Autophosphorylation of the Type II Calmodulin-Dependent Protein Kinase Is Essential for Formation of a Proteolytic Fragment with Catalytic Activity. Implications for Long-Term Synaptic Potentiation†

Ann P. Kwiatkowski‡ and Marita M. King*

Department of Chemistry and The Ohio State Biochemistry Program, The Ohio State University, Columbus, Ohio 43210

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ABSTRACT: Autophosphorylation plays an essential role in proteolytic activation of the type II calmodulin-dependent protein kinase (CaM kinase II). Limited proteolysis of CaM kinase II by trypsin, α-chymotrypsin, and the Ca^{2+}-stimulated neutral protease (calpain) yielded a catalytically active kinase fragment only when the holoenzyme was autophosphorylated prior to proteolysis. Slightly larger, inactive fragments were obtained from nonphosphorylated CaM kinase II, regardless of whether Ca^{2+}/calmodulin or Mg^{2+}/ATP were present or absent. The active fragment exhibited Ca^{2+}/calmodulin-independent kinase activity with kinetic parameters identical with those of the activated holoenzyme. The key autophosphorylation site of CaM kinase II was absent from the active fragment which indicates that proteolysis can effectively uncouple the activation state and Ca^{2+}/calmodulin independence of the kinase from the action of phosphoprotein phosphatases. Because autophosphorylation exerts such a tight control over this irreversible process, proteolytic activation of CaM kinase II by intracellular proteases offers an attractive mechanism for prolonging the effects of Ca^{2+} at the synapse.

The type II calmodulin-dependent protein kinase (CaM kinase II)[1] is a multifunctional protein kinase found most prominently in the brain [reviewed by Nairn et al. (1985), Kennedy et al. (1987), and Schulman and Lou (1989)]. The enzyme purified from rat brain has a native molecular weight of 550000–650000 and is composed of two different subunits, α (M_r 50000) and β/β′ (M_r 60000/58000) (Nairn et al., 1985; Kennedy et al., 1987). Starting from the N-terminus, each of these structurally and functionally related polypeptide chains is composed of a catalytic domain, a regulatory region, and an association domain (Bennett & Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987; Bulleit et al., 1988).

Autophosphorylation of CaM kinase II activates the enzyme by rendering its catalytic site fully accessible to exogenous substrates (Kwiatkowski et al., 1988). Concomitant with activation, autophosphorylation converts CaM kinase II to a Ca^{2+}/calmodulin-independent form (Miller & Kennedy, 1986; Schworer et al., 1986; Lai et al., 1986; Lou et al., 1986). This latter response has been correlated with autophosphorylation of threonine-286 and -287 of the α and β subunits, respectively (Miller et al., 1988; Thiel et al., 1988; Schworer et al., 1988). The physiological role of autophosphorylation is still uncertain, but it was postulated to regulate the intracellular location of CaM kinase II (Bennett et al., 1983; Saitoh & Schwartz, 1985; Willmund et al., 1986), to prolong the effects triggered by the transient Ca^{2+} signal (Miller & Kennedy, 1986; Schworer et al., 1986; Lai et al., 1986; Lou et al., 1986), and to play a role in long-term modulation of synaptic transmissions (LeVine et al., 1985a; Miller & Kennedy, 1986; Lisman & Goldring, 1988).

A regulatory role for autophosphorylation in proteolytic activation of CaM kinase II is suggested by results of the present investigation which show that a catalytically active fragment of the enzyme can be formed by limited proteolysis only after autophosphorylation of the kinase has occurred. CaM kinase II loses all regulatory features in the process and, thus, becomes constitutively active. Because the active fragment is no longer regulated by Ca^{2+}/calmodulin or phosphoprotein phosphatases, this coordination between autophosphorylation and proteolytic activation offers an attractive, tightly controlled mechanism for long-term activation of this enzyme in response to prolonged synaptic stimulation.

Experimental Procedures

Materials. [γ-^{32}P]ATP was purchased from ICN Biomedicals, Inc. The synthetic peptide substrate syntide-2 (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-Lys) (Hashimoto & Soderling, 1987), which is analogous to phosphorylation site 2 of glycogen synthase, was obtained from Biosearch, Inc. Trypsin, α-chymotrypsin, and soybean trypsin inhibitor were purchased from Sigma. Calpain II was a gift from Dr. Ronald Mellgren, Medical College of Ohio.

Protein Purifications. Calmodulin was prepared from frozen bovine brain (Gopalakrishna & Anderson, 1982); fresh bovine brain was used for the purification of synapsin I (Ueda & Greengard, 1977). CaM kinase II was isolated from frozen rat brain as described previously (Kwiatkowski & King, 1987). The enzyme was at least 90% pure and exhibited an apparent V_{max} of 1–4 μmol min^{-1} mg^{-1} with syntide-2.

Standard Kinase Assays. CaM kinase II was assayed for 1 min at 25 °C by measuring the transfer of [^{32}P]P_i from [γ-^{32}P]ATP into syntide-2. The reactions contained the following: kinase (30 nM per average subunit), syntide-2 (20 μM), calmodulin (5 μM), [γ-^{32}P]ATP (100 μM), $MgCl_2$ (10 mM; magnesium acetate replaced $MgCl_2$ for Figure 4), $CaCl_2$ (0.5 mM; calcium acetate replaced $CaCl_2$ for Figure 4), bovine

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*Correspondence should be addressed to this author at the Department of Chemistry, The Ohio State University, 120 West 18th Ave., Columbus, OH 43210.

‡Present address: 723 Light Hall, Vanderbilt University, Nashville, TN 37232-0295.

[1] Abbreviations: CaM kinase II, type II calmodulin-dependent protein kinase; CaM, calmodulin; EGTA, [ethylenebis(oxyethylenenitrilo)]-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

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serum albumin (1 mg/mL), glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). The incorporation of $[^{32}P]P_i$ into syntide-2 was measured by the method of Roskoski (1983). Total kinase activity corresponds to the enzymatic activity of the Ca^{2+}/calmodulin-dependent and/or Ca^{2+}/calmodulin-independent forms of the enzyme measured in the presence of excess Ca^{2+} and calmodulin. When measuring the Ca^{2+}/calmodulin-independent form of the enzyme alone, calmodulin and $CaCl_2$ were replaced by EGTA (0.5 mM). Although syntide-2 was used as the kinase substrate for all of the data shown, similar experiments were carried out with synapsin I (4 μM); this physiologically relevant substrate was assayed as described for syntide-2. For determinations of kinetic parameters, the concentrations of $[^{32}P]$ATP or syntide-2 were varied in the assays from 11.5 to 102 μM and from 2.5 to 25 μM, respectively. In the latter experiments, the data were analyzed by linear regression.

SDS–Polyacrylamide Gel Electrophoresis. For analysis of CaM kinase II and its fragments by SDS–polyacrylamide gel electrophoresis, two parts of a protein sample were mixed with one part of a stop solution containing glycerol (10%), bromophenol blue (0.05%), SDS (0.23%), and Tris (0.23 M, pH 6.8). Phenylmethanesulfonyl fluoride (0.33 mg/mL), trypsin inhibitor (2–20 μg/mL), or EGTA (0.2 M) was included when the stop solution was used to terminate proteolysis by chymotrypsin, trypsin, or calpain, respectively. SDS–polyacrylamide gel electrophoresis (10% acrylamide gels) was carried out according to the method of Laemmli (1970); the protein bands were stained with Coomassie blue.

Analysis of the $[^{32}P]P_i$ Content of the Active Catalytic Fragment. CaM kinase II (0.4 mg/mL) was autophosphorylated for 5 min at 0 °C in the presence of $[^{32}P]$ATP (1.2 mM), $CaCl_2$ (0.5 mM), $MgCl_2$ (10 mM), calmodulin (30 μM), glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). At this point, 0.9 mol of $[^{32}P]P_i$ was incorporated per mole of average subunit. Proteolysis of the autophosphorylated enzyme by trypsin (2 μg/mL) was carried out for 15 min at 25 °C; autophosphorylation was continued concomitant with proteolysis because this enhanced the yield of active fragments. Proteolysis was stopped with the addition of trypsin inhibitor (20 μg/mL). The reaction mixture (0.6-mL total volume) was applied to an Affi-gel blue column (0.25-mL bed volume). The column was washed with buffer (50 mM Hepes, pH 7.5, 0.1 mM EDTA, and 0.3 mM $CaCl_2$), and the active catalytic fragment was eluted with buffer containing 0.5 M NaCl. The fractions were assayed for protein content (Bradford, 1976), protein-bound radioactivity (Corbin & Reimann, 1974), and catalytic activity (Standard Kinase Assays). Phosphoamino acid analyses of the active fragment of CaM kinase II and the autophosphorylated holoenzyme were performed according to Corvera et al. (1988).

Results

Effects of Proteolysis on Subunit Structure and Activity of Native CaM Kinase II. Limited proteolysis of many calmodulin-regulated enzymes has been shown to lead to the generation of calmodulin-independent activities (DePaoli-Roach et al., 1979; Keller et al., 1980; Lin & Cheung, 1980; Niggli et al., 1981; Walsh et al., 1982; Meijer & Guerrier, 1982; Manalan & Klee, 1983). CaM kinase II was added to this list of enzymes when LeVine and Sahyoun (1987) reported that a monomeric kinase domain could be formed by proteolytic cleavage of the enzyme with trypsin and chymotrypsin; the resulting fragment had lost the ability to bind and respond to calmodulin. The results of the present study will document

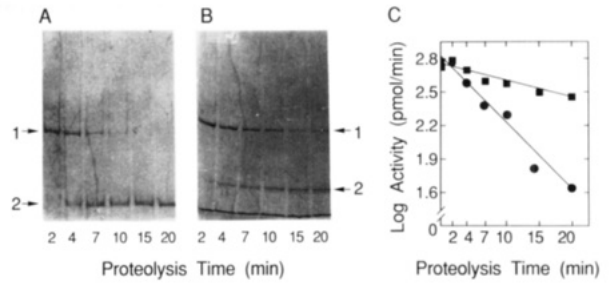

FIGURE 1: Effects of proteolysis on subunit structure and activity of native CaM kinase II. Proteolysis of CaM kinase II (0.4 mg/mL) was carried out at 25 °C in the presence of Hepes buffer (10 mM, pH 7.5) and chymotrypsin (0.5 μg/mL) or trypsin (0.5 μg/mL). At the indicated times, aliquots were removed and analyzed either by SDS–polyacrylamide gel electrophoresis or in standard kinase assays in the presence of Ca^{2+} and calmodulin (see Experimental Procedures). (Panels A and B) Protein staining patterns after incubation with chymotrypsin and trypsin, respectively. The protein bands at arrows 1 and 2 correspond to the α subunit of CaM kinase II and the major proteolytic fragment, respectively. (Panel C) Kinase activity after incubation with chymotrypsin (●) or trypsin (■).

that the process of proteolytic activation is not straightforward where CaM kinase II is concerned; autophosphorylation of the holoenzyme prior to proteolysis is required for expression of enzymatic activity of the proteolytic fragment.

Figure 1 shows the results we obtained for proteolysis of native CaM kinase II (nonphosphorylated holoenzyme) by trypsin and chymotrypsin. The reaction conditions were essentially those described by LeVine and Sahyoun (1987); the enzyme samples were analyzed by SDS–polyacrylamide gel electrophoresis and in standard kinase assays. Cleavage of the kinase by either proteolytic enzyme led to the formation of one major protein fragment of approximately 31 kDa (panels A and B) and a concomitant loss of its Ca^{2+}/calmodulin-stimulated enzymatic activity toward syntide-2 (panel C) or synapsin I (not shown). When assayed in the absence of Ca^{2+} and calmodulin, none of the proteolyzed enzyme samples exhibited kinase activity (not shown).

Inactivation of native CaM kinase II in response to proteolysis shows first-order kinetics (Figure 1C) which suggests that the first cleavage event may be responsible for the loss of enzymatic activity, rather than multiple cleavage steps or a general instability of the proteolytic fragment. Additional support for this hypothesis is provided by the apparent correlation between inactivation of CaM kinase II (Figure 1C) and the loss of the native catalytic subunit (Figure 1A,B).

Proteolytic Activation of Autophosphorylated CaM Kinase II. To test whether ligand-induced conformational changes on CaM kinase II would expose new cleavage sites for the proteases which could yield active, catalytic fragments, proteolysis of native enzyme was carried out in parallel reactions containing Mg^{2+}, Ca^{2+}/calmodulin, and/or Mg^{2+}/ATP; Mg^{2+} and Ca^{2+}/calmodulin are known to change the conformation of CaM kinase II, and Mg^{2+}/ATP can bind to the enzyme even in the absence of Ca^{2+}/calmodulin (King, 1988; King et al., 1988). Added separately, these ligands had no effect on the outcome of the proteolysis reactions; only inactive fragments were formed (not shown). However, when all of the ligands were added simultaneously, and autophosphorylation of CaM kinase II was permitted to occur prior to addition of trypsin or chymotrypsin, a Ca^{2+}/calmodulin-independent kinase activity was formed.

A time course of activation of autophosphorylated CaM kinase II by trypsin is shown in Figure 2. The time-dependent increase in kinase activity upon proteolysis suggests that cleavage of the holoenzyme releases an inhibitory constraint.

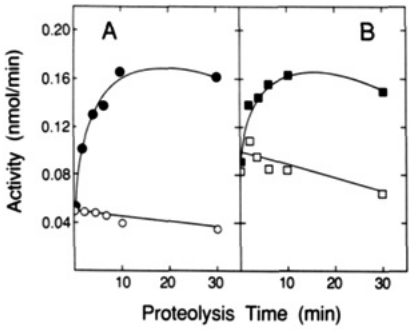

FIGURE 2: Proteolytic activation of autophosphorylated kinase by trypsin. CaM kinase II (0.4 mg/mL) was autophosphorylated at 0 °C in the presence of calmodulin (30 μM), $CaCl_2$ (0.5 mM), ATP (1 mM), $MgCl_2$ (10 mM), glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). After 15 min, small aliquots of trypsin (2 μg/mL) or Hepes buffer (50 mM, pH 7.5) were added to parallel reactions, and the incubation temperature was raised to 25 °C. At the indicated time intervals (time after addition of trypsin or buffer), aliquots were diluted into Hepes buffer (50 mM, pH 7.5) containing trypsin inhibitor (20 μg/mL) and EGTA (0.1 mM). The diluted kinase samples were assayed under standard conditions (see Experimental Procedures). (Panel A) Ca^{2+}/calmodulin-independent activity following incubation in the absence (○) and presence (●) of trypsin. (Panel B) Total kinase activity (Ca^{2+}/calmodulin-dependent and/or -independent activity) following incubation in the absence (□) and presence (■) of trypsin.

The kinase activity plateaued at the same maximum activity regardless of whether Ca^{2+} was present (Figure 2B) in the assays or not (Figure 2A), which indicates that proteolysis of the autophosphorylated enzyme yields a completely Ca^{2+}/calmodulin-independent form. Chelation of Ca^{2+} prior to addition of trypsin did not prevent formation of the active fragment (not shown), which supports the hypothesis that autophosphorylation is the key requirement, not the simultaneous binding of these ligands to CaM kinase II. Although proteolytic activation of autophosphorylated CaM kinase II under similar conditions was recently reported by Colbran et al. (1988), the experiments were carried out exclusively with the autophosphorylated form of the enzyme and no comment was made concerning a possible role of autophosphorylation in this activation process.

Characterization of the Catalytically Active Fragment. The kinetic parameters for the Ca^{2+}/calmodulin-stimulated holoenzyme and the Ca^{2+}/calmodulin-independent proteolytic fragment were determined with syntide-2 as the peptide substrate. Although synapsin I was readily phosphorylated by both forms of CaM kinase II (not shown), the protein substrate was not suitable for the kinetic analysis because it exhibited substrate inhibition. A comparison of the results with syntide-2 (Table I) shows 2-fold changes in the $K_{m,app}$ for ATP and the $V_{max,app}$ which account for the time-dependent increase in kinase activity of Figure 2. The apparent Michaelis–Menten constants for syntide-2 were similar for these two structurally distinct forms of CaM kinase II.

The active and inactive tryptic fragments of CaM kinase II were compared by SDS–polyacrylamide gel electrophoresis (Figure 3). The inactive forms were prepared in the absence and presence of Mg^{2+}, ATP, and Ca^{2+}/calmodulin; autophosphorylated kinase was used in a parallel reaction to yield the active form. Although similar fragment sizes were obtained under all conditions, the active protein, derived from autophosphorylated enzyme, was consistently smaller than all others. The same results were obtained with chymotrypsin (not shown). At present, we cannot explain the discrepancy between our results and those of LeVine and Sahyoun (1987), who obtained an active kinase domain by limited proteolysis of apparently native enzyme and observed that autophosphorylation had no effect on the size of the protein fragment.

Table I: Comparison of Kinetic Parameters of the Holoenzyme and the Active Catalytic Fragment[a]

parameter	holoenzyme	catalytic fragment
$K_{m,app}$ for ATP (μM)	19	36
$K_{m,app}$ for syntide-2 (μM)	2.6	2.9
$V_{max,app}$ (μmol min^{-1} mg^{-1})	1.2	2.1

[a] To prepare the active, catalytic fragment, CaM kinase II (2 μM per average subunit) was autophosphorylated for 15 min at 0 °C in the presence of calmodulin (10 μM), $CaCl_2$ (0.5 mM), [^{32}P]ATP (0.1 mM), $MgCl_2$ (10 mM), glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). Proteolysis was started by the addition of trypsin (0.5 μg/mL). The reaction was carried out for 15 min at 25 °C and terminated by dilution of the kinase into Hepes buffer (50 mM, pH 7.5) containing trypsin inhibitor (5 μg/mL) and EGTA (0.1 mM). The catalytic fragment (30 nM based on the concentration of the average subunit before proteolysis) was assayed under standard assay conditions in the absence of added $CaCl_2$ and calmodulin; nonphosphorylated holoenzyme (30 nM per average subunit) was assayed in the presence of the two effectors (see Experimental Procedures).

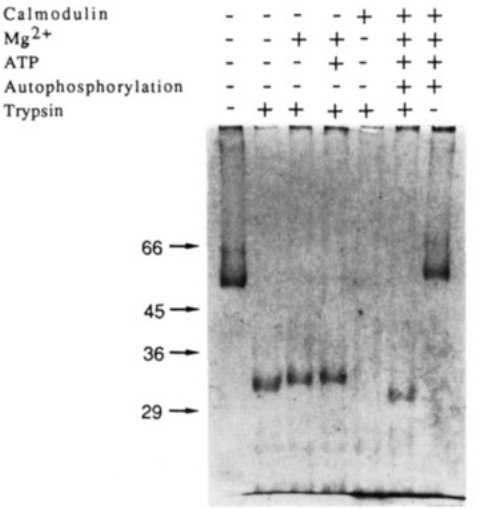

FIGURE 3: Comparison of trypsin-generated proteolytic fragments of CaM kinase II. CaM kinase II (0.4 mg/mL) was incubated for 15 min at 0 °C in the absence or presence of the indicated effectors: $MgCl_2$ (10 mM), $MgCl_2$/ATP (10 mM/2 mM), and/or $CaCl_2$/calmodulin (0.3 mM/60 μM); in the presence of all effectors, CaM kinase II was autophosphorylated. The incubation mixtures also contained glycerol (10%), EDTA (0.1 mM), dithiothreitol (0.1 mM), and Hepes buffer (50 mM, pH 7.5). Where indicated, proteolysis was started with the addition of trypsin (2 μg/mL); the reactions were terminated after 15 min at 25 °C with the addition of a stop mixture, containing trypsin inhibitor (20 μg/mL), and analyzed by SDS–polyacrylamide gel electrophoresis (see Experimental Procedures). Because proteolysis of the nonphosphorylated enzyme in the presence of Ca^{2+}/calmodulin produced rather faint protein bands which are barely visible in this figure, Figure 5 should be consulted for a comparison with the active fragment. The positions of standard mass markers are at the left in kilodaltons: bovine serum albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase (top to bottom).

Proteolysis of autophosphorylated CaM kinase II followed by SDS–polyacrylamide gel electrophoresis and autoradiography of the ^{32}P-labeled fragments indicated that almost all of the radioactive label was associated with six protein bands of less than 29 kDa (not shown). To better quantify the [^{32}P]phosphate content of the catalytically active fragment, the enzyme was autophosphorylated and proteolyzed as described under Experimental Procedures. The active kinase fragment was isolated from the reaction mixture by chromatography over Affi-gel blue and found to contain approximately

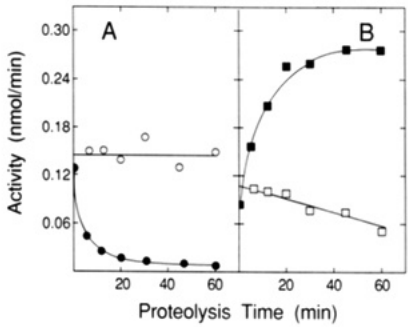

FIGURE 4: Effect of limited proteolysis by calpain on the activity of CaM kinase II. (Panel A) Total kinase activity following incubation of nonphosphorylated enzyme in the absence (○) or presence (●) of calpain. CaM kinase II (0.5 mg/mL) was preincubated for 5 min at 0 °C in the presence of Hepes buffer (10 mM, pH 7.5), glycerol (10%), EDTA (0.1 mM), and dithiothreitol (1 mM). Proteolysis of the kinase (0.4 mg/mL) was initiated by the addition of calpain (0.1 mg/mL) and calcium acetate (5 mM) and carried out at 25 °C; water was added to a parallel control sample. At the indicated times, aliquots of the reactions were diluted into Hepes buffer (50 mM, pH 7.5) containing EGTA (0.2 M) and assayed in standard kinase reactions containing Ca^{2+}/calmodulin (see Experimental Procedures). (Panel B) Ca^{2+}/calmodulin-independent kinase activity following incubation of autophosphorylated enzyme in the absence (□) or presence (■) of calpain. CaM kinase II (0.54 mg/mL) was autophosphorylated for 5 min at 0 °C in the presence of calmodulin (15 μM), calcium acetate (0.5 mM), ATP (1.2 mM), magnesium acetate (10 mM), Hepes buffer (50 mM, pH 7.5), glycerol (10%), EDTA (0.1 mM), and dithiothreitol (1 mM). Proteolysis of the kinase (0.4 mg/mL) was initiated by the addition of calpain (0.2 mg/mL) and calcium acetate (5 mM) and carried out at 25 °C; water was added to a parallel control sample. Aliquots of the reactions were diluted and analyzed as described under panel A except that the kinase assays were carried out in the presence of EGTA.

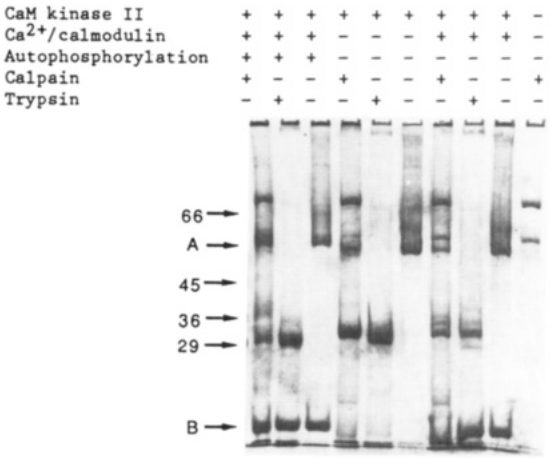

FIGURE 5: Comparison of trypsin- and calpain-generated proteolytic fragments of CaM kinase II. Proteolysis of CaM kinase II was carried out with three different enzyme samples: autophosphorylated kinase in the presence of Ca^{2+}/calmodulin, nonphosphorylated enzyme in the absence of effectors, and nonphosphorylated kinase in the presence of Ca^{2+}/calmodulin. The preincubation conditions were as described for Figure 4, except that the concentrations of kinase and calmodulin were doubled. Where indicated, proteolysis was started with the addition of calpain/calcium acetate (0.2 mg/mL and 5 mM) or trypsin (2 μg/mL). After 30 min (60 min for reactions containing calpain) at 25 °C, the reactions were terminated by addition of stop solution and analyzed by SDS–polyacrylamide gel electrophoresis as described under Experimental Procedures. The positions of standard mass markers are at the left in kilodaltons: bovine serum albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase (top to bottom). Arrows A and B refer to protein bands of unproteolyzed CaM kinase II and calmodulin, respectively.

0.2 equiv of [^{32}P]phosphate distributed between phosphoserine and phosphothreonine. Since active, monomeric fragments are obtained only from autophosphorylated enzyme, this residual amount of [^{32}P]phosphate of the active kinase domain suggests that proteolytic cleavage occurred N-terminal to threonine-286 (α subunit) and threonine-287 (β subunit), the major autophosphorylation sites of CaM kinase II (Miller et al., 1988; Thiel et al., 1988; Schworer et al., 1988).

Proteolytic Activation of CaM Kinase II by Calpain. It has been proposed that the Ca^{2+}-dependent neutral protease, calpain, plays a significant role in producing chemical and structural changes at brain synapses in response to prolonged stimulation (Lynch & Baudry, 1984; Mellgren, 1987). Possible enzyme targets of calpain's action include Ca^{2+}/phospholipid-dependent protein kinase C and Ca^{2+}/calmodulin-regulated calcineurin; both are activated by calpain in vitro and rendered independent of Ca^{2+}/phospholipids and calmodulin, respectively (Inoue et al., 1977; Kishimoto et al., 1983; Tallant et al., 1988). The results of Figure 4B, which show proteolytic activation of autophosphorylated CaM kinase II by calpain, suggest a new target for this physiologically relevant protease. As previously established with trypsin (Figure 2) and chymotrypsin (not shown), proteolytic activation of CaM kinase II was dependent on prior autophosphorylation of the kinase; the native holoenzyme yielded an inactive proteolytic product (Figure 4A). When limited proteolysis of autophosphorylated CaM kinase II by trypsin and calpain was carried out in parallel experiments, the specific activities of the corresponding active fragments were indistinguishable (not shown).

A comparison of the active and inactive proteolytic preparations of CaM kinase II by SDS–polyacrylamide gel electrophoresis showed that the active forms were of smaller size (Figure 5). The small differences in the fragment sizes suggest that the presence of only a few additional amino acid residues may be responsible for the lack of kinase activity of the inactive fragment; in the nonphosphorylated holoenzyme and in the inactive fragment, these additional residues must be tightly associated with the kinase domain as they are totally protected from proteolytic enzymes. Alternatively, the inactive fragment may be nonfunctional because its kinase domain lacks an essential N-terminal region. Compared to trypsin-generated fragments (29.6 and 31.0 kDa), the fragments produced by calpain (30.3 and 31.7 kDa) were only slightly larger (Figure 5), which suggests that the cleavage sites for these proteases are almost adjacent. Peptide mapping and sequencing of all of these proteolytic fragments are required to firmly establish their relationships.

Discussion

Autoinhibitory mechanisms have emerged as a key feature in the regulation of protein kinases; several cases have now been reported in which "internal substrates" or "pseudosubstrate" regions block kinase activity (Flockhart et al., 1980; Kemp et al., 1987; Kennelly et al., 1987; House & Kemp, 1987; Tornqvist & Avruch, 1988; Pearson et al., 1988). We and others have proposed that such autoinhibition is involved in the regulation of CaM kinase II (Hanley et al., 1987; Lai et al., 1987; Kelly et al., 1988; Kwiatkowski et al., 1988; Thiel et al., 1988). To fully relieve the inhibition of the native enzyme, binding of Ca^{2+}/calmodulin and subsequent autophosphorylation of an "internal substrate" appear to be required. The results of the present study show that Ca^{2+}/calmodulin-stimulated autophosphorylation also is a prerequisite for proteolytic activation of CaM kinase II; thus, autoinhibition may be responsible for blocking kinase activity when proteolytic fragments are formed from nonphosphorylated enzyme. To summarize our understanding of the regulation of CaM kinase II activity by Ca^{2+}/calmodulin,

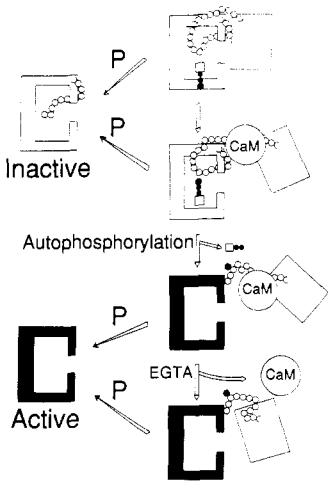

FIGURE 6: Model for the roles of Ca^{2+}/calmodulin, autophosphorylation, and limited proteolysis in regulation of enzymatic activity of CaM kinase II. One subunit of CaM kinase II is shown as a large "C" (catalytic domain) connected via a chain (regulatory region) to a large rectangle (association domain). Darkened catalytic domains indicate the forms of the kinase that are active toward exogenous substrates. CaM and P stand for calmodulin and protease, respectively; the small squares with the attached hexagons represent ATP and ADP. Details of the model are discussed in the text.

autophosphorylation, and proteolysis, a simplified model is depicted in Figure 6 and will be discussed in the following paragraphs.

Activation of nonphosphorylated CaM kinase II by Ca^{2+}/calmodulin promotes nucleotide binding (King, 1988; King et al., 1988) and may be required to properly align ATP and the "internal substrate" for autophosphorylation. Ca^{2+}/calmodulin-induced conformational changes also appear to relieve an allosteric or steric inhibition by the autoinhibitory region immediately C-terminal to the "internal substrate" site (Payne et al., 1988; Colbran et al., 1988). Ca^{2+}/calmodulin-stimulated autophosphorylation and subsequent dissociation of the phosphorylated "internal substrate" then render the catalytic site fully accessible to exogenous substrates (Kwiatkowski et al., 1988).

Prior to autophosphorylation, proteolytic cleavage of CaM kinase II yields inactive fragments regardless of whether Ca^{2+}/calmodulin or Mg^{2+}/ATP are present or not. Concomitant with autophosphorylation of the "internal substrate" and full activation of the protein kinase, a new recognition site for a given protease appears to be exposed because subsequent cleavage leads to the formation of a smaller, active fragment. The autophosphorylated holoenzyme and its active proteolytic fragment both possess Ca^{2+}/calmodulin-independent kinase activity. Phosphorylation of threonine-286 and -287 of the α and β subunits, respectively, is known to correlate with the appearance of the Ca^{2+}/calmodulin independence of the holoenzyme (Miller et al., 1988; Thiel et al., 1988; Schworer et al., 1988); phosphorylation of the same amino acid residues probably couples autophosphorylation to proteolytic activation.

Analysis of the active kinase domain suggested that proteolytic cleavage of the autophosphorylated holoenzyme occurred N-terminal to threonine-286/287. Because cleavage of nonphosphorylated enzymes yielded slightly larger, inactive fragments, it is attractive to speculate that the inactive fragment retained the autoinhibitory region in addition to the kinase domain; the intramolecular arrangement would promote tight binding of such an autoinhibitory tail. The inability of this proteolytic fragment to carry out autophosphorylation further emphasizes the importance of Ca^{2+}/calmodulin in the mechanism of activation of CaM kinase II.

The biochemistry of memory has become an area of intensive research. The most attractive of the proposed mechanisms of information storage is long-term synaptic potentiation (Brown et al., 1988). It appears that a voltage-dependent, transient Ca^{2+} influx through *N*-methyl-D-aspartate receptors is involved in the induction of long-term potentiation, but the molecular mechanisms by which this Ca^{2+} signal is translated into long-term chemical or structural changes at synapses are unknown (Smith, 1987; Kennedy, 1988). Because of high concentrations of CaM kinase II in brain (Bennett et al., 1983) and the ability of this enzyme to become independent of Ca^{2+} following autophosphorylation (Miller & Kennedy, 1986; Schworer et al., 1986; Lai et al., 1986; Lou et al., 1986), several models have been proposed for the involvement of this protein kinase in long-term potentiation and memory storage (Miller & Kennedy, 1986; Willmund et al., 1986; Lisman & Goldring, 1988). However, since the extent of autophosphorylation and Ca^{2+} independence of CaM kinase II can be altered by phosphoprotein phosphatases (LeVine et al., 1985b; Miller & Kennedy, 1986; Lai et al., 1986; Lou et al., 1986; Saitoh et al., 1987), the role of CaM kinase II in signal transduction may be short term unless its Ca^{2+}-independent state can be uncoupled from the action of the phosphatases. One possible mechanism to achieve this uncoupling may be provided by proteolytic cleavage of autophosphorylated CaM kinase II by the Ca^{2+}-activated calpain. A particularly appealing feature of this speculative mechanism is the requirement of prior autophosphorylation of CaM kinase II which can place the irreversible activation of this enzyme under unusually tight physiological control.

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Registry No. ATP, 56-65-5; Ca, 7440-70-2; CaM kinase, 95567-89-8; syntide-2, 108334-68-5.

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