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Purification and Characterization of High Ca^{2+}-Requiring Neutral Proteases from Porcine and Bovine Brains

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ABSTRACT: Porcine and bovine brain high Ca^{2+}-requiring neutral proteases were purified to homogeneity by the same isolation procedures, and their properties were compared. A high degree of similarity existed between the two proteases. The purification procedures included ion-exchange chromatography on DEAE-cellulose, hydrophobic chromatography on phenyl-Sepharose CL-4B, second DEAE-cellulose chromatography, second phenyl-Sepharose CL-4B chromatography, and gel filtration on Ultrogel AcA 34. Both purified enzymes were composed of M_r 75 000 and 29 000 subunits, as shown by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Both enzymes required 250 μM Ca^{2+} for half-maximal activity and 700 μM Ca^{2+} for maximal activity. Sr^{2+} and Ba^{2+}, but not Mg^{2+} or Mn^{2+}, also activated both enzymes but not as effectively as Ca^{2+}. Both enzymes displayed maximum activity at pH 7.5–8.0. Leupeptin, antipain, and *trans*-epoxysuccinyl-L-leucylagmatine inhibited both enzymes. Neurofilament triplet proteins and microtubule-associated proteins were extensively hydrolyzed by both proteases, but tubulin and actin were not hydrolyzed. The amino acid compositions of the two proteases were very similar. Antisera against bovine brain protease cross-reacted with porcine brain protease when examined by immunoelectrotransfer blot techniques.

Calcium-activated neutral proteases (CANPs)[1] have been identified in many tissues of various species (Murachi et al., 1981; Ishiura, 1981). Recently, two forms of CANP which differ in their Ca^{2+} requirement, CANPI requiring low Ca^{2+} and CANPII requiring high Ca^{2+}, have been recognized in several tissues (Mellgren, 1980; Dayton et al., 1981; DeMartino, 1981; Kubota & Suzuki, 1982; Croall & DeMartino, 1983; Inomata et al., 1983; Malik et al., 1983; Otsuka & Tanaka, 1983; Yoshimura et al., 1983). However, the physiological significance of the existence of such a dual enzyme system is not known, and the relationship between the two forms of CANP is still not clear. It is suggested that CANPI, which requires micromolar levels of Ca^{2+}, is the physiologically active form of CANP. However, CANPI is a minor form in most tissues (Mellgren, 1980; Dayton et al., 1981; DeMartino, 1981; Kishimoto et al., 1981; Kubota & Suzuki, 1982; Croall & DeMartino, 1983; Inomata et al., 1983; Murachi, 1983; Otsuka & Tanaka, 1983; Yoshimura et al., 1983). At present, it remains unknown which form, CANPI or CANPII, plays a more important role in the cell. It seems reasonable that the major form of CANP must play an important role in the cell. There are also discrepancies as to the subunit composition of CANPIIs. Some investigators have suggested that proteases are heterodimers composed of M_r 70 000–80 000 and 18 000–30 000 subunits (Dayton et al., 1976; Suzuki et al., 1979; Truglia & Stracher, 1981; Tsuji & Imahori, 1981; Hataway et al., 1982; Kubota & Suzuki, 1982; Mellgren et al., 1982; Wheelock, 1982; Inomata et al., 1983; Otsuka & Tanaka, 1983; Yoshida et al., 1983; Yoshimura et

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[1] Abbreviations: CANP, calcium-activated neutral protease; CANPI, low Ca^{2+}-requiring protease; CANPII, high Ca^{2+}-requiring protease; SDS, sodium dodecyl sulfate; MAPs, microtubule-associated proteins; E-64, *trans*-epoxysuccinyl-L-leucylagmatine, a thiol protease inhibitor; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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al., 1983; Kubota et al., 1984). Others have reported that proteases contain only one polypeptide chain of M_r 73 000–80 000 (Ishiura et al., 1978; Azanza et al., 1979; Kubota et al., 1981; Croall & DeMartino, 1983; DeMartino & Croall, 1983; Malik et al., 1983). Although in some instances these discrepancies may result from tissue- or species-specific differences and/or differences in the methodologies used for purification, in other cases discrepancies exist for enzymes from single sources (Azanza et al., 1979; Kubota & Suzuki, 1982; Mellgren et al., 1982; Inomata et al., 1983). To clarify these problems, it is important to purify CANPs by the same method from a single organ of different species.

In nervous tissues, CANPs have been detected in both vertebrate (Lasek & Hoffman, 1976; Schlaepfer & Micko, 1978; Schlaepfer & Freeman, 1980; Tashiro & Ishizaki, 1982; Zimmerman & Schlaepfer, 1982; Kamakura et al., 1983) and invertebrate (Gilbert et al., 1975; Pant et al., 1979; Pant & Gainer, 1980) species, which selectively degrade neurofilaments. It has also been suggested that CANPs are involved in the regulation of glutamate receptors in synaptic membranes (Baudry & Lynch, 1980; Baudry et al., 1981), activation of protein kinase C (Inoue et al., 1977; Kishimoto et al., 1983), turnover of brain fodrin (Siman et al., 1984), and Wallerian degeneration of the peripheral nerve (Schlaepfer, 1977). Thus, it was indicated that CANPs may play important roles in nervous tissues. However, although there have been several reports on the partial purification of these enzymes from brain (Guroff, 1964; Zimmerman & Schlaepfer, 1982; Kishimoto et al., 1983) and neuroblastoma cells (DeMartino & Croall, 1982), little has been done to completely purify and characterize these enzymes except for the work of Malik et al. (1983), who purified two forms of CANP (CANPs I and II) from bovine brain by casein–Sepharose affinity chromatography in the presence of Ca^{2+}. They reported that CANPI was a major form in bovine brain and both the purified CANPI and CANPII contained only one polypeptide chain of M_r 78 000. The former result is not in accordance with the results of Kishimoto et al. (1981) and Murachi et al. (1983), who showed that brain contained far more CANPII activity than CANPI activity. Previously, we demonstrated that during casein–Sepharose affinity chromatography CANPII was autoproteolyzed and converted to CANPI (Kubota et al., 1981; Suzuki et al., 1981). Moreover, Croall and DeMartino (1983, 1984) recently reported that during casein–Sepharose affinity chromatography a M_r 30 000 subunit was autoproteolyzed and CANPII was isolated as a monomer of M_r 80 000. Therefore, to clarify the above-mentioned problems, it is of importance to purify CANPs without using casein–Sepharose affinity chromatography. In the present study, we attempted to purify CANPs from porcine and bovine brains by the same method without using casein–Sepharose affinity chromatography. We have found that both porcine and bovine brains contain CANPI activity, but at only about one-seventieth of the CANPII activity level.

Experimental Procedures

Materials. Casein (nach Hammarsten) was obtained from Merck. Leupeptin, antipain, and pepstatin were obtained from The Peptide Institute, The Protein Research Foundation, Osaka, Japan. E-64 (Hanada et al., 1978) was kindly provided by Dr. Hanada of Taisho Pharmaceutical Co., Tokyo, Japan. DEAE-cellulose (DE-52) was a product of Whatman. Phenyl-Sepharose CL-4B and Ultrogel AcA 34 were obtained from Pharmacia. Horseradish peroxidase conjugated goat anti-rabbit IgG was the product of Cappel, Cochranville, PA. All other reagents were of analytical grade and obtained from Wako Pure Chemicals or Nakarai Chemicals, Tokyo, Japan.

Buffers. The following buffers were used in the chromatographic procedures for the purification of CANPs: 20 mM Tris-HCl containing 5 mM EDTA and 5 mM 2-mercaptoethanol, pH 7.5 (buffer A); 20 mM Tris-HCl containing 5 mM EDTA, 5 mM 2-mercaptoethanol, and 0.3 M NaCl, pH 7.5 (buffer B).

Chromatographic Procedures. Purification of the CANPs required five column chromatographic procedures in the following order: first DEAE-cellulose, first phenyl-Sepharose CL-4B, second DEAE-cellulose, second phenyl-Sepharose CL-4B, and Ultrogel AcA 34. All chromatographic procedures were performed at 4 °C. Flow rates, column dimensions, and buffer systems are given under Results.

Protease Assay. Protease activity was measured with alkali-denatured casein as substrate. The standard assay mixture (0.5 mL) contained 0.24% alkali-denatured casein, 25 mM 2-mercaptoethanol, 6 mM $CaCl_2$, and 20 mM Tris-HCl (pH 7.5). The reaction was started by the addition of the enzyme solution and stopped by the addition of 10% trichloroacetic acid after incubation at 30 °C for 30 min. The solution was centrifuged at 3000*g* for 10 min. The absorbance at 280 nm of the supernatant was measured against a control which had been treated similarly but without Ca^{2+}. One unit of enzyme activity was defined as the amount of enzyme which catalyzed an increase of 1.0 absorbance unit at 280 nm in 60 min under our standard assay conditions. The CANP inhibitor assay was performed similarly. The inhibitor samples were preincubated with CANP at 30 °C for 5 min, and the decrease in enzymatic activity was determined.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence or absence of SDS was performed according to published methods (Laemmli, 1970; Davis, 1964). Isoelectric focusing was carried out in thin-layer polyacrylamide gels containing Ampholine, with a pH gradient of 4.0–6.5 (obtained from LKB, Bromma, Sweden). The anode electrode solution was 0.1 M glutamic acid in 0.5 M H_3PO_4, and the cathode electrode solution was 0.1 M β-alanine. The gels were run and stained for protein according to the manufacturer's manual. The isoelectric point was estimated with a p*I* calibration kit from Pharmacia.

Amino Acid Composition. The purified CANPIIs were dialyzed against water and then lyophilized. Hydrolysis was performed in 6 N HCl at 110 °C for 24 h. Amino acid analysis was performed with a Hitachi Model 835-50 amino acid analyzer.

Protein Determination. Protein concentration was determined spectrophotometrically using $A_{280nm}^{0.1\%} = 1.0$.

Preparation and Purification of Antibodies. About 1 mg of purified CANPII from either porcine or bovine brain was emulsified in an equal volume of Freund's complete adjuvant and injected subcutaneously into rabbits. Subsequent injections of CANPII were prepared without Freund's complete adjuvant and administered over a period of several months. Rabbits injected in this way produced antisera with high titers against porcine and bovine brain CANPIIs, respectively. The IgG fractions were precipitated from the antisera by 40% ammonium sulfate saturation and further purified by affinity chromatography. An affinity column with CANP as the ligand was prepared by coupling 2 mg of purified CANPII to 1 g of CNBr-activated Sepharose 4B according to the manufacturer's recommendations. The IgG fractions were applied to an affinity column (1 × 3 cm), and then the bound IgG was eluted from the column with 0.2 M glycine hydrochloride buffer, pH 2.5.

Immunoelectrophoretic Blotting. CANPIIs were subjected to electrophoresis on SDS–polyacrylamide gels as described above and then transferred to nitrocellulose paper according to the published method (Towbin et al., 1979). The blotted proteins were incubated with affinity-purified antibodies against CANPIIs and detected with horseradish peroxidase conjugated goat anti-rabbit IgG (Hawkes et al., 1982).

Preparation of Actin, Neurofilaments, and Neurotubules. Actin was purified from rabbit skeletal muscle according to the published method (Spudich & Watt, 1971). Neurofilaments were purified from porcine brain according to the published method (Runge et al., 1981). Tubulin and microtubule-associated proteins (MAPs) were purified from porcine brain according to the published method (Shelanski et al., 1973).

Substrate Specificity. The substrate specificity for exogenous proteins of CANPIIs was tested under the following assay conditions: 20 mM Tris-HCl, pH 7.5, 6 mM Ca^{2+}, 5 mM 2-mercaptoethanol, 10–20 μg of an exogenous protein, and 0.2–0.4 μg of CANPII. The reaction was started by the addition of the protease. Aliquots (40 μL) were taken after a 5-min incubation (neurofilaments) or 60-min incubation (actin and neurotubules) at 30 °C, and the reaction was stopped by the addition of SDS and 2-mercaptoethanol; then, after heating at 100 °C for 2 min, each sample was subjected to SDS–polyacrylamide gel electrophoresis. The gels for neurofilaments and neurotubules were 7.5% polyacrylamide, and the gels for actin were 10% polyacrylamide.

Results

Purification of Porcine and Bovine Brain Calcium-Activated Neutral Proteases. All steps were carried out at 4 °C unless otherwise stated. Porcine and bovine brain CANPIIs were purified by the same procedures, respectively. The results obtained with bovine brain were almost the same as those with porcine brain, and so only the results obtained for porcine brain are presented.

Step 1: Preparation of Crude Extract. Porcine brains were obtained from a local slaughterhouse and carried to the laboratory on ice. After the meninges and clots had been carefully removed, the brains were minced and homogenized with 3 volumes of buffer A with a Polytron grinder at medium speed for 60 s. Each homogenate was centrifuged at 10000*g* for 30 min, and the supernatant, termed "crude extract", was retained.

Step 2. The crude extract was applied to a column of DEAE-cellulose (5 × 70 cm) equilibrated with buffer A. After the column had been washed with 3 L of buffer A, the adsorbed protease was eluted at a flow rate of 100 mL/h with a 4-L linear concentration gradient of NaCl (0–0.8 M) in buffer A (Figure 1). Fractions of 20 mL were collected. Two peaks of CANP were observed, one at 0.1–0.2 M NaCl (peak I protease) and the other at 0.3–0.4 M NaCl (peak II protease). The peak I protease (CANPI), the minor form, required 10 μM Ca^{2+} for half-maximal activity, and the peak II protease (CANPII), the major form, required 250 μM Ca^{2+} for half-maximal activity. As shown in Figure 1, brain contained CANPI activity (83 units), but at only about one-seventieth of the CANPII activity level (5883 units). The active fractions (hatched area in Figure 1) of CANPII were pooled.

Step 3. The pooled fractions from step 2 were loaded onto a column (2.5 × 28 cm) of phenyl-Sepharose CL-4B equilibrated with buffer B. After the column was washed with 400 mL of buffer B, it was eluted at a flow rate of 30 mL/h with 500 mL of buffer A. Fractions of 9.8 mL were collected.

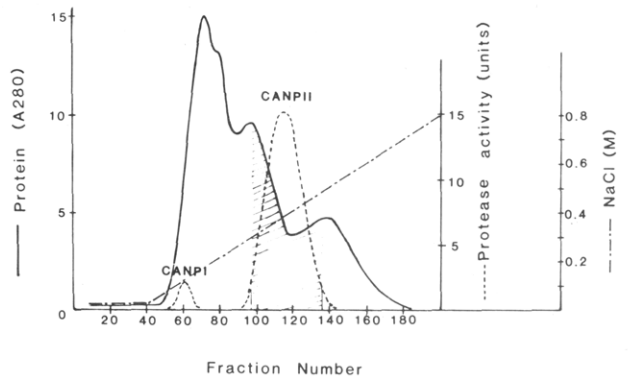

FIGURE 1: First DEAE-cellulose chromatography of CANP from porcine brain. Chromatography on DEAE-cellulose was carried out as described in the text (step 2). Aliquots of each fraction were assayed for protease activity as described under Experimental Procedures. Absorbance at 280 nm (—); protease activity (···); NaCl gradient (–·–).

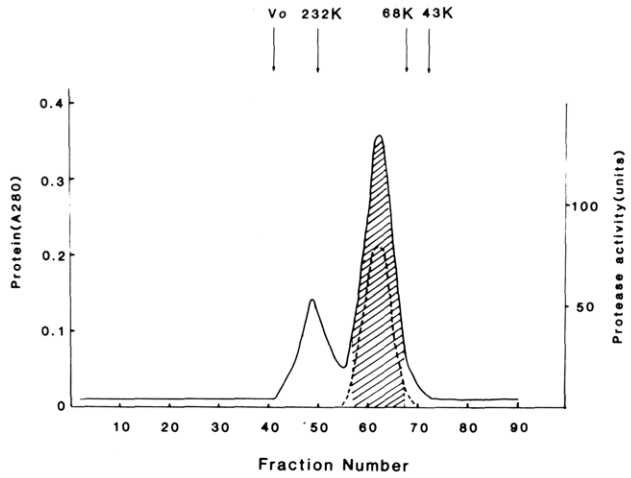

FIGURE 2: Elution profile from an Ultrogel AcA 34 column. The active fractions from step 5 were pooled and subjected to Ultrogel AcA 34 chromatography as described in the text (step 6). Absorbance at 280 nm (—); protease activity (– – –). The column was calibrated with proteins of known molecular weights: catalase, M_r 232 000 (232K); bovine serum albumin, M_r 68 000 (68K); and ovalbumin, M_r 43 000 (43K). The void volume (V_0) was determined with blue dextran.

Active fractions (fractions 22–36) were pooled.

Step 4. The pooled fractions from step 3 were loaded onto a column (2.6 × 35 cm) of DEAE-cellulose equilibrated with buffer A. After the column had been washed with 600 mL of buffer A, the adsorbed protease was eluted at a flow rate of 100 mL/h with a linear concentration gradient of NaCl (0–0.8 M) in buffer A. Fractions of 9.8 mL were collected. Active fractions (fractions 28–39) were collected.

Step 5. The pooled fractions from step 4 were loaded onto a column (1.8 × 34 cm) of phenyl-Sepharose CL-4B equilibrated with buffer B. After the column was washed with 250 mL of buffer B, it was eluted at a flow rate of 30 mL/h with 250 mL of buffer A. Fractions of 9.8 mL were collected. Active fractions (fractions 13–19) were pooled.

Step 6. The pooled fractions from step 5 were precipitated with 60% saturated ammonium sulfate at 4 °C. The precipitate was dissolved in 2 mL of buffer A and then subjected to gel filtration on an Ultrogel AcA 34 column (2.5 × 90 cm) equilibrated with buffer A. Elution was performed with the same buffer at a flow rate of 30 mL/h (Figure 2). Fractions of 4 mL were collected, and the active fractions (hatched area in Figure 2) were pooled.

The procedures employed to isolate the CANPIIs from porcine and bovine brains are summarized in Table I. The

Table I: Purification of CANPIIs from Porcine and Bovine Brains

protease	step[a]	total act. (units)	protein (mg)	sp act. (units/mg)	purification (x-fold)	yield (%)
porcine brain CANP (3.9 kg)	1	*b*	116309			
	2	5883	2560	2.30	1	100
	3	3773	96.0	39.3	17.1	64.1
	4	2865	48.8	58.7	25.5	48.7
	5	2186	22.1	98.9	43.0	37.2
	6	1560	9.4	166.0	72.2	26.5
bovine brain CANP (4.2 kg)	1	*b*	123670			
	2	6174	2663	2.32	1	100
	3	4126	112	36.8	15.9	66.8
	4	3266	52.7	62.0	26.7	52.9
	5	2538	25.4	99.9	43.1	41.1
	6	1886	11.2	168.4	72.6	30.5

[a] The steps are: (1) crude extract, (2) first DEAE-cellulose chromatography, (3) first phenyl-Sepharose chromatography, (4) second DEAE-cellulose chromatography, (5) second phenyl-Sepharose chromatography, and (6) Ultrogel AcA 34 chromatography. [b] The presence of a CANP inhibitor in these fractions prevents accurate measurement of CANP activity.

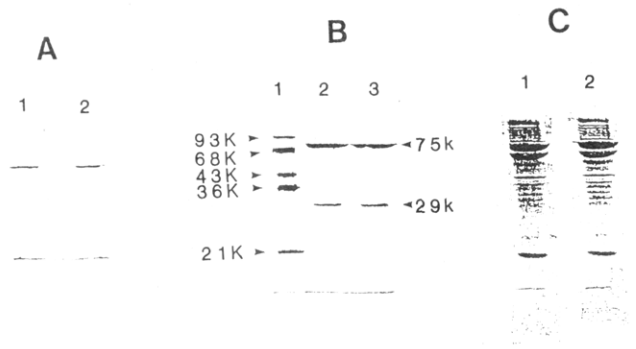

FIGURE 3: Polyacrylamide gel electrophoresis of the purified enzymes in a nondenaturing gel (7.5%) (A) and SDS (15%) (B) and the first DEAE-cellulose column fractions in the SDS gel (15%) (C). Migration is from top to bottom. (A) Lane 1, 10 μg of purified porcine CANP; lane 2, 10 μg of purified bovine CANP. (B) Lane 1, molecular weight markers: phosphorylase *b* [93 000 (93K)], bovine serum albumin [68 000 (68K)], ovalbumin [43 000 (43K)], lactate dehydrogenase [36 000 (36K)], and soybean trypsin inhibitor [21 000 (21K)]; lane 2, 10 μg of purified porcine CANP; lane 3, 10 μg of purified bovine CANP. (C) Lane 1, 50 μg of the first DEAE-cellulose column fractions of porcine brain; lane 2, 50 μg of the first DEAE-cellulose column fractions of bovine brain.

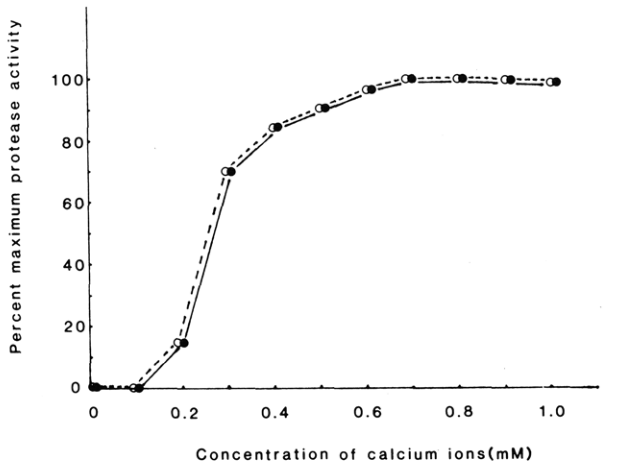

FIGURE 4: Effect of Ca^{2+} concentration on the activity of the purified porcine and bovine CANPs. Prior to determination of the Ca^{2+} requirement of the purified CANPs, the samples were dialyzed against 20 mM Tris-HCl buffer (pH 7.5) containing 5 mM 2-mercaptoethanol and 0.1 mM EDTA. Porcine CANP (○); bovine CANP (●).

final CANPII preparations were purified over 72.2-fold with a 26.5% yield (porcine brain) and 72.6-fold with a 30.5% yield (bovine brain), respectively.

Characterization and Comparison of the Purified Enzymes. The molecular weights of the two CANPIIs were determined by comparing their elution positions on Ultrogel AcA 34 with those of molecular weight standard proteins. Porcine brain CANPII was eluted at a position corresponding to a molecular weight of 100 000 (Figure 2). Bovine brain CANPII was also eluted at a position corresponding to a molecular weight of 100 000 (not shown). The electrophoretic banding patterns of porcine and bovine brain CANPIIs in the absence and presence of SDS are shown in Figure 3. Disc gel electrophoresis of the purified enzymes without SDS showed one protein band for each (Figure 3A). SDS–polyacrylamide gel electrophoresis showed that both enzymes consisted of M_r 75 000 and 29 000 subunits (Figure 3B). Both CANPIIs had isoelectric points of 4.6, as determined by thin-layer isoelectric focusing (graphic data not shown). Both CANPIIs had an optimal pH of 7.5–8.0 (not shown). Calcium requirements of the two CANPIIs are shown in Figure 4. Both porcine brain CANPII and bovine brain CANPII were half-maximally activated at approximately 250 μM and fully activated at 700 μM. Both CANPIIs were slightly activated at 1 mM and fully activated at 5 mM by Sr^{2+}. Ba^{2+} at 10 mM could slightly activate both CANPIIs, but Mg^{2+} and Mn^{2+} had no effect. Leupeptin, E-64, and antipain (all at 10 μg/mL) were effective inhibitors of both CANPIIs. *N*-Ethylmaleimide at 1 mM and iodoacetamide at 1 mM completely inhibited both CANPIIs, while soybean trypsin inhibitor at 100 μg/mL and pepstatin at 50 μg/mL has no effect. These data indicated that both enzymes are thiol proteases. The amino acid compositions of both CANPIIs are shown in Table II. Both CANPIIs were found to have very similar compositions. Table II also shows the reported amino acid compositions of CANPIIs from porcine (Dayton et al., 1976) and chicken (Ishiura et al., 1978) skeletal muscle and rat kidney (Yoshimura et al., 1983) for comparison. The data for the two CANPIIs from porcine and bovine brains are in general agreement with those of rat kidney CANPII. However, in comparison with porcine and chicken muscle CANPIIs, the two CANPIIs from the present study contain relatively large amounts of tyrosine and relatively small amounts of glycine and serine.

The activity of both CANPIIs toward neurofilament triplet proteins (200K, 160K, and 68K), actin, and neurotubules (MAPs and tubulin) was monitored by SDS–polyacrylamide gel electrophoresis. As shown in Figure 5, neurofilament triplet proteins and MAPs were degraded extensively, but tubulin and actin were not. When the porcine and bovine CANPIIs were gel electrophoresed, transferred to nitrocellulose membranes, and then detected with affinity-purified antibodies against these CANPIIs, each antibody stained heterologous CANPII

Table II: Amino Acid Compositions of Porcine and Bovine Brain CANPs Compared with Those of CANPs from Porcine and Chicken Skeletal Muscle and Rat Kidney

	residues/molecule				
	porcine brain CANPII[a]	bovine brain CANPII[b]	rat kidney CANPII[c]	porcine skeletal muscle CANPII[d]	chicken skeletal muscle CANPII[e]
$CysO_3H$	30.4 ± 0.8[f]	30.0 ± 0.6[f]	ND[g]	14	ND[g]
aspartic acid	108.1 ± 2.8	113.1 ± 3.6	100	100	70
threonine	48.6 ± 1.0	48.0 ● 1.3	41	56	32
serine	61.4 ± 1.2	56.9 ± 1.6	76	107	61
glutamic acid	131.4 ± 3.8	129.8 ± 3.8	124	139	72
proline	44.3 ± 1.2	32.3 ± 0.8	32	44	36
glycine	95.1 ± 2.8	94.4 ± 2.7	79	141	117
alanine	70.0 ± 2.2	69.1 ± 1.9	60	80	62
half-cystine	ND[g]	ND[g]	14	ND[g]	16
valine	46.6 ± 0.9	41.2 ± 1.1	41	38	38
methionine	19.1 ± 0.2	17.5 ± 0.5	17	31	4
isoleucine	48.6 ± 0.9	52.0 ± 1.4	57	45	23
leucine	97.7 ± 0.6	97.7 ± 0.6	85	80	51
tyrosine	30.5 ± 0.9	26.5 ● 0.5	28	13	10
phenylalanine	47.4 ± 1.2	50.1 ± 1.4	52	41	24
lysine	54.9 ± 1.7	54.0 ± 1.8	51	64	33
histidine	14.7 ± 0.6	12.6 ± 0.3	15	20	10
arginine	57.9 ± 1.7	56.2 ± 1.7	50	44	24
tryptophan	ND[g]	ND[g]	12	ND[g]	10
total	1005.7 ± 22.6	982.0 ± 25.4	934	1061	693

[a,b] Figures are means plus or minus standard deviations for three separate analyses done on different preparations. [c] Taken from Yoshimura et al. (1983). [d] Taken from Dayton et al. (1976). [e] Taken from Ishiura et al. (1978). [f] Determined by performic acid oxidation. [g] ND, not determined.

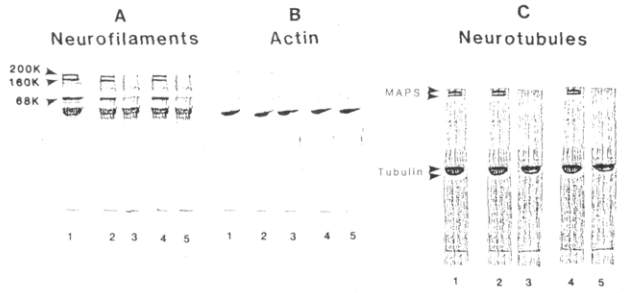

FIGURE 5: SDS–polyacrylamide gel electrophoresis pattern showing the effect of porcine and bovine CANPs on the degradation of cytoskeletal proteins [neurofilaments, actin, and neurotubules (MAPs, tubulin)]. The cytoskeletal proteins were digested individually with porcine or bovine CANP as described under Experimental Procedures. A control sample of each protein containing 6 mM Ca^{2+} but no CANP was also incubated for 5 min (neurofilaments) or 60 min (actin and neurotubules) at 30 °C. (A) Neurofilaments (NF) (200K, 160K, and 68K): lane 1, NF (15 μg); lane 2, NF + 6 mM Ca^{2+}; lane 3, NF + 6 mM Ca^{2+} + porcine CANP (0.3 μg); lane 4, NF + 6 mM Ca^{2+}; lane 5, NF + 6 mM Ca^{2+} + bovine CANP (0.3 μg). (B) Actin: lane 1, actin (10 μg); lane 2, actin + 6 mM Ca^{2+}; lane 3, actin + 6 mM Ca^{2+} + porcine CANP (0.2 μg); lane 4, actin + 6 mM Ca^{2+}; and lane 5, actin + 6 mM Ca^{2+} + bovine CANP (0.2 μg). (C) Neurotubules (NT): lane 1, NT (18 μg); lane 2, NT + 6 mM Ca^{2+}; lane 3, NT + 6 mM Ca^{2+} + porcine CANP (0.35 μg); lane 4, NT + 6 mM Ca^{2+}; and lane 5, NT + 6 mM Ca^{2+} + bovine CANP (0.35 μg).

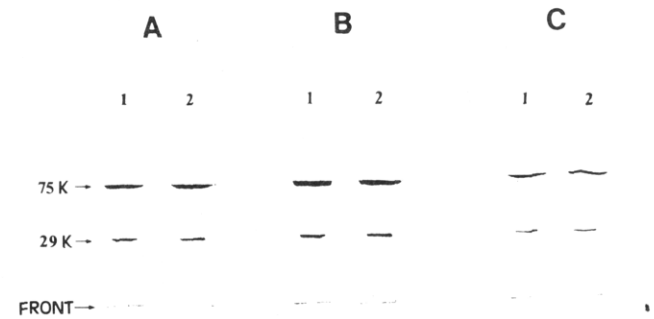

FIGURE 6: Immunoblotting of brain CANPs with affinity-purified antibody for porcine or bovine CANP. The purified porcine and bovine CANPs and first DEAE-cellulose column fractions of porcine and bovine brains were subjected to 15% SDS–polyacrylamide gel electrophoresis (see Figure 3B,C) and then immunoblotted with affinity-purified antibody for porcine or bovine CANP. Horseradish peroxidase coupled goat anti-rabbit IgG and 4-chloro-1-naphthol were used for visualization (see Experimental Procedures). (A) Lane 1, immunoblot of purified porcine CANP with antibody for porcine CANP; lane 2, immunoblot of purified porcine CANP with antibody for bovine CANP. (B) Lane 1, immunoblot of purified bovine CANP with antibody for bovine CANP; lane 2, immunoblot of purified bovine CANP with antibody for porcine CANP. (C) Lane 1, immunoblot of first DEAE-cellulose column fractions of porcine brain with antibody for porcine CANP; lane 2, immunoblot of first DEAE-cellulose column fractions of bovine brain with antibody for bovine CANP.

as well as homologous CANPII (Figure 6A,B). When the first DEAE-cellulose column fractions of porcine and bovine brains (Figure 3C) were used as the test antigens, M_r 75 000 and 29 000 bands were stained by respective antibodies (Figure 6C).

Discussion

We purified the major forms of CANP (CANPII) from porcine and bovine brains to homogeneity by the same method without using casein–Sepharose affinity chromatography. The results clearly showed that the two enzymes had very similar properties. Both enzymes are heterodimers, composed of M_r 75 000 and 29 000 subunits. The two enzymes required 250 μM Ca^{2+} for half-maximal activity (Figure 4). This was in agreement with the results for CANPIIs from bovine brain (Malik et al., 1983), bovine heart (Croall & DeMartino, 1984), rat heart (Croall & DeMartino, 1983), and rat liver (DeMartino & Croall, 1983). Both porcine and bovine brain CANPIIs in the present study were much more sensitive to Ca^{2+} than those from rat brain (Guroff, 1964), canine cardiac muscle (Mellgren, 1980), rabbit skeletal muscle (Mellgren et al., 1982; Inomata et al., 1983), chicken skeletal muscle (Ishiura et al., 1978), porcine skeletal muscle (Dayton et al., 1976), and human platelets (Truglia & Stracher, 1981; Yoshida et al., 1983), but much less sensitive to Ca^{2+} than CANPIIs from rat brain (Zimmerman & Schlaepfer, 1982), chicken smooth muscle (Hathaway et al., 1982), and neuroblastoma cells (DeMartino & Croall, 1982). The reasons for these differences in Ca^{2+} sensitivity are not clear.

Both CANPIIs purified here from porcine and bovine brain hydrolyzed neurofilament triplet proteins and MAPs but did not hydrolyze tubulin or actin (Figure 5). CANPs which cleaved neurofilament triplet proteins were reported by other authors in rat brain (Zimmerman & Schlaepfer, 1982), rat sciatic nerve (Schlaepfer & Micko, 1978; Kamakura et al., 1983), bovine brain (Malik et al., 1983), squid axoplasm (Pant et al., 1979; Pant & Gainer, 1980), and in the soluble fraction of rat spinal cord (Schlaepfer & Freeman, 1980; Ishizaki et al., 1983). The observation that both CANPIIs in the present study hydrolyzed MAPs was consistent with the results of Ishizaki et al. (1983), Sandoval and Weber (1978), and Malik et al. (1983). Similar to findings of Klein et al. (1981) and Sandoval and Weber (1978), both CANPIIs purified here did not hydrolyze tubulin. However, Malik et al. (1983) observed that CANP purified from bovine brain hydrolyzed tubulin. The reason for this difference is not clear.

We have prepared CANPs from both porcine and bovine brains more than 7 times and have always found that CANPII is the major form in brain. This is in accordance with the results of Kishimoto et al. (1981) and Murachi et al. (1981, 1983), who showed that rat brain contained far more CANPII activity than CANPI activity, but contradicted the report of Malik et al. (1983), who showed that CANPI is a major form in bovine brain. Malik et al. (1983) used casein–Sepharose affinity chromatography in the presence of 30 mM Ca^{2+} as one of their purification steps. Our previous finding that CANPII was autoproteolyzed and converted to CANPI during casein–Sepharose affinity chromatography in the presence of Ca^{2+} (Kubota et al., 1981; Suzuki et al., 1981) may possibly explain their result that CANPI was the major form in bovine brain.

The physiological significance for the fact that CANPII requiring an unphysiological Ca^{2+} level is the major form in porcine and bovine brains and the relationship between CANPI and CANPII are unclear at present. The relatively high Ca^{2+} concentration necessary for activation of CANPII is strange. We previously proposed that CANPII can be converted by limited autolysis to CANPI (Kubota et al., 1981; Suzuki et al., 1981), and this hypothesis was supported by other investigators (Hathaway et al., 1982; Mellgren et al., 1982). However, a major problem with this hypothesis is the fact that the conversion process (i.e., CANPII → CANPI) requires prohibitively high concentrations of Ca^{2+} (Kubota et al., 1981; Suzuki et al., 1981). Recently, Coolican and Hathaway (1984) reported that phosphatidylinositol reduces the Ca^{2+} concentration required for autolysis of CANP from bovine aortic smooth muscle and autolysis may be an important regulatory step in the formation of physiologically active CANP in smooth muscle. Whether this is also the case for porcine and bovine brain CANPs must wait further investigations for an answer.

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Registry No. CANP, 78990-62-2; Ca, 7440-70-2; Sr, 7440-24-6; Ba, 7440-39-3.

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Molecular Basis of the Oxygen Exchange from CO_2 Catalyzed by Carbonic Anhydrase III from Bovine Skeletal Muscle†

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ABSTRACT: The exchange of ^{18}O from CO_2 to H_2O in aqueous solution is caused by the hydration–dehydration cycle and is catalyzed by the carbonic anhydrases. In our previous studies of ^{18}O exchange at chemical equilibrium catalyzed by isozymes I and II of carbonic anhydrase, we observed simple first-order depletion of ^{18}O from CO_2 with the ^{18}O distribution among the species $C^{18}O^{18}O$, $C^{16}O^{18}O$, and $C^{16}O^{16}O$ described by the binomial expansion (i.e., a random distribution of ^{18}O). Using membrane-inlet mass spectrometry, we have measured ^{18}O exchange between CO_2 and H_2O catalyzed by native zinc-containing and cobalt(II)-substituted carbonic anhydrase III from bovine skeletal muscle near pH 7.5. The distributions of ^{18}O in CO_2 deviate from the binomial expansion and are accompanied by biphasic ^{18}O-exchange patterns; moreover, we observed regions in which ^{18}O loss from CO_2 was faster than ^{18}O loss from HCO_3^-. These data are interpreted in terms of a model that includes ^{18}O loss from an enzyme–substrate or intermediate complex. We conclude that more than one ^{18}O can be lost from CO_2 per encounter with the active site of isozyme III, a process that requires scrambling of oxygens in a bicarbonate–enzyme complex and cycling between intermediate complexes. This suggests that the rate of dissociation of $H_2{}^{18}O$ (or $^{18}OH^-$) from isozyme III is comparable to or faster than substrate and product dissociation.

The loss of ^{18}O from CO_2 in aqueous solution is caused by the hydration–dehydration cycle and is catalyzed by carbonic anhydrase (Mills & Urey, 1940):

$$C^{18}O^{18}O + H_2{}^{16}O \rightleftharpoons HC^{18}O^{18}O^{16}O^- + H^+ \rightarrow C^{18}O^{16}O + H_2{}^{18}O \quad (1)$$

Here, $H_2{}^{18}O$ is very greatly diluted by solvent $H_2{}^{16}O$, and the loss of ^{18}O from CO_2 is considered irreversible. In our previous studies of ^{18}O exchange at chemical equilibrium catalyzed by isozymes I and II of carbonic anhydrase (Silverman et al., 1979; Tu & Silverman, 1977), we observed rates of depletion of ^{18}O from CO_2 that were monophasic and first order with the ^{18}O distribution in CO_2 described by the binomial expansion at all times. We describe here two new observations for ^{18}O exchange catalyzed by isozyme III from bovine skeletal muscle. First, the ^{18}O exchange is biphasic, described by the sum of two first-order rates; and second, the ^{18}O distribution among labeled CO_2 is not described by the binomial expansion at times before isotopic equilibrium. We concluded that there is scrambling of oxygens in an intermediate, enzyme–substrate complex and that more than one ^{18}O can be lost to solvent per encounter of CO_2 with the active site of carbonic anhydrase III. These conclusions are based on observations of ^{18}O contents of HCO_3^- as well as CO_2 and three criteria for nonrandom ^{18}O distribution, which we describe. The atom fractions of ^{18}O in CO_2 were measured with a mass spectrometer using a membrane inlet.

Although this is the first report of the emergence of nonrandom ^{18}O distributions in catalysis by an isozyme of carbonic anhydrase, such an effect has been thoroughly studied for various phosphatases. Boyer et al. (1977) have described this

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