Calmodulin Binding Domains: Characterization of a Phosphorylation and Calmodulin Binding Site from Myosin Light Chain Kinase†

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ABSTRACT: A protein kinase phosphorylation site in chicken gizzard myosin light chain kinase (MLCK) has been identified, and a synthetic peptide analogue of this site has been shown to be a high-affinity calmodulin binding peptide as well as a substrate for cyclic AMP dependent protein kinase. Phosphorylation of the site in MLCK is diminished when reactions are done in the presence of calmodulin. A fragment of MLCK containing the phosphorylation site was shown to have the amino acid sequence Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg-Ala-Ile-Gly-Arg-Leu-Ser-Ser. The interaction of calmodulin with a synthetic peptide based on this sequence was characterized by using circular dichroism and fluorescence spectroscopies and inhibition of calmodulin activation of MLCK. The peptide–calmodulin complex had an estimated dissociation constant in the range of 1 nM, underwent spectroscopic changes in the presence of calmodulin consistent with the induction of an α-helical structure, and interacted with calmodulin with an apparent 1:1 stoichiometry. Studies with other synthetic peptide analogues indicated that the phosphorylation of the serine residues diminished the ability of the peptide to interact with calmodulin even though the serines are not required for calmodulin binding. On the basis of the primary and secondary structural characteristics of these peptide analogues, a potential calmodulin binding region in another calmodulin binding protein, the γ subunit of rabbit skeletal muscle phosphorylase kinase, was identified. Overall, these studies provide a model for calmodulin binding domains in structurally diverse calmodulin binding proteins that contain clusters of basic residues within potential amphiphilic α-helical structures and provide a well-defined precedent for how a calmodulin binding domain and a phosphorylation site could be structurally related.

Calcium has a variety of biological functions, and a number of different structures in proteins appear to have evolved to allow various types of calcium binding [for a review, see Van Eldik et al. (1982)]. Although it is not possible yet to predict with any degree of certainty what type of calcium binding structure might be associated with a given biological role of calcium, a trend has begun to emerge from the detailed analysis of the family of calcium binding proteins referred to as calcium-modulated proteins. Calmodulin is a member of this family of structurally and functionally related calcium-modulated proteins, appears to be ubiquitous among eukaryotes, has multiple calcium binding sites in a single polypeptide chain, and has a number of biochemical activities. Because of calmodulin's apparent ubiquitous distribution among eukaryotes, multiple in vitro activities, and well-characterized chemical properties, it has become a standard of comparison for other calcium-modulated proteins. Although numerous proteins have been shown to interact with calmodulin in a calcium-dependent or -independent manner [for a review, see Klee & Vanaman (1982)], for only a few, such as myosin light chain kinase (MLCK),[1] phosphorylase kinase, phosphodiesterase, and NAD kinase, is there sufficient evidence for a physiological role in regulating cell function.

The structural features that allow calmodulin to interact with and modulate the activities of these enzymes and other binding proteins are being studied by using diverse, complementary approaches including physical and chemical studies [e.g., see Olwin et al. (1984), Marshak et al. (1985), Lukas et al. (1985), Malencik et al. (1982), and Newton et al. (1984)], comparative biochemical analyses (Watterson et al., 1984), and, more recently, site-specific mutagenesis studies (Roberts et al., 1985). Functional studies of calmodulins of known amino acid sequence and methylation state have shown that a limited number of conservative amino acid sequence differences among calmodulins can result in quantitative differences in the interaction with some enzymes but not others (Roberts et al., 1984). It remains to be determined whether these differences in activity reflect the utilization of different domains on the calmodulin molecule or whether these enzymes utilize similar domains on calmodulin but differ in the strictness of their structural requirements for interaction and activation. Therefore, concurrent with studies of how calmodulin structure is related to function, it is imperative that attempts be made to determine how structure is related to function in physiologically relevant calmodulin binding proteins.

Chicken gizzard MLCK is a calmodulin binding protein that can be obtained in sufficient quantities for chemical characterization, and the interaction of rabbit skeletal muscle and turkey gizzard MLCKs with calmodulin has been examined

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[1] Abbreviations: ATP, adenosine 5′-triphosphate; BOC, N^{α}-*tert*-butoxycarbonyl; CaM, calmodulin; cAMP, adenosine cyclic 3′,5′-phosphate; CD, circular dichroism; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography; MLCK, myosin light chain kinase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; ODS, octadecylsilanyl; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane.

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rather extensively (Olwin et al., 1984; Blumenthal et al., 1984; Malencik et al., 1982). Further, the reports (Conti & Adelstein, 1981; Ikebe et al., 1985) that cyclic nucleotide dependent protein kinases can readily phosphorylate gizzard MLCKs at a site that is affected by calmodulin interaction provide a convenient starting point in attempts to identify a potential calmodulin binding domain. In this report, we summarize our studies on the identification and characterization of a cyclic AMP dependent protein kinase phosphorylation site in chicken gizzard MLCK that appears to be a calmodulin binding domain and demonstrate that a homologous structure is found in another calmodulin binding protein, the γ subunit of skeletal muscle phosphorylase kinase.

Materials and Methods

General Procedures. Chicken gizzard MLCK was prepared according to Adelstein and Klee (1981), and chicken gizzard calmodulin was prepared essentially as described previously (Van Eldik & Watterson, 1979). The catalytic subunit of cyclic AMP dependent protein kinase was a generous gift from Dr. J. Corbin, Vanderbilt University. Trifluoroethanol was from Alpha Products (Danvers, MA). Buffers and other reagents were analytical grade. Acetonitrile for HPLC was from Burdick and Jackson, and water was from a MILLI-Q apparatus (Millipore, Inc.).

Amino acid analyses of peptide and protein hydrolysates were done with an LKB 4400 instrument or by precolumn derivatization with phenyl isothiocyanate (Bidlingmeyer et al., 1984) and analysis on a Waters PicoTag HPLC system. Automated Edman degradations were done on an Applied Biosystems 470A sequencer as described previously (Lukas et al., 1984). PTH-amino acid derivatives were identified by reversed-phase HPLC as described (Zimmerman et al., 1978) with modifications as reported previously (Lukas et al., 1984). Protein and peptide concentrations were determined by amino acid analysis after hydrolysis in 6 N HCl at 115 °C for 20–24 h.

Gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was done with 12.5% (w/v) acrylamide gels using mini-gel apparati described previously (Burgess et al., 1984). Autoradiography was done with Cronex film and a Cronex intensifying screen.

Phosphorylation by Protein Kinases. Phosphorylation of chicken gizzard MLCK by the catalytic subunit of cAMP-dependent protein kinase was done in both the presence and absence of calmodulin. Parallel experiments contained 0.2 mg/mL MLCK, 4 mM EGTA, 7 mM magnesium acetate, 55 μM [γ-^{32}P]ATP (2300 cpm/pmol), and 40 mM Tris, pH 7.4. Reactions done in the presence of calmodulin also contained 0.08 mg/mL chicken gizzard calmodulin and 4.2 mM $CaCl_2$. Reactions were initiated by the addition of 2 μg/mL catalytic subunit and allowed to proceed for 3 h at room temperature. Background phosphorylations were done similarly with no catalytic subunit added. Incorporation of radioactivity into MLCK was determined by spotting reaction aliquots taken at various time intervals onto phosphocellulose papers as described previously (Glass et al., 1978). For preparative work, chicken gizzard MLCK (1.19 mg) was dissolved in a buffer containing 30 mM $MgCl_2$ and 90 mM Tris, pH 7.3, to a concentration of 1.0 mg/mL. To this solution were added 0.476 mL of water, 0.357 mL of 1 mM [γ-^{32}P]ATP (522 cpm/pmol), and 0.357 mL of a 0.1 mg/mL solution of the catalytic subunit of cAMP-dependent protein kinase. After 30 min, a 5-μL aliquot of the reaction was spotted onto phosphocellulose paper for determination of the incorporation of ^{32}P. The reaction mixture was dialyzed against 10 mM ammonium bicarbonate at 4 °C overnight and then lyophilized. The phosphorylated protein was redissolved in 70% formic acid and treated with 7 mg of cyanogen bromide at room temperature for 18 h. The digest was then diluted 10-fold with water and lyophilized. The cleavage products were redissolved in 200 μL of 30% formic acid and then diluted to 1.5 mL with water. Peptides resulting from the cyanogen bromide cleavage were fractionated on a reversed-phase HPLC column (RP-P Synchrom, Linden, IN; 0.46 × 25 cm) eluted with 0.1% (v/v) aqueous trifluoroacetic acid (solvent A) and 0.05% (v/v) trifluoroacetic acid in acetonitrile (solvent B). The elution gradient was programmed as follows: 10 min, % B = 10; 60 min, % B = 60; 70 min, % B = 60.

MLCK activity was measured by incorporation of ^{32}P into a synthetic peptide (KKRPQRATSNVFAM) based upon the amino acid sequence of the phosphorylation site of chicken gizzard light chains (Pearson et al., 1984). Briefly, the assays were done in 25 mM MOPS, 7.5 mM 2-mercaptoethanol, 10 mM magnesium acetate, and 0.1 mM Ca^{2+}, pH 7.0. The peptide substrate concentration was 55 μM. Inhibition experiments were done with 8 nM calmodulin and various concentrations of inhibitor peptide. Assays were run in duplicate for an incubation time of 20 min and included control incubations without substrate or without added calmodulin. Incorporation of ^{32}P was determined by immobilization of the phosphopeptide on phosphocellulose papers.

Peptide Synthesis. Peptides were assembled on a *p*-methylbenzhydrylamine resin (USB) in a Beckman 990 synthesizer. A single coupling program employing dichloromethane and 2-propanol washes was used. A 5:1 (mol/mol) ratio of BOC-amino acids to resin amino groups was used, and dicyclohexylcarbodiimide was the activating agent. Protected amino acid derivatives included an *N*-formyl group on the indole of tryptophan. Peptide carboxamides were liberated by treatment with hydrogen fluoride–anisole, 9:1 (v/v), for 45 min at 0 °C.

Peptides were purified by chromatography on carboxymethylcellulose (CM-52) in pH 5.5 ammonium acetate buffers. Further purification was achieved by reversed-phase HPLC on a Waters μBondapak ODS column (0.79 × 30 cm) eluted with gradients of 0.1% (v/v) aqueous trifluoroacetic acid and acetonitrile. After chromatography, peptide fractions were lyophilized and then redissolved in water. The concentration of peptide stock solutions was determined after hydrolysis in 6 N HCl and amino acid analysis. Synthetic peptides gave the expected amino acid compositions, and structures were confirmed by automated Edman degradation. Peptide solutions used in enzyme assays were obtained by dilution of the stock solution with the appropriate assay buffer.

The indole *N*-formyl protecting group on tryptophan was removed by treatment of the peptide in 90% (v/v) dimethylformamide with 2.0 M (final concentration) piperidine. After incubation at room temperature for 30 min, the solution was neutralized with glacial acetic acid and diluted with 1 volume of water. The resulting solution was then chromatographed by HPLC as described above to recover the deprotected peptide. Ultraviolet absorption spectra of the peptide before and after deprotection indicated the removal of the protecting group by a shift in absorption maxima from 305 to 281 nm as noted earlier (Prystowsky et al., 1981).

Spectroscopic Measurements. CD spectra were measured in a Jasco J-500 spectropolarimeter equipped with a DP-500N processor. Each spectrum was averaged over 8–16 scans, and final spectra were corrected for specious signals generated by the solvent. Difference CD spectra were calculated as de-

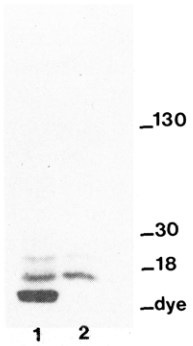

FIGURE 1: Autoradiogram of phosphorylated peptides separated on a 12.5% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. Lanes 1 and 2 contain labeled peptides from the cyanogen bromide digests of MLCK which was phosphorylated by catalytic subunit in the absence (lane 1) or presence (lane 2) of calmodulin.

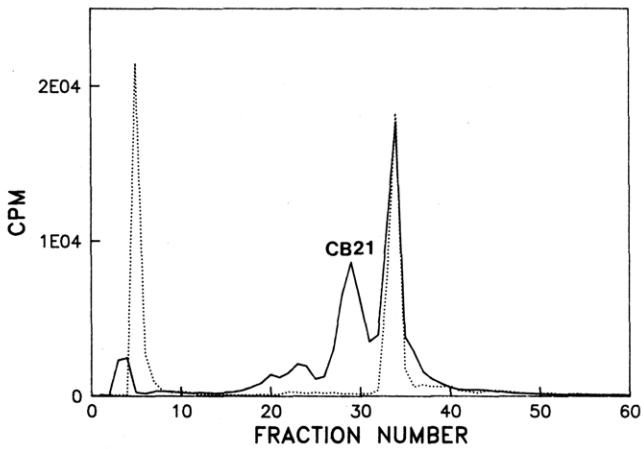

FIGURE 2: HPLC trace of radioactive peptides from cyanogen bromide digest of MLCK. Chicken gizzard MLCK was phosphorylated in the presence or absence of calmodulin as described under Materials and Methods. The labeled proteins were digested with cyanogen bromide, and approximately the same quantity of radioactivity [(2.6–3.4) × 10^5 cpm] was loaded onto the column. The solid line represents the digest of MLCK phosphorylated in the absence of calmodulin, and the dotted line represents the digest of MLCK phosphorylated in the presence of calmodulin (see Figure 1). The eluate was collected as 1-min fractions and the radioactivity in each measured by Cherenkov counting in a Beckman LS 7500 counter. Total recovery of radioactivity was 44–53%.

scribed previously (McDowell et al., 1985), and data were expressed as mean residue ellipticity. Helical contents were estimated according to Chen and Yang (1971).

Fluorescence measurements were made on an SLM 4800 phase fluorometer. Tryptophan from the peptide or peptide–calmodulin complex was excited at 295 nm to minimize the contribution from tyrosine (in calmodulin) to the emission spectra. Excitation and emission bandwidths were, respectively, 2 and 4 nm. Spectra were corrected for the wavelength dependence of detector response. Fluorescence anisotropy was measured either in the SLM 4800 instrument or by photon counting on an SLM 8000 fluorometer. For these experiments, the exciting light (300 nm) was vertically polarized and passed through a slit with 1-nm band-pass. Processing of the fluorescence data was done as described (McDowell et al., 1985).

Results

Chicken gizzard myosin light chain kinase was phosphorylated by the catalytic subunit of cAMP-dependent protein kinase to a maximal stoichiometry of 3.0–3.2 mol of ^{32}P per mole of MLCK. However, in the presence of calcium and calmodulin, incorporation of 0.6–0.8 mol of ^{32}P per mole of kinase was obtained upon phosphorylation by the catalytic subunit. Analysis of the labeled MLCK preparation by SDS–polyacrylamide gel electrophoresis, followed by autoradiography of the gel, revealed a major phosphorylated protein band at approximately M_r 130000 that coincided with Coomassie Blue staining material. Incubation of MLCK in the absence of catalytic subunit under the same conditions resulted in the incorporation of 0.20–0.28 mol of phosphate, while in the presence of calmodulin less than 0.10 mol of radiolabeled phosphate was incorporated per mole of kinase. Thus, the presence of calmodulin reduces the rate of incorporation of radioactivity as well as the total amount of radioactivity incorporated into MLCK in the presence or absence of catalytic subunit.

Cleavage of phosphorylated chicken gizzard MLCK with cyanogen bromide resulted in at least three distinct phosphorylated peptides as shown in Figure 1. The presence of calmodulin during the phosphorylation reaction diminished dramatically the labeling of one peptide, designated CB21 (Figure 1). This peptide could be isolated easily for further characterization by using HPLC for fractionation of the digest (Figure 2). In agreement with the polyacrylamide gel analysis data (Figure 1), the amount of radioactivity (Figure 2) associated with CB21 (peak 1) was diminished in the digest of MLCK phosphorylated in the presence of calmodulin. The other two labeled peptides seen in Figure 1 are found in peak 2 of Figure 2. The major low molecular weight peptide phosphorylated in the absence of calmodulin (CB21), but protected in its presence, was the primary target of characterization because of its potential of being directly involved in the binding of calmodulin by MLCK.

The amino acid composition (molar ratio) of CB21 was the following: Asp, 1,3; Thr, 1.3; Ser, 2.9; Glu, 2.8; Gly, 3.8; Ala, 3.6; Val, 1.5; Ile, 1.0; Leu, 1.5; His, 1.0; Lys, 2.6; Arg, 3.0; homoserine, 0.4. When peptide CB21 was subjected to automated Edman degradation, the following amino acid sequence was obtained: Ala-Arg-Arg-Lys-Trp-Gln-Lys-Thr-Gly-His-Ala-Val-Arg-Ala-Ile-Gly-Arg-Leu-Ser-Ser-Ser. The amount of PTH-Ser identified at each of the last three successful cycles of Edman degradation was, respectively, 76, 84, and 57 pmol followed by three cycles in which no clear identification of a PTH-amino acid was made. On the basis of the amino acid composition and the Edman degradation data, a tentative assignment of a Ser-Ser-Ser sequence was made. However, the placement of the third serine must be considered highly tentative. Current studies with overlap peptides and cDNA sequences should confirm or refute this tentative assignment. An unequivocal determination of the phosphorylation site of peptide CB21 could not be demonstrated because phosphorylated serines and threonines and their degradation products are not efficiently recovered with the gas phase sequencer technology (Murray et al., 1983). However, on the basis of the known specificity of cAMP-dependent protein kinase (Kemp et al., 1977), the first of the three serine residues following Arg-Leu is the likely phosphorylation site. In previous studies of cyanogen bromide digests of chicken gizzard MLCK, we had observed that the mixture of peptides recovered from the cleavage reaction retained the ability to inhibit at low concentrations (<10 nM) the calmodulin activation of MLCK (D. M. Watterson and T. J. Lukas, unpublished results). Due to limitations in material, however, the inhibitory activity of a purified peptide from the cyanogen

Table I: Calmodulin Binding Synthetic Peptides Based on the Amino Acid Sequence of Gizzard Myosin Light Chain Kinase

peptide code	structure
RS20	RRKWQKTGHAVRAIGRLSSS
KS18	KWQKTGHAVRAIGRLSSS
RL17	RRKWQKTGHAVRAIGRL

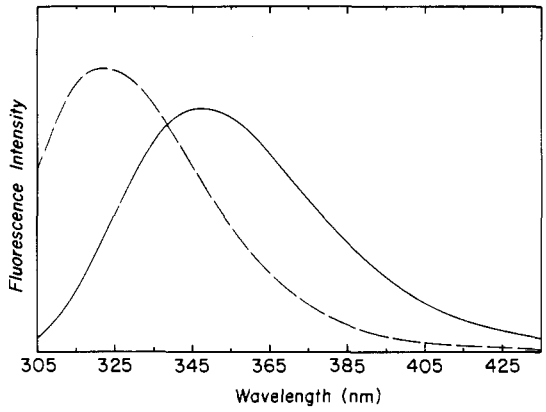

FIGURE 3: Corrected fluorescence emission spectra of RS20 in aqueous solution (—) and bound to the Ca^{2+}–calmodulin complex (– –). All experiments were conducted at 25 °C in a 0.05 M KCl and 0.025 M MOPS buffer, pH 7.0, containing 10^{-4} M Ca^{2+}.

bromide digest of unphosphorylated MLCK was not attempted. We, therefore, synthesized a peptide, referred to as RS20 (Table I), based on the elucidated amino acid sequence of CB21.

Peptide RS20 was tested for inhibition of calmodulin activation of gizzard MLCK. Significant inhibition was observed at peptide concentrations as low as 10 nM. The inhibition of activation by RS20 was completely reversed when the calmodulin concentration was increased. The dissociation constant for inhibition was calculated from the kinetic data by the method described by Cox et al. (1985). The K_d value determined from the data is 1.6 ± 0.3 nM. Analysis of the data also indicated that the calmodulin titration of MLCK activity gives a Hill coefficient of 1.4 in the presence and absence of RS20.

Previous studies of the binding of chicken gizzard MLCK to calmodulin have shown that a tryptophan residue of MLCK undergoes a change in environment when calmodulin is bound to the enzyme (Malencik et al., 1982). Similarly, a direct interaction between RS20 and calmodulin was demonstrated by analysis of the change in the fluorescence properties of the tryptophan residue in the peptide in the presence and absence of calmodulin. As seen in Figure 3, the corrected emission spectrum undergoes a blue shift and an increase in F_{max} in the presence of Ca^{2+} and calmodulin. A fluorescence anisotropy titration of the binding of RS20 to calmodulin is shown in Figure 4. The maximal anisotropy appears at a calmodulin:peptide ratio of 0.93, implying stoichiometry not greater than 1:1. The shape of the curve suggests that at a calmodulin concentration of 10^{-8} M (equivalent to the peptide concentration) there is no free peptide, which would be consistent with a dissociation constant of 10^{-9} M for the complex.

In addition to RS20 exhibiting spectral changes in the presence of calmodulin that are consistent with studies of calmodulin and intact MLCK, peptide RS20, like MLCK, could also serve as a substrate for the catalytic subunit of cAMP-dependent protein kinase. Peptide RS20 was phosphorylated by the protein kinase under conditions similar to those used for MLCK. A time course of the phosphorylation (Figure 5) indicated that up to 0.75 mol of phosphate per mole

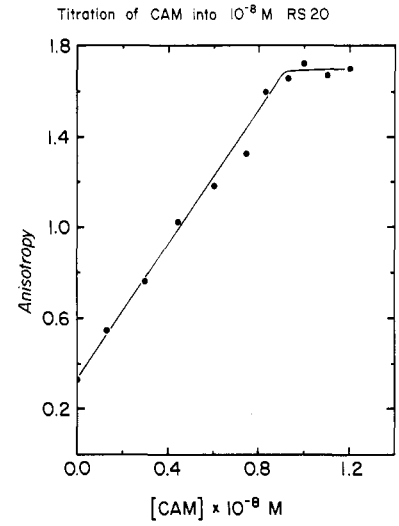

FIGURE 4: Fluorescence anisotropy titration of peptide binding to calmodulin. RS20 was dissolved at 10^{-8} M (from dry weight) in 0.15 M KCL and 0.025 M MOPS, pH 7.0, containing 10^{-4} M Ca^{2+} and 0.02 M PEG 2000. The PEG 2000 was added to minimize adsorption of the peptide to glass. Calmodulin was dissolved in the same solvent and added as indicated on the plot. Fluorescence anisotropy was measured as described (McDowell et al., 1985) with excitation at 300 nm and tryptophan emission isolated from Rayleigh and Raman scattering by use of a Schott WG 340 cutoff filter. The anisotropy of tryptophan emission of the free peptide was 0.033 and of the fully bound peptide 0.170.

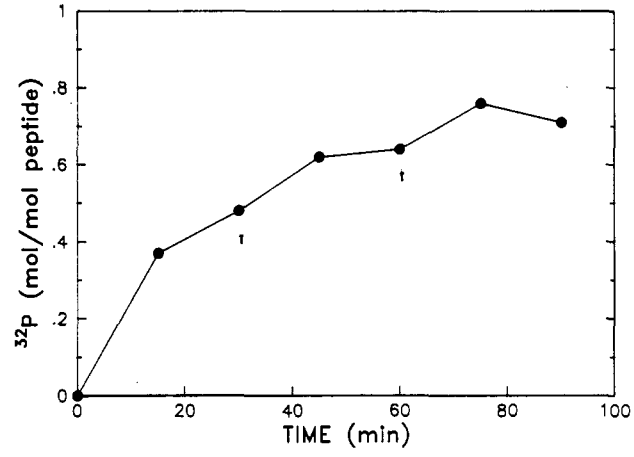

FIGURE 5: Phosphorylation of peptide RS20 by the catalytic subunit of cAMP-dependent protein kinase. The peptide at 62.5 μM was incubated in 30 mM Tris buffer, pH 7.4, containing 12.5 mM magnesium acetate, 1 mM EGTA, and 250 μM [γ-^{32}P]ATP (100 cpm/pmol). The reaction was initiated by addition of 2.9 μg/mL kinase. Two additional aliquots of kinase were added as indicated by the arrows on the plot. A control sample of RS20 was incubated for the same length of time in buffer without added ATP or kinase. The peptides were then recovered from the reaction by HPLC on a reversed-phase column as described under Materials and Methods.

of peptide was incorporated. Analysis of tryptic peptides generated from phosphorylated RS20 indicated that phosphate was incorporated into the serine residues at the carboxy terminus of the peptide (data not shown). Interestingly, the mixture of phosphorylated and unphosphorylated RS20 was found to be diminished in its ability to inhibit calmodulin activation of MLCK (Figure 6), indicating a decreased ability to interact with calmodulin. Since the peptide was not phosphorylated stoichiometrically, a quantitative comparison of the activity of phosphorylated RS20 with the unmodified peptide was not possible.

Although phosphorylation of the site appears to affect interaction with calmodulin, the phosphorylation site serines are

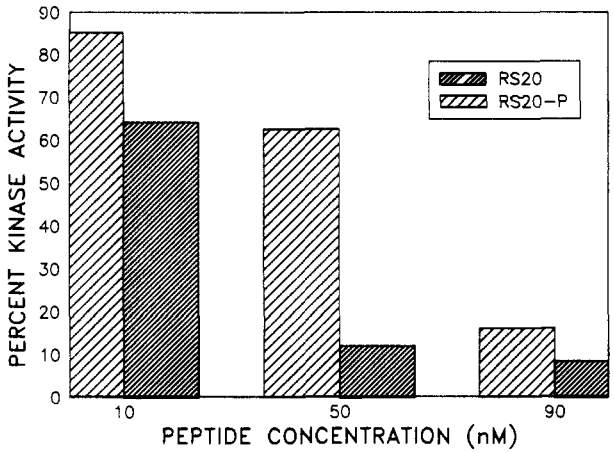

FIGURE 6: Comparison of the inhibitory activity of phosphorylated RS20 (RS20-P) with that of the unmodified peptide (RS20). Peptides RS20 and RS20-P were assayed as described under Materials and Methods. As stated in the text, the RS20-P preparation contained 0.75 mol of ^{32}P per mole of peptide.

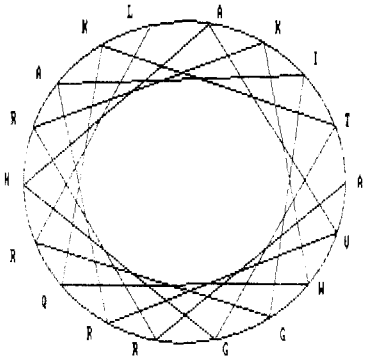

FIGURE 7: Helical wheel projections for the peptide CB21 minus the carboxy-terminal serines.

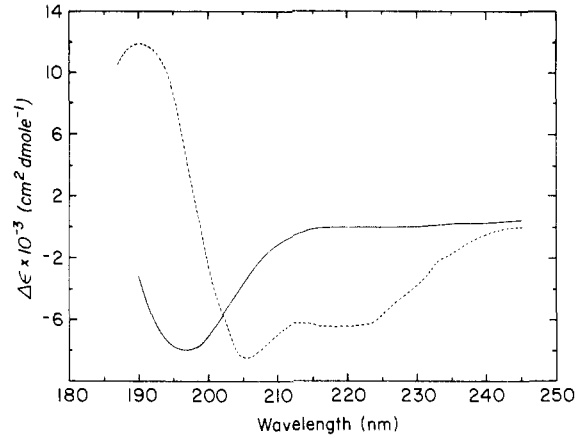

FIGURE 8: Far-UV CD spectra at 25 °C of peptide RS20. The peptide was dissolved in water, and spectra were measured in the presence (---) and absence (—) of the Ca-calmodulin complex. The former spectrum was recorded as the difference spectrum of Ca^{2+}-calmodulin + RS20 minus Ca^{2+}-calmodulin. For all experiments with Ca^{2+}-calmodulin, the complexes were first formed in water and then diluted to 1.5 mL with 100 mM phosphate buffer, pH 7.0, to give a final concentration of 10^{-5} M calmodulin and 10^{-4} M Ca^{2+}, without or with peptide (10^{-5} M). A 0.5-cm CD cell was used in all experiments. The CD spectrum of the protein-bound peptide is identical with that for RS20 dissolved in 70% trifluoroethanol.

Table II: Comparison of Calmodulin Binding Peptides

peptide code	structure
RS20	RRKWQKTGHAVRAIGRLSSS
γRL17	REIVIRDPYALRPLRRL[a]
γRL24	RRVKPVTREIVIRDPYALRPLRRL[b]
M13	KKRWKKNFIAVSAANRFKKISSSGALM[c]

[a] Peptide made in this study but based on the amino acid sequence of residues 329–345 of the γ subunit of rabbit skeletal muscle phosphorylase kinase (Reimann et al., 1984). [b] Peptide made in this study but based on the amino acid sequence of residues 322–345 of the γ subunit of phosphorylase kinase. [c] Calmodulin binding peptide from rabbit skeletal muscle myosin light chain kinase (Blumenthal et al., 1985).

not required for calmodulin binding. Peptide RL17 (Table I) lacks the COOH-terminal serines but, like RS20, is able to inhibit calmodulin activation of MLCK, and this inhibition is reversed by the addition of excess calmodulin. In contrast, the cluster of basic residues at the amino terminus of RS20 appears to play a major role in peptide binding. Peptide KS18 (Table I) lacks the two arginines found at the amino terminus of RS20 and is about 8-fold less effective (K_d = 13.5 ± 0.5 nM) than RS20 as an inhibitor of the calmodulin activation of MLCK. Thus, it appears that RS20 is the minimal amino acid sequence required for both a binding and a phosphorylation site. We did not pursue as part of these studies the possible structural consequences seen after phosphorylation of RS20, although differences in the conformation of the peptide when it is bound to calmodulin are clearly a possibility based on the spectroscopy studies reported here.

Recently, several laboratories have suggested that model peptides that bind to calmodulin with high affinity contain a potential amphiphilic helix structure (Cox et al., 1985; McDowell et al., 1985). A two-dimensional projection (Schiffer & Edmunson, 1967) of CB21 (Figure 7) indicates that CB21 and RS20 contain a potential amphiphilic structure that has four arginine residues and one lysine on its polar face. The apolar face of the helix contains tryptophan, valine, isoleucine, and an alanine residue. Analysis of the circular dichroism spectrum of RS20 (Figure 8) indicates that it exists as a random coil in aqueous solution but undergoes a transition to about 60% α helix in the presence of trifluoroethanol or Ca^{2+} and calmodulin. Thus, RS20 has an amino acid sequence that has the potential of forming an amphiphilic helix and actually assumes an α-helical structure upon binding to calmodulin.

In an attempt to test the general occurrence of this calmodulin binding structure or homologues of it in proteins of known or predicted amino acid sequence, electronic data bases of structures (National Biomedical Research Foundation, May 1985) and the recent literature (Medline) were searched, respectively, for homologies in primary structure or reports of amino acid sequences of calmodulin binding proteins. The protein data base did not yield another protein with significant amino acid sequence homology. Among recent structures of protein kinases, however, a 17-residue segment of the amino acid sequence (Reiman et al., 1984) of the γ subunit of skeletal muscle phosphorylase kinase, a calmodulin binding protein, was found to be homologous to the calmodulin binding peptides RS20 and RL17. Therefore, a peptide (γRL17) corresponding to this segment of the γ subunit was synthesized (Table II) and tested for its ability to inhibit calmodulin activation of MLCK under conditions identical with those used for MLCK and RL17. The peptide had calmodulin inhibitory activity only at concentrations 1000-fold higher than RS20 or RL17. However, several residues upstream (toward the amino terminus) from the γRL17 segment are basic amino acids that, based on the results with KS18, might contribute to a possible interaction with calmodulin. Thus, more extended regions of the γ subunit sequence were synthesized on the basis of the known primary and secondary structural features of RS20 and RS17. The larger peptide, γRL24, contains seven additional

residues from the sequence of the γ subunit at the amino terminus of γRL17 (Table II). When tested for inhibition of the calmodulin activation of MLCK, this peptide was a significantly better inhibitor than γRL17. The K_d estimated from the inhibition data was 20 nM. Thus, a region of γ subunit which contains a potential calmodulin binding domain encompasses residues 322–345.

Discussion

A 20-residue peptide (RS20) corresponding to part of the amino acid sequence of a phosphorylation site from chicken gizzard MLCK has been shown to have the structural requirements necessary for an effective calmodulin binding structure and cAMP-dependent protein kinase phosphorylation site. While the phosphorylation site and the calmodulin binding site are overlapping in the amino acid sequence of MLCK, the phosphorylatable serine residues are not, within the limitations of the assays used, part of the minimal calmodulin binding site. The cluster of basic amino acids at the amino terminus of the site, however, is required for effective calmodulin binding activity. Although it is possible that more extended amino acid sequences may have greater or more selective calmodulin binding activity, quantitative calmodulin binding activity appears, under the conditions of our assays, to be found in a specific 18–20-residue peptide. Analysis of the secondary structure of the peptide in the presence of calmodulin and calcium demonstrated an α-helical structure that is consistent with earlier studies of model compounds. Examination of the amino acid sequence of the γ subunit of phosphorylase kinase in light of the secondary structural properties of RS20 and its analogues revealed a potential calmodulin binding site. This latter structure was shown to be a calmodulin binding peptide on the basis of its ability to inhibit calmodulin activation of MLCK under conditions identical with those used for the assay of RS20. Overall, these studies suggest that there may be at least one type of calmodulin binding domain in different calmodulin binding proteins that is not readily recognized from primary structural features alone but may be apparent from an analysis of secondary structural characteristics.

Previous studies of the phosphorylation of turkey gizzard (Conti & Adelstein, 1981), chicken gizzard (Ikebe et al., 1985), and platelet MLCK (Nishikawa et al., 1984) suggested that one of the sites phosphorylated by the cAMP-dependent protein kinase might be in close proximity to the calmodulin binding site of the enzyme. Our studies of gizzard MLCK phosphorylated in the presence and absence of calcium and calmodulin are consistent with these observations since the phosphorylated cyanogen bromide peptide CB21 is not found in digests of the enzyme which had been phosphorylated in the presence of calmodulin. Foyt et al. (1985) have mapped phosphorylated peptides as well as calmodulin binding fragments of chicken gizzard MLCK generated by proteolytic cleavage. Our results are consistent with their suggested location of a cAMP-dependent protein kinase phosphorylation site on the carboxy-terminal side of the calmodulin binding domain. In our studies, we approached the problem directly by digesting phosphorylated chicken gizzard MLCK with cyanogen bromide and locating a 21 amino acid segment of the molecule that contains one of the phosphorylation sites for cAMP-dependent protein kinase and exhibits the structural features necessary for calmodulin binding. A synthetic peptide based on this amino acid sequence bound to calmodulin with high affinity, inhibited the calmodulin activation of MLCK, and could be phosphorylated by the catalytic subunit of cAMP-dependent protein kinase. Although the exact stoichiometry of phosphate incorporation into MLCK differs between our studies and earlier reports, this could be due to differences in experimental conditions or phosphate content of the purified protein [for a discussion of this for other protein kinases, see Flockhart & Corbin (1982)].

Structural studies of rabbit skeletal muscle MLCK (Blumenthal et al., 1985) have localized a calmodulin binding domain at the carboxy terminus of the molecule. In contrast to the chicken gizzard MLCK, the calmodulin binding site from skeletal muscle MLCK apparently is not a phosphorylation site. However, it is homologous in primary and secondary structure to peptides CB21, RS20, and γRL24 described in this work. Analysis of the possible secondary structure of the calmodulin binding peptides from chicken gizzard and rabbit skeletal muscle MLCK reveals that both exhibit considerable helix potential and contain extended (at least 17 residues) basic amphiphilic regions. Characterization of the binding of RS20 to calmodulin by tryptophan fluorescence indicated a change in environment for the peptide, and analysis of circular dichroism spectra indicated the induced formation of α helix upon binding to calmodulin. These properties have recently been ascribed to high-affinity calmodulin binding model peptides such as mellitin (Cox et al., 1985), mastoparans (McDowell et al., 1985), and related synthetic peptides (Cox et al., 1985), as well as the synthetic peptide based on the calmodulin binding fragment of skeletal muscle MLCK (Blumenthal et al., 1985).

To further substantiate the hypothesis that RS20 forms an amphiphilic helix, we calculated the mean hydrophobic moment ($\hat{\mu}_H$) of the peptide as described by Eisenberg et al. (1982). The mean hydrophobic moment for the entire peptide was 0.54, but considerably higher values (0.68) are observed if one considers an 11 amino acid segment centered on residue 13. Thus, consonant with the characteristics of model calmodulin binding peptides (Cox et al., 1985; McDowell et al., 1985; Comte et al., 1985), RS20 is a "surface-seeking" peptide. Although the Chou–Fasman algorithm predicts only a short helical segment in residues 10–15 and the Robson method (Garnier et al., 1978) predicts essentially zero helix in RS20, the CD data obviously support the predictions made on the basis of the hydrophobic moment calculations. However, it is important to note that the hydrophobic moment, per se, is not the sole determinant of binding to calmodulin [see discussion by McDowell et al. (1985)].

In contrast to the model or exogenous calmodulin inhibitor peptides, the segments of the MLCKs from gizzard or skeletal muscle which contain binding peptides are more hydrophilic and basic than the mastoparans or mellitin. Peptide RS20 contains 7 of 20 hydrophilic amino acids (35%) but only 4 hdyrophobic residues (20%). The mastoparans, on the other hand, have 21–28% hydrophilic amino acids and 36–50% hydrophobic residues. If calmodulin binding domains within a protein structure were predominantly hydrophobic, one would anticipate less surface exposure of the amino acid side chains required for contact and effective binding of calmodulin, with a resultant capability for signal transmission in response to calcium fluxes. Therefore, the interaction of calmodulin with its target enzymes may involve strong electrostatic elements, especially in early steps in the mechanism, which arise from the charged residues presented by calmodulin binding domains such as those found in myosin light chain kinases. Mechanistically, the binding of calmodulin to such exposed domains with resultant induction of a more helical structure may perhaps relieve intramolecular interactions of the kinase which prevent productive substrate binding or catalysis.

It is not clear, based on the results of this study, at what stage of the proposed mechanism phosphorylation may be exerting its effect. Clearly, more quantitative and detailed studies of stoichiometrically phosphorylated peptides and their analogues are required before statements concerning the mechanism can be made. However, the observation that phosphorylation of RS20 decreased the ability of the peptide to inhibit the calmodulin activation of MLCK demonstrates the feasibility of such studies and provides a precedent for a possible structural relationship among calmodulin binding and phosphorylation sites (Watterson et al., 1984). From a physiological perspective, previous studies of gizzard MLCK phosphorylated by cAMP-dependent protein kinase (Conti & Adelstein, 1981) have suggested that phosphorylation may be involved in the regulation of kinase activity through the attenuation of calmodulin activation. Similarly, studies of other nonmuscle MLCKs from brain (Hathaway et al., 1981), platelet, and trachea (Nishikawa et al., 1983) have suggested a regulatory phosphorylation site. Our results indicate that this site may be localized to a domain in chicken gizzard MLCK that includes cyanogen bromide fragment CB21. Since this fragment contains a threonine and two or three serines, it also may be phosphorylated by other kinases such as protein kinase C. The elucidation of these phosphorylation sites as well as calmodulin binding domains from other enzymes will be useful in attempts to delineate the significance of phosphorylation as a regulatory mechanism for calmodulin-dependent enzymes.

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Registry No. RS20, 100113-40-4; γRL17, 100113-41-5; γRL24, 100113-42-6; M13, 100165-35-3; MLCK, 51845-53-5; protein kinase, 9026-43-1; phosphorylase kinase, 9001-88-1.

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