame, N^{α}-*p*-toluenesulfonyl-L-arginine methyl ester; TLME, N^{α}-*p*-toluenesulfonyl-L-lysine methyl ester; TLCK, N^{α}-*p*-toluenesulfonyl-L-lysine chloromethyl ketone; *p*NPGB, *p*-nitrophenyl *p*-guanidinobenzoate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/80/0419-5316$01.00/0

same instability as that observed with native 7S-NGF (Jeng et al., 1979). In contrast, the 116000 molecular weight form of NGF which we have isolated is highly stable even in dilute solution (Young et al., 1978). These results are not presented to draw attention to the differences between our high molecular weight form of NGF and 7S-NGF but rather to set the stage for a comparison of their similarities in light of more recent studies (Young, 1979) and results of the present paper.

In 1969, Greene et al. (1969) observed that 7S-NGF possesses esterolytic activity toward certain N^{α}-substituted arginine and lysine methyl esters (e.g., Tame and TLME). They further found that when 7S-NGF was diluted from high to low protein concentrations, the maximum velocity of substrate hydrolysis was achieved only after a lag phase of lower velocity. Greene et al. (1969) attributed this behavior to progressive dissociation of NGF with concomitant appearance of enzyme activity (i.e., the enzyme activity of 7S-NGF was postulated to be inhibited in the high molecular weight 7S-NGF complex and subsequently expressed only when the protein dissociates). However, these authors did not examine the length of the lag phase as a function of protein concentration. When this is done, as described below, the lag phase cannot be explained by dissociation.

Recent studies in our laboratory have shown that, like 7S-NGF, the stable 116000 molecular weight form of NGF also hydrolyzes Tame and TLME and further that it can activate plasminogen, with concomitant lysis of a fibrin clot. Moreover, inhibition studies with DFP placed this species of NGF in the general class of serine proteases (Orenstein et al., 1978).

The observation that the 116000 molecular weight form of NGF possesses arginine esterase activity led us to study the kinetics of Tame hydrolysis. Like 7S-NGF, our form of NGF also exhibits a lag phase which occurs prior to establishment of the maximum catalytic rate, *but* the length of the lag phase increases greatly as the enzyme concentration is reduced. Thus, the lag phase cannot be explained simply by dissociation, since the half-life of a dissociation reaction is first order in, and therefore independent of, enzyme concentration. Further studies revealed that the 116000 molecular weight form of NGF exists largely, if not entirely, as a zymogen when it is isolated from the mouse submandibular gland. Upon dilution of this protein from a high concentration ($\sim$1 mg/mL) to much lower concentrations ($\sim$1 μg/mL), the enzyme undergoes autocatalytic self-activation. This behavior, not dissociation, is responsible for the lag phase observed in Tame hydrolysis, and it probably accounts for the lag phase noted with 7S-NGF (Young, 1979).

The observation that simple dilution of the 116000 molecular weight form of NGF is sufficient to induce autocatalytic activation bears directly upon work from Dunn's laboratory on the role of Zn(II) in the structure of 7S-NGF (Pattison & Dunn, 1975, 1976a,b; Au & Dunn, 1977). These authors reported that 7S-NGF contains 1–2 g-atoms of Zn(II) per mol (values ranged from 1.7 to 2.0) and that removal of this ion by chelation greatly increases arginine esterase activity. In later studies, these authors concluded that the activation reaction [resulting from removal of Zn(II)] is a reversible equilibrium reaction (Pattison & Dunn, 1976b).

In the study to be presented below, we have examined the role of Zn(II) in the structure and function of the NGF-zymogen, and all results indicate that, like the kinetic lag phase, the role of Zn(II) should be reconsidered. Specifically, we find that autocatalytic activation of the NGF zymogen is strictly under the control of Zn(II). As long as this ion is bound to the zymogen, autocatalysis is completely prevented. Once Zn(II) is removed, either by high dilution of the protein or by chelation, the autocatalytic activation reaction is initiated, and this process is irreversible. We have further studied the stoichiometric relation between the protein, Zn(II) and the active site serine. Results indicate 1 g-atom of Zn(II) is bound per mol of zymogen, and 1 mol of active site serine per mol of the fully autocatalytically activated enzyme.

Experimental Procedures

Reagents. Double glass distilled water was used throughout and all buffer solutions were treated with a 0.01% solution of diphenyl thiocarbazone (Fisher) and Chelex-100 (Bio-Rad) to remove metal ions. Buffer salts were reagent grade. Tame and TLCK were obtained from Sigma Chemical Co., *p*NPGB was from Vega Chemicals, Phoenix, AZ, DFP was from Pfaltz and Bauer, Stamford, CT, [^{3}H]DFP was from New England Nuclear, trypsin (2× recrystallized) was from Worthington, and ovalbumin and ribonuclease A were from Pharmacia. *p*-Nitrophenol was an Eastman product. NGF was prepared as previously described, and its molecular weight was taken to be 116000 (Young et al., 1978).

Physical Measurements. Protein concentrations were measured by absorbance at 280 nm. In an earlier study, the extinction coefficient of NGF was estimated to be 1.92 mL mg^{-1} cm^{-1} based upon amino acid analysis (Young et al., 1978). To obtain a more accurate value for this parameter, we calibrated a Brice-Phoenix differential refractometer (λ = 436 nm) with standard solutions of ribonuclease A and ovalbumin which had been exhaustively dialyzed against 0.1 M potassium phosphate, pH 7.0. The extinction coefficients (280 nm) of ribonuclease and ovalbumin were taken to be 0.695 and 0.735 mL mg^{-1} cm^{-1}, respectively (Sherwood & Potts, 1965; Cunningham & Neunke, 1959). If we assume that the refractive index increments of the three proteins are closely similar, this procedure yields an extinction coefficient for NGF of 1.42 $\pm$ 0.08 (SD) mL mg^{-1} cm^{-1}.

Active-site titrations of trypsin were performed by the method of Chase & Shaw (1967) at λ = 402 nm and pH 8.33. To determine the molar extinction coefficient of *p*-nitrophenol at this pH with our spectrophotometer, we recrystallized this compound twice from methanol–water and dried it in vacuo over $CaCl_2$. A value of 1.698×10^3 M^{-1} cm^{-1} was obtained.

Enzyme activity assays were performed with a Cary Model 15 recording spectrophotometer at λ = 247 nm with 1–2 mM Tame as substrate. The molar absorbance of N^{α}-*p*-toluenesulfonyl-L-arginine was taken to be 540 at λ = 247 nm (Hummel, 1959). Equation 1 was used to analyze the kinetics of autocatalytic activation of NGF (Young, 1979). Here v_{act},

$$\ln \frac{v_{act} - v_t}{v_t} = kAt + \ln \frac{v_{act} - v_0}{v_0} \qquad (1)$$

v_t, and v_0 are the reaction velocities (Tame as substrate) of fully activated enzyme, the velocity at time t, and the velocity at t = 0. A is the total protein concentration, and k is the second-order rate constant for activation. Values of k and v_0 were obtained from the slope and intercept, respectively, of plots of the left side of eq 1 vs. t.

Metal Analyses. Quantitative metal analyses were performed with a Perkin-Elmer atomic absorption spectrometer.

Results

Zn^{II}–NGF Zymogen Stoichiometry. On the basis of earlier findings of Pattison & Dunn (1975) that 7S-NGF contains tightly bound zinc ion, we have measured the stoichiometry of the Zn(II)–NGF zymogen interaction. Protein preparations were dialyzed exhaustively against 0.1 M potassium phosphate

Table I: Stoichiometry of the DFP-NGF Reaction[a]

DFP (M)	reaction time (h)	residual enzyme act. (%)	DFP/NGF (mol/mol)
2.5×10^{-4}	44	7.2	1.10
4.0×10^{-4}	23	8.3	1.10
2.8×10^{-3}	6	<1	1.10[b]

[a] The NGF concentration ranged from 230 to 350 μg/mL in 0.1 M potassium phosphate and 0.06 M EDTA, pH 7.0. After reaction for the indicated times, residual enzyme activity was measured with Tame as substrate, and values are expressed as percent of the control value (without DFP). Specific activity of DFP = 1.0 Ci/mmol. [b] For this reaction, [^{3}H]DFP (1 Ci/mmol) was diluted with cold DFP to give a specific activity of 0.07 Ci/mmol, as determined by reaction of the standard trypsin solution as described in the text.

buffer, pH 7.0, which had been treated with diphenyl thiocarbazone and with Chelex-100. Three different preparations yielded values of 0.88, 1.16, and 1.12 g-atom of Zn(II) per mol of zymogen, respectively (mean = 1.05 ± 0.15 SD).

Reaction of [^{3}H]DFP with Fully Active Enzyme. Earlier studies have shown that both the plasminogen activator activity and the Tame esterase activity of NGF can be inhibited with DFP. Thus, NGF is a member of the general class of serine proteases (Orenstein et al., 1978; Young, 1979).

The following procedure was used to determine the number of DFP-reactive serine residues per molecule of fully enzymically active NGF. To obtain an accurate value for the specific activity of preparations of [^{3}H]DFP, we diluted solutions of this reagent dissolved in propylene glycol gravimetrically with 0.01 M Tris-HCl, pH 8.0, and counted them with Aquafluor (New England Nuclear). The resulting measured specific activity was further checked with solutions of trypsin which had been standardized by reaction with the active site serine titrant *p*NPGB as described under Experimental Procedures. Titration of seven different solutions of trypsin with *p*NPGB yielded a mean value of 0.63 ± 0.03 (SD) mol of *p*NPGB incorporated per mol of protein. Solutions of trypsin were then treated with 0.1 mM [^{3}H]DFP (0.1 M Tris-HCl, pH 8.0) for varying periods of time until the residual enzyme activity (Tame as substrate) was <1% of the control value. Samples were applied to a 1 × 23 cm column of Sephadex G-25 equilibrated with 0.01 M Tris-HCl, pH 8.0, to remove unreacted [^{3}H]DFP. Aliquots were measured for absorbance and radioactivity. By use of the gravimetrically determined specific activity of [^{3}H]DFP, this procedure yielded a value of 0.65 ± 0.04 (SD) mol of [^{3}H]DFP incorporated per mol of trypsin, in excellent agreement with that measured by the *p*NPGB method.

Table I summarizes values for moles of [^{3}H]DFP incorporated per mole of NGF. NGF zymogen was fully converted to active enzyme and reacted with the above standardized solutions of [^{3}H]DFP for varying periods of time. Unreacted [^{3}H]DFP was separated by chromatography upon Sephadex G-25 as described for trypsin. As shown in Table I, a mean value of 1.10 mol of [^{3}H]DFP incorporated per mol of NGF was obtained, and this value did not vary as a function of reaction time or of the initial [^{3}H]DFP concentration employed. To ensure that these values were not falsely low due to slow dephosphorylation of NGF, we measured a sample of NGF which had been reacted with [^{3}H]DFP (and separated from free DFP) for residual enzyme activity and incubated it at room temperature for 48 h. No increase in enzyme activity was observed over time. Therefore, the data of Table I indicate that after the NGF zymogen is fully activated, it contains 1 mol of DFP-reactive serine per mol of protein.

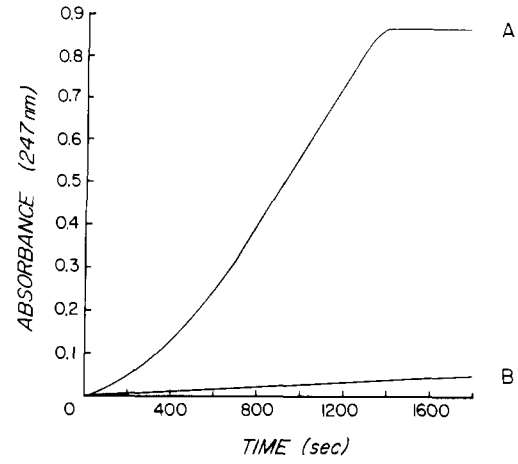

FIGURE 1: Effect of Zn(II) upon the autocatalytic activation reaction. Curve A illustrates the autocatalytic reaction when NGF (640 μg/mL) is diluted to 6.2 μg/mL with 0.1 M Tris-HCl containing 9.6×10^{-5} M EDTA and 2 mM Tame at 25 °C. Curve B shows the enzymic activity when the protein is diluted exactly as in (A) except that the buffer contains 1 mM Zn(II).

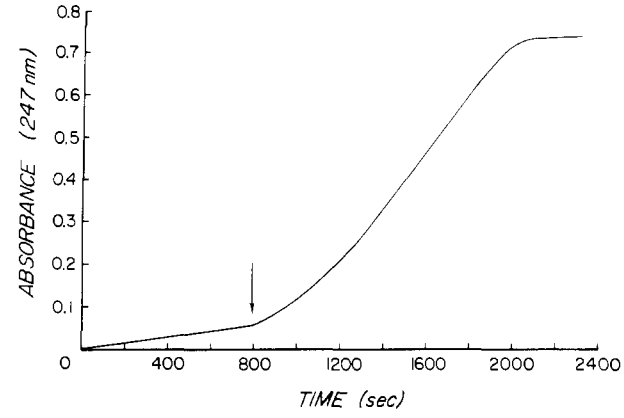

FIGURE 2: Reversal of Zn(II)-induced inhibition of autocatalysis by EDTA. NGF (640 μg/mL) was diluted to 6.2 μg/mL with 0.1 M Tris-HCl and 9.7×10^{-4} M Zn(II), pH 7.0. This solution was incubated at 25 °C for 24 h and enzymic activity measured in the presence of 1 mM Tame. The first part of the curve shows that activity is very low and that autocatalysis is completely inhibited. At the time marked by the arrow, the protein solution was made 2.4 mM in EDTA.

Function of Zn^{II} in the Autocatalytic Activation Reaction. Earlier studies have shown that when the concentration of NGF zymogen is kept high (~100–200 μg/mL), little, if any, activation occurs even after prolonged time periods. On the contrary, when the zymogen is greatly diluted (to 1–20 μg/mL), autoactivation occurs. Furthermore, EDTA can promote autocatalysis at high protein concentrations (Young, 1979). Taken together, these observations suggested that Zn(II) might be responsible for controlling autocatalysis.

Figure 1A illustrates the kinetic profile of Tame hydrolysis which shows that when the zymogen is diluted from high to low concentration into Zn(II)-free buffer, rapid autoactivation occurs. Figure 1B depicts the behavior when the zymogen is diluted to the same low protein concentration in the presence of 1 mM Zn(II). The Tame hydrolytic reaction was followed for 1.5 h (only 30 min of which is shown in Figure 1B), and no activation occurred under these conditions. Thus, even at high dilutions of zymogen, no activation occurs in the presence of 1 mM Zn(II).

The results illustrated in Figure 2 demonstrate that the Zn(II)-induced inhibition of activation can be reversed by EDTA. A solution of the zymogen was diluted to a low concentration (6.2 μg/mL) in the presence of 9.7×10^{-4} M

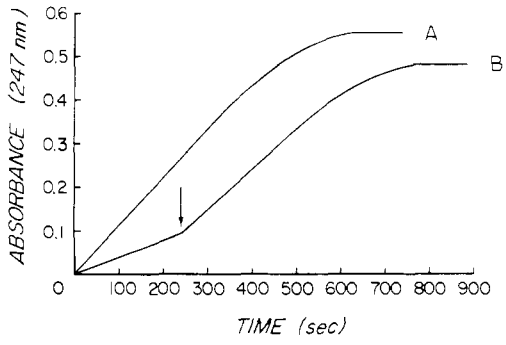

FIGURE 3: Effect of Zn(II) upon enzyme activity following complete autocatalytic activation. (A) NGF (640 μg/mL) was diluted to 6.2 μg/mL with 0.1 M Tris-HCl and 0.1 mM EDTA, pH 7.0, and incubated 1 h at 25 °C. Enzyme activity was then measured with 1 mM Tame. (B) Following substrate exhaustion of the solution used in (A), the solution was made 1 mM in Zn(II) and the enzyme activity measured after adding Tame. At the time indicated by the arrow, this solution was made 2.4 mM in EDTA.

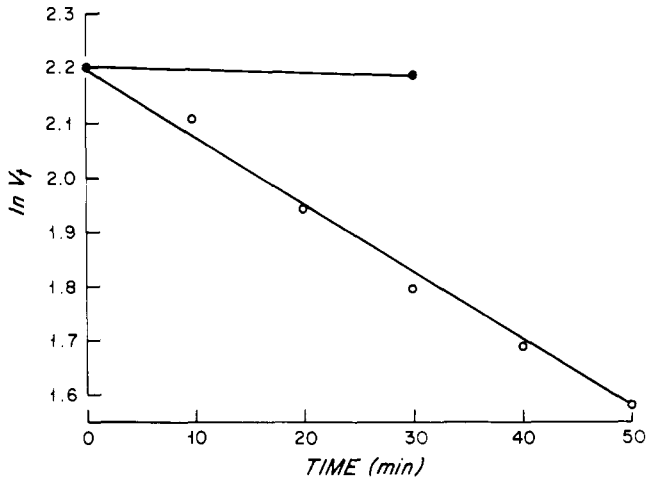

FIGURE 4: Effect of DFP upon enzyme activity of NGF at high and low protein concentrations. (○) NGF, 1.8 μg/mL, dissolved in 0.1 M potassium phosphate buffer, pH 7.0, was treated with 0.9 mM DFP. At the indicated times, samples were withdrawn and made 1 mM in Tame, and the velocity of hydrolysis was measured at 25 °C. (●) Conditions are the same as those above except that 300 μg/mL NGF was treated with 14 mM DFP.

Zn(II) and incubated at 25 °C for 24 h. Even after this prolonged time period, measurements of enzyme activity show that little or no autoactivation of the zymogen has occurred. At the time depicted by the arrow (Figure 2), the protein solution was made 2.4 mM in EDTA. It will be seen that after addition of chelator, the autocatalytic activation reaction is rapidly initiated.

Earlier studies have shown that when activation of the NGF zymogen is initiated by high dilution of the protein, this reaction is irreversible, i.e., it cannot be reversed upon subsequent reconcentration of the enzyme (Young, 1979). This finding is to be expected for an autocatalytic activation process. The results illustrated in Figure 3 further demonstrate that the reaction cannot be reversed by addition of Zn(II). A solution of the protein was diluted to a concentration of 6.2 μg/mL and incubated 1 h at 25 °C and the kinetics of Tame hydrolysis measured. This procedure serves to activate the enzyme fully. As shown in Figure 3A, the lag phase has been eliminated. Following substrate exhaustion of the preparation used in Figure 3A, this solution was treated with 1 mM Zn(II), and additional substrate was added. As shown in the first part of Figure 3B, enzyme activity is somewhat inhibited. At the time indicated by the arrow, the reaction solution was made 2.4 mM in EDTA, and Figure 3B shows that the lag phase is absent. It was not restored by prior addition of Zn(II). Taken together, these results indicate that once the activation reaction has gone to completion, it cannot be reversed by Zn(II). It should be pointed out that the results shown in Figure 3 are obtained no matter how long the activated enzyme is treated with the metal ion (at least up to 24 h).

The following studies indicate that, while the inhibition of activity of the zymogen by Zn(II) is highly specific, inhibition of the fully active enzyme by this ion is probably nonspecific. If we compare the results shown in Figure 1B and Figure 3B, it will be appreciated that the enzyme activity of the zymogen is only ~5% of that of the fully activated protein—both in the presence of 1 mM Zn(II). Consequently, we have compared the enzyme activity of zymogen with that of active enzyme over a wide range of Zn(II) concentrations. With respect to the zymogen, results show that at Zn(II) concentrations of 0.1 μM and higher, autoactivation is completely inhibited. At metal ion concentrations below 0.1 μM, slow activation occurs, the rate of which depends on the Zn(II) concentration. In contrast, the behavior of the activated enzyme under the same conditions is strikingly different. For example, in the presence of 0.1 μM Zn(II), the activated enzyme is inhibited by only 30% compared to its activity in the absence of Zn(II). Furthermore, when the metal ion concentration is progressively raised (from 1×10^{-8} to 0.01 M), the degree of inhibition slowly increases from 17 to 85%. Over this millionfold range of Zn(II) concentration, the inhibition isotherm is very broad and in no way resembles the titration curve which would be expected if the protein–metal ion interaction were characterized by a high association constant such as that shown by the zymogen. Thus, we infer that, while inhibition of autoactivation by Zn(II) is highly specific, inhibition of the activated protein is nonspecific.

Inhibition of NGF Zymogen by DFP. One question which now arises is whether the low level of Tame hydrolytic activity observed with purified zymogen stems from intrinsic activity of the zymogen itself, or from a small amount of already activated enzyme. To approach this problem, we have examined the possibility that DFP might react with the zymogen. In earlier studies, before we appreciated the existence of autoactivation of NGF, we were consistently unable to achieve inhibition of enzyme activity with DFP. Figure 4 shows that when the zymogen is diluted to a low concentration (1.8 μg/mL) at which activation occurs (Young, 1979), it can be inhibited with DFP. Under these conditions, the apparent second-order rate constant for inhibition is 19 L mol^{-1} min^{-1} and the half-life of the reaction is 55 min (at a DFP concentration of 0.9 mM). In contrast, when the protein was prevented from self-activation by keeping the protein concentration high, no detectable inactivation by DFP (14 mM) was observed (Figure 4). This result is fully consistent with the concept of autocatalytic activation since before the proper active-site configuration is generated, the DFP active site serine reaction is strongly inhibited. However, further studies demonstrate that the zymogen can be inhibited by DFP if extraordinarily high concentrations of this reagent and long reaction times are employed. A solution of the zymogen (6.2 μg/mL) was prepared with 0.1 M Tris-HCl, pH 7.5, containing 1 mM Zn(II) to block autocatalytic activation. This solution was made 10 mM in DFP, and aliquots were withdrawn over time for Tame assays. The DFP reaction was followed for 24 h at 25 °C, and from plots of ln (reaction velocity) vs. time a second-order inactivation rate constant of 0.07 L mol^{-1} min^{-1} and a half-life of 18 h were obtained. This

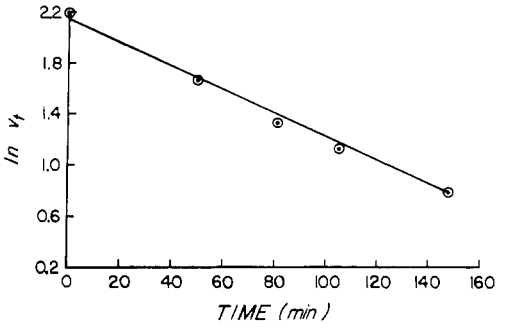

FIGURE 5: Effect of TLCK upon enzyme activity. NGF, 140 μg/mL, dissolved in 0.1 M potassium phosphate, pH 7.0, was treated with 0.12 M EDTA for 1 h at 25 °C to activate the enzyme. This solution was then made 0.04 M in TLCK, and samples were withdrawn for Tame assays as a function of time.

value for the rate constant is nearly 300-fold less than that observed with the fully activated enzyme. These results are consistent with the idea that the zymogen itself possesses a low level of intrinsic enzyme activity with Tame as substrate. If this low level of activity were due to a small amount of contaminating activated enzyme, we would have expected the DFP inactivation rate constants to be identical.

TLCK Inhibition of Activated NGF. The observation that activated NGF is inhibited by DFP suggested that it might also be inhibited by TLCK which alkylates a specific histidine residue at the active site of trypsin (Shaw, 1967). NGF zymogen was activated, treated with 0.04 M TLCK, and assayed over time for Tame activity. Figure 5 presents a plot of ln v_t vs. time which yields an apparent second-order inactivation rate constant of 0.23 L mol^{-1} min^{-1} (half-life = 75 min). Since TLCK can alkylate amino acid residues other than histidine, the above results do not prove that histidine participates in the catalytic reaction of NGF. However, by analogy with other serine proteases, it would not be unexpected to find a specific active-site histidine in this protein.

Discussion

The findings presented above indicate that the NGF zymogen contains 1 g-atom of tightly bound Zn(II) per mol of protein and that the fully autoactivated enzyme contains 1 mol of DFP-reactive serine per mol. Our results on the Zn(II) stoichiometry differ from those of Pattison & Dunn (1975) who reported variable, nonstoichiometric values for the Zn(II) content of 7S-NGF. Our results on the number of DFP-reactive serine residues also do not agree with the reported number of enzymically active subunits per mole of 7S-NGF [see Server & Shooter (1978)]. The results presented here imply that there is only a single active site serine subunit within the structure of the NGF zymogen.

One of the problems pertaining to interpretation of the role of Zn(II) in the structure and function of 7S-NGF is related to the fact that the existence of a NGF zymogen (which can undergo autocatalytic activation) was not established until recently. For example, from their studies on 7S-NGF, Pattison & Dunn (1975, 1976a,b) proposed that the enzymic activity of both the γ subunit and the parent molecule is inhibited by Zn(II). The results presented above indicate that Zn(II) inhibits the enzyme activity of the NGF zymogen, not because it is an enzyme-inhibitory metal but because its presence in the structure prevents autocatalytic activation. Further, Zn(II) is not a potent inhibitor of NGF once it has been fully activated by autocatalysis. For example, when the zymogen has been fully activated, the enzyme retains ~15% of its control activity even in the presence of Zn(II) concentrations as high as 0.01 M. This latter observation is in agreement with results of Pattison & Dunn (1976a,b) who found that 7S-NGF exhibits an affinity for Zn(II) which is 10^5-fold greater than that of its component subunits.

From their studies on the role of Zn(II) in the structure and function of 7S-NGF, Dunn and co-workers (Pattison & Dunn, 1976a,b; Au & Dunn, 1977) concluded that when Zn(II) is removed by EDTA, the protein remains intact (i.e., does not dissociate into subunits). This conclusion has been challenged by Bothwell & Shooter (1978) who have argued that 7S-NGF has no enzymic activity and that dissociation of the γ subunit of the 7S complex is required for expression of proteolytic activity. Results of the present and earlier studies from our laboratory are in agreement with the findings of Pattison & Dunn (1976) and Au & Dunn (1977). For example, under conditions when Zn(II) has dissociated from the zymogen, the protein does not dissociate (Young et al., 1978), yet it is fully catalytically active (Young, 1979).

The foregoing arguments are pertinent to a concept advanced several years ago that (1) the enzyme activity of the γ subunit of 7S-NGF is completely suppressed in the 7S complex, (2) dissociation of the complex is required for expression of enzymic activity, (3) the β subunit is initially synthesized as a larger precursor (a pro β-NGF) and the γ subunit is responsible for enzymically processing this precursor, and (4) the active site of the γ subunit remains complexed with the processed subunit and enzyme activity is thereby inhibited [see Berger & Shooter (1977) for a discussion of this mechanism]. On the basis of studies on the autocatalytic activation reaction (Young, 1979), we pointed out that such a mechanism would appear to be an unlikely one since NGF exists largely as a zymogen following purification from salivary glands. Results of the present study indicate that reaction of the zymogen with DFP proceeds ~300-fold more slowly than reaction of this inhibitor with activated enzyme. This finding suggests that the zymogen, like trypsinogen (Morgan et al., 1972), displays an intrinsic, low level of activity with Tame as substrate and that preparations of the zymogen are not contaminated with small amounts of activated enzyme. Consequently, it is difficult to see how an inactive zymogen could be involved in posttranslational processing of one of its subunits prior to autoactivation.

The observations that NGF exists as a zymogen, that the zymogen undergoes autocatalytic activation, and that autocatalysis is normally inhibited by a metal ion makes this protein an unusual enzyme system. The physiologic purpose of this system is not known, but it could be that Zn(II) serves to act as a control switch and thus to hold the protein in an inactive form until it comes into contact with its naturally occurring substrate.

Acknowledgments

We thank Drs. James Riordan and Takahiro Kumamaru for the atomic absorption studies.

References

Au, A. M.-J., & Dunn, M. F. (1977) *Biochemistry 16*, 3958.
Baker, M. E. (1975) *J. Biol. Chem. 250*, 1714.
Berger, E. A., & Shooter, E. M. (1977) *J. Biol. Chem. 252*, 804.
Bothwell, M. A., & Shooter, E. M. (1978) *J. Biol. Chem. 253*, 8458.
Chase, T., & Shaw, E. (1967) *Biochem. Biophys. Res. Commun. 29*, 508.
Cunningham, L. W., & Nuenke, B. J. (1959) *J. Biol. Chem. 234*, 1447.

Greene, L. A., Shooter, E. M., & Varon, S. (1969) *Biochemistry 8*, 3735.
Hummel, B. C. W. (1959) *Can. J. Biochem. Physiol. 37*, 1393.
Jeng, I. M., Andres, R. Y., & Bradshaw, R. A. (1979) *Anal. Biochem. 92*, 482.
Morgan, P. H., Robinson, N. C., Walsh, K. A., & Neurath, H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3312.
Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977a) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2330.
Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977b) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2672.
Orenstein, N. S., Dvorak, H. A., Blanchard, M. H., & Young, M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 5497.
Pantazis, N. J., Murphy, R. A., Saide, J. D., Blanchard, M. H., & Young, M. (1977) *Biochemistry 16*, 1525.
Pattison, S. E., & Dunn, M. F. (1975) *Biochemistry 14*, 2733.
Pattison, S. E., & Dunn, M. F. (1976a) *Biochemistry 15*, 3691.
Pattison, S. E., & Dunn, M. F. (1976b) *Biochemistry 15*, 3696.
Server, A. C., & Shooter, E. M. (1978) *Adv. Protein Chem. 31*, 339.
Shaw, E. (1967) *Methods Enzymol. 11*, 677.
Sherwood, L. M., & Potts, J. T. (1965) *J. Biol. Chem. 240*, 3799.
Smith, A. P., Greene, L. A., Fisk, H. R., Varon, S., & Shooter, E. M. (1969) *Biochemistry 8*, 4918.
Varon, S., Nomura, J., & Shooter, E. M. (1967) *Biochemistry 6*, 2202.
Young, M. (1979) *Biochemistry 18*, 3050.
Young, M., Saide, J. D., Murphy, R. A., & Blanchard, M. H. (1978) *Biochemistry 17*, 1490.

Carbamate Formation on the ϵ-Amino Group of a Lysyl Residue as the Basis for the Activation of Ribulosebisphosphate Carboxylase by CO_2 and Mg^{2+} †

George H. Lorimer* and Henry M. Miziorko*

ABSTRACT: Ribulosebisphosphate carboxylase (EC 4.1.1.39) from spinach leaves forms a stable complex of enzyme·activator CO_2·Mg·carboxyarabinitol bisphosphate. The CO_2 molecule of this complex does not readily exchange with free CO_2. The enzyme·activator $^{14}CO_2$·Mg·carboxyarabinitol bisphosphate complex was prepared free of unbound $^{14}CO_2$, and a vast molar excess of $^{12}CO_2$ was added. This mixture was added to a solution of diazomethane in methyl Cellosolve. The ^{14}C radioactivity became "fixed" to the enzyme with a yield of about 50%. The fixation of the activator CO_2 to the enzyme by diazomethane treatment occurred in a highly specific manner. The methylated enzyme·activator $^{14}CO_2$ did not undergo exchange with $^{12}CO_2$ and remained bound to enzyme denatured with sodium dodecyl sulfate. The activator $^{14}CO_2$ was bound exclusively to the large catalytic subunit. Prolonged enzymatic hydrolysis (protease from *Streptomyces griseus*) of the methylated enzyme·activator $^{14}CO_2$ followed by amino acid analysis yielded a single peak of ^{14}C radioactivity. The compound in this peak subsequently cochromatographed with genuine N^{ϵ}-(methoxycarbonyl)lysine in a variety of chromatographic systems (ion-exchange chromatography, reverse-phase, high-pressure liquid chromatography, and thin-layer chromatography with two solvent systems). Acid hydrolysis (6 N HCl, 24 h, 115 °C) of the radioactive compound resulted in the volatilization of the ^{14}C. Fixation of the activator CO_2 to the enzyme by diazomethane is due to the esterification of the N^{ϵ}-lysyl carbamate with the formation of N^{ϵ}-(methoxycarbonyl)lysine. Thus activation of ribulosebisphosphate carboxylase is achieved by the formation of a carbamate on the ϵ-amino group of a lysyl residue on the catalytic subunit.

Kinetic (Lorimer et al., 1976; Badger & Lorimer, 1976) and physical studies (Miziorko & Mildyan, 1974) have established that the activation of RuBP[1] carboxylase involves the ordered addition of CO_2[2] and Mg^{2+} (eq 1), with the addition of CO_2 representing the rate-limiting step.

$$\underset{\text{(inactive)}}{\text{enzyme} + CO_2 \rightleftharpoons \text{enzyme}\cdot CO_2 + Mg^{2+}} \rightleftharpoons \underset{\text{(active)}}{\text{enzyme}\cdot CO_2\cdot Mg} \quad (1)$$

The activator CO_2 is distinct from the CO_2 which ultimately becomes fixed during carboxylation. This point has been recently established through kinetic turnover experiments (Lorimer, 1979) and the demonstration (Miziorko, 1979) that a very stable quaternary complex of enzyme·activator CO_2·Mg·2-carboxyarabinitol 1,5-bisphosphate can be formed. CABP is an analogue of 2-carboxy-3-ketoarabinitol 1,5-bisphosphate, which is thought to be the six-carbon intermediate of the carboxylase reaction (Siegel & Lane, 1973; Pierce et al., 1980). CABP binds very tightly to the enzyme with a K_D in the order of 10^{-11} M (Pierce et al., 1980). It is reasonable

† From the Central Research and Development Department, E. I. du Pont de Nemours and Company, Experimental Station E328, Wilmington, Delaware 19898 (G.H.L.), and the Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226 (H. M.M.). *Received May 14, 1980.* This is Central Research and Development Department Contribution No. 2729. This work was in part supported by U.S. Department of Agriculture Grant 5901-0410-9-0339-0 and National Science Foundation Grant PCM-7911474. H.M.M. is a U.S. Public Health Service Research Career Development Awardee (AM 00645).

[1] Abbreviations used: RuBP, ribulose 1,5-bisphosphate; CABP, 2-carboxyarabinitol 1,5-bisphosphate; bicine, *N,N'*-bis(2-hydroxyethyl)-glycine; $NaDodSO_4$, sodium dodecyl sulfate.

[2] CO_2, rather than HCO_3^-, is considered to be the species involved in both activation (Lorimer et al., 1976) and catalysis (Cooper et al., 1969). At the pH values used in the experiments here, HCO_3^- is the predominant species in solution. The term CO_2 will be used to refer to the mixture of CO_2 and HCO_3^-.

0006-2960/80/0419-5321$01.00/0