Calcium/Calmodulin-dependent Protein Kinase II

CHARACTERIZATION OF DISTINCT CALMODULIN BINDING AND INHIBITORY DOMAINS*

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M. Elizabeth Payne§, Yiu-Lian Fong¶, Tomio Ono, Roger J. Colbran¶, Bruce E. Kemp¶, Thomas R. Soderling¶, and Anthony R. Means

From §The Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, ¶The Howard Hughes Medical Institute and the Department of Molecular Physiology and Biophysics, Vanderbilt University Medical School, Nashville, Tennessee 37232, and ¶¶The University of Melbourne, Department of Medicine, and Repatriation General Hospital, Heidelberg, Victoria 3081, Australia

Regulatory domains of the multifunctional Ca\(^{2+}\)/calmodulin-dependent protein kinase II were investigated utilizing synthetic peptides. These peptides were derived from the sequence between positions 281 and 319 as translated from the cDNA sequence of the rat brain 50-kDa subunit (Lin, C. R., Kapiloff, M. S., Durgerian, S., Tatemoto, K., Russo, A. F., Hanson, P., Schulman, H., and Rosenfeld, M. G. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 5962–5966), which contain the putative calmodulin-binding region as well as potential autophosphorylation sites. Peptide 290 to 309 was found to be a potent calmodulin antagonist with an IC\(_{50}\) of 52 nM for inhibition of Ca\(^{2+}\)/calmodulin-dependent protein kinase II. Neither truncation from the amino terminus (peptide 296–309) nor extension in the carboxyl-terminal direction (peptide 294–319) markedly affected calmodulin binding, whereas shortening the peptide from the carboxyl terminus (peptide 290–302) or from both ends (peptide 295–304) resulted in the elimination of this activity. Peptide 281–290 did not bind calmodulin, but was a good substrate for the enzyme, being phosphorylated at Thr-286. Several of the peptides inhibited the kinase in a partially competitive, substrate-directed manner, but were not themselves phosphorylated. These studies identify domains within Ca\(^{2+}\)/calmodulin-dependent protein kinase II which may be involved in 1) inhibition of the kinase in the absence of calmodulin, 2) binding of calmodulin, and 3) the resulting activation. Additionally, it is suggested that phosphorylation of residues flanking these domains may be responsible for the known regulatory effects of autophosphorylation on the properties of the kinase.

Calmodulin (CaM) mediates many of the effects of elevated intracellular calcium. Initial studies on the interactions between CaM and proteins utilized naturally occurring model peptides such as melittin (Comte et al., 1983; Barnette et al., 1983; Seeholzer et al., 1986), mastoparans (Malencik and Anderson, 1983; Barnette et al., 1983), and \(\beta\)-endorphins (Weiss et al., 1986; Giedroc et al., 1983). Within the last 2 years, however, CaM-binding domains have been identified within specific target enzymes. The first such study was by Blumenthal et al. (1985) who localized the CaM-binding domain of rabbit skeletal muscle myosin light chain kinase near the carboxyl terminus. The synthetic 26-residue peptide analog of this sequence inhibited the CaM-dependent activation of skeletal muscle myosin light chain kinase and bound CaM in a calcium-dependent manner with a molar stoichiometry of 1:1 (Klevit et al., 1985). Similar studies have been carried out using chicken gizzard smooth muscle myosin light chain kinase (Lukas et al., 1986; Kemp et al., 1987). A synthetic peptide corresponding to residues 480–501 (deduced from cDNA sequence; Guerriero et al., 1986) inhibited the CaM-dependent activation of myosin light chain kinase (IC\(_{50}\) = 46 nM). In addition, this peptide was also a substrate antagonist with IC\(_{50}\) values of 2.7 and 0.9 \(\mu\)M for myosin light chains and synthetic peptide substrate, respectively (Kemp et al., 1987). When a CaM-independent tryptic fragment of myosin light chain kinase was used to eliminate the possibility of the peptide interacting with CaM, peptide 480–501 inhibited kinase activity with an IC\(_{50}\) of 35 nM (assayed using 2 \(\mu\)M light chains) (Ikebe et al., 1987). These observations on smooth and skeletal muscle myosin light chain kinase suggest that there is an inhibitory domain, which prevents substrate recognition in the absence of CaM, closely associated with the CaM-binding domain (Edelman et al., 1985; Kemp et al., 1987).

Amino acid sequence has recently been derived from cDNA clones of both the 50-kDa (Hanley et al., 1987; Lin et al., 1987) and 60-kDa (Bennett and Kennedy, 1987) subunits of rat brain Ca\(^{2+}\)/CaM-dependent protein kinase II. Hanley et al. (1987) proposed that the CaM-binding domain of Ca\(^{2+}\)/CaM-dependent protein kinase II was located within residues 290–314 of the rat brain 50-kDa subunit and showed that a synthetic peptide corresponding to this region inhibited activation of Ca\(^{2+}\)/CaM-dependent phosphodiesterase and formed a calcium-dependent complex with CaM. Investigation of the Ca\(^{2+}\)/CaM-binding domain of Ca\(^{2+}\)/CaM-dependent protein kinase II is particularly interesting since the kinase can become independent of Ca\(^{2+}\)/CaM following an intramolecular autophosphorylation (Saitoh and Schwartz, 1986; Miller and Kennedy, 1986; Sch worer et al., 1986; Lai et al., 1986; Lou et al., 1986) on threonine residues (Lai et al.,...
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1987). Furthermore, autophosphorylation at additional sites appears to block the interaction of Ca\(^{2+}\)/CaM with Ca\(^{2+}\)/CaM-dependent protein kinase II (Hashimoto et al., 1987). Further, the properties of the CaM-binding domain of Ca\(^{2+}\)/CaM-dependent protein kinase II are more poorly defined by the use of several synthetic peptides. It was also of interest to ascertain whether these peptides exhibited substrate-directed inhibition of the kinase. In addition, putative autophosphorylation sites flanking the CaM-binding and inhibitory domains are identified.

**EXPERIMENTAL PROCEDURES**

**Materials and Methods**—\(\gamma\)-\[^{32}\]P\]ATP was either purchased from ICN Radiochemicals or prepared using the method of WDealer and Johnson (1979) using carrier-free \(^{32}\)PO\(_4\), from the same company. Phosphocellulose (PSI) paper was purchased from Whatman. Sequanol-grade trifluoroacetic acid was from Pierce, and HPLC-grade acetonitrile was from Burdick and Jackson.

**Protein and Peptide Purification**—Rat forebrain Ca\(^{2+}\)/CaM-dependent protein kinase II was purified as described previously (Hashimoto et al., 1987). CaM was purified from bovine brain (Gopala-krishna and Anderson, 1982) or bacterial lysates (Putkey et al., 1980). Bovine brain CaM-dependent phosphodiesterase was purified using the method of Sharma et al. (1980). The Bradford (1976) method was used to determine the protein concentration of the kinase using bovine serum albumin as standard, and the concentration of CaM was assessed either by absorbance \((E_{280\text{nm}} \text{, mg/ml} = 0.18)\) or amino acid analysis.

**Syntide-2**—PLARTLSVAGLPGKK, a peptide analog similar to the sequence surrounding phosphorylation site 2 of glycogen synthase, was synthesized as described previously (Schworer et al., 1986). A synthetic peptide derived from the phosphorylation site of myosin light chain (KKRPQARATSNVFS) was purchased from Peninsula Laboratories Inc. Solid phase synthesis of the peptide derived from Ca\(^{2+}\)/CaM-dependent protein kinase II was accomplished with either Beckman 990 automatic synthesizer or an Applied Biosystems Model 430 machine by the procedures of Hodges and Merrifield (1975). Using benzhydrolyamine resin, the peptides were prepared as the carbamyl-terminal amide forms. Simultaneous deprotection and cleavage from the resin was achieved with anhydrous HF in the presence of 10% anisole at 0°C for 60 min (Stewart and Young, 1966) using a Protein Research Foundation HF apparatus (Osaka, Japan). The peptides were then purified by gel filtration chromatography in the presence of 5.5 M acetic acid followed by either chromatography on SP-Sephadex (50-75) or preparative reverse phase HPLC on a Pharmacia C18 Amicon resin (25-Å pore size) in the presence of 0.1% (v/v) trifluoroacetic acid using a gradient of acetonitrile. Peptide concentrations were determined using a Waters Associates Pico-tag amino acid analyzer.

**Protein Kinase Assays**—Phosphorylation of synthetic peptides and protein substrates was determined using the phosphocellulose paper method of Roskowski (1985). Two procedures were used, depending on the aims of the experiment. Control reactions were performed in which Ca\(^{2+}\)/CaM-dependent protein kinase II, substrate, or Ca\(^{2+}\)/CaM-dependent protein kinase II synthetic peptides were omitted. Method 1: for the determination of the CaM antagonistic effects of synthetic peptides, CaM-like CaM-dependent protein kinase II synthetic peptides, assays were performed in 50 nM HEPES, pH 7.5, 10 mM magnesium acetate, 0.5 mM calcium chloride, 0.4-0.5 mM \[^{32}\]P\]ATP (300-600 cpm/pmol), 250 nM syntide-2 substrate plus the indicated concentrations of CaM and Ca\(^{2+}\)/CaM-dependent protein kinase II synthetic peptide (25-100 total volume). The reactions were run in duplicates and terminated by spotting aliquots onto phosphocellulose papers. Method 2: for the determination of the inhibitory properties of Ca\(^{2+}\)/CaM-dependent protein kinase II synthetic peptides the autophosphorylated, Ca\(^{2+}\)/CaM-independent form of Ca\(^{2+}\)/CaM-dependent protein kinase II was used in an effort to eliminate potential complications due to CaM interactions. Autophosphorylation was achieved by incubation of Ca\(^{2+}\)/CaM-dependent protein kinase II (5.3 nM subunit concentration) on ice in 50 mM HEPES, pH 7.5, containing 10 mM magnesium acetate, 1 mM calcium chloride, 6 mM CaM, and 0.5 mM ATP for 5-8 min. The reaction was terminated by the addition of dilution buffer containing 5 mM EDTA to an appropriate final kinase dilution (Schworer et al., 1986). The subsequent assay was performed as described in Method 1, except that 0.5 mM EDTA was substituted for calcium chloride, CaM was omitted, and various concentrations of Ca\(^{2+}\)/CaM-dependent protein kinase II synthetic peptides were used as substrate. The potential phosphorylation of the Ca\(^{2+}\)/CaM-dependent protein kinase II synthetic peptides was also investigated by this procedure. In these experiments the indicated concentration of Ca\(^{2+}\)/CaM-dependent protein kinase II synthetic peptides was substituted for syntide-2.

**Phosphodiesterase Assay**—Ca\(^{2+}\)/CaM-dependent phosphodiesterase was assayed at 30°C using a modification of a described method (Thompson et al., 1979). The reaction mixture contained 40 nM Tris-HCl, pH 8.0, 5 mM magnesium acetate, 1 mM calcium chloride, 30 mM GMP, 0.15 μM of [\(^{32}\)H]GMP, 1 mM dithiothreitol, 20% glycerol, 0.64 mg/ml bovine serum albumin, 2.38 nM CaM, 50 μM Ca\(^{2+}\)/CaM-deficient phosphodiesterase, and various concentrations of Ca\(^{2+}\)/CaM-dependent protein kinase II synthetic peptides. Following preincubation of the reaction mixture at 30°C, the reaction was initiated by the addition of substrate. After 50 min the reaction was terminated by boiling. Conditions were selected in which the reactions were linear with respect to time.

**RESULTS**

**CaM-Binding Domain in the Ca\(^{2+}\)/CaM-dependent Protein Kinase II**—When the amino acid sequences, deduced from the cDNA sequences, of the 50- (Hanley et al., 1987; Lin et al., 1987) and 60 (Bennett and Kennedy, 1987)-kDa subunits of rat brain Ca\(^{2+}\)/CaM-dependent protein kinase II are compared to the sequences of other protein kinases, certain regions, e.g. the ATP-binding site, are highly conserved. When the sequence comparison is confined to the myosin light chain kinases and phosphorylase kinase γ-subunit, residues 291 to 315 of the 50-kDa subunit and residues 292 to 316 of the 60-kDa subunit show homology to the CaM-binding domains of these other CaM-dependent protein kinases. Hanley et al. (1987) showed that a 25-residue peptide (290-314 of the 50-kDa subunit) did inhibit the CaM-dependent activation of brain cyclic nucleotide phosphodiesterase and produced a Ca\(^{2+}\)-dependent shift in the mobility of CaM in non-denaturing gel electrophoresis. To define further the CaM-binding domain of Ca\(^{2+}\)/CaM-dependent protein kinase II, six peptides were synthesized which contained different portions of the 290-309 50-kDa sequence as well as extensions in both the amino- and carboxyl-terminal directions (see Table I).

Peptide 290-309 was slightly more potent in terms of CaM binding than any of the other peptides. In the presence of a large excess of substrate (250 μM syntide-2) and limiting CaM (100 nM), it inhibited Ca\(^{2+}\)/CaM-dependent protein kinase II with an IC\(_{50}\) of approximately 52 nM (Fig. 1 and Table I). Peptides 296-309 and 294-319 also strongly inhibited the kinase under these conditions with IC\(_{50}\) values of approximately 57 and 77 nM, respectively (Fig. 1 and Table I). In all three cases the inhibition was reversed by addition of excess CaM, indicating that the mechanism of peptide inhibition was through interaction with CaM and not by a direct effect on the kinase. No inhibition was observed using peptides 281-290, 290-302, or 285-304 (Fig. 1). Similar results were obtained using glycogen synthase (1 mg/ml) as the substrate (not shown).

Further evidence that peptides 290-309, 296-309, and 294-319 bind CaM was obtained by two independent methods. First, when CaM-dependent phosphodiesterase was assayed in the presence of limiting CaM (2.38 nM) and saturating substrate, peptides 290–309, 296–309, and 294–319 again showed inhibition (Table I) with IC\(_{50}\) values between 1 and 4 nM. As with Ca\(^{2+}\)/CaM-dependent protein kinase II, peptides 290–302, 295–304, and 281–290 gave no inhibition at concentrations up to 10 μM (not shown).
Determinations of each parameter were made as described under "Experimental Procedures." For measurement of CaM antagonistic activity using Ca<sup>2+</sup>/CaM-dependent protein kinase II, 100 nM CaM and 250 μM syntide-2 substrate were used. In the substrate inhibition experiments 20 μM syntide-2 was used as substrate. CaM antagonistic activity using Ca<sup>2+</sup>/CaM-dependent phosphodiesterase was determined with 2.38 nM CaM. * no inhibition observed. a, not measurable due to significant phosphorylation of peptide 281–290. b, only 32% inhibition at 10 μM peptide 281–290. Results are expressed as the mean ± S.E. The number in parentheses indicates the number of experiments. The diagram at the bottom is a pictorial representation of the adjacent CaM-binding (CaM) and substrate-directed inhibitory (Inhib.) domains with probable overlap of determinations and the flanking phosphorylation (Phos.) sites.

| Peptide      | Sequence                          | Ca<sup>2+</sup>/CaM-dependent protein kinase | Phosphodiesterase, CaM antagonist IC<sub>50</sub> |
|--------------|-----------------------------------|---------------------------------------------|----------------------------------------------|
|              |                                   | IC<sub>50</sub> (nM) | IC<sub>50</sub> (pM) | IC<sub>50</sub> (nM) | IC<sub>50</sub> (pM) |
| 290–309      | LKKFNARRKLIGAILTTMLA              | 52.1 ± 3.4 (7) | 24.4 ± 2.8 (4) | 1.1       |                         |
| 290–302      | LKKFNARRKGA                      | *              | 53.5 ± 4.6 (5) | *          |                         |
| 295–304      | ARRLKIGAIL                       | *              | 199.0 ± 5.7 (3) | *          |                         |
| 296–309      | RRLKIGAILTMLA                    | 57.4 ± 3.5 (7) | 207.0 ± 6.7 (4) | 3.4       |                         |
| 294–319      | NARRKLIGAILTTMLATRNESBKBG        | 77.0 ± 3.8 (7) | 115.0 ± 6.8 (3) | 2.8       |                         |
| 281–290      | MBRQETVDC                       | *              | a              | b          |                         |

"Experimental Procedures." Assays contained indicated concentrations of synthetic peptides. (closed symbols) or 1 μM CaM (open symbols), together with the indicated concentrations of synthetic peptides. O, peptide 290–309; □, □, peptide 296–309; △, Δ, peptide 294–319; ●, ○, peptide 290–302; ▼, ▼, peptide 295–304. Data shown are from a single representative experiment.

**Substrate-directed Inhibition by Peptides**—Smooth muscle and skeletal muscle myosin light chain kinase contain regions associated with their CaM-binding domains that show competitive inhibition against substrates (Edelman et al., 1985; Kemp et al., 1987). It was therefore of interest to determine whether any of the six peptides shown in Table I exhibited substrate-directed inhibition. To avoid any complications due to CaM binding of the peptides, the autophosphorylated form of Ca<sup>2+</sup>/CaM-dependent kinase II, which is active in the presence of excess EGTA (Schworer et al., 1986), was used. When assayed in the presence of excess EGTA, the calcium-independent form of the kinase has a K<sub>c</sub> of about 20 μM for the substrate syntide-2 (Hashimoto et al., 1987). Using these assay conditions, peptides 290–309 and 290–302 inhibited the phosphorylation of syntide-2 with IC<sub>50</sub> values of approximately 25 and 50 μM, respectively (Table I). Peptide 294–319 gave an IC<sub>50</sub> of 115 μM, and peptides 295–304 and 296–309 had IC<sub>50</sub> values of approximately 200 μM. Peptide 281–290 was not tested since it was phosphorylated by the kinase (see below). Under these assay conditions, peptide 294–319 was not significantly phosphorylated.

The kinetic mechanism of the inhibition was next examined as a function of syntide-2 concentration (Fig. 2). With peptides 290–309, 290–302, and 296–309, the intercepts of the Lineweaver-Burk plots (Fig. 2, A–C) with increasing concentrations of peptides intersected close to or on the ordinate, indicative of competitive inhibition. In order to examine the nature of the competitive inhibition, Dixon plots (Fig. 2, D–F) were constructed and found to be nonlinear, indicating partially competitive inhibition for all three peptides. Likewise, slope replots from the Lineweaver-Burk data were also not linear (not shown).

Several control experiments were performed to assess the specificity of the substrate-directed inhibition. When another peptide substrate, K-R-R-P-Q-R-A-T-S-N-V-F-S (modeled after the sequence in smooth muscle myosin light chain), was used in the kinase assay, similar inhibition was obtained (not shown). When the phosphorylation of syntide-2 (20 μM) by cAMP-dependent protein kinase was measured, no inhibition by peptides 290–309 and 290–302 (up to 100 μM) was observed (not shown). However, when a protein substrate such as glycogen synthase (0.15 mg/ml) or casein (1 mg/ml) was used in the assay for Ca<sup>2+</sup>/CaM-dependent protein kinase II, no inhibition by peptide 290–309 (up to 158 μM) or peptide 290–302 (up to 280 μM) was obtained.

**Phosphorylation of Peptides by Ca<sup>2+</sup>/CaM-Dependent Protein Kinase II**—Examination of the amino acid sequences surrounding serine or threonine residues phosphorylated by Ca<sup>2+</sup>/CaM-dependent protein kinase II suggests a consensus sequence of -R-X-X-S/T- (Payne et al., 1983). The essential nature of the arginine three residues amino-terminal of the phosphorylated serine or threonine has been confirmed using synthetic peptides (Pearson et al., 1985; Soderling et al., 1986). Sequences -R-Q-E-T- (283–286) and -R-N-F-S- (311–314) conform to this consensus sequence and were therefore ex-
DISCUSSION

Three important questions concerning mechanisms of regulation of Ca\(^{2+}\)/CaM-dependent protein kinase II are the following: 1) why is the kinase inactive in the absence of Ca\(^{2+}\)/CaM?; 2) how does Ca\(^{2+}\)/CaM activate the kinase; and 3) how does autophosphorylation generate a Ca\(^{2+}\)-independent species of the kinase? Our understanding of each of these areas is enhanced by the results of this investigation which utilized synthetic peptides of the CaM-binding domain and flanking sequences. Although the peptides used in this study were synthesized from the 281-319 amino acid sequence of the 50-kDa subunit of the kinase (Lin et al., 1987), it should be emphasized that the corresponding sequence in the 50-kDa subunit (Bennett and Kennedy, 1987) differs by only a single Asp to Glu substitution at position 289 and several conservative substitutions at the extreme carboxyl terminus.

The 29-amino acid peptide 290–309 was the most potent of the six peptides tested in terms of both CaM-binding and in substrate-directed inhibition of the kinase (Table I). Thus, both of these regulatory properties reside in close proximity to each other in the primary sequence. Although there may be some overlap in amino acid determinants for these two properties, it is clear that different regions of peptide 290–309 are involved. For example, peptide 290–302 did not bind CaM but did show substrate-directed inhibition of the kinase that was almost as potent as peptide 290–309. Likewise, peptide 296–309 bound CaM with about the same affinity as peptide 290–309 but was relatively weak with regard to sub-

strate-directed inhibition. These results indicate that the majority of determinants for CaM binding reside in the carboxy-terminal portion, whereas the determinants of substrate-directed inhibition are localized predominantly in the amino-terminal portion of peptide 290–309 (see bottom diagram of Table I). The 10-residue peptide 295–304, comprising the middle portion of peptide 190–309, did not bind CaM and showed only weak substrate-directed inhibition. This result would be consistent with the above conclusion that the determinants for these two properties of peptide 290–309 are in the carboxy- and amino-terminal sequences, respectively. Alternatively, it is possible that peptide 295–304 was too short to form a stable interaction with CaM.

The kinetic analysis of the substrate-directed inhibition indicated partial-competitive inhibition with respect to the peptide substrate syntide-2 (Fig. 2). The Dixon plots were not linear, indicating that the inhibition was not strictly competitive. This is not surprising since the sequence of peptide 290–309 does not contain the consensus sequence of R-X-S/T found in good substrates of Ca\(^{2+}\)/CaM-dependent protein kinase II. Thus, binding of the inhibitory peptide may not be exactly the same as occurs with a substrate. Inhibition was only observed using peptides as substrates, either syntide-2 or the peptide modeled after smooth muscle myosin light chain. With either skeletal muscle glycogen synthase or casein as substrates, peptides 290–302 and 290–309 did not give inhibition. The explanation for this is not readily apparent but may relate to binding of peptides by glycogen synthase or casein as well as steric problems or multiple binding interactions with the larger protein substrates. Lack of substrate-directed inhibition was probably not due to binding of the peptides by glycogen synthase since CaM antagonism was observed with peptide 290–309 using glycogen synthase as substrate. On the other hand, substrate-directed inhibition was not due to some unusual interaction of the inhibitory peptides with the syntide-2 since phosphorylation of syntide-2 by cAMP-dependent protein kinase was not affected by peptides 290–302 and 290–309.

These results on substrate-directed inhibition by a sequence contiguous to the CaM-binding domain are very similar to analogous experiments using both skeletal muscle (Edelman et al., 1985) and smooth muscle (Kemp et al., 1987) myosin light chain kinases. Both of these enzymes have pseudosubstrate sites next to the CaM-binding sequences. Synthetic peptides containing these pseudosubstrate sites gave either strict competitive inhibition (skeletal muscle kinase) or non-linear competitive inhibition (smooth muscle kinase). The peptides which give substrate-directed inhibition of Ca\(^{2+}\)/CaM-dependent protein kinase II do not contain any apparent pseudosubstrate sequences. The sequence -R-R-K-L- would probably not be a good pseudosubstrate site since the addition of the second arginine residue (R-R-X-S/T) appears to be a negative determinant for Ca\(^{2+}\)/CaM-dependent protein kinase II (Soderling et al., 1986). Additional studies with substitutions of certain residues in peptide 290–302 may further define specificity determinants involved in the substrate-directed inhibition of Ca\(^{2+}\)/CaM-dependent protein kinase II.

Certain structural features are thought to be important for interaction of proteins or peptides with CaM. Peptides with widely divergent functions such as melittin, the mastoparans, and the peptide analogs derived from both smooth and skeletal muscle myosin light chain kinases and phosphorylase kinase all bind CaM with high affinity and share certain structural features thought to be important for interaction with CaM. These include the ability to form an amphipathic a-helix (Barnette et al., 1983; Malencik and Anderson, 1983;
Comte et al., 1983; Maulet and Cox, 1983), clusters of basic residues, and a hydrophobic region adjacent to the basic residues (Malencik and Anderson, 1982; Cox et al., 1985; O’Neil et al., 1987). Protein secondary structure analysis predicted that peptide 290–309, the most potent at binding CaM, would exist as about 80% α-helix (Fig. 3). Extending the peptide from 281 to 319 would reduce the calculated helical content to 59%. The average hydrophathy for the 290–309 sequence was calculated to be 0.0, while that of the 281 to 319 sequence was −0.6 (Garnier et al., 1978). The hydropathy analysis shown in Fig. 3 illustrates that the most hydrophobic region of the sequence 281–319 exists between residues 300 and 307, just carboxyl-terminal to the cluster of basic residues. The CaM-binding domain does not contain any acidic residues, which is important since CaM is an acidic protein. Furthermore, using the algorithm of Garnier et al. (1978), the secondary structure in this area would be predicted to be primarily α-helix (Fig. 3). Thus Ca<sup>2+</sup>/CaM-dependent protein kinase II contains a domain which conforms to the structural requirements thought to be important for CaM-binding proteins. Just amino-terminal to this CaM-binding domain there exists an inhibitory domain that may be responsible for inactivation of the kinase in the absence of Ca<sup>2+</sup>/CaM. Binding of Ca<sup>2+</sup>/CaM may induce α-helical structure, disrupting the inhibitory interaction with the catalytic site and thereby activating the kinase. This hypothesis would be consistent with the models proposed for myosin light chain kinase (Kemp et al., 1987) and may represent a common mechanism for other CaM-activated enzymes.

A unique property of Ca<sup>2+</sup>/CaM-dependent protein kinase II is in the formation of a substantially (60–80%) Ca<sup>2+</sup>-independent form upon Ca<sup>2+</sup>-dependent autophosphorylation (Saitoh and Schwartz, 1985; Miller and Kennedy, 1986; Schworer et al., 1986; Lai et al., 1986; Lou et al., 1986). This is associated with autophosphorylation of a threonine residue (Lai et al., 1987). Subsequent Ca<sup>2+</sup>-independent (i.e. in the presence of EGTA) autophosphorylation of the kinase results in loss of stimulation by Ca<sup>2+</sup>/CaM in the subsequent kinase assay (Hashimoto et al., 1987). The amino acid sequence (peptide 290–309) in the kinase which contains both the CaM-binding and substrate-directed inhibitory domains is flanked on both sides by potential autophosphorylation sequences, -R-Q-E-T- (peptide 283–286) and -R-N-F-S- (peptide 311–314). The peptides containing these sequences were both phosphorylated by Ca<sup>2+</sup>/CaM-dependent protein kinase II. Studies are currently in progress to assess the effects of phosphorylation of these sites on the substrate-directed inhibition and CaM-binding properties of the adjacent sequences.

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