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Ca^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II FROM HEN BRAIN

PURIFICATION AND CHARACTERIZATION

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Abstract—Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II) has been purified from hen whole brain. The enzyme was purified 3000-fold using phosphocellulose and calmodulin-Agarose column chromatography. The specific activity was 200 nmol/min/mg protein. Microtubule associated protein-2 (MAP-2) was used as a substrate to assess the activity of the enzyme during purification and for its characterization. CaM-kinase II consisted of α and β/β' subunits of molecular weights 46,000 and 55,000/52,000, respectively. The ratio of α to β/β' subunits was 3:1 in the enzyme purified from the whole brain. The enzyme exhibited broad substrate specificity and phosphorylated myelin basic protein, MAP-2, histone II, histone VIII, casein, tubulin, myosin light chains, glycogen synthase, and phosvitin in decreasing order. Phosphorylase *b* was phosphorylated at a negligible rate. Autophosphorylation of CaM-kinase II for 10 min in the presence of calcium and calmodulin decreased its total activity to 33%, and calcium/calmodulin-independent activity reached 30% after 1 min and then dropped to 14% after 10 min of autophosphorylation. The K_m value of ATP was 19 ± 1.3 μM, and the $K_{0.5}$ values of calcium and calmodulin were 4.4 ± 0.5 and 3.0 ± 0.5 μM, respectively. The latter were determined using myelin basic protein as the substrate. CaM-kinase II exhibited great differences in the calmodulin requirement for phosphorylation of MAP-2, histone II and myelin basic protein. MAP-2 required the least amount of calmodulin for its phosphorylation. Autophosphorylation of CaM-kinase II resulted in decreased mobility of the α-subunit but apparently not of the β/β' subunits in sodium dodecyl/sulfate-polyacrylamide gel. Antiserum was raised against the CaM-kinase II α subunit and used for testing cross-reactivity of hen brain enzyme with that of other species. The antiserum which reacted with both α and β subunits of hen brain CaM-kinase II cross-reacted with only the α subunit of rat, mouse, rabbit, cat, dog, pig and human brain samples. The purified hen brain CaM-kinase II is a multifunctional enzyme and resembled rat brain CaM-kinase II in several properties. Immunocross-reactivity suggested that there was similarity in the α but not the β/β' subunits of the hen brain enzyme and the brain enzyme of other species.

The Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase II†) is a multifunctional protein kinase and has a broad substrate specificity. It is immunocytochemically found in nerve endings, axons, dendrites, neuronal somata, and postsynaptic densities [1]. CaM-kinase II differs from Ca^{2+}/calmodulin-dependent protein kinases I and III in molecular weight, substrate specificity, and site of phosphorylation [2, 3]. CaM-kinases I and III have limited substrate specificity [4]. CaM-kinase I is found in high concentration in brain [5] and phosphorylates efficiently a neuron-specific protein, protein III, and a site on synapsin I distinct from that phosphorylated by CaM-kinase II. Similarly, CaM-kinase III is found in high concentrations in the pancreas and skeletal muscle, and phosphorylates only a 100 kDa endogenous protein. Ohmstede *et al.* [6] have purified another Ca^{2+}/CaM-dependent protein kinase that is comprised of subunits of 65 and 67 kDa, phosphorylates synapsin I, and is particularly abundant in cerebellar granule cells.

The subcellular distribution of CaM-kinase II varies in different tissues and is presumably the result of different functions and isozymes of this enzyme in these tissues [7]. CaM-kinase II occurs in soluble as well as membrane-bound form. Thus, most of the activity of CaM-kinase II is found in the cytosolic fraction in rat kidney (100%), liver (94%), and heart (82%), and in the particulate fraction in cerebrum (88%) and testes (84%). The ratio of cytosolic to membrane-bound CaM-kinase II is reversed in the newborn rat brain, with four times more cytosolic than membrane-bound form [8]. The two subunits of CaM-kinase II, referred to as α and β [9], are present in different ratios in the cytosol and membrane-bound form in different regions of brain. The ratios of α:β are 3:1 and 1:4 in rat forebrain and cerebellum, respectively [9, 10]. Tryptic fingerprinting of α subunits from the forebrain and cerebellum reveals a possible difference in their structure, whereas β subunits in these two

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† Abbreviations: CaM, calmodulin; MAP-2, microtubule associated protein-2; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; and *St. aureus* V_8 protease, *Staphylococcus aureus* V_8 protease.

brain regions are indistinguishable [11]. This suggests the existence of at least two types of CaM-kinase II enzymes in rat brain [4].

CaM kinase II phosphorylates a number of proteins including microtubule associated protein-2 (MAP-2), Tau, neurofilaments, and tubulin, and is abundant in microtubule preparations [12]. This enzyme can regulate microtubule assembly either by interacting directly with the microtubules or by phosphorylating MAP-2 at different sites. MAP-2 is one of the best substrates for CaM kinase II, and the phosphorylation of MAP-2 by CaM kinase II or cAMP-dependent protein kinase II (AMP-kinase II) reduces its ability to stimulate microtubule assembly and causes preassembled microtubules to dissociate [13, 14]. Both MAP-2 and Tau are phosphorylated by CaM-kinase II, copurify with tubulin through several cycles of purification, and are similar in their primary sequence [14–16]. The two proteins, however, differ in their cellular localization in that Tau is localized in axons and MAP-2 is found in dendrites [16].

Previous studies in this laboratory have shown that organophosphorous compound-induced delayed neurotoxicity (OPIDN) by tri-*o*-cresyl phosphate (TOCP) [17] increases phosphorylation of 55–60 kDa proteins in brain synaptosomal cytosol, synaptosomal membranes, myelin membranes, and brain supernatant, and of MAP-2 in the brain supernatant of adult chickens [18, 19]. This was also accompanied by an increased autophosphorylation of CaM-kinase II [20]. Rostas *et al.* [21] have purified CaM-kinase II from hen brains and reported some of its properties. However, they used buffer containing calcium during homogenization and purification, which may have caused some proteolysis of the enzyme.

We report here the purification and characterization of CaM-kinase II from adult chicken brain. The enzyme was purified by a different procedure, and buffers containing ethylene glycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and EDTA were used during homogenization and other steps, wherever possible to minimize any proteolysis of the enzyme. In addition, the enzyme was also characterized in greater detail. MAP-2 was used as a substrate for most of the procedures, since this was a good substrate for this enzyme and is known to play a great role in assembly and disassembly of microtubules. The availability of a pure CaM-kinase II and its antibodies should help in studying the mechanism of its increased activity in delayed neurotoxicity in hens.

MATERIALS AND METHODS

Chemicals

Materials were purchased from the following sources: piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES), calmodulin, calmodulin-Agarose, soybean trypsin inhibitor, histone II, histone VIII, myelin basic protein, phosvitin, phosphorylase *b*, glycogen synthase, myosin light chains, dephosphorylated casein, diisopropylphosphorofluoridate, anticalmodulin raised in goat, rabbit antigoat IgG and goat antirabbit IgG (Sigma Chemical Co., St. Louis, MO); ATP and calmodulin-Sepharose (Pharmacia LKB Biotechnology AB, Uppsala, Sweden), dithiothreitol (DTT) (Bachem Inc., Torrance, CA); sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) reagents and Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA), *Staphylococcus aureus* V_8 protease (ICN Biomedicals, Inc., Costa Mesa, CA); leupeptin (Chemicon, Temecula, CA), and [γ-^{32}P]ATP and ^{125}I-calmodulin (Du Pont Co., Boston, MA).

Purification of CaM-dependent kinase II

Phosphocellulose chromatography. Female White leghorn hens (*Gallus gallus* domesticus), 14–18 months old and weighing approximately 1.5 kg, were used for the purification of this enzyme. They were supplied with feed and water *ad lib*. The birds were anesthesized with CO_2 and killed by decapitation. Purification of CaM-kinase II was performed at 4°. The brains were homogenized quickly in a Potter–Elvehjem homogenizer (5 passes) with 4 vol. buffer A [100 mM PIPES, pH 6.9, 10 mM EDTA, 10 mM EGTA, 0.5 mM DTT, 50 mg/L soybean trypsin inhibitor, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/L leupeptin]. The homogenate was centrifuged at 100,000 *g* for 1 hr and the supernatant was loaded on a phosphocellulose column (1.5 × 10 cm) equilibrated with buffer B (30 mM PIPES, pH 6.9, 10% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, 20 mg/L soybean trypsin inhibitor, 0.3 mM PMSF, 2 mg/L leupeptin) at a flow rate of 30 mL/hr. The column was washed with buffer B containing 0.19 M NaCl at a flow rate of 20 mL/hr and eluted with the same buffer containing 0.32 M NaCl. The fractions were read at 280 nm.

Calmodulin affinity chromatography. The selected fractions were pooled, and brought to 5 mg/L with leupeptin and 0.5 mM with $CaCl_2$. The resulting materials were mixed with calmodulin-Sepharose 4B/Agarose (10 mL) equilibrated with buffer C (50 mM Tris · HCl, pH 7.1, 10% glycerol, 0.32 M NaCl, 0.5 mM $CaCl_2$, 1 mM DTT, 0.3 mM PMSF, 5 mg/L leupeptin) for 3 hr and packed into a column. The column was washed with buffer C containing 0.4 M NaCl (250 mL) and then with buffer C containing 0.2 M NaCl (30 mL) at a flow rate of 20 mL/hr. The column was then eluted with buffer D (50 mM Tris · HCl, pH 7.1, 10% glycerol, 0.2 M NaCl, 2 mM EGTA, 1 mM EDTA, 1 mM DTT, 0.3 mM PMSF, 2 mg/L leupeptin) in 2-mL fractions. The fractions containing CaM-kinase II activity were pooled, made 0.5 M with sucrose, and kept at −70°.

Assay of CaM-kinase II activity

The reaction mixture (100 μL) contained 50 mM Tris · HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.2 mm $CaCl_2$, 10 μg calmodulin, substrate, CaM-kinase II, 50 μM [γ-^{32}P]ATP (0.4 nCi/pmol). The reaction mixture was preincubated for 1 min at 30° before starting the reaction by adding ATP solution. The blank contained 0.2 mM EGTA in place of calcium and calmodulin in the reaction mixture. The reaction was allowed to run for 1 min at 30° and then stopped by adding either 50 μL Stop solution (0.125 M Tris · HCl, pH 6.8, 9% SDS, 3 mM

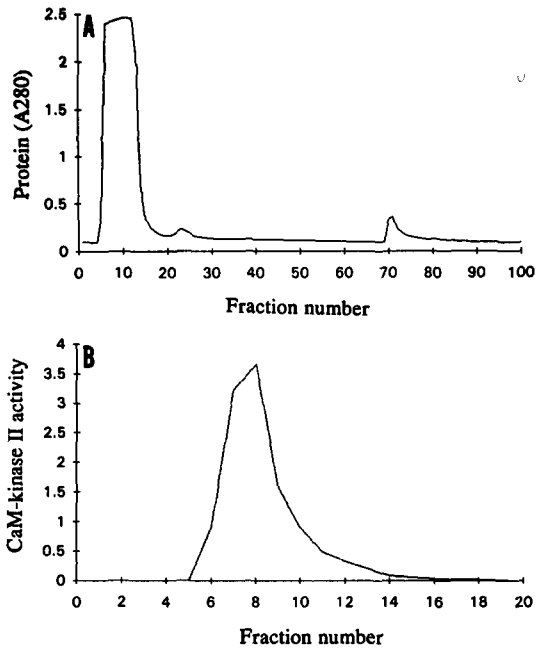

Fig. 1 (A) Phosphocellulose column chromatography of Ca^{2+}/CaM-dependent protein kinase II. The brain supernatant was loaded on a PC column (1.5 × 10 cm), washed, and eluted as described under Materials and Methods. Fractions (10 mL) were collected and read at 280 nm. Fractions 70–78 were pooled and processed for CaM-Agarose column chromatography. (B) CaM-Agarose column chromatography. The pooled fractions from the PC column containing $CaCl_2$ were mixed with CaM-Agarose (6 mL) for 3 hr and packed into a column (1.5 × 10 cm). The column was washed with buffer containing 0.4 M NaCl and then eluted with buffer containing EGTA as described under Materials and Methods. Fractions (2 mL) were collected and used for determining Ca^{2+}/calmodulin-dependent protein kinase activity. Activity is expressed as nmol/min/mL of each fraction.

EDTA, 10% 2-mercaptoethanol, 20% glycerol, trace of bromophenol blue) or 10 μL Stop solution (0.2 M EDTA and 10 mM ATP, pH 7.0). The reaction mixture containing 50 μL Stop solution was heated in boiling water for 2 min, and 75 μL was used for SDS–PAGE. The gels were stained with Coomassie Blue and autoradiographed using two intensifying screens. The relevant bands were excised from the gel and counted in the liquid scintillation fluid. The reaction mixture containing 10 μL Stop solution with EDTA and ATP was used for measuring total [γ-^{32}P]ATP incorporation in the presence of substrate. Fifty microliters of reaction mixture was used for spotting on P81 Whatman filter paper and washed as described by Roskoski [22]. This was followed by liquid scintillation counting. Substrate MAP-2 was omitted from the reaction mixture for autophosphorylation of CaM-kinase II, and the radioactivity of 50 μM [γ-^{32}P]ATP was doubled (0.8 nCi/pmol).

^{125}I-Calmodulin binding

^{125}I-Calmodulin binding to subcellular fractions (150 μg) or purified CaM-kinase II (5 μg) was carried out by the method of Carlin *et al.* [23] with some modifications. The pH of the washing buffer was 7.1 and ^{125}I-calmodulin (2.5×10^6 cpm), prepared by the method of Bolton and Hunter [24], was used in 50 mL of buffer for treating the gel.

Partial peptide mapping

MAP-2 (6 μg) was phosphorylated and subjected to SDS–PAGE in 7.5% acrylamide gel. The gel was stained with Coomassie Blue and dried, and MAP-2 bands were rehydrated in 1 mL of 62.5 mM Tris · HCl, pH 6.8, 0.1% SDS, 1 mM EDTA, 2.5% 2-mercaptoethanol. After 1 hr, the gel pieces were loaded on a 12.5% acrylamide gel and overlayed with 50 μL of α-chymotrypsin (5 μg) or *St. aureus* V_8 protease (5 μg). Electrophoresis was performed at 30 mamp through the stacking gel and 50 mamp through the resolving gel. MAP-2 was proteolyzed during electrophoresis through the stacking gel, which took more than 1 hr. The gel was stained with Coomassie Blue and autoradiographed in the presence of intensifying screens. Partial peptide mapping of CaM-kinase II was also performed by the same method with minor modifications. CaM-kinase II (0.4 μg) was autophosphorylated for 10 min for maximum phosphorylation and subjected to SDS–PAGE. Both the subunits were separately excised from the gel, after autoradiography, and processed for partial peptide mapping as described for MAP-2.

Tryptic peptide mapping

MAP-2 (6 μg) was phosphorylated at 50 μM concentrations of [γ-^{32}]ATP, subjected to SDS–PAGE in 7.5% acrylamide gel, stained with Coomassie Blue, and dried. The labeled MAP-2 was proteolyzed completely by L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin, and subjected to electrophoresis in one direction and chromatography in the other direction [25]. Electrophoresis (600 V, 45 min) and chromatography were performed on 15 × 20 cm cellulose-coated TLC plates. Tryptic peptide mapping of α and β subunits of autophosphorylated CaM-kinase II (0.4 μg) was done in the same way as MAP-2 after removing related protein bands from the gel.

Raising of antibodies

CaM-kinase II (60 μg) was subjected to SDS–PAGE [26] in 7.5% acrylamide gel and then electrophoretically transferred onto a nitrocellulose sheet (0.2 μM) for 1 hr at 100 V [27]. CaM-kinase II was stained with Coomassie Blue, and then α subunit bands were excised from the nitrocellulose sheet. The latter were dissolved in 0.5 mL of dimethyl sulfoxide. This CaM-kinase II solution was injected subcutaneously two times in complete Fruend's adjuvant and once in incomplete Fruend's adjuvant at 1-month intervals. The antibodies were finally boosted after 15 days with the same amount of enzyme in incomplete Fruend's adjuvant. The birds were bled after 8 days. Antiserum was heated at 56° for 20 min before using.

Table 1. Summary of purification of Ca^{2+}/calmodulin-dependent protein kinase II

Fraction	Protein (mg)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Recovery (%)	Purification (fold)
Homogenate	1620	0.07	111.8	100	1
Supernatant	302	0.28	83.5	74.7	4
PC column	16.7	6.1	101.8	91.1	88.3
Calmodulin-Agarose column	0.19	206.0	38.7	34.6	2942.8

N-terminal amino acid sequence

CaM-kinase II (20 μg) was dialyzed against deionized water containing 0.3 mM PMSF and 2 mg/L leupeptin, lyophilized, and dissolved in 0.1 mL of water. CaM-kinase II was precipitated with 90% ethanol at −20°, pelleted at 12,000 *g* for 30 min, subjected to SDS–PAGE, and electrophoretically transferred onto Immobilon P (PBDF). The protein was stained with Coomassie Blue R-250 and analyzed for N-terminal amino acid sequence by Edman degradation using an automatic gas phase sequencer (Applied Biosystems 477A protein sequencer).

Other procedures

Protein was estimated by the method of Peterson [28] or Bradford [29] using bovine serum albumin as the standard, and SDS–PAGE was performed according to the method of Laemmli [26].

RESULTS

Purification of CaM-kinase II

The protein profile obtained from the phosphocellulose column is shown in Fig. 1A. A major portion of protein passed through the column on loading and washing with buffer B (550 mL) containing 0.19 M NaCl. The pH of buffer B was 6.9, and an increase in pH seemed to leave more colored protein on the column. The column was eluted with buffer containing 0.32 M NaCl, and the peak fractions were used for CaM-Agarose column chromatography. The concentration of NaCl was not lowered to 0.2 M NaCl as in other procedures [9, 15], since CaM-kinase II was able to bind CaM-Agarose at a 0.32 M NaCl concentration in the presence of 0.5 mM $CaCl_2$. Washing of the CaM-Agarose column with buffer C containing 0.5 M NaCl decreased the yield of enzyme without affecting its purity. The fractions containing CaM-kinase II during elution from CaM-Agarose were detected by absorbance at 280 nm, and the selected fractions were used for estimating the kinase activity (Fig. 1B). There was 1500- to 3000-fold purification of the enzyme (Table 1). This depended on the initial kinase II activity in the homogenate, which varied from 0.07 to 0.17 nmol/min/mg protein in different batches. This procedure is different from that of Rostas *et al.* [21]. The latter used buffers containing calcium during homogenization, which might result in some proteolysis of CaM-kinase II subunits, and the brain supernatant was directly loaded on the CaM-Sepharose 4B column without prior partial purification of the enzyme through some other column. The enzyme was then purified further on a gel filtration column.

SDS–PAGE and autophosphorylation of CaM-kinase II

Purification of CaM-kinase II at different steps of the procedure was assessed by SDS–PAGE and autophosphorylation of brain homogenate, brain supernatant, PC column fraction, and purified CaM-kinase II (Fig. 2). Silver staining of the gel showed that CaM-kinase II was comprised mainly of the α subunit (Fig. 2A). The ratio of α/β subunits was 3:1 in the pooled enzyme, but it varied from 1.6:1 to 4:1 in different fractions from the same CaM-Agarose column during elution with buffer D. Rostas *et al.* [21] reported the ratio of α/β as 1.85 ± 0.17 by silver staining. Autophosphorylation of purified CaM-kinase II exhibited a doublet for both the α and β subunits of CaM-kinase II (Fig. 2B). Autophosphorylation of the CaM-kinase II for increasing periods yielded a single band for the α subunit (Fig. 3). Thus autophosphorylation of CaM-kinase II decreased the mobility of the α subunit (Fig. 3) but, obviously, not of the β/β′ subunits as shown in rat brain CaM-kinase II by McGuiness *et al.* [9]. Rostas *et al.* [21] also found a distinct decrease in the mobility of the α subunit but not in the β subunit of hen brain CaM-kinase II.

Maximum autophosphorylation of the α and β subunits was achieved in 6–8 min at 30°, and 50% or less was complete in 1 min (Fig. 3). The maximum incorporation of ^{32}P was approximately 2 and 8 mol/mol of CaM-kinase II α and β subunits, respectively. The α/β ratio by autoradiography after maximum autophosphorylation was 0.98 ± 0.04 against 1.92 ± 0.26 reported by Rostas *et al.* [21]. The observation of a single or double band for the α subunit, however, depended on some fine modification of its conformation since CaM-kinase II of the same batch sometimes exhibited a single band even after 1 min of autophosphorylation under apparently identical conditions. The molecular weights of α, β, and β′ subunits by SDS–PAGE on 7.5% acrylamide gel were 46,000, 55,000, and 52,000, respectively.

^{125}I-Calmodulin binding and immunocytochemical reaction with anti-CaM-kinase II

^{125}I-Calmodulin bound to both α and β subunits

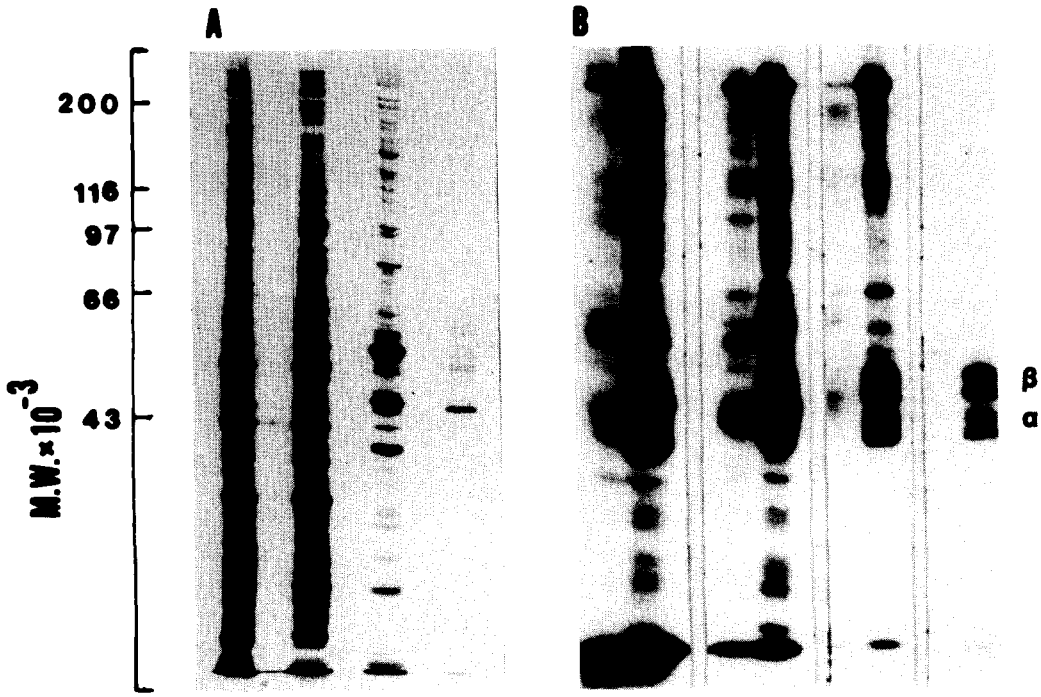

Fig. 2. SDS–PAGE and autophosphorylation of different fractions. (A) (1) Brain homogenate, 8.1 μg; (2) supernatant, 8.2 μg; (3) PC column fraction, 2.1 μg; and (4) CaM-kinase II, 1.1 μg were subjected to SDS–PAGE (7.5% acrylamide gel) and silver nitrate staining as described under Materials and Methods. (B) The fractions (1, 2) brain homogenate, 324 μg; (3, 4) supernatant, 82 μg; (5, 6) PC column fraction, 42 μg; and (7, 8) CaM-kinase II, 0.25 μg, were phosphorylated in the presence of EGTA (1, 3, 5, 7) or calcium (2, 4, 6, 8) as described under Materials and Methods. The phosphorylated proteins were subjected to SDS–PAGE (7.5% acrylamide gel), Coomassie Blue staining, and autoradiography in the presence of two Du Pont intensifying screens.

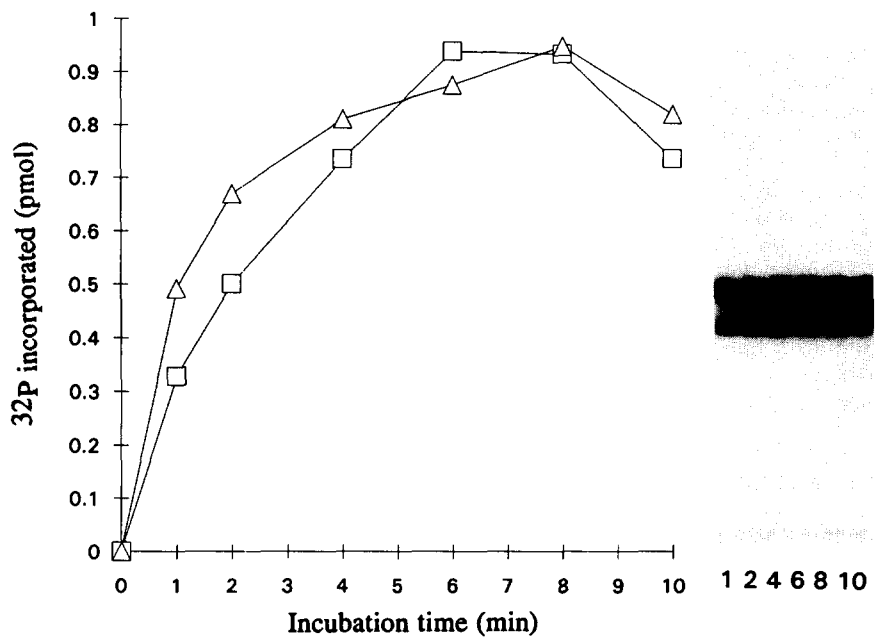

Fig. 3. Ca^{2+}/CaM-dependent kinase II autophosphorylation for different periods. CaM-kinase II was phosphorylated for 1, 2, 4, 6, 8, and 10 min at 30° as described in the legend to Table 2. The phosphorylated samples were subjected to electrophoresis, staining, and autoradiography. Then α and β/β′ subunits were excised from the gel and counted in the liquid scintillation counter. ^{32}P incorporation is given in pmol for α (□) and β (△) subunits.

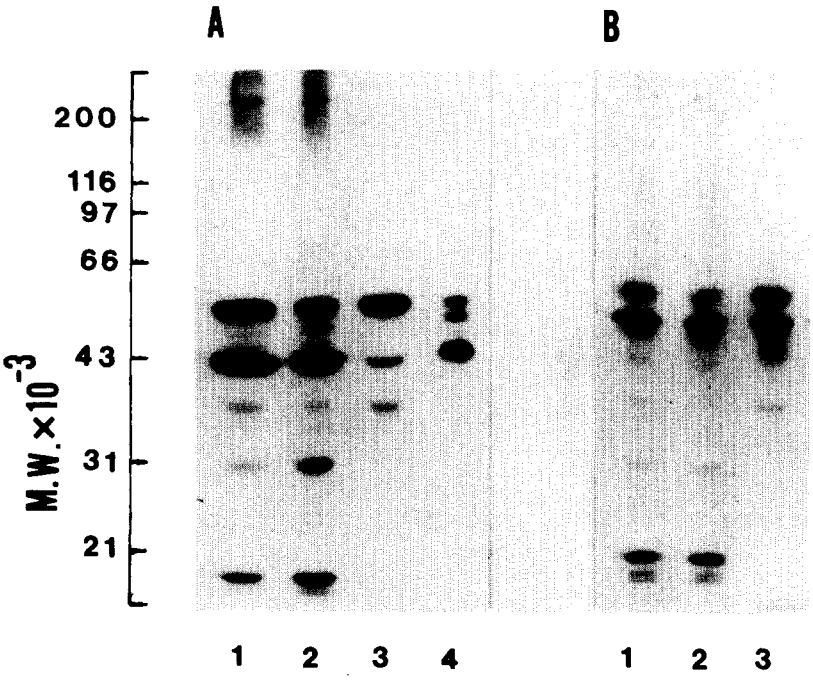

Fig. 4. ^{125}I-Calmodulin binding and immunocytochemical reaction with CaM-kinase II. Brain homogenate, 150 μg (1); 100,000 *g* particulate, 150 μg (2); and 100,000 *g* supernatant, 150 μg (3) were used for determining both calmodulin binding (A) and immunocytochemical reaction with anti-CaM-kinase II (B). CaM-kinase II (5 μg) was used only for calmodulin binding (4). The proteins were subjected to SDS–PAGE in 7.5% acrylamide gels and the gels were directly used for ^{125}I-calmodulin binding. For immunocytochemical reaction, the proteins were electrophoretically transferred to a nitrocellulose sheet and developed using anti-CaM-kinase II and ^{125}I-protein A as described under Materials and Methods.

of CaM-kinase II in brain homogenate, the particulate fraction, and purified enzyme. However, ^{125}I-calmodulin, which showed a comparable reaction with the β subunit in brain supernatant, showed only a weak one with the α subunit (Fig. 4A). The presence of the α subunit, therefore, was examined further by immunoblotting analysis using anti-CaM-kinase II. The antibodies against CaM-kinase II reacted equally well with the α subunit in all three fractions—homogenate, particulate, and supernatant (Fig. 4B). The reaction of anti-CaM-kinase II with low molecular weight proteins in brain homogenate, supernatant, and PC column fractions was probably due to degradation products of CaM-kinase II, since antibodies against CaM-kinase II were raised by using the α subunit and not the whole purified enzyme. However, cross-reactivity of the antibody with other proteins in these fractions could not be excluded.

^{125}I-Calmodulin showed two bands of β subunits in the purified enzyme and only one in the homogenate, particulate, and supernatant fractions. This appeared to suggest that β′ subunit was probably formed by the degradation of the β subunit during purification, even though sequencing of cDNA clones from rat brain has shown that the β′ subunit carries a deletion of amino acid residues 378–392 in the aligned sequence of the β subunit [30].

Substrate specificity of CaM-kinase II

Various substrates were used to examine the substrate specificity of the purified enzyme from hen whole brain (Table 2). MAP-2, histone II, histone VIII, casein (dephosphorylated), and myeline basic protein were good substrates for this enzyme, and myelin basic protein showed the highest rate of phosphorylation. Other substrates including phosvitin, tubulin (α, β), glycogen synthase, and myosin light chains were phosphorylated at lower rates. There was overestimation of the phosphorylation of tubulin because the subunits of tubulin and CaM-kinase II overlapped. Phosphorylase *b* was phosphorylated at a negligible rate. Ten micrograms of calmodulin was used in the reaction mixture (0.1 mL) while assessing the specificity of CaM-kinase II for different substrates. This was necessary because the phosphorylation of at least two substrates (MAP-2 and histone II) had different calmodulin requirements.

Calmodulin requirement for MAP-2 and histone II phosphorylation

MAP-2 and histone II showed a great difference in calmodulin requirements for their optimum phosphorylation (Fig. 5). There was significant phosphorylation of MAP-2 along with auto-

Table 2. Substrate specificity of Ca^{2+}/calmodulin-dependent protein kinase II

Subsrate	Amount (μg)	Specific activity (%)
MAP-2	3	100
Histone II	10	66.3
Histone VIII	10	58.5
Myelin basic protein	10	211.5
Phosvitin	40	10.7
Tubulin	40	21.5
Phosphorylase *b*	40	1.2
Glycogen synthase	10	18.5
Myosin light chains	40	20.4
Casein (dephosphorylated)	40	50.1

The substrates were incubated with purified enzyme (0.12 μg) for 1 min at 30°. The reaction mixture contained 50 mM Tris · HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 65 mM NaCl, 0.2 mM $CaCl_2$, and 10 μg calmodulin. The control included 0.2 mM EGTA in place of calcium and calmodulin. The reaction was stopped by 50 μL of Stop solution, the reaction mixture was heated for 2 min in boiling water, and 75 μL was used for SDS–PAGE as described under Materials and Methods. The specific activity of kinase II for MAP-2 was 166 nmol/min/mg protein. Results are the averages of two separate experiments done in duplicate.

phosphorylation of CaM-kinase II without adding calmodulin to the reaction mixture containing the enzyme and MAP-2 (Fig. 5, lane 1). Increasing calmodulin from 2 to 10 μg in the reaction mixture did not increase the phosphorylation (lane 3, 4). The addition of trifluoperazine (50 μM), however, completely inhibited the phosphorylation of MAP-2 and autophosphorylation of the enzyme. This suggested the presence of calmodulin in MAP-2 but not in the CaM-kinase II preparation, since there was no autophosphorylation of CaM-kinase II in the absence of calmodulin or MAP-2 (data not shown). In contrast, myelin basic protein (data not shown) and histone II were not phosphorylated in the absence of calmodulin (lane 6), and an increase of calmodulin from 2 to 10 μg further enhanced the phosphorylation of histone II (lane 8, 9). Furthermore, the inclusion of histone II in the reaction mixture containing MAP-2 also inhibited the phosphorylation of MAP-2 and autophosphorylation of CaM-kinase II in the absence of calmodulin (data not shown). SDS–PAGE of 10 μg MAP-2, and 1 and 5 μg calmodulin followed by silver or Coomassie Blue staining did not identify the presence of calmodulin in the MAP-2 preparation (data not shown). It is plausible that a trace of calmodulin present in the MAP-2 preparation was sufficient for some phosphorylation of MAP-2, when the latter was present alone without histone II in the reaction mixture. A band of 120 kDa is always obtained below MAP-2. This protein, although present in trace amounts in the MAP-2 preparation, as evident from Coomassie staining, is highly phosphorylated along with MAP-2.

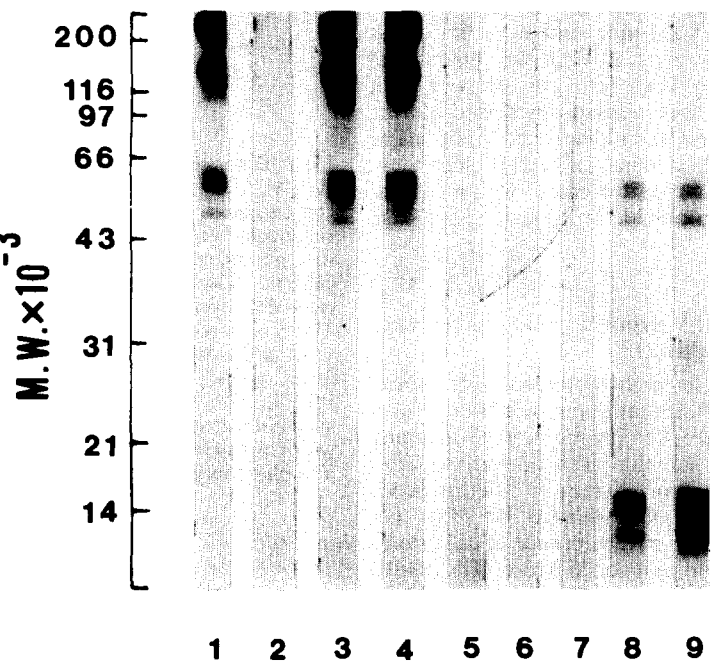

Fig. 5. Calmodulin requirement for MAP-2 and histone II phosphorylation by CaM-kinase II. Phosphorylation of MAP-2 (3 μg) and histone II (10 μg) was performed as described in the legend to Table 2 with different concentrations of calmodulin, 50 μM trifluoperazine, or 0.2 mM EGTA. Lanes 1–5: phosphorylation of MAP-2; lanes 6–9: phosphorylase of histone II. Lanes 1 and 6, absence of calmodulin; 2 and 7, addition of trifluoperazine; 3 and 8, 2 μg of calmodulin; 4 and 9, 10 μg of calmodulin; and lane 5, 0.2 mM EGTA in place of calcium and calmodulin.

Kinetics of phosphorylation of MAP-2 or myelin basic protein by CaM-kinase II

Phosphorylation of MAP-2 was performed at different concentrations of calcium and ATP to determine the K_m and $K_{0.5}$ values of ATP and free calcium concentration, respectively (Fig. 6, A and B). In contrast, the $K_{0.5}$ of calmodulin was determined for myelin basic protein instead of MAP-2 (Fig. 6C), since the $K_{0.5}$ value of calmodulin for MAP-2 was so low that significant phosphorylation of the latter occurred even without adding any calmodulin to the reaction mixture. The composition of the reaction mixture was modified to determine the $K_{0.5}$ value of free calcium. The main difference was in the concentration of EGTA (1 mM) and pH (7.3) of the reaction mixture. Free calcium concentration was calculated as described by Fabiato and Fabiato [31]. The half-maximal concentration of MAP-2 was $19 \pm 1.3\,\mu M$ and the $K_{0.5}$ values for calcium and calmodulin were 4.4 ± 0.5 and $3.0 \pm 0.5\,\mu M$, respectively (Table 3).

Effect of autophosphorylation on CaM-kinase II activity

Purified enzyme was autophosphorylated for 1–10 min at 30°, and MAP-2 phosphorylation was estimated in the presence and absence of Ca^{2+} and calmodulin at different periods. There was no phosphorylation of MAP-2 in the absence of calcium and calmodulin. The enzyme, however, was not phosphorylated further in the absence of calcium by adding EGTA. Continuous autophosphorylation of CaM-kinase II decreased its total activity, in that activity was 33% of its initial activity at the end of 10 min. The Ca^{2+}/CaM-independent activity was 30% after 1 min and 14% after 10 min of autophosphorylation (Fig. 7). This showed that Ca^{2+}/CaM-independent activity increased during the first minute and then decreased upon more phosphorylation. Also, the Ca^{2+}/CaM-dependent activity of the enzyme decreased without autophosphorylation, up to 62% at the end of a 10-min incubation.

Peptide mapping and N-terminal amino acid sequence of CaM-kinase II subunits

Partial proteolysis by α-chymotrypsin or *St. aureus* V_8 protease showed different peptide patterns for α and β subunits (Fig. 8A). Tryptic peptide mapping of CaM-kinase II subunits was performed after autophosphorylation for 10 min in the presence of Ca^{2+}/calmodulin. CaM-kinase II was not phosphorylated further in the absence of calcium by adding EGTA to the reaction mixture. The tryptic peptide pattern of α and β/β′ subunits could be easily distinguished from each other (Fig. 8, B and C).

CaM-kinase II did not show any peak when PVDF membrane containing the α subunit was used for amino acid sequencing in the automatic gas phase sequencer. This suggested that the hen brain CaM-kinase II α subunit may have a blocked N-terminal amino acid. The same method has been used previously in this laboratory to determine the N-terminal sequence of several cytochrome P450s purified from hen liver [32, 33]. The β/β′subunits were not used for amino acid sequencing.

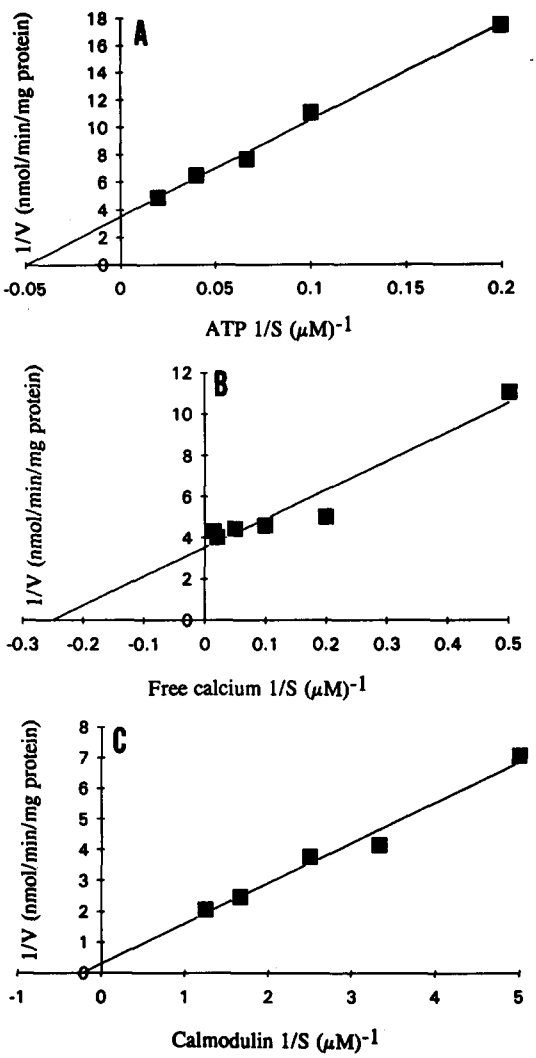

Fig. 6. Determination of kinetic parameters of CaM-kinase II. (A) MAP-2 (3 μg) was phosphorylated at different concentrations of ATP. The concentrations of calcium, and calmodulin were 100 and 3 μM, respectively. (B) MAP-2 was phosphorylated at various concentrations of free calcium. The concentrations of EGTA, ATP, and calmodulin were 1 mM, 50 μM, and 1.5 μM, respectively. The concentration of total calcium was varied from 0.91 to 1.1 mM to obtain a 1 to 100 μM free calcium concentration in the reaction mixture. The reaction mixture contained PIPES, pH 7.3, instead of Tris · HCl, pH 7.5, in the standard reaction mixture. (C) Myelin basic protein (10 μg) was phosphorylated at various concentrations of calmodulin. The phosphorylation was carried out as described in the legend of Table 2.

Peptide mapping of phosphorylated MAP-2

Partial proteolysis of phosphorylated hen brain MAP-2 by α-chymotrypsin and *St. aureus* V_8 protease

Table 3. Summary of the kinetic properties of Ca^{2+}/CaM-dependent protein kinase II

Property	Substrate	Value (μM)
K_m (ATP)	MAP-2	19 ± 1.3
$K_{0.5}$ (free calcium)	MAP-2	4.4 ± 0.5
$K_{0.5}$ (calmodulin)	Myelin basic protein	3.0 ± 0.5
$K_{0.5}$ (calmodulin)	MAP-2	*

K_m and $K_{0.5}$ values were determined as described in the legends of Fig. 6. Results are means ± SEM from three experiments.

* $K_{0.5}$ for calmodulin was very low when MAP-2 was used as a substrate. MAP-2 was phosphorylated to a great extent without adding any calmodulin to the reaction mixture. This was attributed to the presence of a trace of calmodulin in the MAP-2 preparation, since trifluoperazine (50 μM) completely inhibited the phosphorylation of MAP-2.

showed one (18 kDa) and five (49, 27, 21.5, 17.5, 14.5 kDa) major peptides, respectively (Fig. 9A). Four peptides ranging from 9 to 23 kDa are also obtained after proteolysis of rat and bovine MAP-2 by *St. aureus* V_8 protease [15, 34]. In contrast to the apparent similarity among hen, rat, and bovine MAP-2 on the basis of partial proteolysis, two-dimensional tryptic peptide mapping of hen (Fig. 9B) and rat [35] brain MAP-2 appeared to exhibit a different pattern. More close comparison of hen, rat, and bovine MAP-2, however, would require their peptide analysis under the same conditions.

Cross-reactivity of CaM-kinase II between hen and other species

Polyclonal antibody to the hen brain CaM-kinase II α subunit was used to test cross-reactivity with several species. This antiserum strongly reacted with the brain supernatants (100 μg protein) of the rat, mouse, rabbit, cat, dog, pig, and human, suggesting similarity between the CaM-kinase II of all these species (Fig. 10). Although, the antibody against the α subunit of CaM-kinase II reacted with the β subunit in hen brain supernatant, it did not react with the β subunit of the enzyme from other species.

DISCUSSION

This study was designed to isolate, in a pure form, CaM-kinase II and to prepare its polyclonal antibodies to be used in further studies into the mechanisms of OPIDN [36]. The purification procedure used two columns and was derived by modification of the procedures of Goldenring *et al.* [37] and Hashimoto *et al.* [38]. The inclusion of a gel filtration column, Sephacryl S-400 column (1.6 × 100 cm), after phosphocellulose column chromatography did not improve significantly the purity of CaM-kinase II preparation. The major protein band that was removed by gel filtration chromatography from the phosphocellulose column effluent did not appear in the purified enzyme even without using the Sephacryl S-400 column. Therefore, CaM-kinase II purified by the two-column procedure was used for further investigation. The Sephacryl S-

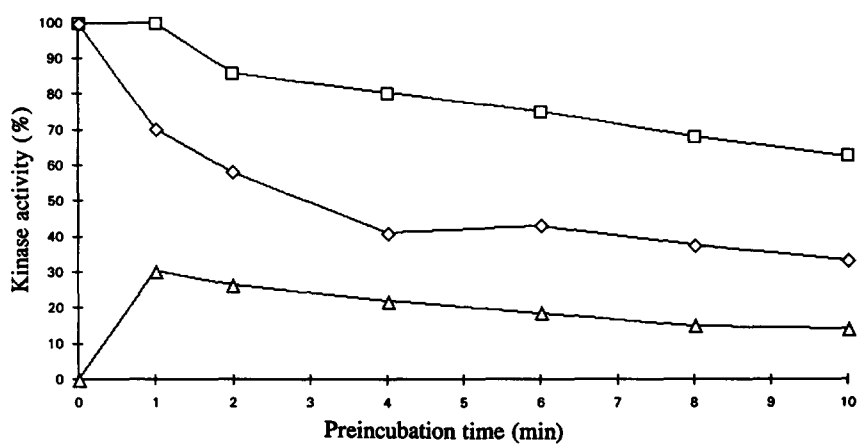

Fig. 7. Effect of autophosphorylation on Ca^{2+}-dependent (total) and Ca^{2+}-independent CaM-kinase II activity. Phosphorylation of MAP-2 was carried out by 0.4 μg of CaM-kinase II as described in the legend of Table 2. The enzyme was autophosphorylated by incubating at 30° with calcium, calmodulin, and [γ-^{32}P]ATP for different periods. This was followed by the addition of MAP-2 for determination of total activity (◇), and EGTA and MAP-2 for the determination of calcium-independent activity (△). Control reactions were also carried out (□), where the enzyme was incubated for different periods in the absence of ATP and the phosphorylation was performed by sequential addition of MAP-2 and [γ-^{32}P]ATP. Every reaction was accompanied by a blank reaction in which 0.2 mM EGTA was included from the beginning. The reactions were terminated by Stop solution, and then the reaction mixture was heated for 2 min in boiling water, and subjected to SDS–PAGE. The phosphorylated MAP-2 bands were excised from the gel and counted in the liquid scintillation counter. One hundred percent enzyme activity was 180 nmol/min/mg protein.

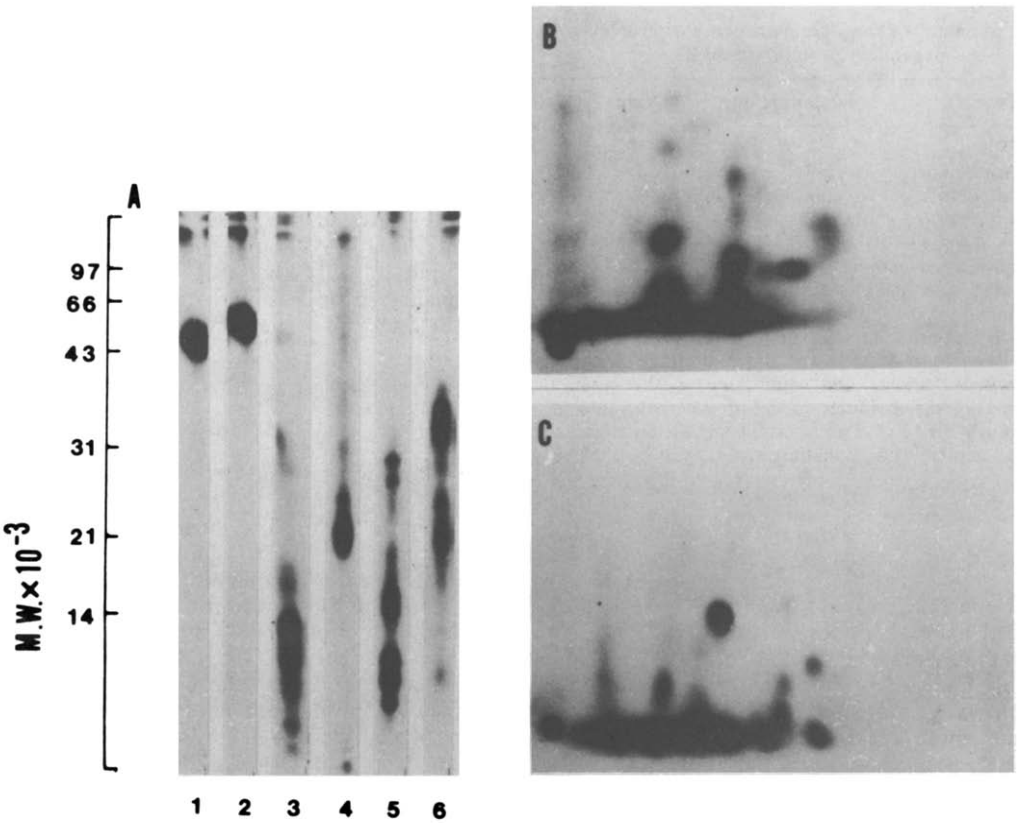

Fig. 8. (A) Partial peptide mapping of phosphorylated CaM-kinase II. CaM-kinase II (0.4 μg) was phosphorylated at 30° for 10 min and subjected to SDS–PAGE. The bands were excised after autoradiography, and rehydrated in 1 mL of 62.5 mM Tris · HCl, pH 6.8, 0.1% SDS, 1 mM EDTA, 2.5% 2-mercaptoethanol. After 1 hr, the gel pieces were loaded on a 12.5% acrylamide gel and overlayed with 50 μL of α-chymotrypsin (5 μg) or *St. aureus* V_8 protease (5 μg). Electrophoresis was carried out at 30 mamp in the stacking gel and 50 mamp in the resolving gel. The gel was stained, dried, and autoradiographed. Lane 1, untreated CaM-kinase II α subunit; 2, untreated CaM-kinase II β/β′subunit; 3, α subunit treated with α-chymotrypsin; 4, β/β′ subunit treated with α-chymotrypsin; 5, α subunit treated with *St. aureus* V_8 protease; and 6, β/β′ subunit treated with *St. aureus* V_8 protease. (B and C) Tryptic peptide mapping of Ca^{2+}/CaM-dependent kinase II. CaM-kinase II (0.4 μg) was phosphorylated for 10 min in the absence of any substrate as described in the legend of Table 2. Phosphorylated CaM-kinase II was subjected to SDS–PAGE, staining, and autoradiography. The autophosphorylated α and β/β′ subunits were excised from the gel and completely digested with TPCK-treated trypsin and lyophilized. The resulting material was subjected to electrophoresis (600 V, 45 min) [25] in one direction and chromatography in the other direction on 15 × 20 cm cellulose-coated TLC plates as described in Materials and Methods. Panels B and C are the autoradiographs of tryptic peptides of α and β/β′ subunits, respectively.

400 column (1.6 × 100 cm), when used, was equilibrated with 30 mM PIPES, pH 7.0, 10% glycerol, 0.1 mM EGTA, 0.1 mM EDTA, 0.32 M NaCl, 0.3 mM PMSF, 2 mg/L leupeptin. The flow rate was 10 mL/hr. The concentration of NaCl in the equilibrium buffer was kept at 0.32 M, since CaM-kinase II appeared in the void volume at 0.2 M NaCl concentration. However, Rostas *et al.* [21] used 0.2 M NaCl in their buffer for gel filtration chromatography of hen brain CaM-kinase II. The discrepancy might be ascribed to the difference in the purification procedures.

Variation in the α/β ratio (1.6 to 4.0) in different fractions from the CaM-Agarose column was probably because of the purification of CaM-kinase II from the whole brain, which contained α and β subunits in different proportions in different regions [9]. The molecular weights of the α, β, and β′ subunits of hen brain kinase II (46,000, 55,000 and 52,000) were also lower than those of mammalian brain [14]. The immunostaining of hen and rat brain supernatants after SDS–PAGE and electrophoretic transfer also supported a slightly lower molecular weight for at least the α subunit of hen brain CaM-kinase II (Fig. 10). In contrast, Rostas *et al.* [21] have reported similar molecular weights for rat and adult chicken brain CaM-kinase II subunits.

Brain homogenate, 100,000 *g* pellet, supernatant, and purified CaM-kinase II were examined for ^{125}I-calmodulin binding. Brain supernatant exhibited a

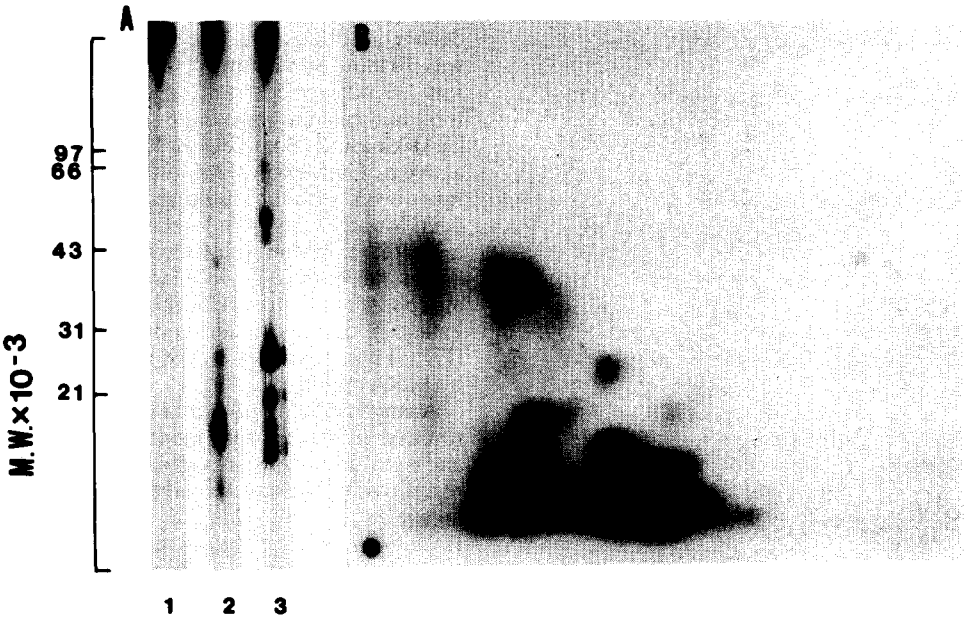

Fig. 9. (A) Partial peptide mapping of phosphorylated MAP-2. MAP-2 (6 μg) was phosphorylated by purified CaM-kinase II for 1 min and subjected to SDS–PAGE in 7.5% acrylamide. The stained band of MAP-2 was used for partial peptide mapping as described in the legend of Fig. 8A. After SDS–PAGE, the gel was stained, dried, and autoradiographed. Lane 1, MAP-2; 2, MAP-2 treated with α-chymotrypsin; and 3, MAP-2 treated with *St. aureus* V_8 protease. (B) Tryptic peptide mapping of phosphorylated MAP-2. MAP-2 (6 μg) was phosphorylated for 1 min and subjected to SDS–PAGE in 7.5% acrylamide gel, stained, and dried. The labeled MAP-2 bands were excised from the gel and processed for complete digestion by trypsin as described in the legend of Fig. 8B.

weak binding of the α subunit with labeled calmodulin as compared to brain particulate fraction (100,000 *g* pellet) (Fig. 4). The ratio of ^{125}I-calmodulin binding to the α subunit in brain supernatant and particulate was about 1:10. This weak binding was not due to a decreased amount of α subunit, since anti-CaM-kinase II showed a comparable reaction with this subunit in both fractions (Fig. 4). This suggested limited accessibility of calmodulin to its binding site in the α subunit. It seemed that CaM-kinase II existed in different conformations in the soluble and membrane-bound form, and only a fraction of the soluble kinase II could bind to ^{125}I-calmodulin.

Autophosphorylation of CaM kinase II in the presence of calcium and calmodulin increased [39, 40] its binding to calmodulin moderately, and further phosphorylation by adding EGTA results in the loss of ^{125}I-calmodulin binding [14]. It is possible that a greater portion of hen brain soluble CaM-kinase II was present in the phosphorylation form that was unable to bind calmodulin. It is interesting to point out that there was 20–25% Ca^{2+}/CaM-independent protein kinase activity in the brain supernatant.

^{125}I-Calmodulin binding and reaction with anti-CaM-kinase II showed a single band of the β subunit in the brain homogenate, particulate, and supernatant fractions, whereas autophosphorylation and ^{125}I-calmodulin binding always exhibited a doublet β/β′ in the purified enzyme. This suggested that the β′ subunit was a degradation product of the β subunit. This is consistent with the results of McGuinness *et al.* [9], but is in contrast to the report that the β′ subunit is not a degradation product of the β subunit of rat brain CaM-kinase II and is formed by the deletion of amino acid residues 378–392 in the aligned sequence of the β subunit [30]. Further work including molecular cloning and sequencing is required to identify the CaM-kinase II β′ subunit in the hen brain.

Hen brain Ca^{2+}/CaM-dependent protein kinase II showed great phosphorylating activity for MAP-2 and myelin basic protein, which was also observed in rat brain CaM-kinase II purified by Goldenring *et al.* [37]. Hen brain CaM-kinase II phosphorylated histone VIII (arginine-rich) at 58% of the rate for MAP-2, while Goldenring *et al.* [37] found only 6% in their rat brain CaM-kinase II. Tubulin was phosphorylated very poorly by hen brain CaM-kinase II. The measured rate of phosphorylation of tubulin, 21% of MAP-2, was an over-estimation, since the tubulin band overlapped with the autophosphorylated kinase II subunits. In general, tubulin has been found to be a poor substrate for rat brain CaM-kinase II [9, 15, 41], although some exceptions have been noted [37]. Similarly, hen brain CaM-kinase II phosphorylated both myosin light chains and glycogen synthetase at nearly equal rates, whereas rat brain kinase II phosphorylated glycogen synthase at a very low rate compared to myosin light chains [37]. There are also differences in the substrate specificity of rat brain CaM-kinase II purified in different laboratories. At least some

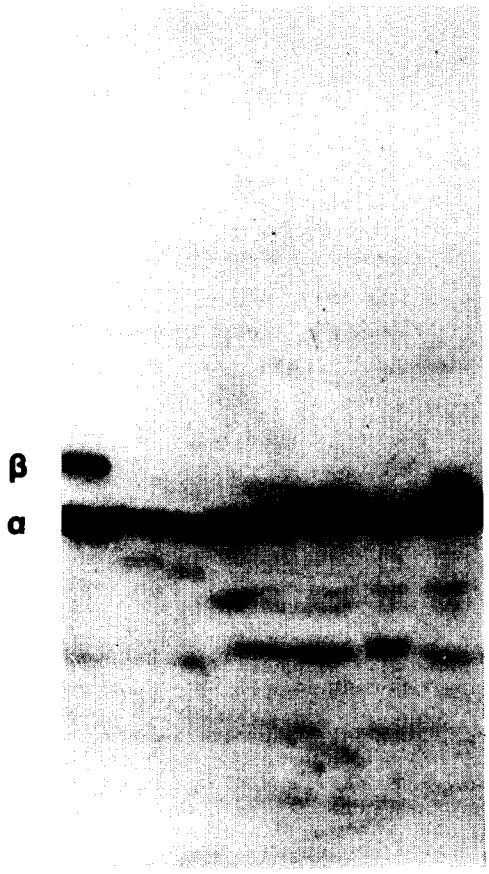

Fig. 10. Cross-reactivity of hen brain CaM-kinase II with other species. The brain supernatants (100 μg protein) of hen (1), rat (2), mouse (3), rabbit (4), cat (5), dog (6), pig (7), and human (8) were subjected to SDS–PAGE and electrophoretically transferred onto a nitrocellulose sheet. The sheet was developed by subsequent treatment with antiserum (1:1000 dilution) against the α subunit of hen brain CaM-kinase II and ^{125}I-protein A as described in Materials and Methods. The immunoblot was exposed to X-ray film in the presence of two intensifying screens.

of these differences might be ascribed to the nature of the substrate. For example, glycogen synthase from different sources showed remarkable differences in its rate of phosphorylation [9]. Similarly, we found a 100% increase in the phosphorylation of myelin basic protein after keeping it at −70° for 5 months. Other assay conditions, e.g. amount of calmodulin, could also lead to differences in the substrate specificity of the same CaM-kinase II in different laboratories. We found that 2 μg calmodulin was required for optimal phosphorylation of MAP-2, whereas 10 μg was required for histone II.

Autophosphorylation of CaM-kinase II in the presence of calcium and calmodulin caused a continuous decline in the total activity of enzyme determined in the presence of calcium and calmodulin. In contrast, the calcium-independent activity increased in the first minute and then declined continuously. The total period of incubation was 10 min. Similar results have been reported in rats [38, 42], except that there was a greater fall in total and calcium-independent activity of hen brain CaM-kinase II. Some decrease observed in the total activity of hen brain CaM-kinase II with incubation time even without autophosphorylation has also been found in rats [38]. Rostas *et al.* [21] also found a decrease in the CaM-kinase II activity in the brains of both 2-day-old chicks and hens after autophosphorylation. Autophosphorylation of CaM-kinase II decreased the mobility of the α subunit (Fig. 3) but, obviously, not of β/β′ subunits as shown by McGuinness *et al.* [9].

Autophosphorylation of hen brain CaM-kinase II resulted in the incorportation of approximately 2 and 8 mol of phosphorus per mol of α and β subunits, respectively. The stoichiometry of ^{32}P incorporation in the α subunit is close to that observed by Hashimoto *et al.* [38] (1.6 mol/mol) and different from that found (0.3 mol/mol) by Yasugawa *et al.* [43] in rat brain CaM-kinase II. The incorporation of 2 mol of ^{32}P in the α subunit seemed to be supported by the results obtained from tryptic peptide mapping of site mutants of α subunits of rat brain CaM-kinase II, where Thr 286 and Ser 279 were found to be the major sites for phosphorylation in decreasing order [44]. As in the case of the α subunit, incorporation of ^{32}P in hen brain CaM-kinase II β subunit was much greater (8 vs 1.8 mol/mol) than that reported by Yasugawa *et al.* [43], but the ratio of ^{32}P incorporation between β and α subunits in the rat (6:1) is closer to that observed in hen brain CaM-kinase II (4:1).

CaM-kinase II from hen brain exhibited K_m values of 19 and 3.0 μM for ATP and calmodulin, respectively. The K_m value for ATP was within the range of 7–109 μM reported for rat brain CaM-kinase II [5, 37, 45, 46]. The hen brain CaM-kinase II $K_{0.5}$ value (3.0 μM) for calmodulin was, however, much higher than that reported for rat CaM-kinase II [15, 37, 46]. Different rat brain CaM-kinase II $K_{0.5}$ values for calmodulin have been reported depending upon the substrate. For example, the $K_{0.5}$ values using casein, phosvitin, and MAP-2 are 620, 29 and 29 nM respectively [45]. The high $K_{0.5}$ value (3.0 μM) was thought to be due to the nature of the substrate (myelin basic protein) used for phosphorylation. Hen brain CaM-kinase II also had such a low K_m value for MAP-2 that the latter was actively phosphorylated without adding any calmodulin to the reaction mixture. In contrast, myelin basic protein and histone II were not phosphorylated in the absence of calmodulin and seem to have very high $K_{0.5}$ values. Hen brain CaM-kinase II also exhibited a high $K_{0.5}$ value (4.4 μM) for calcium using MAP-2 as a substrate. This is much higher than the values reported, 0.7 to 2.3 μM, for the rat brain [15, 45, 46] and spleen [47]. The reason for such a high $K_{0.5}$ value for calcium is not known. It may be related to some change in the conformation of hen brain CaM-kinase II during purification. However, free calcium concentration is reported to be in the range of 1–10 μM in the stimulated state [48].

Partial as well as tryptic peptide maps of autophosphorylated α and β/β' subunits of hen brain CaM-kinase II suggested that they are very similar yet distinct proteins. Similar results have been obtained after tryptic peptide mapping of iodinated rat brain CaM-kinase II [10, 37]. There are two consensus phosphorylation sequences in the α subunit and four in the β/β' subunit of rat brain CaM-kinase II [14]. A similar situation was expected in hen brain kinase II, and the difference in the fingerprints of α and β/β' subunits reflected the difference in the phosphorylation sites of these subunits.

Hen brain CaM kinase II showed immunological cross-reactivity with brain enzyme from rat, mouse, rabbit, cat, dog, pig, and human, suggesting a close relationship in the primary sequence of this enzyme in different species. This is in contrast to the phenobarbital- and β-naphthoflavone-induced cytochrome P450 isozymes in hen, which showed no or only weak cross-reactivity with isozymes in the rat, mouse, and cat [32, 33].

This report describes the purification and characteristics of hen brain Ca^{2+}/CaM-dependent kinase II. Hen brain CaM-kinase II resembled rat brain CaM-kinase II in many properties such as molecular weight of subunits, broad substrate specificity, autophosphorylation of subunits, alteration in enzyme activity on autophosphorylation, decrease in the mobility of subunits on autophosphorylation, and immunocross-reactivity between α subunits. The major differences between these two species were the high $K_{0.5}$ value of calcium for hen brain CaM-kinase II, the absence of immunochemical cross-reactivity between β/β' subunits, and no apparent decrease in the mobility of β/β' subunits on autophosphorylation. The absence of immunocross-reactivity between β/β' subunits of hen and rat brain CaM-kinase II suggest a major difference in their primary structure.

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