Characterization of a Brain Calcium-Activated Protease That Degrades Neurofilament Proteins†

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ABSTRACT: A Ca^{2+}-dependent protease was prepared from rat brain by using DEAE-Sephadex, Sephadex G-200, and substrate affinity chromatography. Degradation of neurofilament proteins was determined by measuring the changes in radioactivity of electrophoretically separated bands of radioiodinated neurofilament proteins. The apparent K_m values for 68 000- (P68), 150 000- (P150), and 200 000- (P200) dalton neurofilament proteins are 3.9×10^{-8} M, 4.4×10^{-8} M, and 8.2×10^{-8} M, respectively. Proteolytic activity is dependent upon Ca^{2+} concentration with threshold and saturation values of 10^{-6} and 10^{-4} M, respectively. The enzyme is also inactivated by preincubation with Ca^{2+}. Similar Ca^{2+} concentrations cause activation and inactivation of enzyme, but the process of inactivation is intrinsically slower than the process of activation. The enzyme is sensitive to thiol protease inhibitors, is activated by Sr^{2+}, Ba^{2+}, Mn^{2+}, and La^{3+} at 1–10 mM, and has an optimal pH range of 7.4–8.0.

Neurofilaments (NF)[1] are linear fibrillar structures with a diameter of about 10 nm which are found throughout neurons and their processes. Mammalian NF are composed of the so-called NF triplet proteins with molecular weights of approximately 68 000 (P68), 150 000 (P150), and 200 000 (P200), respectively (Anderton et al., 1978; Liem et al., 1978; Schlaepfer & Freeman, 1978). NF comprise the major ultrastructural component of the axonal cytoskeleton (Friede & Samorajski, 1970; Friede et al., 1971) and probably represent a principal determinant in the development and maintenance of neuronal shape and of the axonal conduit. Their prominence in slow axonal transport (Hoffman & Lasek, 1975; Griffin et al., 1978; Mori et al., 1979) reflects the continuous replenishment of axonal NF following synthesis in the cell body. The nature of their degradation and turnover is unknown, although there is a rapid dissipation of radioactivity from labeled NF proteins upon reaching the axonal termini (Lasek & Black, 1977). It is speculated that NF degradation may be coupled to the relatively high local calcium concentrations in axonal termini (Lasek & Hoffman, 1976; Lasek & Black, 1977).

A calcium-mediated disruption of NF has been documented in rat peripheral nerve (Schlaepfer, 1971; Schlaepfer & Micko, 1978, 1979). The accompanying loss of NF triplet proteins suggests that this phenomenon may be due to a calcium-activated proteolysis. Calcium-dependent breakdown of invertebrate NF proteins from the giant axons of squid (Pant et al., 1979) and *Myxicola* (Gilbert et al., 1975; Lasek et al., 1979) has also been attributed to calcium-activated protease(s) on the basis of protein loss from electrophoretic profiles. However, the enzymatic nature of NF degradation hitherto has not been fully demonstrated through quantitative studies.

In the present study, a highly enriched calcium-activated protease has been obtained from rat brain. A sensitive method has been developed to assess the kinetics of NF degradation, and the relative rates have been determined for the initial step in the enzyme-catalyzed degradation of the three NF proteins. In addition, other properties of the NF protease have been established, including the activation and inactivation of enzyme by calcium.

Experimental Procedures

Isolation of Neurofilament Proteins. Intact segments of bovine spinal cords were obtained within 15 min of slaughter, stripped of their covering meninges and nerve roots, and immersed in ice-cold buffer (100 mM NaCl, 2.5 mM EDTA, 2.5 mM EGTA, and 1% dextrose in 1 mM phosphate buffer, pH 7.0). Spinal cords were split longitudinally, and gray matter was scraped from the interior surface. Tissues were finely minced and washed for 1 h in several changes of hypotonic media (2.5 mM EDTA + 2.5 mM EGTA in 1 mM phosphate buffer, pH 7.0). The swollen and macerated tissues were admixed with 20 mL of 225 mM NaCl/25 g of tissues, homogenized in a 40-mL Dounce homogenizer with 5 strokes of A and B pestles (clearance, 0.003–0.006 and 0.001–0.003 in., respectively), and centrifuged in a Beckman SW 27 rotor (r_{av} = 4.13 in.) at 80000*g* for 20 min at 4 °C. Cloudy supernatant was collected, and the precipitate was rehomogenized with a volume of 100 mM NaCl equal to the withdrawn supernatant and recentrifuged. The combined supernatants were centrifuged at 80000*g* for 4 h. Pelleted neurofilaments were suspended in 8 M urea and spun overnight (12–14 h) at 80000*g*. The floating lipid layer was removed, and NF proteins (3–4 mg/mL) were aliquoted and stored at −70 °C.

Iodination of Neurofilament Proteins. Aliquots (100 μL) of bovine NF proteins were combined with an equal volume of 10 mM Hepes buffer, pH 7.4, and mixed thoroughly with 15 μL of $Na^{125}I$ (17 μCi/μL, IMS 30, Amersham) and 100 μL of 5 mg/mL Chloramine T for 1 min at 4 °C. Iodination was terminated by addition of 100 μL (11.1 mg/mL) of sodium metabisulfite. Iodinated proteins were separated by gel filtration chromatography with a PD-10 column (1.4 × 5.5

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[1] Abbreviations: BSA, bovine serum albumin; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, *N*-ethylmaleimide; NF, neurofilament(s); PCMB, *p*-(chloromercuri)benzoate; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3CCOOH, trichloroacetic acid; TLCK, 1-chloro-3-(tosylamido)-7-amino-L-2-heptanone hydrochloride; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone.

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cm, Pharmacia) equilibrated with 10 mM Hepes buffer, pH 7.4. Noncovalently bound ^{125}I was removed by equilibration with 0.1 M NaI for 1 h and rechromatographed on a fresh PD-10 column equilibrated with HNEED (10 mM Hepes, pH 7.4, 10 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 1 mM DTE). Radioiodinated NF preparations had a protein concentration of 0.33 mg/mL and specific activities of 6.7×10^7 cpm/mL (44 μCi/mg). Freshly radioiodinated NF preparations were used for each experiment. Radioactivity was monitored with an LKB 1275 Minigamma counter which had 68% counting efficiency with ^{129}I as a standard; the absolute background count was 90 cpm.

Preparation of Crude Enzyme Fraction from Rat Brain. Sprague-Dawley male rats weighing 250–300 g were perfused with ice-cold isotonic saline and HNEED. Brain slices (40 g from 14 rats) were swollen in HNEED for 1 h and homogenized at low speed in 1 volume of HNEED with five to ten strokes of the Teflon pestle in a no. 24 Duall homogenizer. The homogenate was centrifuged in a Sorvall SS 34 rotor (r_{av} = 4.25 in.) at 48000g for 1 h, yielding about 35 mL of supernatant with a protein concentration of 9–10 mg/mL.

Preparation of Affinity Chromatographic Column. Radiolabeled NF proteins (^{125}I-labeled NF) were coupled to activated Sepharose 4B following the methods suggested by Pharmacia. Activated Sepharose 4B (2 g) was swollen and washed in a sintered glass filter funnel with 200 mL of 1 mM HCl. The washed gel was suspended in 6 mL of coupling buffer (0.1 M $NaHCO_3$–0.5 M NaCl) containing 20 mg of ^{125}I-labeled NF proteins in a sealed test tube, and the mixture was gently shaken end over end overnight at 4 °C. The ^{125}I-labeled NF–Sepharose 4B was washed with 100 mL of coupling buffer, and unreacted groups on the gel were blocked by treating with 12 mL of 0.1 M ethanolamine (pH 8) for 2 h. The ^{125}I-labeled NF–Sepharose 4B was washed with 30 mL each of 0.1 M acetate buffer (pH 4) containing 1 M NaCl and 0.1 M borate buffer (pH 8) containing 1 M NaCl. This wash cycle was repeated 3 times. Washed ^{125}I-labeled NF–Sepharose 4B contained approximately 37 μg of protein/mL of wet gel.

Incubation Conditions, Analysis of Samples, and Assay for Enzyme Activity. For incubations, glass test tubes (12 × 75 mm) that had been soaked in 1 mM EGTA and rinsed several times with deionized water were used; all incubations were made in HNEED. A typical incubation mixture contained enzyme, substrate, and Ca^{2+} in a total volume of 400–1000 μL. Incubations with very low total protein concentration were carried out in the presence of 0.2% BSA. A zero time sample was withdrawn, and reactions were initiated by addition of Ca^{2+}. Aliquots (40 μL) were taken at various time intervals, and the reaction was terminated by injecting the reaction mixture into 15 μL of solubilizing buffer (5% $NaDodSO_4$, 20 mM EGTA, 200 mM DTE, 50% sucrose, and 0.5% bromophenol blue in 50 mM Tris-HCl buffer, pH 6.7) followed by heating for 5 min at 100 °C. The zero time sample was treated in the same way except that an equivalent amount of Ca^{2+} was added after the heat inactivation. A 20–30-μL aliquot was taken from each sample for $NaDodSO_4$ gel electrophoresis using a Tris–glycine buffer system (Laemmli, 1970) at 50 mA/gel plate and at room temperature. Radioactivity applied to each gel lane was determined by measuring total counts per minute before and after each aliquot was taken out from the reaction mixture. Autoradiograms were made from the dried gels by direct contact with Kodak No-Screen X-ray films. Hand tracings of the autoradiograms were superimposed on the stained dried gels in order to identify the three prinicipal radioiodinated NF proteins bands. In a given electrophoretic lane, the NF protein bands were cut out, all other parts of the lane were subdivided, and each piece was counted separately. The total radioactivity of each lane was determined by summing the activity of all pieces. The extent of the degradation of the NF proteins (P200, P150, and P68) at a given time was estimated by the relation $[F_{P200}(0) - F_{P200}(t)]/F_{P200}(0)$ where $F_{P200}(t)$ represents the fraction of radioactivity found in the P200 electrophoretic band (the ratio of the counts per minute of the P200 band to the total counts per minute of the gel lane) at time t. Cl_3CCOOH-soluble degradation products were measured concurrently by withdrawing additional 40-μL aliquots which were added to 10 μL of 2% (w/v) BSA and immediately precipitated with the addition of 50 μL of 4% (w/v) Cl_3CCOOH and spun in a microfuge (Beckman) for 5 min. The ratio of the radioactivity of the supernatant to the total radioactivity of the aliquot represents the combined products of the breakdown of all three NF proteins. One unit of the enzyme activity was then defined as the amount of enzyme which catalyzes 1% degradation of NF proteins in 1 h as determined by Cl_3CCOOH precipitation.

Table I: Isolation of Calcium-Activated Protease from Rat Brain

fraction	protein (mg)	total activity (units)	sp act. (units/mg)
crude supernatant	323	1034	3.2
DEAE-Sephadex	12.8	468	36.6
G-200 Sephadex	1.1	364	330.9
^{125}I-labeled NF–Sepharose 4B	0.06	271	4524

Determination of Free Calcium Ion Concentration. Ca–EGTA buffer with the free Ca^{2+} concentration ranging from 10^{-8} to 10^{-2} M was prepared by varying the concentration of total Ca^{2+} in a solution containing 0.67 mM EGTA, 10 mM Hepes (pH 7.4), and 10 mM NaCl. Calcium concentrations below 10^{-4} M were estimated from the absorbance of Arsenazo III (Sigma) at 652 nm and by using the dissociation constant of the dye, 1.95×10^{-6} M/L [calculated by using data in Bauer (1981)]. A calcium ion electrode (F-2112, Radiometer Copenhagen) was used to measure the free Ca^{2+} concentrations between 10^{-2} and 10^{-6} M.

Results

Purification of Calcium-Activated Protease. Crude supernatant of brain homogenate was adsorbed on a DEAE-Sephadex column (2.4 × 7.5 cm) equilibrated with HNEED. After the column was washed with 60 mL of HNEED, the enzyme was eluted with 100 mL of HNEED by using a linear concentration gradient of NaCl from 0.01 to 0.5 M (Figure 1A). The active fractions were pooled, concentrated in a vacuum, and subjected to gel filtration on a G-200 Sephadex column (1.5 × 95 cm). Elution was carried out with the same buffer, and active fractions were pooled (Figure 1B). For the last purification step, we developed a new method of affinity chromatography by using an ^{125}I-labeled NF–Sepharose 4B column (1.2 × 4.5 cm) equilibrated with HNEED containing 10^{-5} M Ca^{2+}. The pooled active fraction from the G-200 Sephadex column was adjusted to 10^{-5} M Ca^{2+} and immediately applied to the affinity column. The column was then washed with 18 mL of the above calcium buffer, and the enzyme was eluted with HNEED containing 4 mM EGTA (Figure 1C). The procedures employed to isolate the enzyme are summarized in Table I. The enzyme was purified greater than 1400-fold by starting from crude supernatant, with a yield

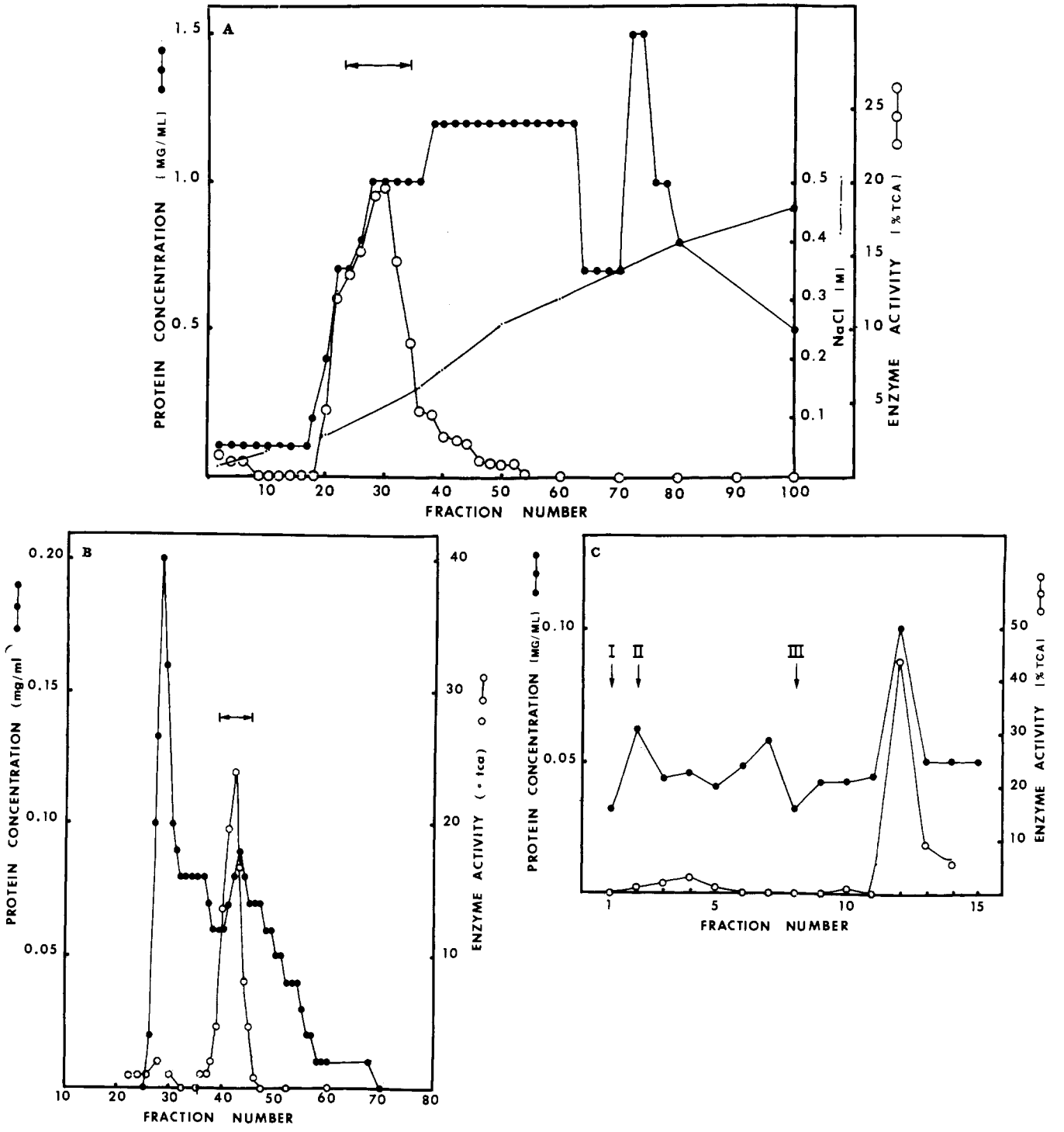

FIGURE 1: (A) Fractionation of rat brain protease on DEAE-Sephadex. Crude supernatant (35 mL, 9–10 mg/mL) was applied to a column (2.4 × 7.5 cm) in HNEED and eluted with a linear gradient of NaCl (0.01–0.5 M) with a flow rate of 0.2 mL/min and in 1.2-mL fractions. Enzyme activity was determined by Cl_3CCOOH precipitation of ^{125}I-labeled NF proteins (see text). (B) Fractionation of protease on Sephadex G-200. The active fraction from DEAE-Sephadex was applied to a Sephadex G-200 column (1.5 × 95 cm) and eluted with HNEED with a flow rate of 4.2 mL/h in 2.2-mL fractions. Active fractions 39–45 were combined. Ratio of elution (92.5 mL) to void volume (55 mL) was 1.69. (C) Affinity chromatography of protease on ^{125}I-labeled NF–Sepharose 4B. The column (1.5 × 5.0 cm) was equilibrated with HNEED containing 10^{-5} M Ca^{2+}. At I, the enzyme was applied to the column. At II, the column was washed with the same calcium buffer. At III, the enzyme was recovered by elution with HNEED containing 4 mM EGTA. Fractions of 0.6 mL were collected.

of 0.02%. The enzyme obtained after ^{125}I-labeled NF–Sepharose 4B column chromatography showed major bands at approximately 100, 80, 70, and 30 kilodaltons upon NaDodSO$_4$–polyacrylamide gel electrophoresis.

Kinetics of Degradation of Neurofilament Proteins by the Enzyme in the Presence of 1 mM Ca^{2+}. The electrophoretogram of bovine NF proteins separated by 7.5% NaDodSO$_4$–polyacrylamide gel electrophoresis and stained with Coomassie blue is shown in Figure 2a, and the corresponding autoradiogram of radioiodinated NF proteins with the same triplet pattern is shown in Figure 2b. The distribution of radioactivity among the various components as a function of time has been determined and is presented in Table II. The sum of the radioactivity lost from the three individual NF protein bands at any given time can be accounted for almost entirely by the corresponding rise in the radioactivity found

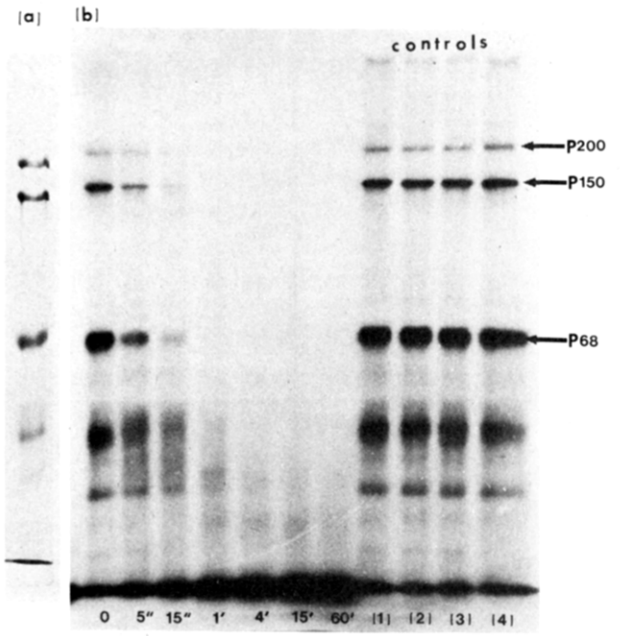

FIGURE 2: (a) Coomassie blue stained $NaDodSO_4$ 7.5% gel electrophoretogram of bovine NF triplet proteins. (b) Autoradiogram of the $NaDodSO_4$ electrophoretogram of the samples taken at various times from the incubation mixture consisting of enzyme (12 μg/mL), ^{125}I-labeled NF proteins (0.33 mg/mL; 2.2×10^7 cpm/mL), and 1 mM Ca^{2+}. The control incubations were carried out for 120 min and contained (1) no Ca^{2+} but an equivalent amount of Mg^{2+}, (2) no enzyme, (3) heat-inactivated enzyme, or (4) Ca^{2+}, enzyme, and 1 mM PCMB.

Table II: Distribution of Radioactivity as a Function of Time in Electrophoretograms following Incubation of ^{125}I-Labeled NF and Purified Enzyme in the Presence of 1 mM Calcium[a]

time (s)	F_{200}	F_{150}	F_{68}	F_{LMWC}	F_{HMWC}
0	0.042	0.093	0.184	0.555	0.125
5	0.035	0.065	0.097	0.698	0.106
15	0.025	0.036	0.058	0.796	0.085
60	0.015	0.016	0.029	0.883	0.056

[a] The various fractions, F, were calculated by dividing the counts per minute for each portion by the total counts per minute for all the portions. F_{LMWC} = fraction of components with M_r less than 68 000. F_{HMWC} = fraction of components with M_r greater than 68 000 excluding the P200, P150, and P68 NF proteins.

in the region of lower molecular weight components. From Table II, the percent degradation for each NF protein can be calculated as a function of time. For example, 50% of P68 is degraded in about 5 s and over 80% in 1 min whereas no degradation of any NF protein band occurs in the controls after 120-min incubation (Figure 2). From the controls, we conclude that the enzyme is heat labile and requires intact thiol groups and Ca^{2+} for its activity.

Initial Rate of Breakdown of Each Triplet NF Protein as a Function of the Total Substrate Concentration. Incubation mixtures contained enzyme (12–25 μg/mL), 1 mM Ca^{2+}, and a variable total substrate concentration (0.4×10^6–4.0×10^6 cpm/mL; 6–60 μg/mL). Aliquots were taken at time intervals of 0, 10, 30, and 60 s. For each NF protein band, the initial rate was estimated from the initial slope of the change in counts per minute per milliliter as a function of time for different total substrate concentrations. The Lineweaver–Burk plots for the three proteins show straight lines with almost the same intercepts and with average apparent K_m values (from three independent experiments) of 3.9×10^{-8} M, 4.4×10^{-8} M, and 8.2×10^{-8} M for P68, P150, and P200 proteins, respectively. Note that the K_m values indicate the enzyme–substrate affinities for P68 and P150 proteins to be nearly the same and about twice the affinity for P200.

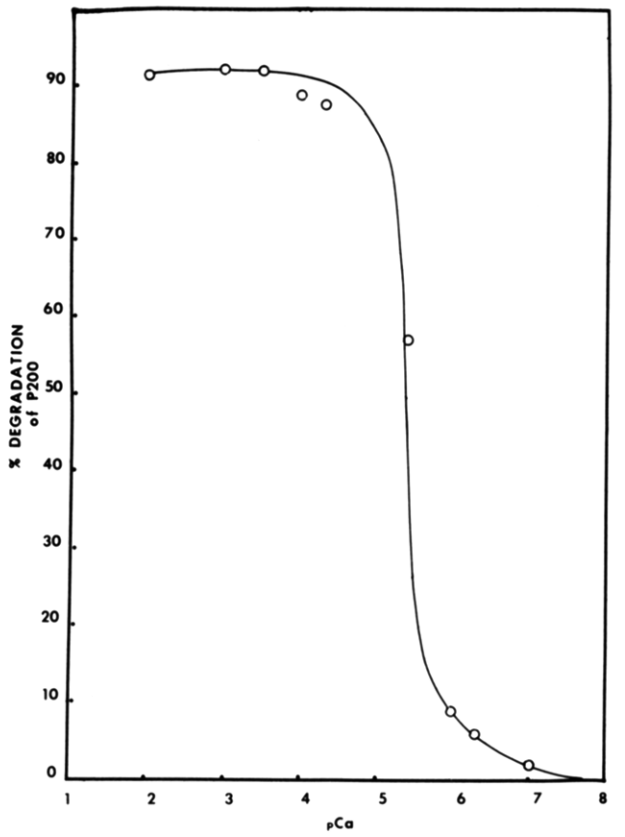

FIGURE 3: Ca^{2+} concentration dependence of the proteolytic activity of the enzyme. Incubation of enzyme (25 μg/mL) and ^{125}I-labeled NF proteins (2.2×10^7 cpm/mL) was carried out in HNEED containing free Ca^{2+} concentration from 10^{-8} to 10^{-2} M. Aliquots were taken at 1 h and analyzed for NF degradation by the method of $NaDodSO_4$ gel electrophoresis and autoradiography.

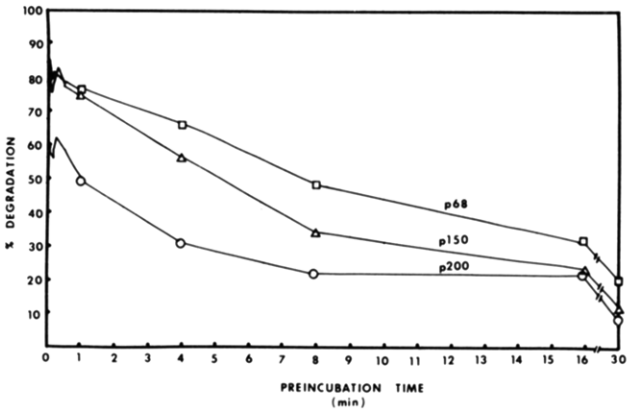

FIGURE 4: Time dependence of the inactivation of enzyme by Ca^{2+}. Aliquots of freshly prepared enzyme (25 μg/mL) were preequilibrated with 1 mM Ca^{2+} for varying lengths of time after which incubations were started with the addition of substrate (2.2×10^7 cpm/mL) and terminated after 15 s. The extent of Ca^{2+} inactivation of the enzyme was estimated by comparing the degradation with control (i.e., without preequilibration with Ca^{2+}).

Dependence of Enzyme Activity on Calcium Ion Concentration. Incubations of enzyme and ^{125}I-labeled NF were carried out in media with free Ca^{2+} concentration ranging from 10^{-2} to 10^{-8} M (Figure 3). The threshold for calcium ion activation is about 10^{-6} M, and saturation occurs at 10^{-4} M. Half-maximal degradation is reached in 1 h at a Ca^{2+} concentration of 4×10^{-6} M. When aliquots of enzyme alone were

preequilibrated with 1 mM Ca^{2+} for varying lengths of time and then tested for their proteolytic activity, an initial rise of activity for 5–10 s followed by the decline of activity was observed (Figure 4); e.g., at 1 min and at 1 h, 10% and 84% inactivation of the enzyme was observed. This inactivation was also dependent on calcium ion concentration. When the aliquots of enzyme were preequilibrated with varying concentrations of Ca^{2+} (10^{-2}–10^{-8} M) for a fixed time (1 h) followed by incubations with substrate and 1 mM Ca^{2+}, the inactivation increased monotonically with the Ca^{2+} concentration; e.g., 10^{-4} M Ca^{2+} produced 50% inactivation.

Inhibitors and Simulators of the Enzyme and Other Properties. PCMB, NEM, antipain, and leupeptin (all 0.1 mM) were effective inhibitors of the enzyme, while TLCK and TPCK were only partially effective at 1 mM and PMSF at 1 mM had no effect. Other metal ions, Sr^{2+}, Ba^{2+}, Mn^{2+}, and La^{3+}, all at 1 mM activated the enzyme but less effectively than Ca^{2+}. The enzyme was active at pH 5.8–9.0 with optimum activity at pH 7.4–8.0. This enzyme preparation also degraded [^{125}I]casein and [^{125}I]F-actin while no detectable degradation was observed with [^{125}I]BSA.

Discussion

Characterization of calcium-activated proteolytic activity was conducted on highly enriched fractions. Our rates of protein degradation were measured in seconds and far exceed those reported previously for calcium-activated proteases. Use of rat brain as a source of enzyme enabled the isolation of calcium-activated protease to be conducted under optimal conditions. Perfusion of rat tissues served to remove enzyme inhibitor(s) present in rat serum (U.-J. P. Zimmerman and W. W. Schlaepfer, unpublished results), reduced overall tissue calcium, permeated tissue with calcium chelators, and quickly equilibrated tissue to lower temperatures. A major drawback in the use of rat brain, however, is the relatively limited amount of starting material which is available.

Our active enzyme fraction eluted as a single peak by affinity chromatography as calcium concentrations were reduced from 10^{-5} M to less than 10^{-7} M. Nevertheless, the enzyme fraction revealed several bands by $NaDodSO_4$ electrophoresis. Similar bands were obtained when anion-exchange chromatography was substituted as the final preparative step. Since the active enzyme fraction has a molecular weight of about 100 000 by gel filtration, it is unlikely that the multiple polypeptides are separate components of the enzyme moiety. Instead, it seems more likely that they represent multiple molecular weight forms, possibly degradative products, of the same protein. Kubota (Kubota et al., 1981) suggested that his calcium-activated protease from chicken skeletal muscle using a casein-coupled Sepharose column was slightly different (76 000 daltons) from the one (80 000 daltons) prepared by Ishiura (Ishiura et al., 1978), who had used a second DEAE column. The difference was attributed to conversion of high to low molecular weight forms during chromatography.

Bovine NF proteins can be harvested in large quantities, are stable when aliquoted and stored at −70 °C, and provide a uniform substrate for quantitation of enzymatic activity. It has been possible to isolate individual NF proteins but only in medium containing $NaDodSO_4$ (Hogue-Angeletti et al., 1982). Our enzyme preparation loses its enzymatic activity in the presence of $NaDodSO_4$. Furthermore, we have found that purified NF proteins are insoluble and/or unstable in the absence of $NaDodSO_4$. Therefore, we have used a substrate consisting of the three NF proteins in a fixed mole ratio. Our present methodology enables us to follow the kinetics of degradation of the three coexisting NF proteins simultaneously by using the radioactivity of each as a measure of its concentration. Thus, for the kinetics of the NF protein degradation, we determined apparent K_m values, the ratios of which provide relative reactivities.

Our electrophoretic analysis indicates that the three NF proteins are highly susceptible to calcium-activated proteolysis and that the primary step of degradation is that of a limited proteolysis cleaving interior bonds. Continued proteolysis results in small peptide fragments that appear as a Cl_3CCOOH-soluble fraction. Accordingly, Cl_3CCOOH precipitation does not measure the primary degradative step in NF proteolysis but rather a collective series of intermediate reactions. Furthermore, Cl_3CCOOH precipitation is a relatively insensitive monitor of NF proteolysis. Although considerable breakdown (i.e., 50%) of P68 NF protein occurs in 5 s, only very limited degradation is evidenced by Cl_3CCOOH precipitation at 1 min (1–2%).

It is essential to use very short incubation times in order to obtain good initial rates of Ca^{2+}-activated degradation of NF proteins. In this way one can avoid the complication due to the slower Ca^{2+}-catalyzed inactivation of the enzyme and the competition for the enzyme in later degradative steps. Initial rate studies thus allow one to use a simple kinetic model for the NF protein degradation in which the three primary reactions are coupled by the competition of the three initial proteins for the free enzyme in forming enzyme–substrate complexes. Such a model predicts the saturation curves and the straight line Lineweaver–Burk plots as reported.

The Ca^{2+} concentration dependence of both activation and inactivation processes shows similar sigmoid curves with the same threshold Ca^{2+} concentration of 10^{-6} M and saturation concentration of 10^{-4} M. The only difference between the two processes seems to be that the rate of inactivation is 10–100 times slower than that of proteolysis, as seen in Figure 4, which shows an initial rise of activity for 5–10 s prior to the decline of activity. These observations suggest that the enzyme is fully functional at micromolar concentrations of calcium and that the activation of NF protease by Ca^{2+} may be a transient step attuned to a fluctuating level of intracellular Ca^{2+} concentration and with a built-in mechanism for self-regulation. Transient and short-lived rises in intracellular Ca^{2+} concentration would favor degradation of NF proteins whereas more prolonged elevations of Ca^{2+} concentration would tend to degrade the enzyme itself. The successful binding of the enzyme to the ligand in the affinity column equilibrated with 10^{-5} M Ca^{2+} also strongly supports the above conclusion that the enzyme is activatable at a very low concentration of calcium.

In a preliminary study, effects on the Ca^{2+}-activated degradation rates of pH, inhibitors, other metal ions, and temperature dependence were examined by using crude brain supernatant. The rates were much slower, due in part to the presence of an endogenous inhibitor known to be present in the crude supernatant. Nevertheless, the order of reactivity of the three NF proteins was always the same. The large increase in the proteolytic rate for each NF protein resulting from the enrichment of enzyme strongly suggests that we are dealing with a single enzyme specific for NF degradation rather than with several enzymes. Furthermore, similar behavior of all three NF proteins, such as the same threshold Ca^{2+} concentration for activation and for inactivation, provides additional support for the view that the NF proteolysis observed is mediated by a single enzyme.

The NF proteases described in this report bear close resemblance to the calcium-activated protease of rat brain de-

scribed by Guroff (1964). The Guroff enzyme was also a neutral thiol protease which degraded casein and an unidentified endogenous substrate from brain. It is quite possible that the latter substrate included NF proteins in part, although a relatively insensitive assay method (i.e., Cl_3CCOOH solubility) was used to detect calcium-activated proteolysis in the Guroff study. More recent studies have identified additional calcium-activated neutral thiol protease in skeletal muscle (Meyer et al., 1964; Belocopitow et al., 1965; Drummond & Duncan, 1968; Huston & Krebs, 1968; Reddy et al., 1975; Dayton et al., 1976; Ishiura et al., 1978), uterine smooth muscle (Puca et al., 1977), platelets (Phillips & Jakabova, 1977), Ehrlich ascites tumor cells (Nelson & Traub, 1981), and rat brain (Inoue et al., 1977). In some cases, the enzyme has been demonstrated in several tissues (Puca et al., 1977; Inoue et al., 1977); however, in most instances, the relationship between these enzymes of similar properties remains speculative. It is of interest that many calcium-activated proteases reveal very similar properties, including activation of Sr, Mn, and Ba, inhibition by thiol but not serine inhibitors, and inactivation by calcium (Schlaepfer et al., 1981). The latter phenomenon may be due to autoproteolysis, as based upon the disappearance of protein bands from electrophoretograms of enzyme preparations following preincubation with calcium (Dayton et al., 1976). This view is also supported by the present study in which the pCa of inactivation was shown to approach that of enzyme activation.

Participation of calcium-activated protease in the turnover of NF proteins would require neuronal localization of both substrate and enzyme. Our quantitative studies of calcium-activated NF protease which has been highly enriched from tissue homogenates cannot address the question of enzyme localization. On the other hand, the isolated enzyme bears a very strong resemblance to tissue factor(s) which cofractionate with isolated NF (Schlaepfer & Freeman, 1980) and to suspected calcium-activated proteases in the axoplasm of rat nerve fibers (Schlaepfer & Micko, 1979; Kamakura et al., 1981). The latter have been characterized by studying biochemical and morphological alterations of NF following an influx of calcium into isolated nerve fibers under different incubational conditions (Schlaepfer et al., 1981). It is suggested that the presence of NF protease in the axoplasm of neurites may reflect the ubiquitous transit of macromolecules en route toward synaptic endings from biosynthetic loci in neuronal perikarya. Furthermore, it is suspected that very low levels of intraaxonal calcium render the enzyme inactive under physiological conditions during the period of axonal transport.

The hypothesis that axoplasmic calcium-activated protease is operative in NF turnover is based in part on studies of NF in axonal transport (Lasek & Hoffman, 1976). These studies have shown that radiolabeled NF triplet proteins move in synchrony down the axon and accumulate proximal to a nerve ligature but are lost rapidly upon reaching the axonal termini at active synaptic junctions. These findings have led to conjecture that an axoplasmic protease degrades NF proteins at axonal termini, possibly by coupling proteolysis with local events at nerve endings, such as the transient calcium influx which accompanies neurotransmission. At present, this theory is attractive but entirely speculative. Nevertheless, the theory is supported by our findings of an enzyme in neural tissues which rapidly degrades NF proteins and can be activated by micromolar levels of calcium.

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Purification and Characterization of a New Mammalian Serum Protein with the Ability To Inhibit Actin Polymerization and Promote Depolymerization of Actin Filaments†

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ABSTRACT: A protein with capacity to bind G-actin and the ability to inhibit polymerization and promote depolymerization of actin filaments has been isolated from the serum of rabbit. The protein, SAIP (for serum actin inhibitory protein), has been purified by affinity chromatography of serum over actin–Sepharose followed by protein fractionation with ammonium sulfate and chromatography over DEAE-cellulose. Five milligrams of purified SAIP is obtained from 100 mL of serum. Rabbit SAIP is resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis into two closely related polypeptides of 60 000 and 56 000 daltons, respectively (ratio 5.7:1). Each of these polypeptides consists of two isoelectric variants. SAIP binds to monomeric actin with a stoichiometry of 1:1 and a K_d of 0.12 μM. The SAIP–actin complex binds to DNase I. Actin polymerization is completely inhibited by incubation of actin with an equal concentration of SAIP. At equimolar concentrations to F-actin, SAIP induces complete depolymerization of the actin filaments. SAIP is also present in calf serum.

Actin is a contractile–structural protein present in all eukaryotic cells. Upon polymerization, the monomeric form of actin (G-actin)[1] forms double-stranded helical filaments (F-actin). These filaments are a major component of the cell cytoskeleton and play an important role in cell motility [for reviews, see Clarke & Spudich (1977), Hitchcock (1977), Korn (1978), and Lindberg et al. (1979)]. The way cells polymerize actin depends on their function. Tissues specialized in contraction–relaxation, such as striated and possibly smooth muscle, organize the actin filaments in stable structures (i.e., thin filaments). On the other hand, the diversity and transient character of many of the structural and contractile functions in which actin plays a role in nonmuscle cells requires the frequent polymerization and depolymerization of actin in these cells. The assembly of actin can be regulated at different levels including filament nucleation and the addition and loss of actin monomers to elongating and steady-state filaments [for review, see Hitchcock-De Gregori (1980)].

The nucleation of actin filaments has been shown to be promoted by a complex of spectrin–actin–band 4.1 isolated from human erythrocytes (Lin & Lin, 1979; Ungewickell et al., 1979) and also by a complex of spectrin–actin obtained from sheep erythrocytes which blocks the slow-growing end of the actin filaments (Brenner & Korn, 1980). More recently several proteins capping the fast growing end of the actin filaments (i.e., capping proteins) have been isolated from sources as diverse as *Acanthamoeba* (Isenberg et al., 1980), *Physarum* (Hasegawa et al., 1980), and chicken intestinal epithelial cells (Craig & Powell, 1980; Glenney et al., 1980, 1981). This class of proteins also has the property to enhance nucleation of actin filaments (Isenberg et al., 1980; Hasegawa et al., 1980; Craig & Powell, 1980; Glenney et al., 1981), and some of them, like gelsolin, villin, and fragmin, show the ability to sever the actin filaments, producing short nonsedimentable oligomers of actin in the presence (Yin & Stossel, 1979; Hasegawa et al., 1980; Glenney et al., 1981) or absence of Ca^{2+} (Isenberg et al., 1980). Actin nucleation can also be controlled by proteins which bind to G-actin preventing polymerization. One of these proteins, profilin (Carlsson et al., 1977), seems to prevent actin nucleation specifically without inhibiting filament elongation (Reichstein & Korn, 1979). The inhibitory effect of profilin on actin nucleation can be reversed by α-actinin (Blikstad et al., 1980), short fragments of actin filaments (Reichstein & Korn, 1979), and a cytochalasin binding complex isolated from erythrocytes (Grumet & Lin, 1980). Other proteins binding to G-actin in addition to inhibiting the nucleation of actin also prevent filament elongation and cause depolymerization of the filaments. To this group of proteins belongs DNase I (Lazarides & Lindberg, 1974), which has been found complexed to G-actin in rat pancreatic juice (Mannherz & Rohr, 1978) and the 65K and 62K polypeptides isolated recently from human granulocytes (Southwick & Stossel, 1981).

Blood plasma and sera from various mammals have been shown to have the capacity to depolymerize actin filaments. Human serum contains a Ca^{2+}-dependent enzyme which promotes catalytically the depolymerization of actin filaments (Norberg et al., 1979). On the other hand a 92 000-dalton protein has been isolated from pig plasma which binds stoi-

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[1] Abbreviations: G-actin, globular actin; F-actin, filamentous actin; IEF, isoelectric focusing; SAIP, serum actin inhibitory protein; DNase I, deoxyribonuclease I; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; DTT, dithiothreitol; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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