Purification and Characterization of Two Forms of Ca\(^{2+}\)-activated Neutral Protease from Calf Brain*

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Two forms (CANP1 and CANP2) of a calcium-activated neutral protease (CANP) have been purified, 1,950- and 1,250-fold, respectively, to near homogeneity from calf brain. The purification procedure involves ammonium sulfate fractionation of the brain cytosol followed by chromatography on DEAE-Sephacel, hydroxylapatite, and \(\alpha\)-casein-CH-Sepharose 4B affinity gel. A protein with apparent \(M_r = 17,000\) co-purifies with each of the proteases. This protein was separated by chromatography on a reactive red-120 agarose. Preliminary experiments indicate that, in the absence of this protein, the activity of each of the proteases was reduced. These observations raise the possibility that the 17,000-Da protein may regulate the activity of these proteases.

Each of the proteases have similar apparent \(M_r = 78,000\) as judged on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Except for casein, hemocyanin, and hemoglobin, no other exogenous proteins tested were significantly hydrolyzed by either of the proteases. [\(\text{methyl-}^{14}\text{C}\)]\(\alpha\)-Casein or methemoglobin was routinely used as a substrate for both of the enzymes. The endogenous proteins, neurotubules (microtubule-associated proteins and tubulin), neurofilament triplet proteins and desmin from smooth muscle were extensively hydrolyzed by both of the proteases. A marked difference was found in their requirement for CaCl\(_2\). CANP1 was maximally active at 700 \(\mu\)M while CANP2 exhibited highest activity at 2 \(\mu\)M CaCl\(_2\). Both displayed maximum activity at pH 7.5, although the overall pH profiles were slightly different. Among the actinomycete protease inhibitors, antipain, leupeptin, and pepstatin, leupeptin was highly effective in inhibiting the activity of both enzymes. Both of the proteases were also inhibited by sulphydryl modifying agents. Metal ions other than CaCl\(_2\) were poor activators of the activity of either protease.

Among the nonlysosomal class, the CANPs\(^1\) have received a great deal of attention in recent years. They have been identified in several tissues such as erythrocytes (1), platelets (2, 3), uterus (4), chick oviduct (5), liver (6), brain (7), cultured neuroblastoma cells (8), Erlich ascites tumor cells (9), and muscle (10–12). There have also been several reports of calcium-activated proteolytic activity, in both invertebrate (13–15) and vertebrate nerves, which selectively degrade neurofilament (16, 17). Calcium-activated proteolytic activity has also been implicated: 1) in the regulation of the glutamate receptors in neuronal (synaptic) membranes (18, 46); 2) turnover of neurofilament proteins at nerve endings (47); and 3) Wallerian degeneration of peripheral nerves (48).

Despite the emphasis on the possibly important role played by the calcium-activated proteolytic activity in the brain and nerves, as mentioned above, little has been done to completely purify and characterize this enzyme(s). For example, purification of the CANP from the brain was attempted earlier by Guroff (7) and Inoue et al. (19) but these studies were lacking by the important fact that no criteria was presented to assess the purity of the enzyme. In addition, no specificity of this protease toward endogenous proteins was demonstrated. Partial purification of a single CANP from rat brain (20) and two forms of CANP from cell cultures of neuroblastoma (8) was also reported recently but in none of these studies was this enzyme(s) fully characterized. This background, as well as our recent observations (21) on the degradation of fibrous proteins by crude CANP from calf brain, stimulated us to undertake the purification and characterization of this protease(s). Some aspects of this work were published in abstract form (22).

**EXPERIMENTAL PROCEDURES**

**Materials**

\(\alpha\)-Casein, Tris, phenylmethylsulfonyl fluoride, trypsin inhibitor, soybean, \(p\)-chloromercuribenzoate, iodoacetic acid, and \(N\)-ethylmaleimide were purchased from Sigma Chemical Co. Sephacryl S-200, DEAE-Sephacel, and activated CH-Sepharose 4B were from Pharmacia. Hydroxylapatite was from Calbiochem. [\(\text{methyl-}^{14}\text{C}\)]\(\alpha\)-Casein and [\(\text{methyl-}^{14}\text{C}\)]methemoglobin were from New England Nuclear and Dimerscint scintillation mixture was from National Diagnostics. Leupeptin and antipain were from Beckman while pepstatin was from Boehringer Mannheim. Aff-Gel 501, DEAE-Bio-Gel A, and Chelex 100 were from Bio-Rad. Calmodulin from rat testes and antibody to calmodulin from sheep was obtained from Caabco, Inc., Houston, TX. The exogenous proteins hemoglobin, hemocyanin, thyroglobulin, cytochrome, myoglobin, insulin, ovalbumin, and \(\alpha\)-casein were purchased from Sigma Chemical Co. An electrophoresis calibration kit, containing low molecular weight proteins, provided by Pharmacia Fine Chemicals, was used as standard.

**Methods**

**Buffers**—The following buffers were used in the chromatographic procedures for the purification of the proteases: 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, and 2 mM DTT (buffer A); 0.1 M KCl, 0.01 M PO\(_4\),...
pH 7.5, 3 mM EDTA, and 2 mM DTT (buffer B); 0.1 M NaCl, 50 mM Tris-HCl, pH 7.5 and 2 mM DTT (buffer C).

Measurement of Proteolytic Activity—The proteolytic activity was measured by the hydrolysis of \([\text{methyl-}^{14}\text{C}]\)-casein or methemoglobin. Each assay contained 50 mM Tris-HCl, pH 7.5, 1 mM or 5 \(\mu\)M CaCl\(_2\), and 10 \(\mu\)g of \(\alpha\)-casein or methemoglobin with specific activity of 0.35 \(\mu\)Ci/ml of labeled substrate in a final volume of 75 \(\mu\)l. Assays were carried out at 37 °C for 1 min and were then stopped by the addition of 175 \(\mu\)l of cold 10% trichloroacetic acid containing 25 mg/ml bovine serum albumin. The assays were centrifuged for 2 min in an Eppendorf centrifuge. The supernatant was separated and added to 5 ml of Dimecine scintillation mixture. Proteolytic activity was determined by the number of trichloroacetic acid-soluble counts/min in such a sample after subtraction of control assay (in an identical reaction mixture but without the addition of the protease).

Chromatographic Procedures—For the purification of the CANP(s), three different columns were required in the following sequence: 1) DEAE-Sephacel, 2) hydroxylapatite, and 3) affinity chromatography on \(\alpha\)-casein-CH-Sepharose 4B. Details of column dimensions, flow rates, and method of concentrating the active peaks are given under "Results."

The K-phosphate or KCl concentration in the eluted fractions was determined using a radiometer conductivity meter with a double platinum titrating electrode. The eluate from each column was monitored with a Uvicord-S UV monitor (LKB) at a wavelength of 280 nm.

Polyacrylamide Gel Electrophoresis—SDS-slab gel electrophoresis, using 5–15% linear gradient polyacrylamide gels, was carried out as previously described (23). Polyacrylamide slab gel electrophoresis under nondenaturing conditions was performed according to the method of Davis et al. (24) but with the following modifications: the running gel consisted of a 5–25% acrylamide gradient; the stacking gel buffer was pH 6.2; the electrode buffer, stacking gel, and running gel all contained 2 mM EDTA and 2 mM DTT; and the bromphenol blue was added to the samples rather than to the electrode buffer. At the end of the run, the gel was silver stained according to the procedure of Merril et al. (25).

Protein Determination—Protein determination at all stages was done using a radiometer conductivity meter with a double platinum titrating electrode. The eluate from each column was monitored with a Uvicord-S UV monitor (LKB) at a wavelength of 280 nm.

Purification of the Calp Brain Ca\(^{2+}\)-activated Neutral Protease(s) It was difficult to purify the protease(s) with conventional chromatographic procedures. For example, our initial efforts to purify the protease by a combination of steps 1–4 (see Table I) followed by Affi-Gel 501 (organonemical agarose) and Sephacryl S-200 resulted in impure preparations with only about 63-fold purification of the enzyme (data not shown). In another approach, after step 5 (Table I), DEAE-Bio-Gel A was used. Although this resulted in 149-fold purification of the proteases, the preparation was still contaminated with several unidentified proteins (data not shown). Finally, affinity chromatography using \(\alpha\)-casein-CH-Sepharose 4B after step 5 was found to be suitable for the purification of both of the proteases. The details of each step is described below. All steps were carried out at 4 °C unless otherwise stated.

Step 1: Extract—Calf brains were obtained from a local slaughterhouse immediately after slaughter and were brought back to the laboratory on ice. The meninges were carefully removed and the cerebral cortex was aspirated into a flask through a Pasteur pipette. The cortex was removed from the flask and was frozen in aluminum foil at −80 °C until ready to use. For a typical preparation, 800–900 g of the cortex were thawed and suspended to three times its volume in an extraction medium consisting of 0.15 mM KCl, 50 mM Tris-HCl, pH 8.0, 2 mM EDTA, and 2 mM DTT. It was then homogenized in a Teflon-glass homogenizer with 5–6 up- and-down strokes with a motor-driven drill press at 1400 rpm. The homogenate was stirred gently at 4 °C for 1.5 h and was then spun at 13,000 × g for 20 min. The supernatant was used to prepare cytosol and the pellet was discarded.

Step 2: Cytosol—The supernatant from step 1 was spun at 87,000 × g (average) for 60 min in a Beckman L2-65B ultracentrifuge. The supernatant, which is referred to as the cytosol, was carefully decanted and the pellet was discarded.

Step 3: Ammonium Sulfate Fractionation—Solid ammonium sulfate was added gradually to the cytosol while the mixture was stirred gently. The solution was brought to 30% saturation and stood for 10–15 min at 0 °C. The cloudy suspension was centrifuged at 27,000 × g for 10 min. The supernatant was brought to 65% by further addition of solid ammonium sulfate, left standing at 0 °C for 10–15 min, and centrifuged at 27,000 × g for 15 min. The pellet was dissolved and dialyzed overnight in buffer A.

Step 4: DEAE-Sephacel Chromatography—The dialyzed protein from step 3 was dialyzed 1:1 with buffer B to bring the conductivity down. It was then loaded onto a column (4 × 20 cm) of DEAE-Sephacel which was previously equilibrated with 0.17 M KCl in buffer A. After washing the column with

**Table I**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Yield</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract</td>
<td>18,750.0</td>
<td>1,286,250.0</td>
<td>68.6</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2. Cytosol</td>
<td>11,774.0</td>
<td>1,131,481.4</td>
<td>96.1</td>
<td>88.0</td>
<td>1.4</td>
</tr>
<tr>
<td>3. 30–65% (NH(_4)(_2)){\text{SO}}(_4) of cytosol</td>
<td>6,077.8</td>
<td>777,568.4</td>
<td>128.0</td>
<td>60.5</td>
<td>1.9</td>
</tr>
<tr>
<td>4. DEAE-Sephacel</td>
<td>501.2</td>
<td>943,258.4</td>
<td>1,882.0</td>
<td>73.3</td>
<td>27.4</td>
</tr>
<tr>
<td>5. Hydroxylapatite</td>
<td>177.7</td>
<td>553,144.6</td>
<td>3,112.8</td>
<td>45.0</td>
<td>45.4</td>
</tr>
<tr>
<td>6. (\alpha)-casein-CH-Sepharose 4B (mM CANP)</td>
<td>0.31</td>
<td>41,605.6</td>
<td>134,211.5</td>
<td>3.2</td>
<td>1,956.4</td>
</tr>
<tr>
<td>7. (\alpha)-casein-CH-Sepharose 4B ((\mu)M CANP)</td>
<td>1.2</td>
<td>102,519.5</td>
<td>85,432.9</td>
<td>8.0</td>
<td>1,245.4</td>
</tr>
</tbody>
</table>

* Activity units are expressed as micrograms of \(\alpha\)-casein digested/h.
Calcium-activated Neutral Proteases

1000 ml of equilibration buffer, it was then eluted at a flow rate of 60 ml/h with 1100 ml of a continuous gradient of 0.17-0.5 M KCl made up in buffer A. The eluted fractions were assayed for protease activity using [methyl-14C]a-casein, as described under "methods." Three peaks of proteolytic activity were observed, indicating the possible presence of multiple species of CANP (Fig. 1). All three activity peaks were eluted at 0.184 to 0.330 M KCl. It may be noted that none of the peaks possessing proteolytic activity corresponded to any discrete peaks but, rather, were associated with the trailing portion of the major peak. The active fractions (hatched area in Fig. 1) were pooled and solid ammonium sulfate was added to 40% saturation. Precipitated protein was removed by centrifugation at 27,000 x g for 10 min. The supernatant was brought to 75% saturation with ammonium sulfate and centrifuged at 27,000 x g for 15 min. The pellet was dissolved and dialyzed overnight in buffer B. This step resulted in about 27-fold increase in specific activity over the extract (Table I).

Step 5: Hydroxylapatite Chromatography—The dialyzed protein from step 4 was loaded onto a column (3 x 12 cm) of hydroxylapatite which was previously equilibrated with buffer B. After washing the column with 250 ml of buffer B, it was eluted with an 800-ml linear gradient of 0.01-0.15 M PO₄, made up in buffer B. The eluted fractions were assayed for the protease activity. The protease(s) eluted from 0.04-0.08 M PO₄ (Fig. 2). Again, it may be noted that the proteolytic activity did not correspond to any discrete peak but was associated with the trailing portion of the major peak. The active fractions (hatched area, Fig. 2) were pooled and concentrated by ultrafiltration on an Amicon YM-10 membrane. The concentrated protein (3-5 mg/ml) was dialyzed overnight against a large excess of buffer C + 2 mM EDTA. This step resulted in an about 45-fold increase in specific activity over the extract (Table I).

Step 6: a-Casein-CH-Sepharose 4B Affinity Chromatography—The dialyzed sample from step 5 was brought to a final concentration of 32 mM CaCl₂. It was immediately applied to a column (1 x 3.0 cm) of a-casein-CH-Sepharose 4B, which as previously equilibrated with buffer C + 30 mM Ca²⁺, at a flow rate of 0.75 ml/min. The column was washed with 30 ml of the equilibration buffer followed by 3 ml of buffer C. The column was then eluted with 30 ml of buffer C containing 10 mM EGTA at a flow rate of 1.5 ml/min and passed through a column of Chelex 100 arranged in tandem. The eluted fractions were assayed at high (1 mM) and low (5 µM) Ca²⁺ for protease activity. It was observed that the leading active fraction was maximally active at high Ca²⁺ whereas the following fractions were maximally active at low Ca²⁺. The high and low requiring Ca²⁺-activated proteases will be referred to as CANP1 and CANP2, respectively. A typical elution profile from this column can be seen in Fig. 3. It was only in this step that the proteolytic activity corresponded to a discrete

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*Fig. 1. Ion exchange chromatography of CANP from calf brain cortex.* Chromatography on DEAE-Sephacel was carried out as described in the text (step 4). Aliquots of each fraction were assayed for the protease activity as described under "Methods." Absorbance at 280 nm (●●●●●●●); Ca²⁺-activated proteolytic activity (○○●○); KCl gradient (---).

*Fig. 2. Elution profile from hydroxylapatite column.* The active fractions from Fig. 1 (hatched area) were pooled and ammonium sulfate fractionation was performed as described in the text (see step 4). The dialyzed protein, in buffer B, was subjected to hydroxylapatite as described in the text (step 5). Absorbance at 280 nm (●●●●●●●); Ca²⁺-activated proteolytic activity (○○●○); PO₄ gradient (---).

*Fig. 3. Elution profile from a-casein-CH-Sepharose 4B column.* The active protein pool from Fig. 2 was loaded onto an affinity column of a-casein-CH-Sepharose 4B (for details, see steps 5 and 6 in the text). The bound proteases were eluted with EGTA. The leading fraction contained CANP1 whereas the others contained CANP2. Absorbance at 280 nm (●●●●●●●); Ca²⁺-activated proteolytic activity (with 1 mM Ca²⁺) (○○●○).
peak (hatched area, Fig. 3). The active fractions were used as the source of the purified proteases.

Purity and Characterization of the Proteases

The purification data of a typical preparation presented in Table I were reproducible from preparation to preparation. CANP1 and CANP2 were purified from the bovine brain cortex extract with approximately 1,950- and 1,250-fold purification in 3 and 8% yield, respectively. SDS-polyacrylamide gel electrophoresis of the purified proteases revealed that both peaks were identical. They consisted of one major polypeptide with M, = 78,000 and a minor band with an apparent M, = 17,000 (Fig. 4, lanes 7 and 8). Staining of the gel with silver (25) indicated no other contaminating proteins (data not shown).

In order to establish whether or not the 78,000- and 17,000-Da polypeptides observed in SDS-polyacrylamide gels of the purified CANP1 and CANP2 together constitute the active proteases or if the 17,000-Da protein was a contaminant, we investigated by using reactive red-120 agarose. The protein pool containing the active proteases, from step 5 (see Table 1), + 0.5 M NaCl was applied to a column (1 x 3.0 cm) of reactive red-120 agarose equilibrated with buffer 1 (50 mM Tris-HCl, pH 7.5, 10 mM EGTA, 2 mM DTT). It was then washed with approximately 30 ml of buffer 1 + 0.5 M NaCl followed by elution with buffer 1. The unretarded fractions and those that were eluted with buffer 1 were monitored by SDS-PAGE. As shown in Fig. 5, eluted fractions with buffer 1 contained the proteases [lanes 8–10]. All of the contaminating proteins and some of the proteases were not bound to the column [lanes 6 and 7]. The significant point to note in this figure is that the 17,000-Da protein did not co-purify with the major 78,000-Da protein. It must be remembered that this component did co-purify with the 78,000-Da protein on the a-casein affinity column (see above). However, when the reactive red column was washed with 8 M urea, the 17,000-Da protein was eluted, indicating strong binding of this component with reactive red (data not shown). When the fractions containing the 78,000-Da component were assayed, the specific activity was significantly reduced, thus indicating the necessity of the 17,000-Da component for maximal activity of the proteases. The addition of rat testes calmodulin to the protease also had no effect on the activity. In addition, the migration rates of the 17,000-Da protein and calmodulin on SDS-PAGE were different (Fig. 5, lanes 1–4). In the case of casein-purified enzyme, where the 78,000- and 17,000-Da proteins were present, the addition of calmodulin antibody had no effect on the activity of either protease (data not shown). These results indicate that neither protease was a calmodulin-regulated system.

**Fig. 5.** SDS-polyacrylamide gel electrophoresis of protein samples from reactive red-120 agarose. The hydroxylapatite pool (see step 5 in the text) was subjected to the reactive red-120 agarose as described in the text. Lane 1, purified CANP2 (2 µg); lanes 2 and 11, calmodulin (5 µg); lane 3, CANP1 (1.2 µg); lane 4, calmodulin (10 µg); lane 5, molecular weight standards (see Fig. 4); lane 6, hydroxylapatite pool (25 µg); lane 7, peak fraction from the wash (for details, see text) (25 µg); lanes 8–10, active fractions eluted from reactive red-120 agarose column with zero salt (see text). The doublet below the protease band and above the calmodulin band has been determined to be a gel artifact which has been seen occasionally.

**Fig. 4.** SDS-polyacrylamide gel electrophoresis of protein samples taken at each stage of purification of the CANPs. The protein samples to be used for electrophoresis were prepared as described under "Methods." Lane 1, extract (50 µg); lane 2, cytosol (50 µg); lane 3, 65% saturated (NH)2SO4 pellet of the cytosol (for details, see text) (50 µg); lane 4, DEAE-Sepharose pool (25 µg); lane 5, hydroxylapatite pool (25 µg); lane 6, unbound protein from a-casein-CH-Sepharose 4B column (25 µg); lane 7, leading fraction (CANP1, millimolar Ca++ requiring) eluted with EGTA from the a-casein-CH-Sepharose 4B column (1.5 µg); lane 8, fraction following the leading fraction (CANP2, micromolar Ca++ requiring) eluted with EGTA from the a-casein-CH-Sepharose 4B column (2 µg); lane 9, molecular weight standards: (a) phosphorylase b (M, = 94,000); (b) bovine serum albumin (M, = 67,000); (c) ovalbumin (M, = 43,000); (d) carbonic anhydrase (M, = 30,000); (e) soybean trypsin inhibitor (M, = 20,100); (f) α-lactalbumin (M, = 14,400).

**Fig. 6.** Effect of time at different temperatures on the hydrolysis of [14Cl]casein by CANP2. The assays were performed at different temperatures in the presence of 5 µM free CaCl2 for various lengths of time as shown. For other conditions, see “Methods.” Similar results were obtained when [14Cl]methemoglobin was used as the substrate.
proteases exhibited very low activity below pH 5. The loss in pH 6.8 and 7.8 whereas the pH optimum for CANPB was activity beyond pH 8.5 for CANPl was gradual whereas a sharper decline in activity was observed with CANP2. The pH optimum for CANPl was broad and ranged between pH 6.8 and 7.8 whereas the pH optimum for CANP2 was sharp with maximum activity at pH 7.5 (Fig. 8). Both of the proteases exhibited very low activity below pH 5. The loss in activity beyond pH 8.5 for CANP1 was gradual whereas a sharper decline in activity was observed with CANP2. CANP1 was maximally active at 700 μM Ca²⁺ while CANP2 achieved maximum activity at 2 μM Ca²⁺ (Fig. 9). Since Ca²⁺ was essential for the activity of both proteases, the effect of some other divalent cations on the activity was also studied. As shown in Table II, both of the proteases exhibited very little but differential activity with various metal ions as compared to Ca²⁺. The only exception was Ba²⁺ where the activity with CANP1 was 34%. The addition of Mg²⁺ in the presence of Ca²⁺ did not affect the activity of either enzyme, indicating the inability of these metals to replace Ca²⁺ for the activity of either protease (data not shown). Both of the proteases were substantially hydrolyzed in the presence of Ca²⁺ in 2 to 5 min at 37 °C. However, when the substrate, casein, was present, the rate of hydrolysis of the proteases was significantly reduced. This may be due to a protective effect of the substrate on the autolysis of the enzymes (data not shown). Table III shows the effect of several protease inhibitors. Among the actinomycete protease inhibitors, leupeptin blocked the activity of both proteases in a differential manner.

**TABLE II**

<table>
<thead>
<tr>
<th>Divalent cation</th>
<th>CANPl</th>
<th>CANP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>4.0</td>
<td>8.7</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>8.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>3.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Sr²⁺</td>
<td>10.5</td>
<td>14.9</td>
</tr>
<tr>
<td>Co²⁺</td>
<td>3.8</td>
<td>3.5</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>3.4</td>
<td>6.2</td>
</tr>
<tr>
<td>Ba²⁺</td>
<td>33.9</td>
<td>5.7</td>
</tr>
<tr>
<td>Al³⁺</td>
<td>0</td>
<td>13.3</td>
</tr>
</tbody>
</table>

**TABLE III**

<table>
<thead>
<tr>
<th>Additions</th>
<th>CANPl</th>
<th>CANP2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protease activity</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Control</td>
<td>cpm</td>
<td>%</td>
</tr>
<tr>
<td>Leupeptin (3.3 μg/ml)</td>
<td>134</td>
<td>72.0</td>
</tr>
<tr>
<td>Leupeptin (13.3 μg/ml)</td>
<td>57</td>
<td>88.5</td>
</tr>
<tr>
<td>Pepstatin (13.3 μg/ml)</td>
<td>480</td>
<td>0</td>
</tr>
<tr>
<td>Antipain (33.3 μg/ml)</td>
<td>329</td>
<td>39.6</td>
</tr>
<tr>
<td>Antipain (13.3 μg/ml)</td>
<td>147</td>
<td>70.4</td>
</tr>
<tr>
<td>Aprotinin (0.02 TIU)⁴</td>
<td>482</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin inhibitor (13.3 μg/ml)</td>
<td>490</td>
<td>0</td>
</tr>
<tr>
<td>Phenylmethylsulfonyl fluoride (0.1 mM)</td>
<td>431</td>
<td>13.1</td>
</tr>
<tr>
<td>p-Chloromercuribenzoate (0.1 mM)</td>
<td>143</td>
<td>71.1</td>
</tr>
<tr>
<td>N-Ethylmaleimide (1.0 mM)</td>
<td>176</td>
<td>64.5</td>
</tr>
<tr>
<td>Iodoacetic acid (1.0 mM)</td>
<td>109</td>
<td>78.0</td>
</tr>
</tbody>
</table>

⁴ Aprotinin concentration is given in trypsin inhibitor units (TIU).
Antipain exerted more effect on CANPl while pepstatin was protease. Both proteases were inhibited by sulfhydryl while aprotinin and trypsin inhibitor had no effect on either the least effective with either protease. Phenylmethylsulfonyl acid) although the degree of inhibition of each protease was different. These data provide indications that each protease is a thiol enzyme. We have also observed that the rate of loss of activity of both proteases depends on the concentration of DTT. Both purified proteases lost activity within a few hours if the DTT concentration was below 1 mM. However, in the presence of 2 mM DTT, activity was maintained for at least 72 h (data not shown).

The activity of the proteases toward the partially purified endogenous brain proteins, neurofilament triplet proteins (P200, P170, P70) and neurotubules (microtubule-associated proteins and tubulin), and the smooth muscle intermediate filament protein, desmin, was measured on SDS-PAGE. As can be seen in Fig. 10, all of these proteins were extensively degraded. In addition, the activity of CANP2 was also tested with exogenous proteins, hemoglobin, hemocyanin, thyroglobulin, cytochrome, myoglobin, insulin, ovalbumin, and α-casein. Among these proteins, only α-casein, hemocyanin, and hemoglobin were hydrolyzed significantly (data not shown).

**DISCUSSION**

The procedure to purify CANP1 and CANP2 from calf brain cortex described here yielded nearly homogeneous enzymes as judged by SDS-gel electrophoresis. Both forms of the protease were purified about 1950- and 1250-fold, respectively. The yield of the CANP2 (micromolar Ca++ requiring) was generally four to five times higher than CANP1 (millimolar Ca++ requiring). This would indicate abundance of CANP2 in the brain. Although small amounts of both the proteases were purified, low concentrations of the enzymes were needed to degrade the substrates. Some aspects of the purification method need special comment. 1) The use of Chelex 100 (33) in tandem with α-casein-CH-Sepharose 4B was very critical since it was observed that CANP1 and CANP2, after their elution from the latter column with 10 mM EGTA, were still contaminated with 1.9 and 0.19 mM free Ca++, respectively. At these levels, free Ca++, if not chelated immediately, was found to cause degradation of both proteases, as seen on SDS-PAGE, with concomitant loss of activity in about 12-16 h at 4 °C (data not shown). 2) The speed with which the proteases from step 5 (see Table 1) were loaded onto and eluted from the α-casein-CH-Sepharose 4B column should be noted. Under these conditions (see “Results”), the binding of the proteases was enhanced and degradation after elution was minimized. 3) To ensure the separation of the two proteases, not more than 10 mg of the hydroxyapatite pool (step 5, Table 1) was loaded onto a 2-ml α-casein-CH-Sepharose 4B column.

Because of the identical SDS-gel pattern and co-migration of the 78,000-Da component of both proteases in a nondenaturing gel (data not shown), it would appear that CANP1 and CANP2 are two different forms of the same enzyme. These forms require different Ca++ concentration for their maximum activity. CANP1 is maximally active at 700 μM whereas CANP2 is maximally active at 2 μM Ca++. The factor(s) that are responsible for different Ca++ requirements are not clear from the data presented here. Whatever causes the difference in the Ca++ requirement does not appear to affect the size and subunit molecular weight of the two proteases. One possibility may be a difference in the charge of the two proteases. This argument is based on the differential elution of CANP1 and CANP2 from the affinity column with EGTA. In other words, it appears that Ca++ binds somewhat weakly with CANP1 in...
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A single, partially purified CANP from rat brain has been reported and the Ca\textsuperscript{2+} concentration required to maximally activate this enzyme ranged from 1–4 mM (8, 19, 20). It was difficult to assess the significance of these results since the physiological level of Ca\textsuperscript{2+} is around 0.1-1.6 mM (9). High Ca\textsuperscript{2+} concentrations were also required for maximum activity of the CANP from skeletal muscle, although, in this system, the enzyme was purified and characterized (34). While our work was in progress, a paper by Mellgren (35) was published where the authors demonstrated the presence of two forms of CANP in canine cardiac muscle. One form (peak I) was maximally active at about 80 μM while the other form (peak II) was at 1.6 mM Ca\textsuperscript{2+}. Subsequently, even in the case of skeletal muscle, a low Ca\textsuperscript{2+}-requiring form of CANP, which was missed earlier, has now been found and purified. The Ca\textsuperscript{2+} concentration required to maximally activate this enzyme was found to be about 90 μM (36). In addition, the size and subunit composition of two forms of CANP from skeletal muscle were found to be similar (36). Two forms of CANP have also been partially purified from neuroblastoma (8), rat liver (6), and several other rat tissues (37). However, only one form of CANP, which was maximally active at 150 μM, had been purified from Ehrlich ascites tumor cells (9). A single CANP was purified from smooth muscle, but, in this system, the Ca\textsuperscript{4+} requirement of the enzyme was complex. The native enzyme was maximally active at 1 mM Ca\textsuperscript{2+} while limited autolysis of the native enzyme substantially reduced the Ca\textsuperscript{2+} requirement. The degraded enzyme exhibited maximum activity at 10 μM Ca\textsuperscript{2+} (38). It is important to note that the Ca\textsuperscript{2+} concentrations required to activate the brain enzymes reported here are significantly lower when compared to their counterparts in muscle (36) or other systems where such enzymes have been partially purified (6, 8, 35).

Both of the proteases were inactivated, differentially, by various sulfhydryl modifying agents and actinomycete inhibitors. In general, the degree of inhibition by these compounds was greater with CANP1 compared to CANP2 (Table III). Nevertheless, it may be possible to classify both as thiol proteases (39). The relative activities of both of the proteases with metal ions other than Ca\textsuperscript{2+} were low but different for each protease (Table II). This is in contrast to the peak I protease from skeletal muscle (micromolar Ca\textsuperscript{2+} requiring) where Mg\textsuperscript{2+} and Mn\textsuperscript{2+} also activated this enzyme significantly (36). Although both of the proteases presented here were maximally active at pH 7–7.5, the overall shape of the pH curves was different. Despite the fact that both of the proteases are similar in size (subunit composition) but, based on the differences in their properties, as discussed above, it is likely that there may be structural differences between them. Evidence supporting this conclusion has recently been presented on the two forms of CANP from skeletal muscle (36).

Each of the brain proteases purified here consist only of a polypeptide of 78,000 Da, since the minor protein of 17,000 Da can be separated away under nondenaturing conditions (Fig. 5). Therefore, it is obvious that the 17,000-Da protein is not a subunit of either of the proteases but simply co-purified with them. While this manuscript was in preparation, a paper by DeMartino and Elmanthal (40) was published. These investigators used calmodulin from brain prepared according to the conventional methods. It was claimed by these authors that such a calmodulin preparation contained another protein, called CDPR (calcium-dependant protease regulator), that activated the partially purified CANPs (peaks I and II) from rat liver, while calmodulin purified on a phophatase-Sepharose affinity column had no effect on the activity of these proteases. These investigators subsequently partially purified the activating factor (CDPR) from bovine brain and the molecular mass of this factor was found to be between 16,000 and 22,000. When conventionally prepared calmodulin from bovine brain (41) was added to the 78,000-Da protein eluted from a reactive red-120 agarose column (see Fig.), it stimulated the activity of the proteases approximately 2–3-fold (data not shown). Thus, it appears that a cofactor is needed to achieve maximum activity of each enzyme. It appears that the 17,000-Da protein that co-purifies with each of the proteases described here may fulfill this requirement. This cofactor is not calmodulin (see “Results”). Work is now in progress to provide further insight into how the 17,000-Da protein is involved in activating the two proteases.

It may be important to note that the two forms of the CANP purified from skeletal muscle (36) and a single CANP purified from tumor cell (9) and smooth muscle (38) are heterodimers, each of M\textsubscript{s} = 110,000 with a subunit composition of M\textsubscript{i} = 72,000–80,000 and 29,000–30,000. In the case of skeletal muscle, this subunit composition has been further confirmed by cross-linking experiments (42). In contrast, both of the brain proteases purified here appear to be monomeric enzymes with a single polypeptide of M\textsubscript{i} = 78,000.

The degradation of the endogenous brain proteins, neurofilament triplet polypeptides, and neurotubule proteins (microtubulin-associated proteins and tubulin), by the purified proteases was similar to our previous observations where crude protease(s) was used (21). Thus, it appears that the proteases purified here have a high specificity for these proteins. The degradation of tubulin is of particular interest since in none of the previously published works, where partially purified brain CANP was used, was this observed (43, 44). In addition to the above proteins, desmin, the subunit protein of smooth muscle intermediate filaments, was also hydrolyzed. Coincidentally, P70 (one of the subunit proteins of neurofilaments) and desmin are the major proteins that cause neurofilaments and smooth muscle intermediate filaments, respectively, to assemble (these are also referred to as cytoskeleton) (45). The degradation of these key proteins, from unrelated cell types, by the purified brain proteases strongly suggests the specificity of these enzymes for cytoskeleton. It may be pertinent to mention here that the CANP purified from Ehrlich ascites tumor cells also degraded desmin from smooth muscle but had no degrading effect on the other cytoskeletal proteins, namely neurofilament triplet proteins and tubulin (9). The reason for these differences is not clear.

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