Regulatory Domain of Calcium/Calmodulin-dependent Protein Kinase II

MECHANISM OF INHIBITION AND REGULATION BY PHOSPHORYLATION*

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Regulatory mechanisms of rat brain Ca**/calmodulin-dependent protein kinase II (CaM-kinase II) were probed using a synthetic peptide (CaMK-(281-309)) corresponding to residues 281-309 (α-subunit) which contained the calmodulin (CaM)-binding and inhibitory domains and also the initial autophosphorylation site (Thr**). Kinetic analyses indicated that inhibition of a completely Ca**/CaM-independent form of CaM-kinase II by CaMK-(281-309) was noncompetitive with respect to peptide substrate (syntide-2) but was competitive with respect to ATP. Interaction of CaMK-(281-309) with the ATP-binding site was independently confirmed since inactivation of proteolyzed CaM-kinase II by phenylglyoxal (tM = 7 min) was blocked by ATP analog plus Mg** or by CaMK-(281-309). In the presence of Ca**/CaM, CaMK-(281-309) no longer protected against phenylglyoxal inactivation, consistent with our previous observations (Colbran, R. J., Fong, Y.-L., Schworer, C. M., and Soderling, T. R. (1988) J. Biol. Chem. 263, 18145–18151) that binding of Ca**/CaM to CaMK-(281-309) 1) blocks its inhibitory property, and 2) enhances its phosphorylation at Thr**. The present study also showed that phosphorylation of CaMK-(281-309) decreased its inhibitory potency at least 10-fold without affecting its Ca**/CaM-binding ability. Thus, CaM-kinase II is inactive in the absence of Ca**/CaM because an inhibitory domain within residues 281-309 interacts with the catalytic domain and blocks ATP binding. Autophosphorylation of Thr** results in a Ca**/CaM-independent form of the kinase by disrupting the inhibitory interaction with the catalytic domain.

Calcium/calmodulin-dependent protein kinase II (CaM-kinase II) is a multifunctional protein kinase which is distributed widely in many tissues and organisms (for reviews see Refs. 1-4). Within the last 2 years the cDNA sequences have been synthesized and purified as described (12). Synthetic peptide analogs of various regions of the α-subunit of the kinase have been used to define more precisely the CaM-binding domain to residues 296-309 in this subunit (9, 10). Payne et al. (9) also investigated whether synthetic peptide analogs of this region could inhibit Ca**-independent forms of CaM-kinase II and might therefore represent an inhibitory domain. The analog of residues 290-309 was shown to inhibit (IC** = 24 μM) the autoprophosphorylated, partially Ca**-independent form of the kinase in a partially competitive manner with respect to synthetic peptide substrate (9). Subsequent studies have demonstrated that extension of this peptide at the amino terminus to give the peptide 281-309 results in a 20-fold more potent inhibition (11, 12). However, a kinetic mechanism for the inhibition was not determined in these latter studies.

Investigation of the inhibitory domain of CaM-kinase II is particularly interesting because autophosphorylation has a profound effect on its regulatory properties. Purified CaM-kinase II activity is totally dependent on the addition of Ca**/CaM to the assay. However, following Ca**/CaM-dependent autophosphorylation the kinase becomes partially Ca**/CaM-independent, exhibiting 50–90% of total activity in the presence of EGTA (13-16). Recent results have demonstrated that autophosphorylation at threonine 286 in the α-subunit (threonine 287 in β-subunit) correlates with the generation of Ca**/CaM-independent kinase activity (17-19). Thus, this regulatory autophosphorylation site is within the inhibitory domain of the kinase, as defined using synthetic peptides (see above).

The present work suggests that the mechanism for interaction between the inhibitory and catalytic domains of CaM-kinase II in the absence of Ca**/CaM does not appear to be strictly pseudosubstrate in nature as has been suggested for several other protein kinases (reviewed in Ref. 20). In addition, data are presented indicating that autophosphorylation at threonine 286 results in partial Ca**-independence of CaM-kinase II by reducing the effectiveness of the inhibitory domain.

EXPERIMENTAL PROCEDURES

Proteins—The sources of CaM-kinase II (rat forebrain), CaM, glyogen synthase, and chymotrypsin were as described previously (12).

Peptide Synthesis—The substrate peptide syntide-2 (Pro-Leu-Ala-Arg-Thr-Leu-Ser-Val-Ala-Gly-Leu-Pro-Gly-Lys-lys) was synthesized and purified as described (21). Synthetic peptides corresponding to various regions of the α subunit of rat brain CaM-kinase II (7) were synthesized and purified as described (12). The sequences are
as follows: CaMK-(281-309), Met-His-Arg-Gln-Glu-Thr-Val-Asp-Cys-Leu-Lys-Lys-Phe-Asn-Ala-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Met-Leu-Ala; CaMK-(290-309), Leu-Lys-Lys-Phe-Asn-Ala-Arg-Lys-Leu-Lys-Gly-Ala-Ile-Leu-Thr-Thr-Met-Leu-Ala. Concentrations were determined using a Waters Associates Pico-Tag amino acid analyzer.

**Protein Kinase Assays**—Assays were performed essentially as described previously (12) except that 0.005% Triton X-100 was included. Lines of best fit ($r^2 > 0.98$) were computed from the experimental data of the kinetic analyses by the method of least squares using the RS/1 program (BBN Software Products Corp.).

**Limited Proteolysis of CaM-Kinase II—** CaM-kinase II (100–500 µg/ml) was autophosphorylated and then subjected to limited proteolysis with chymotrypsin as described previously (12) except that 50 µM ATP was used in the autophosphorylation reaction.

**Stoichiometric Phosphorylation of CaMK-(281-309)—** CaMK-(281-309) (40 µM) was incubated for 30 min at 30 °C with 50 mM HEPES, pH 7.5, containing 10 mM magnesium acetate, 0.5 mM [γ-32P]ATP (60–200 cpm/pmol), 0.01% Triton X-100, 67 µM CaM, and 130 nM native CaM-kinase II. The reaction was stopped by the addition of trichloroacetic acid (10% final concentration). The trichloroacetic acid pellet contained all the CaMK-(281-309), and the concentration of CaMK-(281-309) was determined by amino acid analysis, and the stoichiometry of phosphorylation was calculated from the radioactivity in the sample. A control (non-phosphorylated) sample of CaMK-(281-309) was treated similarly except that calcium chloride was replaced with an IC50 of 0.4 mM and further purified by reverse phase HPLC on a C-18 column (Beckman) developed with a gradient of 20–40% acetonitrile in 0.1% trifluoroacetic acid over 50 min. The trichloroacetic acid pellet was then dissolved in 70% formic acid and further purified by reverse phase HPLC on a C-18 cartridge (Baxter Healthcare Corp.) equilibrated in 0.1% trifluoroacetic acid, washed with equilibration buffer, and eluted with 40% acetonitrile in 0.1% trifluoroacetic acid.

**Materials—** [γ-32P]ATP was obtained from Du Pont-New England Nuclear. Triton X-100 was from Bio-Rad. Phenylglyoxal and AMP-PCP were obtained from Sigma and Boehringer Mannheim, respectively. The sources of other materials were described previously (12).

**RESULTS**

**Kinetic Analyses of Inhibition**—The synthetic peptide corresponding to residues 281–309 of the α-subunit of rat brain CaM-kinase II (CaMK-(281–309)) has been shown to inhibit a proteolyzed Ca2+/CaM-independent form of CaM-kinase II with an IC50 of 0.9 µM (12) using either syntide-2 or glycosyn synthase as substrate. In addition, this peptide has been shown to inhibit Ca2+/CaM-independent autophosphorylation and Ca2+/CaM-independent phosphorylation of synapsin I by an autophosphorylated Ca2+-independent form of CaM-kinase II (11). However, the kinetic mechanism of inhibition was not investigated in either of these studies.

Proteolyzed CaM-kinase II was assayed using fixed [ATP] (0.4 mM) and variable concentrations of CaMK-(281–309) (0–2 µM) and syntide-2 (0–60 µM). When the data were analyzed by double-reciprocal (Lineweaver-Burk) plots, the computed lines of best fit intersected at the x-axis (Fig. 1, top) indicating that the mechanism of inhibition is noncompetitive with respect to syntide-2. When the slopes of the fitted lines were replotted against the concentration of CaMK-(281–309), a straight line was obtained and a mean KI of 0.21 ± 0.04 µM was calculated from three similar experiments (data not shown). CaMK-(281–309) was also shown to inhibit proteolyzed CaM-kinase II by a noncompetitive mechanism with respect to glycosyn synthase substrate (data not shown).

In order to further investigate the mechanism of inhibition by CaMK-(281–309) proteolyzed kinase was also assayed at several concentrations of CaMK-(281–309) (0.1 µM) and ATP (20–1000 µM) using fixed [syntide-2] (500 µM). Double-reciprocal plots intersected at the y-axis (Fig. 1, bottom), indicating that the mechanism of inhibition is competitive with respect to ATP. Slope replots again gave a linear relationship, and a mean KI of 0.16 ± 0.01 µM was calculated from three similar experiments (data not shown). Similar results were obtained when glycosyn synthase was used as the phosphate acceptor substrate rather than syntide-2 (data not shown).

Similar analyses to those described above were performed with CaMK-(290–309) and indicated that CaMK-(290–309) inhibited proteolyzed CaM-kinase II by a mechanism which was noncompetitive with respect to ATP and competitive with respect to syntide-2 (data not shown). These results confirm and extend previous data (9) obtained using autophosphorylated, Ca2+-independent CaM-kinase II.

**Protection of the ATP-binding Site by CaMK-(281-309)—**
Since the inhibitory mechanism of CaMK-(281–309) is unusual in that it is competitive with ATP rather than the protein/peptide substrate, we wanted to further probe the interaction of CaMK-(281–309) with the ATP-binding site using an independent approach. Phenylglyoxal is a reagent which modifies primarily arginine residues and has been used to modify and inactivate CaM-kinase II (22). Incubation of native CaM-kinase II with phenylglyoxal rapidly inactivated the kinase (85% inactive after 15 min) only in the presence of Mg\(^{2+}\), Ca\(^{2+}\), and CaM. Inclusion of ADP in the incubations protected against the inactivation, suggesting that modification occurs at the adenine nucleotide binding site. Inactivation was also shown to be associated with the incorporation of 1 mol of phenylglyoxal/mol kinase subunit (22). Similar experiments were performed in this laboratory using native CaM-kinase II yielding essentially identical results (not shown).

When proteolyzed CaM-kinase II was incubated with phenylglyoxal, rapid inactivation occurred with only 10–15% of original activity remaining after 15 min (Fig. 2). Inactivation was unaffected by the presence or absence of Mg\(^{2+}\) (not shown) or Ca\(^{2+}\)/CaM (Fig. 2, bottom). In the presence of Mg\(^{2+}\) and absence of phenylglyoxal approximately 25% inactivation was observed (Fig. 2), probably due to the thermal lability of the kinase. Somewhat greater inactivation (44 ± 5%, n = 6) in the absence of phenylglyoxal was observed in the absence of Mg\(^{2+}\) (not shown). Inclusion of the nonhydrolyzable ATP analog AMP-PCP in the incubations protected against inactivation by phenylglyoxal. In the presence of Mg\(^{2+}\), 1 mM AMP-PCP protected approximately 60% of the inactivation by phenylglyoxal (Fig. 2). 4 mM AMP-PCP completely protected the kinase from phenylglyoxal inactivation in the presence of Mg\(^{2+}\), but was without effect on inactivation in the absence of Mg\(^{2+}\) (not shown). Thus, inactivation by phenylglyoxal appeared to be caused by reaction at the ATP-binding site of the proteolyzed kinase.

Since CaMK-(281–309) inhibits proteolyzed CaM-kinase II competitively with respect to ATP and CaMK-(290–309) inhibits competitively with respect to syntide-2, the effects of these inhibitors on inactivation by phenylglyoxal were investigated. Fig. 2 shows that CaMK-(281–309) (10 μM) protected the proteolyzed kinase from inactivation, whereas CaMK-(290–309) (10 μM) was without significant effect. Furthermore, Ca\(^{2+}\) and a molar excess of CaM (30 μM) reversed the protection afforded by CaMK-(281–309), restoring inactivation by phenylglyoxal (Fig. 2, bottom). In an additional experiment, 250 μM syntide-2 had no significant effect on the inactivation by phenylglyoxal (not shown). Thus, both CaMK-(281–309) and AMP-PCP protect the proteolyzed kinase from inactivation by phenylglyoxal, suggesting that they may interact with the catalytic domain at a similar location.

Effect of Phosphorylation on the Inhibitory Properties of CaMK-(281–309)—Ca\(^{2+}\)/CaM-dependent autophosphorylation of threonine 286 correlates with transition of the kinase to the partially Ca\(^{2+}\)/CaM-independent form (17–19). Since threonine 286 is located within the inhibitory peptide CaMK-(281–309), the effect of phosphorylation on the inhibitory properties of this peptide was investigated. CaMK-(281–309) has been shown to be a poor substrate for proteolyzed CaM-kinase II in the presence of EGTA but
becomes a relatively good substrate in the presence of a molar excess of Ca\textsuperscript{2+}/CaM (12). CaMK-(281–309) was phosphorylated by CaM-kinase II, and the phosphorylated peptide was purified by reverse-phase HPLC (see "Experimental Procedures"). Phosphorylation attained a maximal stoichiometry of approximately 0.8 mol/mol.

The properties of purified phospho-CaMK-(281–309) were compared with a sample of CaMK-(281–309) which had been treated in an identical manner except that excess EGTA was added and CaM was omitted from the phosphorylation reaction (see "Experimental Procedures"). The phosphorylation of CaMK-(281–309) was equally effective at binding CaM, as determined by their abilities to antagonize the activation of native CaM-kinase II by a limiting concentration of CaM (Fig. 3, top). However, there was a striking difference in their inhibitory properties toward proteolyzed CaM-kinase II. As shown in Fig. 3 (bottom), phospho-CaMK-(281–309) was approximately a 10-fold less potent inhibitor (IC\textsubscript{50} = 3.2 \mu M) than dephospho-CaMK-(281–309) (IC\textsubscript{50} = 0.7 \mu M) using syntide-2 as substrate. In an experiment performed using glycogen synthase as substrate, 2.5 \mu M dephospho-CaMK-(281–309) inhibited the kinase 65% whereas 2.5 \mu M phospho-CaMK-(281–309) only inhibited the kinase 26% (data not shown).

**DISCUSSION**

The present data indicate that the mechanism involved in the suppression of CaM-kinase II activity in the absence of Ca\textsuperscript{2+}/CaM is somewhat different than for other protein kinases. Three observations suggest that the inhibitory domain contained within residues 281–309 (epsilon-subunit rat brain kinase) interacts with the catalytic domain and restricts access to the ATP-binding site. Firstly, the inhibition of proteolyzed CaM-kinase II by a synthetic peptide analog of this region (CaMK-(281–309)) is by a mechanism which is non-competitive with respect to syntide-2 (synthetic peptide substrate) but is competitive with respect to ATP (Fig. 1). Secondly, a residue (presumably an arginine) in, or close to, the ATP-binding site of the native kinase reacts with phenylglyoxal producing inactivation only when Ca\textsuperscript{2+}/CaM (and Mg\textsuperscript{2+}) are added (22). Limited proteolysis generates a Ca\textsuperscript{2+}/CaM-independent kinase and removes the CaM-binding and inhibitory domains of the kinase (12, 23) and also exposes this residue in the ATP-binding site to phenylglyoxal even in the presence of EGTA (Fig. 2). Thirdly, this reactive residue in the proteolyzed kinase can be protected by the addition of CaMK-(281–309), and this protection is reversed by CaM\textsuperscript{2+}/CaM (Fig. 2). Binding of Ca\textsuperscript{2+}/CaM to CaMK-(281–309) has previously been shown to block its inhibitory properties (12).

Several published reports suggest that binding of Ca\textsuperscript{2+}/CaM to native CaM-kinase II affects the interaction with adenine nucleotides. Shields et al. (33) reported that the kinase in synaptic junctions could only be photoaffinity labeled with [\alpha-\textsuperscript{32}P]ATP in the presence of Ca\textsuperscript{2+}/CaM. In addition, ADP has been used to protect the kinase from inactivation by phenylglyoxal (22) and 5'-p-fluorosulfonylbenzoyl adenosine (34). In both cases addition of Ca\textsuperscript{2+}/CaM decreased by 10-fold the concentration of ADP required to give half-maximal protection from inactivation (22, 24). These data can be explained by our observations that the inhibitory domain of the kinase restricts access to the ATP-binding site of the kinase in the absence of Ca\textsuperscript{2+}/CaM.

It is interesting that the inhibitory domain of CaM-kinase II appears to act by a different kinetic mechanism than that of other protein kinases. It was first suggested that the regulatory subunit/domain of cyclic AMP- and cyclic GMP-dependent protein kinases suppresses the activity of constitutively active catalytic subunit/domain by interacting with the catalytic site, mimicking the interaction with phosphate acceptor substrates (24, 25). Such a pseudosubstrate inhibitory domain is also thought to be responsible for suppressing the activity of myosin light chain kinase (26–28) and Ca\textsuperscript{2+}/phospholipid-dependent protein kinase (29) in the absence of Ca\textsuperscript{2+}/CaM and diacylglycerol, respectively (for discussion see Ref. 20). According to the pseudosubstrate hypothesis, the amino acid sequence of the inhibitory domain is homologous to the consensus substrate phosphorylation sequence for that kinase, generally with the replacement of the phosphorylated serine or threonine by a non-phosphorylatable amino acid. Synthetic peptide analogs of the inhibitory domains of myosin light chain kinase and Ca\textsuperscript{2+}/phospholipid-dependent protein kinase have been shown to inhibit their respective kinases by a mechanism competitive with the phosphate acceptor substrate, and non-competitive with ATP. However, the amino acid sequence of the inhibitory domain of CaM-kinase II does not show any obvious homology with consensus phosphorylation sequences (-Arg-X-X-Ser/Thr-) (30, 31) except for the residues surrounding threonine 286, which cannot be interacting with the active site as a substrate since CaMK-(281–309) is not a good substrate for proteolyzed kinase in the presence of EGTA (12). Furthermore, the synthetic peptide analog of the inhibitory domain (CaMK-(281–309)) appears to interfere with the ATP-binding site of the kinase (see above). The reasons for this difference in mechanism of action of the inhibitory domain are not completely clear. It is possible that the 290–309 region interacts with the protein substrate binding site, which would explain the competitive mechanism for inhibition by CaMK-(290–309) (9). Extension at the amino terminus to CaMK-(281–309) might then also lead to interaction with the ATP-binding site, which must be in close proximity to the protein substrate binding site, resulting in a higher affinity for the interaction. Recent data obtained using the peptide CaMK-(281–289) have indicated that some inhibitory determinants are contained within this peptide which cannot be explained by it acting as an alternative substrate (12). The relatively weak inhibition by CaMK-(281–289) observed using 1 mM syntide-2 as substrate (12) may be due to interaction of the peptide at the ATP-binding site. Thus, the inhibitory domain of CaM-kinase II may contain elements that interact with both substrate- and ATP-binding sites. If CaM-kinase II shows an ordered reaction mechanism, with binding of Mg-ATP preceding binding of protein substrate, this could account for the competitive inhibition with ATP. Such an ordered mechanism has been described for cyclic AMP-dependent protein kinase (32).

The inhibitory domain of CaM-kinase II is located in close proximity to two previously identified functional domains of the kinase. The CaM-binding domain has been defined to residues 296–309 (9, 10), and threonine 286 has been identified as the initial site of Ca\textsuperscript{2+}/CaM-dependent autophosphorylation, which correlates with the generation of the partially Ca\textsuperscript{2+}-independent form of the kinase (17–19). Therefore, it is likely that there are extensive interactions between these three functional domains. It has been shown that the binding of Ca\textsuperscript{2+}/CaM to CaMK-(281–309), which may result in a conformational change such as induction of an \alpha-helix (35, 36), relieves the inhibition of proteolyzed CaM-kinase II (12). CaMK-(281–309) has also been shown to be a poor substrate for the proteolyzed kinase in the presence of EGTA, but phosphorylation at threonine 286 is enhanced approximately 10-fold by binding of Ca\textsuperscript{2+}/CaM to the peptide (12). In the present study, the phosphorylated form of CaMK-(281–309)
was isolated and shown to be a much less potent inhibitor of proteolyzed CaM-kinase II than the non-phosphorylated peptide, although the two forms were equally potent in their ability to bind CaM (Fig. 3). The 10-fold decrease in inhibitory potency that occurs following phosphorylation may be an underestimation since the stoichiometry of phosphorylation was only approximately 0.8.

These data provide additional support to a recently presented model for the regulation of CaM-kinase II by Ca\(^{2+}\)/CaM and autophosphorylation (4, 12). Additional synthetic peptide analogs will be used to further test further aspects of this model. However, perhaps the best evidence for or against the model will be obtained using site-directed mutagenesis approaches.

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