Active Catalytic Fragment of Ca\textsuperscript{2+}/Calmodulin-dependent Protein Kinase II

PURIFICATION, CHARACTERIZATION, AND STRUCTURAL ANALYSIS*

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We report the purification and characterization of an active catalytic fragment of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II, derived from autophosphorylation and subsequent limited chymotryptic digestion of the purified rat forebrain soluble kinase. The purified fragment was completely Ca\textsuperscript{2+}/calmodulin-independent, existed as a monomer, and phosphorylated synapsin I at the same sites as does the native form of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II. Recent studies with the purified fragment revealed a more than 10-fold increase in \( V_{max} \) and a 50% decrease in \( K_m \) for synthetic peptide substrates, compared with native Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II. No \(^{32}\)P-labeled autophosphorylated residues were detected in the purified active fragment, indicating that the autophosphorylation sites were not contained within this fragment. Comparative studies of this active fragment (30 kDa) and its inactive counterpart (32-kDa fragment) revealed certain structural details of both fragments. Calmodulin-overlay study, immuno blot analysis, and direct amino acid sequencing suggested that both fragments contain the entire NH\textsubscript{2}-terminal catalytic domain and were generated by distinct cleavage within the regulatory domain. The putative cleavage sites for both fragments are discussed.

Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is a multifunctional protein kinase that is one of the important mediators of Ca\textsuperscript{2+}-dependent signal transduction (reviewed in Refs. 1-3). Recent investigations have indicated an essential role for CaM kinase II in the regulation of neurotransmitter release (4-6) and in the induction of long term potentiation (7, 8). CaM kinase II has been identified in various tissues, and it exists most abundantly in brain (9). The soluble rat brain holoenzyme is a large multimeric structure (molecular mass of 550-650 kDa), composed of 10-12 \( \alpha \) (50 kDa) and \( \beta/\beta' \) (60/58 kDa) subunits, whose ratio varies with the brain region examined (10-13). The primary structures of four rat brain CaM kinase II subunits have been deduced from cDNA clones (14-19). All subunits have a catalytic domain in their NH\textsubscript{2}-terminal half, followed by a regulatory domain that includes autoinhibitory and calmodulin-binding regions and an association domain toward the COOH-terminal end. All three domains, in particular the catalytic and regulatory domains, are highly homologous among the subunits (19). The major differences are several amino acid insertions located between the regulatory domain and the association domain. The activity of native CaM kinase II is Ca\textsuperscript{2+}/calmodulin-dependent, but the enzyme becomes partially Ca\textsuperscript{2+}/calmodulin-independent after autophosphorylation at Thr-286 (\( \alpha \))/Thr-287 (\( \beta \)) (20-22). This feature has been confirmed by site-directed mutagenesis using the brain \( \alpha \) subunit cDNA clone (23-25). The autophosphorylation at this threonine residue may be a mechanism to prolong the effect of a transient intracellular Ca\textsuperscript{2+} increase in response to stimulation in vivo (26-29).

Recent studies have shown that a catalytically active, Ca\textsuperscript{2+}/calmodulin-independent fragment of CaM kinase II can be produced by limited proteolysis after autophosphorylation (30, 31) and it has been used in some in vitro studies (30-34), but the fragment has not been purified to homogeneity. Limited proteolysis of CaM kinase II without prior autophosphorylation has been shown to generate a slightly larger, inactive fragment (31). The structural relationships between this inactive fragment, the active fragment, and the native form of CaM kinase II have not been determined. Site-directed mutagenesis of the brain \( \alpha \) subunit cDNA clone has produced forms of the kinase only partially independent of Ca\textsuperscript{2+}/calmodulin (23, 35).

We now report a procedure for the generation and purification of an active fragment of rat brain soluble CaM kinase II. Comparative studies of this fragment and the inactive counterpart provided structural details of both fragments, which should help in understanding the autoregulatory mechanism of CaM kinase II in vivo.

EXPERIMENTAL PROCEDURES

Materials—CaM kinase II was purified from rat forebrain essentially as described (12), except that hydroxylapatite chromatography was added to the purification procedure. Synapsin I was prepared from bovine brain as described (36). Catalytic subunit of cyclic AMP-dependent protein kinase and calmodulin were gifts from A. Horiuchi and A. C. Nairn (The Rockefeller University). [\( \gamma \textsuperscript{32}\)P]ATP and \( \textsuperscript{32}\)P-labeled calmodulin were purchased from Du Pont-New England Nuclear. TLCK-treated \( \alpha \)-chymotrypsin was purchased from Worthington. Aprotinin was purchased from Miles (West Haven, CT). A synthetic peptide (CaMK-(281-291)) (corresponding to residues 281-
291$^2$ of the subunit (20), a CaM kinase II inhibitory peptide (CaMKIP) (a nonphosphorylatable analog corresponding to residues 281-302 of the subunit in which Thr-286 is substituted by alanine (5)), and a bovine synapsin I phosphorylation site 3 peptide (Syn I-site 3) (corresponding to residues 587-609 of bovine synapsin I with a tyrosine residue added to the NH$_2$ terminus (37, 38)) were compared by the Protein Sequencing Facility of the Rockefeller University and the Protein and Nucleic Acid Chemistry Facility of Yale University. The concentrations of peptides were determined by amino acid analysis.

**Protein Kinase Assays**—Kinase activity was measured as described (12), except that the peptides CaMK-(281-291) or Syn I-site 3 were used as substrate and the reactions were carried out for 1 min. Ca$^{2+}$/calmodulin-independent activity was measured in the presence of 1 mM EGTA without Ca$^{2+}$/calmodulin. For kinetic studies, 200 mM [gamma-$^32$P]ATP was used, and the reactions were carried out for 15 s. Kinetic parameters were derived from double-reciprocal plots.

**One-dimensional Phosphopeptide Analysis of Synapsin I**—Analysis was performed as described (39).

**Generation of the Active and the Inactive Fragments of CaM Kinase II**—Generation of the active fragment of CaM kinase II was performed by a modification of a procedure described previously (30). CaM kinase II was first autophosphorylated for 10 min at 0°C in a reaction mixture containing 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mM EGTA, 1.5 mM CaCl$_2$, 25 μg/ml calmodulin, and 500 μM ATP. After 10 min, TLCK-treated a-chymotrypsin was added to the incubation mixture without terminating the autophosphorylation reaction. Continued autophosphorylation during proteolysis resulted in an increased production of the active fragment, compared with the condition in which autophosphorylation was terminated before proteolysis. The incubation with a-chymotrypsin was continued for 60 min at 0°C. The reaction was terminated by addition of an excess amount of aprotinin (10-fold excess by weight), EGTA, and EDTA (final concentration, 20 mM). A protease (a-chymotrypsin) substrate (CaM kinase II) ratio of 1:2 was employed in order to provide for complete digestion of the kinase, generation of maximal Ca$^{2+}$/calmodulin-independent activity, and total elimination of Ca$^{2+}$/calmodulin-dependent activity. Generation of the inactive fragment was performed by proteolysis without prior autophosphorylation. CaM kinase II was incubated with a-chymotrypsin for 60 min at 0°C in 50 mM HEPES, pH 7.5, containing 1 mM EGTA, and the reaction was terminated by addition of an excess amount of aprotinin (10-fold excess by weight). The same protease/substrate ratio was used as that for the generation of the active fragment. In some experiments, inactive fragment was generated under the same incubation conditions, as generation of the active fragment, except that CaCl$_2$ was excluded from the reaction mixture.

**2H-Labeled Calmodulin Overlay**—These studies were performed as described (40).

**Antibody Production and Immunoblot Analysis**—Polyclonal antibodies (G-301) were raised against a synthetic peptide corresponding to amino acid residues 281-302 of the subunit of rat brain CaM kinase II after conjugation to bovine thyroglobulin. Antibodies were affinity-purified with the aid of a peptide-CH-Sepharose 4B column. Immunoblot analysis was performed as described (41) using alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution) as a secondary antibody (Promega Biotec, Madison, WI). Protein concentration was measured by Peterson's modification (46) of the method of Lowry et al. (47) with bovine serum albumin as standard.

**RESULTS**

**Generation and Purification of the Active 30-kDa Fragment**—The active fragment of CaM kinase II was generated as described under "Experimental Procedures." The complete reaction sequence was performed at 0°C rather than at 30°C for the following reasons. At 0°C, Ca$^{2+}$/calmodulin-independent kinase activity rose dramatically for 30 min and remained constant for 150 min after the addition of a-chymotrypsin. In contrast, when the reactions were performed at 30°C, the Ca$^{2+}$/calmodulin-independent kinase activity reached a maximum value at 15 min, which was only half of that observed at 0°C, and then declined gradually (data not shown). As shown in Fig. 1A, native CaM kinase II was completely digested upon termination of the reaction after 60 min at 0°C. A prominent 30-kDa protein band was visible, whereas no α or β subunit bands were detected (Fig. 1A, lanes 1 and 2). The reaction mixture was applied to a fast protein liquid chromatography Mono Q HR 5/5 anion-exchange column and eluted with a linear gradient of NaCl (0-1.0 M). The Ca$^{2+}$/calmodulin-independent kinase activity eluted as a single peak (Fig. 1B). The peak fractions contained only the 30-kDa protein band, completely separated from a-chymotrypsin, aprotinin, calmodulin, and any other digested fragments (Fig. 1A, lanes 3-7). It was concluded that this 30-kDa fragment possessed Ca$^{2+}$/calmodulin-independent kinase activity. From 0.6 mg of native CaM kinase II, 0.12 mg of the purified active fragment was obtained. The recovery of the activity in the purified fraction was about 70%, relative to the activity in the reaction mixture applied to the column. When dialyzed immediately and stored in glycerol-containing buffer (25 mM Blue R-250, Protein concentration was measured by Peterson's modification (46) of the method of Lowry et al. (47) with bovine serum albumin as standard.

**FIG. 1. Purification of the active 30-kDa fragment of CaM kinase II.** CaM kinase II (0.6 mg) was first autophosphorylated as described under "Experimental Procedures" and subsequently digested by TLCK-treated a-chymotrypsin (0.3 mg) for 60 min at 0°C. The reaction was terminated by the addition of an excess amount of aprotinin, EGTA, and EDTA. The reaction mixture was applied to a fast protein liquid chromatography Mono Q HR 5/5 anion-exchange column equilibrated with 20 mM Tris/HCl, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol. After a 5-column volume wash with the same buffer, proteins were eluted with a linear gradient of NaCl (0-1.0 M) and fractions of 0.5 ml were collected. A, a 20-μl aliquot of the reaction mixture prior to proteolysis (lane 1) and after proteolysis (lane 2) and of column fractions 49-53 shown in B (lanes 7-9, respectively) was mixed with 5 μl of 5× SDS sample buffer and subjected to SDS-PAGE using 12% acrylamide gels. Protein bands on gels were visualized by using Coomassie Brilliant Blue R-250. a, β subunit of CaM kinase II; b, α subunit of CaM kinase II; c, the active 30-kDa fragment; d, calmodulin; e, a-chymotrypsin; f, aprotinin. B, elution profile from the Mono Q HR 5/5 column. Each fraction was assayed for kinase activity in the absence of Ca$^{2+}$/calmodulin using 11 μM CaM kinase-(281-291) as substrate (filled circles). The solid line shows absorbance at 280 nm, and the broken line shows NaCl concentration. The large absorbance peak observed in fractions 46-49 is due to ATP and/or ADP.

$^2$ The numbering of amino acid residues in this communication is based on the sequence of the α subunit of rat brain CaM kinase II (14) unless otherwise noted.
Tris/HCl, pH 7.5, 0.5 mM EGTA, 0.5 mM EDTA, 50% glycerol) at -20 °C, the purified fragment retained 60-80% of the initial activity after 1 week.

**Characterization of the Purified Active Fragment**—The purified active fragment migrated with a molecular mass of 30 kDa on SDS-PAGE under reducing conditions (Fig. 1A). The fragment eluted at a position corresponding to a molecular mass of a 24-kDa protein on a Superose 12 HR 10/30 gel filtration column (data not shown), clearly indicating the monomeric structure of the fragment. It phosphorylated synapsin I, with a stoichiometry close to 2.0, like native CaM kinase II (data not shown). Fig. 2 shows phosphorylation of synapsin I (Fig. 2A) and a one-dimensional phosphopeptide map of phosphorylated synapsin I (Fig. 2B). The active fragment and native CaM kinase II produced a 35-kDa phosphopeptide (Fig. 2B, lanes 1 and 2), which contains phosphorylation sites 2 and 3 of synapsin I (37, 39, 48). A 10-kDa phosphopeptide was produced by cyclic AMP-dependent protein kinase (Fig. 2B, lane 3), which contains phosphorylation site 1 (37, 39, 48). Phosphorylation of synapsin I by the active fragment was inhibited by CaMKIP (data not shown). These results indicate that the substrate specificity of native CaM kinase II is conserved in the active fragment.

Kinetic parameters of the purified active fragment were compared with those of native CaM kinase II (Table I), using the synthetic peptide substrates CaMK-(281-291) and Syn I-site 3. The reactions were performed under conditions in which maximal enzyme activity was observed, i.e. in the presence of Ca"/calmodulin for native CaM kinase II and in the absence of Ca"/calmodulin for the active fragment. The active fragment exhibited a 40-50% decrease in \( K_m \) and a 10-13-fold increase in \( V_{max} \), compared with the native enzyme. The overall catalytic efficiency showed 19- and 12-fold increases for CaMK-(281-291) and Syn I-site 3, respectively, compared with those values of native CaM kinase II. An increase in \( V_{max} \) for synthetic peptide substrate was in agreement with previous observations using unpurified reaction mixtures containing the active fragment (30, 31). In contrast, when synapsin I was used as substrate, both \( K_m \) and \( V_{max} \) increased, and the catalytic efficiency was virtually unchanged (data not shown).

**Comparative Studies of the Active 30-kDa Fragment and the Inactive 32-kDa Fragment**—As previously observed (31), proteolysis of CaM kinase II by \( \alpha \)-chymotrypsin without prior autophosphorylation produced a slightly larger fragment that had no kinase activity. The inactive fragment migrated with a molecular mass of 32 kDa on SDS-PAGE under reducing conditions (Fig. 3A, lane 4). It was completely devoid of catalytic activity even in the presence of Ca"/calmodulin (Fig. 3B, lane 4). In contrast, the total Ca"/calmodulin-independent kinase activity of the active fragment was increased more than 10-fold after proteolysis of the autophosphorylated kinase and had no residual Ca"/calmodulin-dependent kinase activity (Fig. 3B, lanes 1 and 2). In fact, the active fragment showed 38% less kinase activity in the presence than in the absence of Ca"/calmodulin. The presence of calmodulin alone did not affect the activity, but Ca" alone reduced the activity to the same extent as Ca"/calmodulin. This apparent “suppression” of the kinase activity by Ca" was also observed in the purified preparations of the active fragment (data not shown).

Since the major autophosphorylation site, Thr-286, is located within the regulatory domain, the presence or absence of autophosphorylated residues in the active fragment provides important structural information. The absence of the major autophosphorylation site in the active fragment has been suggested (31). We tested for the presence of this autophosphorylation site, using \([\gamma^{32P}]ATP\) in the autophosphorylation reaction (Fig. 4). After proteolysis, several \([\gamma^{32P}]\)-labeled bands smaller than 30 kDa were detected (Fig. 4A, lane 2), but no radioactivity was detected in the purified 30-kDa preparations, and even after a long exposure of the autoradiogram (Fig. 4, A, lanes 3-6, and B). This demonstrates that the specific threonyl residue that had been autophosphorylated in native CaM kinase II (Thr-286) was not contained within the active fragment.

Neither the active 30-kDa fragment nor the inactive 32-kDa fragment bound \([\gamma^{32P}]\)-labeled calmodulin in the presence or absence of Ca" in gel overlay studies (Fig. 5). The absence of \([\gamma^{32P}]\)-calmodulin binding to the active fragment was in agreement with previous observations (30). A faintly reactive 24-kDa protein band was detected in the active 30-kDa fragment reaction mixture after a long exposure of the autoradiogram (Fig. 5, lane 2), but was not detected in a purified fraction of the active 30-kDa fragment (data not shown). These results indicate that the critical residues required for high affinity binding to calmodulin (residues 305-309) (49) are absent in both the active 30-kDa and the inactive 32-kDa fragments.

In order to further define the COOH-terminal region of the active 30-kDa and the inactive 32-kDa fragments, immunoblot analysis was performed using affinity-purified anti-CaM kinase II peptide antibodies (G-301), which recognize an epitope corresponding to amino acid residues 281-302 of the \( \alpha \) subunit. For this experiment, the purified forms of 30-kDa and 32-kDa fragments were used. The inactive 32-kDa fragment was purified on a Mono S HR 5/5 cation-exchange column. The initial buffer was 20 mM HEPES, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, and 10 mM 2-mercaptoethanol, and proteins were eluted with a linear gradient of NaCl (0-1.0 M). The inactive 32-kDa fragment was identified by absorbance at 280 nm and SDS-PAGE (data not shown). The antipeptide antibodies detected the inactive 32-kDa fragment, but not the active 30-kDa fragment (Fig. 6, lanes 2 and 3). This indicates that at least a portion of the corresponding...
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Comparison of kinetic parameters for native CaM kinase II and for the active fragment

Kinase activity was measured in the presence of Ca\(^{2+}\)/calmodulin for native CaM kinase II (130 ng) or in the absence of Ca\(^{2+}\)/calmodulin for the purified active fragment (15 ng). CaM-K(281–291) peptide (16–268 \(\mu\)M final concentration) or Syn 1-site 3 peptide (10–328 \(\mu\)M) was used as a substrate. Incubation was performed for 15 s at 30 °C. Kinetic parameters, \(K_m\) and \(V_{max}\), were derived from double-reciprocal plots. The data represent the mean ± S.E. of four independent experiments. The data for the turnover number (\(k_{cat}\)) and catalytic efficiency (\(k_{cat}/K_m\)) were calculated by using the mean values of \(K_m\) and \(V_{max}\) for each substrate. The turnover number of native CaM kinase II was calculated in terms of each subunit of the enzyme.

|                  | Native CaM kinase II | Active fragment |
|------------------|----------------------|----------------|
|                  | \(K_m\) \(\mu\)M | \(V_{max}\) \(\mu\)mol/min/mg | \(k_{cat}\) 1/min | \(k_{cat}/K_m\) 1/min/\(\mu\)M |
| CaM-K(281–291)   | 136 ± 21.0          | 8.8 ± 1.4        | 462              | 3.4                |
| Syn 1-site 3     | 130 ± 5.8           | 7.0 ± 0.6        | 368              | 2.8                |

The peptide sequence (281–302) is absent in the active 30-kDa fragment but is retained in the inactive 32-kDa fragment. The reactivity of the inactive 32-kDa fragment with this antibody provides clear evidence that this fragment was derived from native CaM kinase II.

In order to determine whether the limited chymotryptic digestion resulted in cleavage of residues from the NH\(_2\)-terminal of the kinase subunits, the purified active 30-kDa and inactive 32-kDa fragments were subjected to SDS-PAGE and electrophoretically transferred to PVDF membranes, which were then subjected to direct amino acid sequence analysis. No sequence was obtained from either sample. The NH\(_2\)-terminal residue of the \(\alpha\) subunit of the postsynaptic density CaM kinase II was previously reported to be acetylated (N-acetyl-alanine) and thus blocked to Edman degradation (50). We also subjected the purified \(\alpha\) and \(\beta\) subunits of soluble CaM kinase II to NH\(_2\)-terminal amino acid sequence analysis after transfer to PVDF membranes, and no sequence was obtained from either subunit. This indicated that the NH\(_2\)-terminal amino acids of both the active 30-kDa and the inactive 32-kDa fragments, as well as those of the \(\alpha\) and \(\beta\) subunits of native CaM kinase II, were blocked. Taken together, these data suggest that \(\alpha\)-chymotrypsin did not cleave the NH\(_2\)-terminal portion of native CaM kinase II under the conditions employed in this study, and that both fragments contained the intact NH\(_2\)-terminal catalytic domain.

Since there are a limited number of methionine residues in the \(\alpha\) and \(\beta\) subunits of native CaM kinase II from the NH\(_2\) terminus through the end of the regulatory domain, we performed in situ CNBr cleavage of the active 30-kDa and the inactive 32-kDa fragments after transfer to PVDF membranes, and subjected the peptide mixtures to direct amino acid sequence analysis. Fig. 7 shows a summary of the sequence analysis. For the active 30-kDa fragment, amino acid residues corresponding to the sequences of peptide 1 (starting from Gly-130) and peptide 2 (starting from Leu-252) were detected at successive cycles of Edman degradation, but those corresponding to peptide 3 (starting from His-282) were not detected. When OPA treatment was performed after the fourth cycle, only the residues corresponding to the sequence of peptide 2 were detected, and there was a significant drop in the yield of the released amino acid after the 20th cycle. For the inactive 32-kDa fragment, amino acid residues corresponding to the sequences of peptides 1–3 were detected at each cycle. For the sequence of peptide 3, amino acids through Phe-293 were clearly detected, and small amounts of amino acids were also detected through Leu-299, although not unequivocally (data not shown). No amino acid residues corresponding to the sequence of peptide 4 (starting from Leu-308) were detected in either the active 30-kDa or the inactive 32-kDa fragment. These results suggest that the COOH terminus of the active 30-kDa fragment is Ile-271 (\(\alpha\))/Val-272 (\(\beta\)), and that the major portion of the inactive 32-kDa fragment terminates at Phe-293, with a minor portion (roughly 15–20%) of the fragment extending to an alternative site of chymotryptic cleavage at Leu-299.

**DISCUSSION**

In this report, we described a procedure for the generation and purification of the active catalytic fragment of rat brain soluble CaM kinase II. The proteolytic generation of the active fragment required prior autophosphorylation as described previously (31). Autophosphorylation was reported not to be necessary to produce a catalytic fragment from CaM kinase II derived from postsynaptic density (51). The disparity of these observations may be due to a difference in the autoregulatory mechanism of the soluble and postsynaptic density CaM kinase II, or may be due to some other fundamental difference in these two forms of CaM kinase II. The purified active fragment should provide a useful tool for the
Fig. 4. Absence of autophosphorylated residues in the purified active 30-kDa fragment. Generation and purification of the active 30-kDa fragment was essentially the same as described in the legend to Fig. 1, using 0.16 mg of CaM kinase II and 0.08 mg of TLCK-treated a-chymotrypsin, except that [γ-32P]ATP (4.3 × 10^4 cpm/μmol) was used. A, autoradiography after SDS-PAGE of 20 μl of each sample just before proteolysis (lane 1), after proteolysis (lane 2), or from column fractions of Mono Q HR 5/5 anion-exchange chromatography (lanes 3–6) correspond to fractions 38–41 in B, respectively. a, β subunit of CaM kinase II; b, α subunit of CaM kinase II. B, elution profile from the Mono Q HR 5/5 column. Each fraction was assayed for kinase activity in the absence of Ca^2+/CaM (open circles) as substrate (filled circles) and was measured for 32P radioactivity (open circles). The broken line shows NaCl concentration.

Fig. 5. Comparison of 125I-labeled calmodulin binding to native CaM kinase II, the active 30-kDa fragment, and the inactive 32-kDa fragment. Native CaM kinase II (2.3 μg) (lanes 1 and 4), the active 30-kDa fragment (lanes 2 and 5), and the inactive 32-kDa fragment (lanes 3 and 6), both of which were generated from 4 μg of CaM kinase II under the conditions described in the legend to Fig. 3, were subjected to SDS-PAGE using 12% acrylamide gels, and then electrophoretically transferred to a nitrocellulose membrane. The membrane was incubated with 125I-labeled calmodulin in the presence (lanes 1–3) or absence (lanes 4–6) of Ca^2+, and the binding of 125I-labeled calmodulin was visualized by autoradiography. a, β subunit of CaM kinase II; b, α subunit of CaM kinase II.

Fig. 6. Immunoblot analysis of native CaM kinase II, the active 30-kDa fragment, and the inactive 32-kDa fragment using anti-CaM kinase II peptide antibodies. Native CaM kinase II (0.1 μg) (lane 1), the active 30-kDa fragment (0.16 μg) purified by Mono Q HR 5/5 anion-exchange column chromatography (lane 2), and the inactive 32-kDa fragment (0.1 μg) purified by Mono S HR 5/5 cation-exchange column chromatography (lane 3), were subjected to SDS-PAGE using 12% acrylamide gels and transferred to a nitrocellulose membrane. The membrane was incubated with the anti-CaM kinase II peptide antibodies, G-301 (1:2000 dilution of 2.1 mg/ml solution). Bound antibodies were visualized by the second incubation with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:5000 dilution). a, β subunit of CaM kinase II; b, α subunit of CaM kinase II; c, the inactive 32-kDa fragment.

Comparative studies of the active 30-kDa fragment and the inactive 32-kDa fragment provided certain structural details of both fragments (Fig. 8).

1) The purified active 30-kDa fragment did not contain autophosphorylated residues. Initial autophosphorylation occurs at Thr-286, which is responsible for the generation of Ca^2+/calmodulin-independent activity in the autophosphorylated kinase (20–22). The reaction time employed in the current study may have been long enough for autophosphorylation to occur not only at Thr-286 but also at Ser-279 (21). The COOH-terminal cleavage site for the active 30-kDa frag-
Thus, the calmodulin-binding region (residues 296–309) (52), and more specifically, the "critical" residues required for high affinity binding to calmodulin (residues 305–309) (49), are not contained within the inactive 32-kDa fragment. This fragment, however, does contain a considerable portion of residues 281–302.

3) Since both the active 30-kDa and the inactive 32-kDa fragments contain the intact NH2-terminal catalytic domain of the native subunits, it is clear that each fragment was generated by distinct cleavage within the regulatory domain. The results of amino acid sequence analysis after in situ CNBr cleavage indicate that the COOH terminus of the active 30-kDa fragment is Ile-271 (α)/Val-272 (β). They also suggest that the COOH terminus of the major portion of the inactive 32-kDa fragment is Phe-293 and that a small portion of it may extend to Leu-299. The suggested amino acid sequences of both fragments (30-kDa fragment: 1–271; 32-kDa fragment: major, 1–293 and minor, 1–299) are consistent with the results of functional studies described above. Autophosphorylation of the kinase prior to proteolysis should have induced a critical conformational change of native CaM kinase II, which in turn exposed another preferred cleavage site for α-chymotrypsin and thus led to the generation of the smaller active 30-kDa fragment instead of the larger inactive 32-kDa fragment. The heterogeneity of the COOH terminus of the inactive 32-kDa fragment may reflect the difference of the susceptibility to chymotrypsin between the α and β subunits of the native enzyme.

Recent investigations have revealed the existence of an autoinhibitory sequence within the regulatory region of CaM kinase II (30, 52, 53), which may be involved in the regulation of CaM kinase II activity. Other protein kinases, such as myosin light chain kinase and protein kinase C, contain analogous regions termed pseudosubstrate sequences (54–58). Based on the studies using synthetic peptides, the autoinhibitory region of CaM kinase II has been proposed to reside within amino acid residues 281–309 (30, 52, 53). Binding of Ca2+/calmodulin to this region and subsequent autophosphorylation at Thr-286 seem to relieve the inhibitory constraint of this region on the catalytic domain. Thus, the autoinhibitory region may be responsible for maintaining the enzyme in an inactive form in the absence of Ca2+/calmodulin. The results of this study indicate that the entire autoinhibitory sequence is not present within the active 30-kDa fragment and that the inactive 32-kDa fragment retains at least a portion of the autoinhibitory sequence (residues 281–293 or 281–299). The observation that the inactive 32-kDa fragment cannot be activated even in the presence of Ca2+/calmodulin suggests that the presence of the retained portion of the autoinhibitory sequence, and perhaps additional residues on the NH2-terminal side of this sequence (residues 272–280), maintains the enzyme in the inactive form. These results provide direct support for the concept that at least a certain portion of the proposed autoinhibitory sequence is responsible for the autoregulation of CaM kinase II in situ.

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REFERENCES

1. Nairn, A. C., Hemmings, H. C., Jr., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–976
2. Schulman, H. (1988) Adv. Second Messenger Phosphoprotein Res. 22, 39–112
3. Nairn, A. C., Hemmings, H. C., Jr., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–976
4. Horiiuchi, A. and Nairn, A. C. (1985) J. Biol. Chem. 260, 11218–11225
5. Mische, S., Fernandez, J., and Fernandez, J. (1985) J. Biol. Chem. 260, 11218–11225
6. Nairn, A. C., Hemmings, H. C., Jr., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–976
7. Schulman, H. (1988) Adv. Second Messenger Phosphoprotein Res. 22, 39–112
8. Horiiuchi, A. and Nairn, A. C. (1985) J. Biol. Chem. 260, 11218–11225
9. Mische, S., Fernandez, J., and Fernandez, J. (1985) J. Biol. Chem. 260, 11218–11225
10. Nairn, A. C., Hemmings, H. C., Jr., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–976
11. Horiiuchi, A. and Nairn, A. C. (1985) J. Biol. Chem. 260, 11218–11225
12. Mische, S., Fernandez, J., and Fernandez, J. (1985) J. Biol. Chem. 260, 11218–11225
13. Nairn, A. C., Hemmings, H. C., Jr., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–976
14. Horiiuchi, A. and Nairn, A. C. (1985) J. Biol. Chem. 260, 11218–11225
15. Mische, S., Fernandez, J., and Fernandez, J. (1985) J. Biol. Chem. 260, 11218–11225
16. Nairn, A. C., Hemmings, H. C., Jr., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–976
17. Horiiuchi, A. and Nairn, A. C. (1985) J. Biol. Chem. 260, 11218–11225
18. Mische, S., Fernandez, J., and Fernandez, J. (1985) J. Biol. Chem. 260, 11218–11225
19. Nairn, A. C., Hemmings, H. C., Jr., and Greengard, P. (1985) Annu. Rev. Biochem. 54, 931–976
3. Colbran, R. J., Schworer, C. M., Hashimoto, Y., Fong, Y.-L., Rich, D. P., Smith, M. K., and Soderling, T. R. (1989) Biochem. J. 258, 313-325
4. Linas, R., McGuinness, T. L., Leonard, C. S., Sugimori, M., and Greengard, P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8257-8261
5. Nichols, R. A., Sihra, T. S., Czernik, A. J., Nairn, A. C., and Greengard, P. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 5851-5855
6. Laemmli, U. K. (1970) Nature 227, 680-685
7. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265-275
8. Schworer, C. M., Colbran, R. J., and Soderling, T. R. (1989) J. Biol. Chem. 264, 13485-13489
9. Fong, Y.-L., Taylor, W. L., Means, A. R., and Soderling, T. R. (1989) J. Biol. Chem. 264, 16759-16763
10. Hanson, P. I., Kapiloff, M. S., Lou, L. L., Rosenfeld, M. G., and Schulman, H. (1989) Neuron 3, 59-70
11. Waxham, M. N., Aronowski, J., Westgate, S. A., and Kelly, P. T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1273-1277
12. Miller, S. G., and Kennedy, M. B. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7518-7522
13. Levine, H., III, Hunt, D. F., Zhu, N-Z., and Shabanowits, J. (1987) Biochem. Biophys. Res. Commun. 152, 122-128
14. Levine, H., III, Hunt, D. F., Zhu, N-Z., and Shabanowits, J. (1987) J. Biol. Chem. 262, 1104-1109
15. Levine, H., III, and Sambrook, J. (1987) Eur. J. Biochem. 168, 481-486
16. Payne, M. E., Fong, Y.-L., Ono, T., Colbran, R. J., Kemp, B. E., Soderling, T. R., and Means, A. R. (1989) J. Biol. Chem. 263, 7190-7195
17. Colbran, R. J., Schworer, C. M., and Soderling, T. R. (1986) J. Biol. Chem. 261, 8581-8584
18. Colbran, R. J., Fong, Y.-L., Schworer, C. M., and Soderling, T. R. (1988) J. Biol. Chem. 263, 18145-18151
19. Kwiatkowski, A. P., and King, M. M. (1989) Biochemistry 28, 5280-5285
20. Waxham, M. N., Aronowski, J., Westgate, S. A., and Kelly, P. T. (1987) J. Biol. Chem. 262, 4800-4804
21. Fong, Y.-L., and Soderling, T. R. (1990) J. Biol. Chem. 265, 11091-11097