Interaction of Calmodulin with the Calmodulin Binding Domain of the Plasma Membrane Ca^{2+} Pump†

Thomas Vorherr,‡ Peter James,‡ Joachim Krebs,‡ Agnes Enyedi,§ Daniel J. McCormick,∥ John T. Penniston,∥ and Ernesto Carafoli*,‡

Laboratory of Biochemistry, Swiss Federal Institute of Technology (ETH), 8092 Zurich, Switzerland, Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, Minnesota 55905, and National Institute of Haematology and Blood Transfusion, 1113 Budapest, Hungary

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ABSTRACT: Peptides corresponding to the calmodulin binding domain of the plasma membrane Ca^{2+} pump (James et al., 1988) were synthesized, and their interaction with calmodulin was studied with circular dichroism, infrared spectroscopy, nuclear magnetic resonance, and fluorescence techniques. They corresponded to the complete calmodulin binding domain (28 residues), to its first 15 or 20 amino acids, and to its C-terminal 14 amino acids. The first three peptides interacted with calmodulin. The K value was similar to that of the intact enzyme in the 28 and 20 amino acid peptides, but increased substantially in the shorter 15 amino acid peptide. The 14 amino acid peptide corresponding to the C-terminal portion of the domain failed to bind calmodulin. 2D NMR experiments on the 20 amino acid peptides have indicated that the interaction occurred with the C-terminal half of calmodulin. A tryptophan that is conserved in most calmodulin binding domains of proteins was replaced by other amino acids, giving rise to modified peptides which had lower affinity for calmodulin. An 18 amino acid peptide corresponding to an acidic sequence immediately N-terminal to the calmodulin binding domain which is likely to be a Ca^{2+} binding site in the pump was also synthesized. Circular dichroism experiments have shown that it interacted with the calmodulin binding domain, supporting the suggestion (Benaim et al., 1984) that the latter, or a portion of it, may act as a natural inhibitor of the pump.

Calmodulin activates the Ca^{2+} pump of plasma membranes by interacting with a domain next to its carboxy terminus (Shull & Greeb, 1988; Verma et al., 1988). The domain, which contains approximately 30 amino acids, has been identified and sequenced with the help of a bifunctional cross-linker coupled to calmodulin (James et al., 1988). It shows homology to the putative calmodulin binding domains of other calmodulin-modulated proteins (Blumenthal et al., 1985; Lukas et al., 1986; Buschmeier et al., 1987; Harris et al., 1988): the abundance of positively charged and hydrophobic residues in the N-terminal portion of the domain and the general propensity to form an amphiphilic helix are distinctive characteristics. The presence of a tryptophan at approximately the same position in the N-terminal portion of the domain is also worth mentioning: this residue is in fact found even in the adenylate cyclase of *Bordetella pertussis*, whose calmodulin binding domain appears somewhat atypical in that it contains several negatively charged residues (Glaser et al., 1988). One could thus imagine a specific role for the conserved tryptophan in the interaction with calmodulin; indications that this could indeed be the case have already appeared for the case of myosin light chain kinase (Cox et al., 1984; Blumenthal et al., 1988).

Earlier proteolytic work on the purified erythrocyte Ca^{2+} pump (Zurini et al., 1984; Benaim et al., 1984) had led to the suggestion that the calmodulin binding domain functions as an inhibitor of the Ca^{2+}-ATPase: calmodulin would relieve the inhibition by binding to the domain and removing it from the active site of the pump. No indications were provided by the proteolytic work on whether the active site influenced by the domain is the site of aspartic acid phosphorylation and/or ATP binding or a site of Ca^{2+} binding. Recent sequencing work (Verma et al., 1988) has identified a negatively charged sequence immediately N-terminal to the calmodulin binding domain and has led to the suggestion that this may be the (or one of the) Ca^{2+} binding site(s). Thus, the calmodulin binding domain could inhibit the pump by binding to the negatively charged site, preventing Ca^{2+} access to it.

In the work described in this paper peptides corresponding to the calmodulin binding domain of the Ca^{2+} pump and to its putative Ca^{2+} binding site have been synthesized and used to study the interaction of the former domain with calmodulin and with the putative Ca^{2+} binding site N-terminal to it. The interactions have been monitored by following the conformational changes of the peptides with circular dichroism (CD),[1] infrared spectroscopy (IR), nuclear magnetic resonance (NMR), and fluorescence spectroscopy. The role of the conserved tryptophan has been investigated by replacing

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‡ Swiss Federal Institute of Technology.

§ National Institute of Haematology and Blood Transfusion.

∥ Mayo Clinic.

[1] Abbreviations: Boc, tertiary butoxycarbonyl; CD, circular dichroism; DC, dansylcalmodulin; DCC, dicyclohexylcarbodiimide; DQF-COSY, double quantum filtered correlated spectroscopy; DVB, divinylbenzene; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Fmoc, 9-fluorenylmethyloxycarbonyl; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; IR, infrared spectroscopy; MTR, 4-methoxy-2,3,6-trimethylbenzenesulfonyl; MTS, mesitylene-2-sulfonyl; NMR, nuclear magnetic resonance; NOESY, two-dimensional nuclear Overhauser and exchange spectroscopy; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulfonyl; tBu, tertiary butyl; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluorethanol; TFMSA, trifluoromethanesulfonic acid; TOCSY, total correlated spectroscopy.

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it with either alanine or tyrosine residues in the synthetic peptides. The results support the suggesting of a role for the conserved tryptophan in the interaction of calmodulin. They have also shown that the calmodulin binding domain interacts with the putative Ca^{2+} binding domain N-terminal to it.

Materials and Methods

Materials

The reagents used were of the highest purity grade available and were purchased from Fluka AG, Buchs, Switzerland. Sperm whale myoglobin, bovine pancreas ribonuclease, Hepes, dansyl chloride, and EGTA were obtained from Sigma Chemical Co., St. Louis, MO. Dialysis tubes were bought from Spectrum Medical Industries, Los Angeles, CA. For peptide synthesis and sequencing the products of Applied Biosystems, Foster City, CA, were used. The amino acid analysis of the peptides was kindly performed by Dr. G. Frank (Zurich) using the ninhydrin procedure. Hydrolysis was performed at 110 °C for 24 h.

Methods

Preparation of Calmodulin. Calmodulin was isolated from bovine brain as described by Guerini et al. (1984). In the final purification step the protein was passed through a Sephadex G-100 column (2.5 × 100 cm, Pharmacia, Uppsala, Sweden) equilibrated in 100 mM NH_4HCO_3. The calmodulin-containing fractions were pooled, lyophilized, and stored at −70 °C. Calmodulin was made Ca^{2+} free by passing it through a Chelex 100 column (0.9 × 20 cm, Bio-Rad, Richmond, CA) in H_2O. The concentration of the calmodulin samples was determined spectrophotometrically by using the molar extinction coefficient $\epsilon_{276nm} = 1.8$ (Klee, 1977). The concentration of the standard was determined by amino acid analysis. The samples for the NMR measurements were lyophilized twice from D_2O (99.95%, Merck, Darmstadt, FRG) and finally dissolved in 500 μL of D_2O. The final concentration was 2 mM, and the pH was adjusted to 6.0. The $CaCl_2$ concentration was 10 mM, and the sample also contained 0.2 M KCl.

Synthesis of Peptides. The sequences of the peptides synthesized are shown under Results. The crude products of peptides A18, C14, C28A, and C28Y were the kind gift of Dr. B. Riniker, Ciba-Geigy, Basle, Switzerland. The products were obtained after solid-phase synthesis on a Ciba-Geigy-built peptide synthesizer using polystyrene resin (1% DVB) according to the Fmoc/tBu strategy: The Pmc protecting group was used to protect the arginines. Subsequent cleavage was performed in 95% TFA for 35 (A18), 20 (C14), and 65 min (C28A and C28Y) at 30 °C.

Peptide C15 was synthesized on an Applied Biosystems peptide synthesizer Model 430 using the standard cycles for Boc/benzyl strategy and DCC/HOBt activation. In this case the MTS group was used to protect the arginines. Deprotection was performed in a TFA solution containing 10% TFMSA at 0 °C for 30 min.

The synthesis of peptides C20W, C20A, C20AA, and C28W was carried out on an Applied Biosystems peptide synthesizer Model 431 using the Fmoc/tBu strategy with 1-methyl-2-pyrrolidone for coupling and washing according to the standard protocol for this synthesizer. The MTR group was employed to protect the arginines. Cleavage from the support was performed in 95% TFA for 3 h at 50 °C (C20W), for 2.5 h at room temperature and 2 h at 50 °C (C20A and C20AA), and for 1 h at room temperature and 45 min at 50 °C (C28W).

Semipreparative HPLC was used for the purification of the peptides. Characterization was performed by HPLC and amino acid analysis for all of the peptides. In addition, Edman degradation was carried out for peptides C20W, C20A, C20AA, and C28W. Further details on the synthesis, deprotection, and purification procedures are available as supplementary material.

HPLC Analysis and Sequencing of the Purified Peptides. Semipreparative HPLC and analytical HPLC were carried out by using Nucleosil reversed-phase materials packed in Macherey & Nagel columns (Oensingen, Switzerland). The reversed-phase buffers were, buffer A, 0.1% TFA in water, and buffer B, 0.05% TFA and 50% 1-propanol in water. In the case of peptide C28W buffer B had the following composition: 0.02% TFA and 80% acetonitrile in water. The peptides were purified on a 250 × 10 mm C_{18} column (7 μm, 300 Å) on a linear or stepwise linear gradient from buffer A to buffer B. Analytical control was performed on a 80 × 4 mm C_{18} column (3 μm, 100 Å) in the same solvent system. Chromatography was carried out by using LKB equipment (LKB, Uppsala, Sweden). UV detection was performed at 206 nm. The purified peptides were sequenced by using an Applied Biosystems 470A sequencer with 120A on-line PTH detection.

Molecular Weight ($M_{w,obsd}$) Determination. Conventional sedimentation equilibrium experiments were carried out in a Beckmann Model E (Beckmann Instruments, Inc., Irvine, CA) analytical ultracentrifuge (Chervenka, 1969) with absorption optics at 280 nm. The molecular weight was calculated from a corrected plot of ln c vs r^2 (Creeth & Pain, 1967). Partial specific volumes were calculated from the amino acid sequence by using the data of Prakash and Timasheff (1985). Peptides C28W and C28Y were studied in 10 mM ammonium acetate buffer, pH 6, and in 25% TFE in 10 mM ammonium acetate, pH 6. The peptide concentrations were 0.2 mg/mL for C28W and 0.45 mg/mL for C28Y.

Circular Dichroism Experiments. CD spectra of peptides A18, C14, C15, C20W, C28A, C28Y, and C28W, calmodulin, and calmodulin–peptide complexes in solution at pH 7.4 were recorded with a Jasco J-500 spectropolarimeter (Eastern, MD) equipped with an Epson data processor. Measurements were carried out in a far UV jacketed 1-mm quartz cell at 27 °C. Spectra were run at a scanning speed of 50 nm/min and a time constant of 1.0 s over the wavelength range 190–250 nm. Each spectrum was the average of nine scans. The spectra were corrected for spurious signals generated by the solvent and smoothed by digital filtering according to the instructions of the manufacturer. Difference CD spectra were calculated after background correction and smoothing of the appropriate spectra. In the experiments on calmodulin complexes total molar ellipticities were calculated on the basis of the concentration of calmodulin. In the experiments on the complex between peptides A18 and C20W the concentration of the former was used as a basis for the calculations.

The CD spectra of peptides C14, C28a, C28Y, and C28W in solution at pH 5 and pH 6 were obtained by using a Jasco J-600 spectropolarimeter connected to an IBM-AT computer. The solutions were clarified by centrifugation, and peptide concentrations were determined by amino acid analysis. The CD data were recorded in a 0.2-mm quartz cell at 23 °C. The spectra were run at a scanning speed of 20 nm/min and a time constant of 1.0 s over the wavelength range 190–250 nm. Four scans were performed for each spectrum. Smoothing of the base-line-corrected spectra was performed according to the instructions of the manufacturer. The spectra of the reference proteins sperm whale myoglobin (0.15 mg/mL) and bovine pancreas ribonuclease (0.67 mg/mL) were obtained in 10 mM

phosphate buffer, pH 7.0, containing 90 mM NaCl at 23 °C. The CD data were recorded in a 0.1-mm quartz cell at a scan speed of 2 nm/min.

The CD data are expressed as total molar ellipticities. The CD spectra were used to calculate the mean residue ellipticities after base-line correction and analyzed with the program CONTIN developed by Glöckner and Provencher (Provencher & Glöckner, 1981; Provencher, 1982). The data input for the CONTIN program contained one data point per nanometer. α values in the range 0.7–5.2 × 10^{-5} and values for the parameter VAR and OBJ.FCTN in the range 10^{6}–10^{8} were obtained for the peptides, showing that the reference solutions were adequate. The value $\alpha/S(1)$ ranged from 0.16 to 1 × 10^{-5} (value for PRECIS: 1.49 × 10^{-15}), showing that the regularizor term α was indeed small. For the reference proteins α was 2.98 × 10^{-4} and $\alpha/S(1)$ was 2.28 × 10^{-3}.

Infrared Spectroscopy Experiments. IR spectra were recorded in the solid state in KBr. Peptide (0.15–0.35 mg) in 50-mg KBr pellets was applied to a Perkin-Elmer 983 G IR spectrometer (Perkin-Elmer Ltd., Buckinghamshire, England).

Nuclear Magnetic Resonance Measurements. The NMR spectra (360 MHz) were obtained on a Bruker AM 360 (Karlsruhe, FRG) at T = 30 °C. For the 1D NMR experiments usually 500 transients of 8K size were signal-averaged. A 90° observation pulse was used; the residual signal of the solvent was suppressed by a presaturation pulse (t = 1.5 s). 2D DQF-COSY (Rance et al., 1983) or NOESY (Wider et al., 1984) spectra were obtained as described elsewhere (Krebs, 1984, 1986; Krebs, unpublished results). The mixing time for NOESY was 200 ms. The signal in the SER-1 file was reduced by 50% to decrease the t_1 ridges in the spectrum (Otting et al., 1986). Quadrature detection was used in both dimensions. A time domain data matrix of 512 × 2048 points was usually expanded to 1024 × 2048 points by zero filling in the t_1 dimension. Both dimensions of the time domain data matrix were multiplied with a sine-bell window function.

Dansylation of Calmodulin. Calmodulin (1.17 mg) was dissolved in 1 mL of 20 mM NH_4HCO_3, pH 7.5, and the exact concentration (47 μM) determined by UV absorption at 276 nm. Ca^{2+} was added to a final concentration of 1 mM. Stock solution (6.2 μL) of 2.17 mg/mL dansyl chloride (about 95%) in acetone was added to achieve a 47 μM solution of dansyl chloride.

The mixture was kept at room temperature for 2 h and vortexed every 20 min. Prior to the dialysis step the dialysis tube (cutoff of 3500 Da) was incubated in 20 mM NH_4HCO_3 buffer at pH 7.5 and kept in a solution (5 mg/mL) of poly(vinylpyrrolidone) 40 kDa to prevent adsorption phenomena. The compound had to be dissolved in a small volume of acetic acid to obtain a clear solution. The tube was incubated for 20 min and rinsed exhaustively with distilled water and 20 mM NH_4HCO_3 buffer, pH 7.5. Dialysis was carried out overnight against two changes of 5 L of NH_4HCO_3 buffer, pH 7.5 (20 mM NH_4HCO_3). Prior to and after dialysis, the solution was centrifuged to remove dust and solid derivatives formed during the reaction.

Amino acid analysis after reaction, dialysis, and centrifugation indicated a 25 μM solution of dansylcalmodulin. Absorption measurements at 320 nm using a molar extinction coefficient of 3400 M^{-1} cm^{-1} (Chen, 1968) showed incorporation of about 0.35 mol of dansyl chloride/mol of calmodulin. Eighteen microliters of this solution (20 mM NH_4HCO_3 buffer, pH 7.5) in 3 mL of Hepes buffer was used for each titration experiment. The concentration of dansylcalmodulin in all experiments was 140 nM.

Fluorescence Measurements. Fluorescence measurements were performed with a SPEX Fluorolog 1680 (Metuchen, NJ) double-wavelength spectrometer connected to a DM1B coordinator. Quartz cuvettes with a path length of 10 mm and a volume of about 3.5 mL were used. Dilution effects were <3%, and the sample temperature was 26 °C. The fluorescence emission spectra of the tryptophan in the peptides were recorded by exciting at 295 nm with a bandwidth of 4 nm to avoid excitation of the tyrosine residues in calmodulin. The system was buffered with 20 mM Hepes and 130 mM KCl, pH 7.2, containing either 1 mM Ca^{2+} or 1 mM EGTA.

The dansyl moiety of calmodulin was excited at 340 nm, and the peptides dissolved in doubly distilled water were added to the medium containing dansylcalmodulin. The resolution of the excitation monochromator was set at 8 nm. The samples were stirred, and spectra were recorded from 400 to 550 nm. The titrations of fluorescence enhancement or quenching were performed by recording the fluorescence emission at 490 and 500 nm for the Ca^{2+}-bound and EGTA-treated states, respectively. One data point corresponded to fluorescence intensities integrated over a total time of 5 s after equilibration of the mixture.

Determination of the Affinity Constants for the Interaction between Calmodulin and the Calmodulin Binding Peptides. For the determination of the affinity constants two different approaches were followed. The data points were calculated according to the method of Stinson and Holbrook (1978) because the method is suitable to determine the boundary value. The equations

$$K/(1-\alpha) = ([\text{peptide}_T]/\alpha) - [DC_T], \quad \alpha = \frac{F - F_0}{F_\infty - F_0}$$

$$K = [DC][\text{peptide}]/[DC + \text{peptide}], \quad [\text{peptide}] = [\text{peptide}_T] - \alpha[DC_T]$$

used, where α is the fractional degree of saturation of DC, K is the affinity constant, DC is dansylcalmodulin, [peptide] is the concentration of free peptide, $[\text{peptide}_T]$ is the total concentration of peptide added, [DC + peptide] is the concentration of the complex, [DC] is the concentration of unoccupied sites in DC, $[DC_T]$ is the total concentration of dansylcalmodulin, F is the fluorescence intensity of the complex, F_0 is the fluorescence intensity of DC, F_∞ is the fluorescence at the end point (saturation), and F/F_0 is the relative fluorescence intensity. Graphs based on this equation imply a 1:1 stoichiometry of binding and result in straight lines and an intercept of $[\text{peptide}_T]/\alpha = [DC_T]$ when $1/(1-\alpha) = 0$, if $1/(1-\alpha)$ is plotted against $[\text{peptide}_T]/\alpha$. The shape of the curve allows control of the infinite ligand concentration and adjustment of the end point of the titration.

The second approach was derived from the equation of Dandliker et al. (1973).

$$F = F_0 + (F_\infty - F)[\text{peptide}]/K$$

The plot of F against $(F_\infty - F)$[peptide] results in a straight line. The intercept gives the initial fluorescence intensity F_0 as a control. The reciprocal slope yields the value for K.

Both methods require a 1:1 stoichiometric binding of the target and the correct determination of the titration end point. Direct evidence for 1:1 stoichiometric binding of peptide to dansylcalmodulin was obtained from the plot of relative fluorescence (F/F_0) against the ratio $[DC_T]/[\text{peptide}_T]$ for the 20- and 28-residue peptides. The titrations were repeated at least three times, and the figures present single data from one titration as a representative experiment. The complete data are summarized in Table IV.

Table I: Amino Acid Analysis of the Peptides

amino acid	peptides					
	C14	C15	C20W	C28W	C28A	C28Y
D + N	1.3 (1)	1.4 (1)	1.0 (1)	2.3 (2)	2.3 (2)	2.5 (2)
T	0.8 (1)		1.1 (1)	0.8 (1)	1.0 (1)	1.0 (1)
S	2.4 (3)			2.5 (3)	2.9 (3)	2.8 (3)
E + Q	*2* (2)	1.2 (1)	3.1 (3)	3.1 (3)	3.5 (3)	3.3 (3)
G		*2* (2)	*2* (2)	*2* (2)	*2* (2)	*2* (2)
A	1.0 (1)			1.0 (1)	2.2 (2)	1.1 (1)
Y						0.7 (1)
V	1.7 (2)			1.8 (2)	2.3 (2)	2.0 (2)
I	1.8 (2)	1.8 (2)	2.8 (3)	1.9 (3)	3.0 (3)	2.9 (3)
L		2.7 (3)	2.8 (3)	2.7 (3)	2.8 (3)	2.8 (3)
F	1.2 (1)	1.5 (1)	1.3 (1)	2.7 (2)	2.6 (2)	2.6 (2)
K	1.0 (1)		1.0 (1)	0.9 (1)	1.2 (1)	1.1 (1)
R		4.1 (4)	4.0 (4)	3.8 (4)	4.1 (4)	3.9 (4)

[a] Expected ratio in parentheses; tryptophan not determined. The italic values were set as integer figures as references.

Table II: Synthetic Peptides Related to the Calmodulin Binding Domain of the Plasma Membrane Ca^{2+} Pump[a]

A18	E-E-I-P-E-E-E-L-A-E-D-V-E-E-I-D-H-A
C15	L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I
C14	I-Q-T-Q-I-K-V-V-N-A-F-S-S-S
C20W	L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K
C28W	L-R-R-G-Q-I-L-W-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S
C20A	L-R-R-G-Q-I-L-A-F-R-G-L-N-R-I-Q-T-Q-I-K
C20AA	L-R-R-G-Q-I-L-A-A-R-G-L-N-R-I-Q-T-Q-I-K
C28A	L-R-R-G-Q-I-L-A-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S
C28Y	L-R-R-G-Q-I-L-Y-F-R-G-L-N-R-I-Q-T-Q-I-K-V-V-N-A-F-S-S-S

[a] The synthesis procedures are described under Materials and Methods.

RESULTS

Synthesis of Peptides Related to the Calmodulin Binding Domain of the ATPase. The peptides synthesized for the work described in this paper are presented in Table II. In the deduced teratoma sequence (Verma et al., 1988) they correspond to the following sequences, all located near the C terminus of the pump: C15, residues 1100–1114; C14, residues 1114–1127; C20W, residues 1100–1119; C28W, residues 1100–1127. In the four analogues the (conserved) tryptophan in position 1107 was replaced with an alanine (C20A, C28A) or with a tyrosine (C28Y). In peptide C20AA both tryptophan 1107 and the adjoining phenylalanine 1108 were replaced with alanines (C20AA). The acidic peptide N-terminal to the calmodulin binding domain (A18) is also shown in the table.

Circular Dichroism and Infrared Spectroscopy Studies of the Conformation of the Peptides Related to the Calmodulin Binding Domain of the Ca^{2+} Pump and of Their Interaction with Calmodulin. The CD studies were routinely performed in 10 mM ammonium acetate buffer, pH 6, in 2 mM phosphate buffer, pH 7.4, or in 25% TFE solution in either buffer. CD spectra not shown here can be obtained as supplementary material. The secondary structure percentages of the peptides were calculated according to the method of Provencher and Glöckner (1981) (Table III).

The spectrum of the C-terminal portion of the calmodulin binding domain (peptide C14) showed a predominance of β-structure in 25% TFE solution in 10 mM ammonium acetate buffer, pH 6 (data not shown). The limited solubility of this peptide in aqueous buffer at pH 6 led to the formation of aggregates which were removed by centrifugation. The spectrum of the peptide remaining in the supernatant showed only a small percentage of secondary structure (Table III).

Table III: Percentage of Secondary Structure of the Peptides As Derived from the CD Spectra[a]

	% α-helix	% β-sheet	% remainder
C14[b]	2 (0)	65 (16)	33 (84)
C28W[c]	19 (29)	39 (0)	41 (71)
C28A[c]	32 (29)	10 (0)	58 (71)
C28Y[c]	38 (50)	48 (0)	14 (50)
C28W[d]	9 (84)	57 (16)	34 (0)
C28A[d]	12 (76)	51 (4)	37 (20)
C28Y[d]	22 (46)	54 (54)	24 (0)
myoglobin[e]	73 (79)	4 (0)	27 (21)
ribonuclease[e]	12 (23)	54 (40)	34 (37)

[a] The percentages of secondary structure of peptides C14, C28W, C28A, and C28Y in aqueous solution and of the 28 amino acid peptides in the 25% TFE buffer solution were calculated from the CD spectra according to the method of Provencher (1982) using the program CONTIN. The column "% remainder" refers to the percentage of conformations other than α-helix and β-structure contributing to the CD spectrum (e.g., β-turn and random coil conformations). The standard error in the determination of the percentages of the different conformations was <5% except for the 28 amino acid peptides in the pH 6 buffer (standard error 10–12%) and for peptide C28Y in the 25% TFE buffer, pH 6 (standard error 9%). The standard error for the reference proteins was <6.5% except for the portion of myoglobin having conformations other than α-helix or β-structure (standard error 16%). [b] Peptide C14 in 2 mM phosphate buffer and 40 mM KCl, pH 7.4. Values in 10 mM ammonium acetate buffer, pH 6, are in parentheses. [c] The 28 amino acid peptides in 2 mM phosphate buffer, pH 7.4. Values in 10 mM ammonium acetate, pH 6, are in parentheses. [d] The 28 amino acid peptides in 25% TFE in 2 mM phosphate buffer, pH 7.4. Values in 25% TFE in 10 mM ammonium acetate, pH 6, are in parentheses. [e] CD spectra of reference proteins calculated by the program CONTIN. Values in parentheses were obtained from crystallographic data of myoglobin (Kendrew, 1962; Kendrew et al., 1960) and ribonuclease (Kartha et al., 1967).

Improved solvation of peptide C14 in 2 mM phosphate buffer, pH 7.4, containing 40 mM KCl resulted in a spectrum typical of a β-structure; however, the low ellipticities reflected the high tendency to form aggregates in the aqueous system. In pure TFE peptide C14 exhibited an almost ideal helical CD spectrum (data not shown).

Although the 28-residue peptides were soluble at neutral pH, they also tended to aggregate and at concentrations slightly higher than those employed for the CD measurements. Spectra recorded in 2 mM phosphate buffer, pH 7.4, and in 25% TFE solution showed a high proportion of secondary structure, corresponding to a helix plus β-structure arrangement for all 28-residue peptides (Table III). However, at pH 6 a striking difference between the spectra of peptides C28W and C28A and those of C28Y was observed (data not shown). Peptides C28A and C28W showed a predominantly random coil conformation, whereas the Y analogue exhibited a considerable proportion of secondary structure (β-structure). In 25% TFE at pH 6 the difference was less pronounced, due to the high percentage of secondary structure present in all 28-residue peptides (data not shown).

The distinct spectral characteristics of peptides C28W and C28Y in 10 mM ammonium acetate, pH 6, and in 25% TFE called for the determination of their apparent molecular weights (the A analogue was not studied in detail). Approximately 70% of peptide C28Y had an apparent molecular weight ($M_{w,obsd}$) of about 600 000, corresponding to the aggregation of 150–200 molecules in both aqueous buffer and 25% TFE solutions. The portion remaining after removal of the high molecular weight material evidently reflected the molecular weight of the monomer.

As expected from the spectrum of peptide C28W, no aggregated material was detected in 10 mM ammonium acetate, pH 6. The interesting observation was made that peptide C28W existed exclusively as a dimer in 25% TFE. In this case

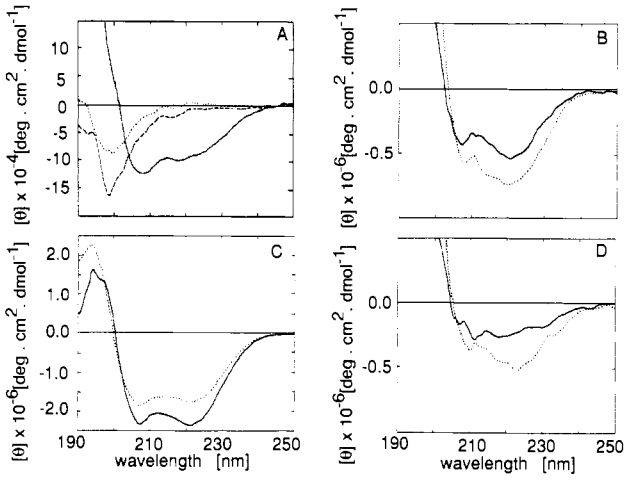

FIGURE 1: (A) CD spectra of peptides of different chain length in 2 mM phosphate buffer, pH 7.4, and 25% TFE in 2 mM phosphate buffer, pH 7.4. The figure shows peptides C15 (···) and C20W (---) in phosphate buffer and peptide C20W (—) in 25% TFE and 2 mM phosphate buffer, pH 7.4. The peptide concentrations in phosphate buffer were 200 μM for C15 and 200 μM for C20W, and that of C20W in 25% TFE and 2 mM phosphate buffer was 170 μM. (B) CD difference spectra of a calmodulin–peptide complex in the presence and absence of Ca^{2+}. The figure shows the difference spectrum of the calmodulin complex with peptide C20W in the presence of Ca^{2+} subtracted from that of Ca^{2+}-bound calmodulin (—) and the difference spectrum of the calmodulin complex with C20W in EGTA solution subtracted from that of calmodulin (···). The spectra were recorded in 2 mM phosphate buffer, pH 7.4, containing 0.38 mM Ca^{2+} and 7.5 mM EGTA, respectively. The concentration of calmodulin after addition of the peptide was 19.5 μM. The concentration of peptide C20W was 33 μM. (C) CD spectra of calmodulin and of the calmodulin–peptide complex in the presence of Ca^{2+}. The figure shows the spectra of calmodulin (···) and of the calmodulin complex of peptide C20W (—). The concentration of calmodulin was 20 μM, decreasing to 19.5 μM after the addition of the peptide. The concentration of peptide C20W was 33 μM. The measurements were performed in 2 mM phosphate buffer, pH 7.4, containing 0.38 mM Ca^{2+}. (D) As in (B), but the peptide was C28W. The concentration of calmodulin after addition of the peptide was 18.8 μM. The concentration of peptide C28W was 23 μM. The concentration of peptide C28W was 23 μM. The spectra were recorded as described under Materils and Methods.

the peptide had an apparent molecular weight of 6300 ± 200 (molecular weight of the monomer, 3270). In contrast to the properties of the 28-residue peptides, peptides C15 and C20W adopted a random coil conformation in the aqueous buffer, pH 7.4 (Figure 1A), but assumed a predominantly helical conformation in 25% TFE (Figure 1A).

The CD studies of calmodulin and the complexes were carried out in aqueous phosphate buffer in the presence of either 375 μM Ca^{2+} or 7.5 mM EGTA. The spectrum observed upon mixing calmodulin and the C-terminal half of the calmodulin binding domain (peptide C14) was the same in the presence and the absence of Ca^{2+}, indicating that no complex formation occurred. A marked increase in α-helical conformation was observed upon addition of peptides C15, C20W, C28W, C28A, and C28Y to calmodulin in the presence of EGTA (Figure 1B,D). In the presence of Ca^{2+} the increase of α-helical conformation in peptides C15, C20W, and C28W in the complex with calmodulin was smaller (Figure 1B,C,D). By contrast, the spectra of peptides C28A and C28Y upon complex formation were virtually identical in the presence and absence of Ca^{2+} (data not shown). Thus, the spectral characteristics of the complex peptide C28W–calmodulin were more closely related to those of the complexes of peptides C15 and C20W than to those of the C28A and C28Y peptide complexes. Evidently, the substitution of the tryptophan had a drastic effect on the conformation of the 28-residue peptides and on the induction of secondary structure in the complex with calmodulin.

The solid-state infrared spectroscopy (IR) work presented in Figure 2 indicates, in agreement with the CD spectra, a β-structure for peptide C14. Typical bands at 1630 and 3280 cm^{-1} in the amide I and amide A regions, respectively, were observed (Figure 2A,D). The discrimination between helix and random coil conformations by IR is not possible because of absorptions around 1660–1670 cm^{-1} for both types of secondary structure. For peptides C15 and C20W only broad bands in this region were present; however, the absence of absorptions at 1630–1640 cm^{-1} and a strong band in the amide A region at 3370 cm^{-1} showed that these peptides did not have a β-pleated sheet structure in the solid state (Figure 2A,B,-D,E). In the case of the 28-residue peptides absorptions typical of a β-pleated sheet and of a random coil/helix structure were present. The contents of β-structure as judged by the amide I peak (see 1630 cm^{-1}) decreased in the order C28Y > C28A, C28W (Figure 2F). The distinct double band in the Y analogue suggests a more defined conformation in contrast to the broad amide I peak of peptide C28A at about 1650 cm^{-1}. The amide A bands showed a more dominant β-structure in peptides C28A and C28Y as compared to peptide C28W (Figure 2C).

^1H NMR Study of the Interaction of Calmodulin with the Synthetic Calmodulin Binding Peptides. As was the case for the circular dichroism studies described above, the high concentrations of peptides necessary for the NMR experiments and the spectral characteristics described below prevented studies of the binding constants. The NMR work, however, has led to the identification of the domain of the calmodulin molecule that interacts with the pump.

Figure 3 compares the aromatic regions of the ^1H NMR spectra of peptide C20W (a) with those of isolated calmodulin (b) and of the calmodulin–peptide complex (c). The figure shows that the ^1H resonances of the free tryptophan of the peptide C20W positioned between 7.4 and 7.6 ppm disappeared completely when calmodulin and the peptide interacted: the spectrum of the isolated peptide showed resonances at chemical shift positions of tryptophan or phenylalanine typical of a random coil conformation (Figure 3a). As calmodulin was added in the presence of Ca^{2+} all of the tryptophan resonances shifted upfield, disappearing under the broad envelope between 7.0 and 7.4 ppm. The resonances of calmodulin were apparently also influenced, as shown by the comparison of the resonances of tyrosine 99 and 138 or phenylalanine 89 in calmodulin alone and in the presence of peptide C20W. The resonance of tyrosine 138 displayed an upfield shift, whereas the resonances of tyrosine 99 and phenylalanine 89 shifted downfield. In Figure 4 the aromatic portion of the experimentally measured C20W–calmodulin complex spectrum is compared with the spectra of the two reactants as added by the computer: the sharp resonances typical of the free peptide disappeared completely concurrent with the broadening of the resonances between 7.0 and 7.4 ppm.

However informative, the 1D NMR experiments described above did not permit the conclusive interpretation of the spectral changes induced by the formation of the complex between peptide C20W and calmodulin. Two-dimensional nuclear Overhauser spectra (NOESY) of calmodulin and of the calmodulin–peptide C20W complex were thus carried out and are compared in Figure 5. Since the intensities of the through-space connectivities observed with this technique are related by a r^{-6} dependence, only interactions of less than 5 Å can be observed (Wüthrich, 1986). In Figure 5A the strong

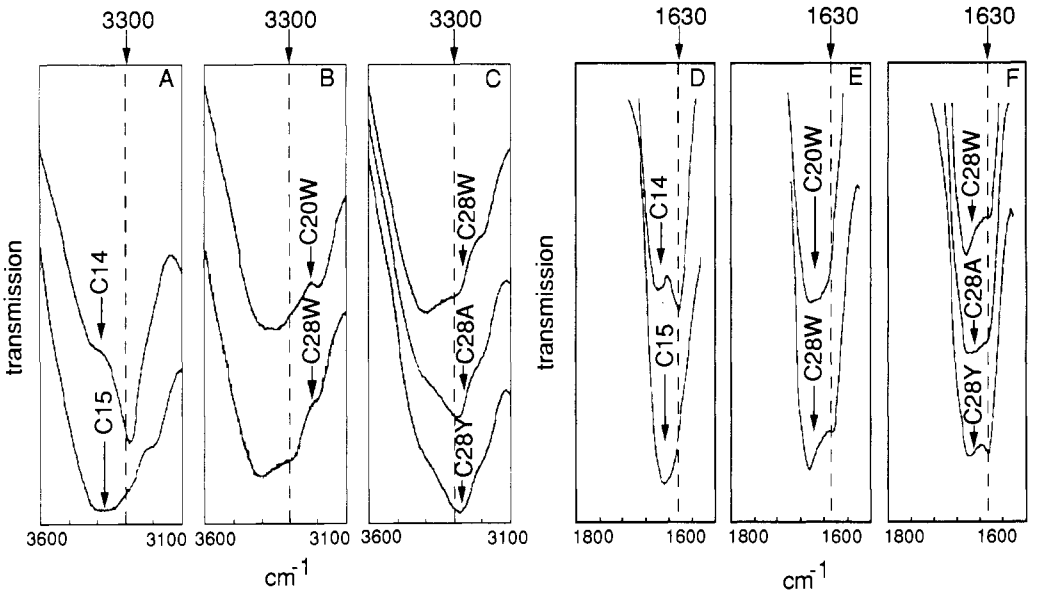

FIGURE 2: (A) Solid-state amide A IR spectra of the C-terminal portion of the calmodulin binding region (peptide C14) and of the N-terminal peptide C15. 0.35 mg of peptide C15 in 50 mg of KBr and 0.25 mg of peptide C14 in 50 mg of KBr were used for the measurements. (B) Solid-state amide A IR spectra of the 20-residue peptide C20W and the 28-residue peptide C28W. 0.18 mg of peptide C20W in 50 mg of KBr and 0.15 mg of peptide C28W in 50 mg of KBr were used for the measurements. (C) Solid-state amide A IR spectra of the calmodulin binding peptide C28W and of the analogues C28A and C28Y. 0.15 mg of peptide C28W in 50 mg of KBr, 0.09 mg of peptide C28A in 50 mg KBr, and 0.07 mg of peptide C28Y in 50 mg of KBr were used for the measurements. (D) Solid-state amide I IR spectra of the C-terminal portion of the calmodulin binding region (peptide C14) and of the N-terminal peptide C15. 0.35 mg of peptide C15 in 50 mg of KBr and 0.25 mg of peptide C14 in 50 mg of KBr were used for the measurements. (E) Solid-state amide I IR spectra of the 20-residue peptide C20W and the 28-residue peptide C28W. 0.18 mg of peptide C20W in 50 mg of KBr and 0.15 mg of peptide C28W in 50 mg of KBr were used for the measurements. (F) Solid-state amide I IR spectra of the calmodulin binding peptide C28W and the analogues C28A and C28Y. 0.15 mg of peptide C28W in 50 mg of KBr, 0.09 mg of peptide C28A in 50 mg of KBr, and 0.07 mg of peptide C28Y in 50 mg of KBr were used for the measurements.

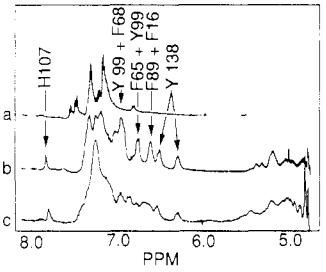

FIGURE 3: Aromatic portions of the ^1H NMR spectra of the calmodulin binding peptides. The figure shows the spectra of the peptide C20W (a), of Ca_4–calmodulin (b) and of the peptide–calmodulin complex (1:1) (c). The spectra were recorded as described under Materials and Methods. The assignments given are based on extensive assignments using COSY, NOESY, and TOCSY 2D NMR techniques (Krebs, 1989, unpublished results) and are in general agreement with those found in the literature (Seamon, 1980; Krebs & Carafoli, 1982; Ikura et al., 1984, 1985; Dalgarno et al., 1984).

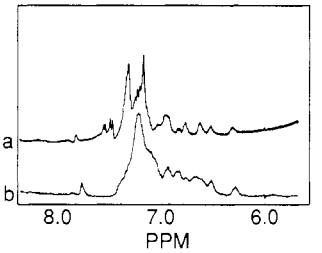

FIGURE 4: Comparison of the ^1H NMR spectra of the computed and measured peptide–calmodulin complex. The figure compares the aromatic portion of the computed 1:1 complex of peptide C20W and Ca_4–calmodulin (a) with the measured complex (b). The spectra were obtained as described under Materials and Methods. The computed spectrum a was obtained by adding the free induction decay of the peptide (see a) to that of Ca_4–calmodulin (see b) before the Fourier transformation.

cross-peaks at 6.3, 6.5, and 7.0 ppm were due to the close interaction between tyrosine 138 and phenylalanine 89. Similar interactions, albeit weaker, could be observed at 6.65 and 7.2 ppm due to the close spatial relationship of phenylalanine 16 with phenylalanine 65. The positions of these two residues mirror-image those of phenylalanine 89 and tyrosine 138 in the N-terminal half of calmodulin. However, the NOESY spectrum of the complex peptide C20W–calmodulin measured under the same conditions (Figure 5B) showed differences in the environment of the pairs phenylalanine 89/tyrosine 138 and phenylalanine 16/phenylalanine 65 in the two halves of calmodulin: the cross-peaks at 6.65 and 7.2 ppm reflecting the through-space connectivity of phenylalanine 16/phenylalanine 65 were still present, at variance with the cross-peaks at 6.3, 6.5, and 7.0 ppm which reflect the interaction of phenylalanine 89 and tyrosine 138 (Figure 5A). This indicated differences in the interactions of the spatially related connectivities of the two pairs of aromatic residues in the C and N half of calmodulin in the complex. Therefore, preferential binding of peptide C20W to the C-terminal half of calmodulin apparently occurs, without interference with the spatial interactions in the N-terminal half. Binding of the peptide to the N-terminal half without influence on the spectrum would in principle be possible. However, this appears unlikely since through-space connectivities are much more sensitive monitors of conformational changes than changes in chemical shift values. No significant differences in the 2D NOESY spectrum of the calmodulin complex were observed when the analogues C20A and C20AA were used instead of peptide C20W (data not shown).

Fluorescence Studies of the Interaction of Calmodulin with the Synthetic Calmodulin Binding Domain and its Analogues. The CD and NMR experiments, while demonstrating unequivocally the formation of the complex between calmodulin and the synthetic binding domain, demanded the use of high

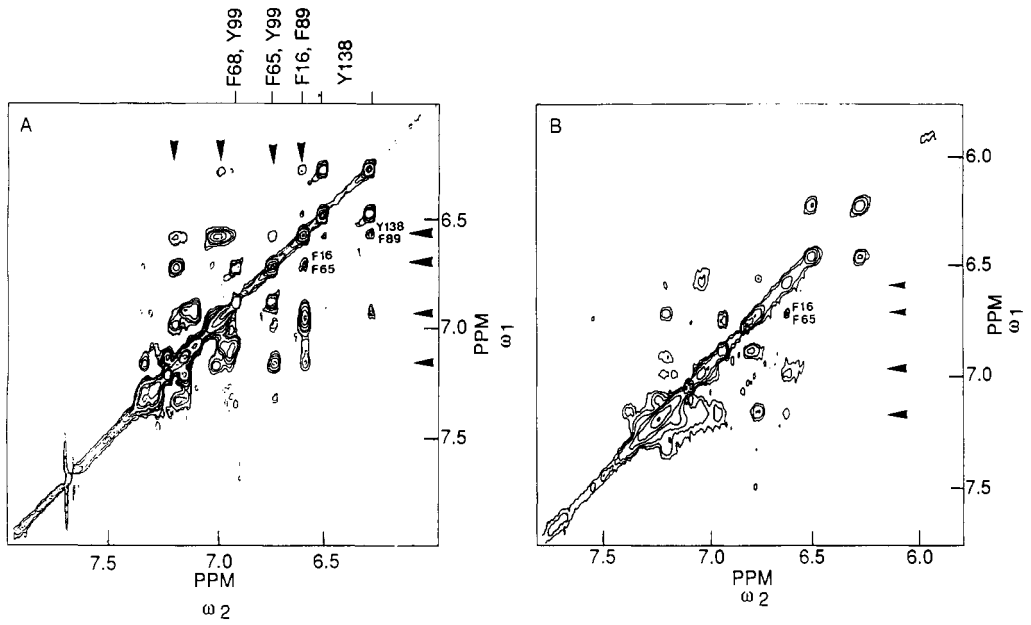

FIGURE 5: 2D NOESY spectra of Ca^4–calmodulin and of calmodulin–C20W peptide complex. The figure shows the aromatic portion of a 2D NOESY spectrum of Ca_4–calmodulin (A) and of the Ca_4–calmodulin–peptide C20W complex (1:1) (B). Both spectra were recorded in D_2O. Details are given under Materials and Methods. The assignments in the figure are in agreement with those given in Figure 3. The arrows indicate the locations of the cross-peaks between tyrosine 138/phenylalanine 89 and phenylalanine 65/phenylalanine 16.

concentrations of calmodulin and binding peptides (e.g., 20 μM in the case of the CD measurements), preventing detailed studies of the affinity of the two components for each other. The fluorescence experiments described below were designed to study the interaction of calmodulin with the peptides at the physiological nanomolar concentration and to analyze in detail the effects of Ca^{2+} and of the removal of tryptophan 1107 from the binding domain. Although significant data on the interaction could be obtained by monitoring the intrinsic tryptophan fluorescence, the weak signal could not be used for measurements of high affinity interactions (nanomolar range). This, as well as the decision to carry out experiments on peptides lacking tryptophan 1107, prompted the use of dansylated calmodulin for most of the fluorescence work. Previous work has shown that the binding and activation of different calmodulin targets (e.g., activation of phosphodiesterase) were not altered by dansylation (Malencik & Anderson., 1982; Kincaid et al., 1982).

The maximum in the emission spectrum of the binding peptides underwent a shift to lower wavelengths, and a slight increase in intensity, upon complex formation with calmodulin, indicating a more hydrophobic tryptophan environment. In the case of C15 the maximum shifted from 365 to 345 nm and in that of C20W, further down, to about 335 nm (data not shown). Only qualitative experiments were carried out by using C28W, revealing a shift of the maximum to about 355 nm. Titration of the tryptophan fluorescence in peptide C28W with dansylated calmodulin resulted in a pronounced decrease of the tryptophan absorption and in a shift in the maximum to 365 nm, indicating energy transfer from the excited tryptophan to the dansyl group of calmodulin. The effect was seen at nanomolar concentrations of dansylcalmodulin in the presence of Ca^{2+}, but required micromolar concentrations in its absence (data not shown).

The affinity constants for the formation of the complexes with the binding peptides were derived from experiments with dansylated calmodulin carried out in 20 mM Hepes buffer, pH 7.2, and 130 mM KCl. The emission spectra of the dansyl group upon excitation at 340 nm exhibited maxima at 508 and 522 nm in the presence of Ca^{2+} and EGTA, respectively. The fluorescence enhancement at 508 nm induced by the addition of Ca^{2+} was 1.7-fold. The spectra of the complexes were obtained by adding variable amounts of the concentrated peptide solution to the buffer containing 140 nM dansylcalmodulin and by measuring the fluorescence emission at 490 and 500 nm to achieve the maximal fluorescence enhancement in the presence of Ca^{2+} and EGTA, respectively. No changes in the spectrum of dansylcalmodulin were induced by the addition of peptide C14, as expected from the results of the CD experiments, which had indicated that no complex formation had occurred with this peptide. Peptide C15 induced fluorescence enhancement, but with a plateau in the micromolar concentration range. In the presence of Ca^{2+} the spectrum showed a blue-shifted maximum at 490 nm with an enhancement factor of 2.5 as compared to that of Ca^{2+}-saturated dansylcalmodulin. A second weaker maximum, but with an enhancement factor of 2.7, appeared at 426 nm. In the absence of Ca^{2+} the emission maxima were at 417 and 482 nm, with enhancement factors of 15.8 and 6.8, respectively (data not shown).

The titration of dansylcalmodulin with peptide C20W was completed in the nanomolar concentration range. In the presence of Ca^{2+} a maximum at 499 nm with an enhancement factor of 1.6 and a smaller peak at 423 nm were observed (Figure 6A). In the absence of Ca^{2+} the completion of the titration required micromolar amounts of peptide C20W: in this case the emission at 423 nm became the dominant maximum (enhancement factor 4.4), and a second maximum was present at 500 nm (enhancement factor 2.3) (Figure 6B). The analogues C20A and C20AA produced a spectrum with essentially the same shape.

Peptide C28W induced only a small fluorescence enhancement and a blue shift of the emission maximum to 500 nm in the presence of Ca^{2+}. A minor enhancement (and a red shift) was observed also at the shorter wavelength peak at about 420 nm. Greater concentrations of the peptide induced a very evident quenching of the fluorescence at the 500-nm emission maximum and the appearance of a second maximum of about 420 nm (Figure 6C). The quenching factor at the 500-nm maximum was 1.4, whereas the enhancement at the

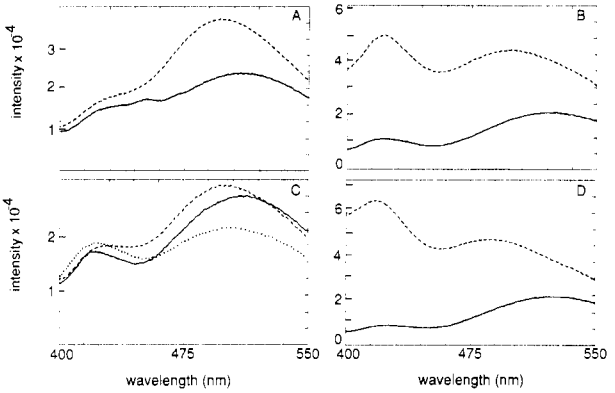

FIGURE 6: (A) Emission spectrum of dansylcalmodulin after complex formation with peptide C20W in the presence of Ca^{2+}. Excitation was performed at 340 nm. The figure shows the emission spectrum of Ca^{2+}-bound dansylcalmodulin (—) and of the peptide C20W–dansylcalmodulin complex in the presence of Ca^{2+} (---). The solution contained 140 nM dansylcalmodulin (—) and 700 nM peptide C20W (---). (B) Emission spectrum of dansylcalmodulin after complex formation with peptide C20W in EGTA solution. The figure shows the emission spectrum of dansylcalmodulin (—) in EGTA solution and of the peptide C20W–dansylcalmodulin complex in EGTA solution (---). The solution contained 140 nM dansylcalmodulin (—) and 16 μM peptide C20W (---). (C) Emission spectrum of dansylcalmodulin after complex formation with peptide C28W in the presence of Ca^{2+}. The figure shows the emission spectrum of Ca^{2+}-bound dansylcalmodulin (—) of the peptide C28W–dansylcalmodulin complex in the presence of Ca^{2+} (first putative binding site occupied) (---) and of the peptide C28W–dansylcalmodulin complex in the presence of Ca^{2+} (second putative binding site occupied) (···). The solution contained 140 nM dansylcalmodulin (—) and 180 nM peptide C28W (---) (first addition). The concentration of C28W was 388 nM after the second addition (···). (D) Emission spectrum of dansylcalmodulin after complex formation with peptide C28W in EGTA solution. The figure shows the emission spectrum of dansylcalmodulin (—) in EGTA solution and of the peptide C28W–dansylcalmodulin complex in EGTA solution (---). The solution contained 140 nM dansylcalmodulin (—) and 6.5 μM peptide C28w (···). All spectra were recorded as desribed under Materials and Methods.

Table IV: Interaction between Calmodulin and the Synthetic Peptides Related to the Calmodulin Binding Domain[a]

peptide	K	peptide	K
C15W	1.7 ± 0.6 μM	C28W	<1 nM
C20W	11 ± 3 nM	C28A	18 ± 4 nM
C20A	30 ± 4 nM	C28Y	15 ± 3 nM
C20AA	160 ± 25 nM		

[a] The reaction medium contained 1 mM Ca^{2+}, 20 mM Hepes buffer, pH 7.2, 130 mM KCl, and 140 nM dansylcalmodulin. The methods for the determination of K and the synthesis of the peptides (see Table II for the sequences) are described under Materials and Methods and in detail in the supplementary material.

shorter wavelength peak was 1.2. In the presence of EGTA significant fluorescence enhancements were only observed at micromolar concentrations of the peptide. No fluorescence quenching at the higher wavelength maximum occurred. The maximum at 420 nm became dominant in the absence of Ca^{2+} (enhancement factor 7.3) (Figure 6D). The shape of the spectrum resembled more that of peptide C20W than those of the peptide analogues C28A and C28Y (see below), pointing to the importance of tryptophan 1107 in determining the binding characteristics of peptide C28W in EGTA.

Affinity Constants for the Interaction between Calmodulin and the Calmodulin Binding Peptides. Peptide C15 bound calmodulin with low affinity (Table IV): the points in the titration curve obeyed the equation discussed under Materials and Methods, resulting in a straight line consistent with a 1:1 binding stoichiometry. The affinity of calmodulin for peptide C20W increased by almost 3 orders of magnitude, to values

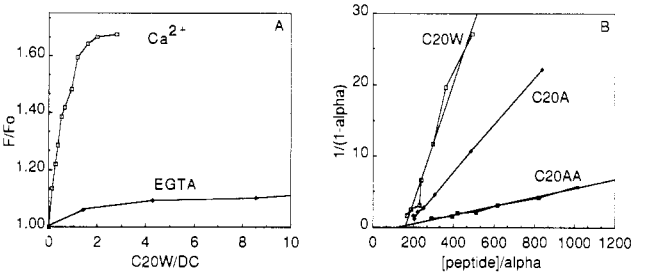

FIGURE 7: (A) Dansylcalmodulin (140 nM) was titrated with the calmodulin binding peptide C20W in the presence and absence of Ca^{2+}. Excitation was performed at 340 nm. The relative fluorescence intensities are plotted against the ratio of the total concentration of peptide C20W and the total concentration of dansylcalmodulin, as given by one representative titration experiment. The data points were recorded as outlined under Materials and Methods. (B) Titration curves of peptides C20W, C20A, and C20AA in the presence of Ca^{2+}. 140 nM dansylcalmodulin was titrated with the corresponding peptides. The data points were recorded, and the fractional degree of saturation of dansylcalmodulin (α) was calculated as outlined under Materials and Methods. The plot of $1/(1-\alpha)$ against the free concentration (nanomolar) of peptide divided by α results in a straight line if a 1:1 complex is formed and the titration endpoint is correctly estimated. The zero intercept on the x axis refers to the total dansylcalmodulin (140 nM) and serves as a control. The reciprocal of the slope gives the affinity constant. The plots describe representative experiments. The calculated K values were 11.5 nM for peptide C20W, 30.5 nM for peptide C20A, and 160 nM for peptide C20AA.

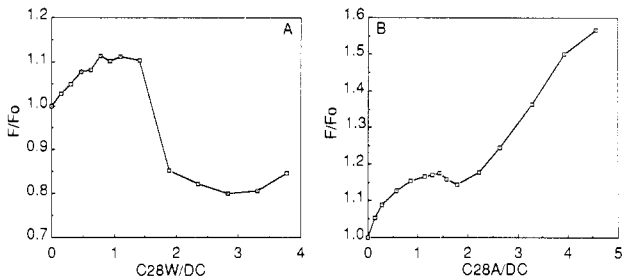

FIGURE 8: Titration of dansylcalmodulin with peptide C28W. (A) Dansylcalmodulin (140 nM) was titrated with the calmodulin binding peptide C28W in the presence of Ca^{2+} in a concentration range permitting detection of two binding sites. Excitation was performed at 340 nm. The relative fluorescence intensities are plotted against the ratio of the total concentration of peptide C28W and the total concentration of dansylcalmodulin in one representative titration experiment. Obviously the binding of a second molecule of peptide C28W to the dansylcalmodulin complex caused an evident quenching of the fluorescence at the end of the titration. (B) Dansylcalmodulin (140 nM) was titrated with the calmodulin binding peptide C28A in the presence of Ca^{2+} in a concentration range permitting detection of binding of two peptide molecules. Excitation was performed at 340 nm. The relative fluorescence intensities are plotted against the ratio of the total concentration of peptide C28A and the total concentration of dansylcalmodulin in one representative titration experiment. The binding of a second molecule of peptide C28A to dansylcalmodulin caused a fluorescence enhancement, resulting in the drastic increase in the relative fluorescence F_0/F. The data points were recorded as outlined under Materials and Methods.

comparable to those observed for the intact enzyme. The strong Ca^{2+} dependence of the binding is demonstrated by the plot of relative fluorescence against the ratio C20W/DC shown in Figure 7A. A 1:1 peptide/calmodulin binding stoichiometry is suggested by the plot. The titration was about 70% complete at a ratio of 1:1 (Figure 7A). The affinities of the analogues C20A and C20AA decreased about 3-fold and 15-fold, respectively, in the presence of Ca^{2+} (Figure 7B and Table IV). When the titrations were performed with the C28 peptides, the binding of a second peptide molecule was detected. In the case of peptide C28W an initial fluorescence enhancement of about 1.1-fold was followed by a strong quenching of the fluorescence for peptide/calmodulin ratios exceeding 1.0

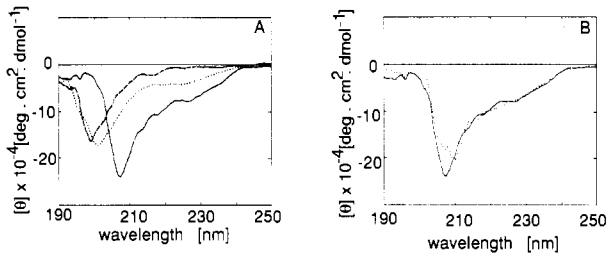

FIGURE 9: (A) CD spectra of the calmodulin binding peptide C20W, of the acidic peptide A18, and of the complex of C20W with A18 in the presence of Ca^{2+}. The figure shows the spectra of peptides A18 (···), C20W (- - -), and their complex (—) in 2 mM phosphate buffer, pH 7.4, containing 0.38 mM Ca^{2+}. The peptide concentrations were 0.3 mM for A18 and 0.20 mM for C20W. In the solution containing the complex the peptide concentrations were 0.15 for A18 and 0.22 mM for C20W. The spectra were recorded as described under Materials and Methods. (B) CD spectra of the complex of the peptides C20W and A18 in the presence and in the absence of Ca^{2+}. The figure shows the spectra of the complex in 2 mM phosphate buffer, pH 7.4, containing 0.38 mM Ca^{2+} (—) and in 2 mM phosphate buffer, pH 7.4, containing 7.5 mM EGTA (···). The peptide concentrations were 0.15 mM for A18 and 0.22 mM for C20W. The spectra were recorded as described under Materials and Methods.

(Figure 8A). The affinity constant estimated for the first phase of the titration, corresponding to a 1:1 peptide/calmodulin ratio was below 1 nM. As was the case for peptide C20W the constant increased to the micromolar range in the absence of Ca^{2+}. The analogues C28A and C28Y had affinity constants in the low nanomolar range in the presence of Ca^{2+} and in the micromolar range in its absence. At a peptide/calmodulin ratio of 1:1 they both exhibited a weak fluorescence enhancement (about 1.2-fold), followed by a dramatic enhancement for ratios exceeding 1:1. Figure 8B documents this behavior for peptide C28A (the curve for peptide C28Y was essentially the same).

Interaction of a Putative Ca^{2+} Binding Site in the Ca^{2+} Pump with the Calmodulin Binding Domain. Previous work with the purified erythrocyte Ca^{2+} pump (Benaim et al., 1984) and on other calmodulin-regulated enzymes (Klee, 1980; Kennelly et al., 1987; Pearson et al., 1988; Foster & Gaeta, 1988) has shown that the calmodulin binding domain is likely to act as an inhibitor of the enzyme. In the absence of calmodulin, this domain would interact with the main body of the pump, preventing substrate (Ca^{2+}?) accessibility. This suggestion has now been corroborated by experiments on the synthetic acidic peptide A18, immediately N-terminal to the calmodulin binding domain, which has been proposed as a probable Ca^{2+} binding site (Verma et al., 1988). Ten of the 18 amino acids in the A18 sequence (residues 1079–1096 in the deduced sequence of the human pump; Verma et al., 1988) are either glutamic or aspartic acid residues. The CD studies shown in Figure 9 show that peptide A18 indeed interacted with peptide C20W, as detected by a red shift of the minimum in the CD spectrum. Peptides A18 and C20W adopted a predominantly random coil conformation when studied separately (see above for the case of C20W), whereas in the complex there was an evident increase in secondary structure. Figure 9A shows the shift upon complex formation in the presence of Ca^{2+}. In its absence the smaller ellipticities and a red shift of the minimum indicate an increase in secondary structure as compared to the spectrum in the presence of Ca (Figure 9B).

Discussion

The calmodulin binding domain of the plasma membrane Ca^{2+} pump interacts with calmodulin in the presence of Ca^{2+} to increase the Ca^{2+} affinity and the maximal velocity of the enzyme (Niggli et al., 1981). Recent sequencing work (Shull & Greeb, 1988; Verma et al., 1988) has shown that the domain is located near the carboxy terminus of the pump: the domain is positively charged and most likely protrudes from the membrane into the aqueous space of the cytosol. Previous work in which the purified erythrocyte Ca^{2+} pump had been labeled with a bifunctional, photoactivable, cleavable cross-linker bound to calmodulin had shown the domain to consist of about 30 amino acids (James et al., 1988). When separated from the main body of the pump by CNBr cleavage, the domain bound to a micro calmodulin column and could be eluted, albeit only partially, with EGTA (James et al., 1988). Thus, the domain was evidently able to bind calmodulin, but a detailed study of the characteristics of the interaction appeared necessary. The synthetic peptides used in the present work correspond to different portions (i.e., lengths) of the domain and could thus define the minimal size necessary to bind calmodulin with adequate affinity. The underlying assumption made here is that the peptide results could be extrapolated to the complete ATPase molecule. While the assumption appears permissible, the possibility that the native enzyme behaves differently, however unlikely, must nevertheless be mentioned.

Although there appears to be little sequence similarity among the calmodulin binding domains in the calmodulin-modulated proteins so far sequenced (Blumenthal et al., 1985; Lukas et al., 1986; Guerriero et al., 1986; Buschmeier et al., 1987; Glaser et al., 1988; Kincaid et al., 1988), some general characteristics appear nevertheless to be shared. As mentioned in the introduction, a tryptophan, or at least an aromatic residue, appears to be frequently conserved in the N-terminal portion of the domain. That the tryptophanyl residue could play a role in the interaction with calmodulin has in fact already been speculated (Cox et al., 1984). A preliminary paper on a synthetic calmodulin binding peptide of myosin light chain kinase describing the substitution of the tryptophan with a cysteine to which a fluorescent label had been attached (Blumenthal et al., 1988) mentions the decrease of the affinity for calmodulin.

In the work described here the interaction of synthetic peptides derived from the calmodulin binding domain of the plasma membrane Ca^{2+} pump with calmodulin were studied by CD, NMR, and fluorescence spectroscopy. Prior to these experiments the secondary structure of the peptides was established by CD and IR measurements. The CD spectra demonstrated that the full-length calmodulin binding domain (peptide C28W) displayed a helix plus β-sheet arrangement, in agreement with the IR studies. The latter were performed in the solid state and must thus be interpreted with caution: the possibility that they are not representative of solution conformations, albeit unlikely, must be considered. CD and IR spectra of peptides C20W and C14 showed that the N-terminal portion of the calmodulin binding domain developed a helical conformation when dissolved in 25% TFE and that the C-terminal half of the domain exhibited a predominantly β-structure.

CD studies of the peptides plus calmodulin established conclusively the formation of a complex. The CD difference spectra of the complex with peptide C20W revealed the induction of a helical conformation within the peptide (Figure 1B) since peptide C20W adopted an essentially random coil conformation in buffer at neutral pH. Peptide–calmodulin complexes could be observed even in the presence of EGTA, evidently due to the high concentrations required. In fact, the conformational changes upon complex formation as compared

to the CD spectrum of calmodulin were even greater in the presence of EGTA than in Ca^{2+} solution. Nevertheless, the CD spectra of the peptide–calmodulin complexes in the presence and absence of Ca^{2+} were nearly identical in terms of shape and ellipticities.

The helical content of calmodulin increases upon binding Ca^{2+} (Klee, 1977). Considering the CD spectra of the peptide calmodulin complexes in the presence of Ca^{2+}, the additional increase in helicity of the complexes in the EGTA solution (Figure 1B,D) can be attributed with a high degree of likelihood to the induction of secondary structure in calmodulin.

The 1D and 2D NMR experiments of the peptide–calmodulin complexes have demonstrated that the C-terminal half of calmodulin interacted preferentially with the peptides. 1D NMR studies on myosin light chain kinase have led to the conclusion that both halves of calmodulin are involved in the binding (Klevit et al., 1985; Blumenthal et al., 1988). At variance with myosin light chain kinase, the plasma membrane Ca^{2+} ATPase can be activated by the C-terminal tryptic half of calmodulin (Guerini et al., 1984). Thus, the cases of the plasma membrane Ca^{2+} pump and of myosin light chain kinase could be intrinsically different. However, the detection of changes due to direct interactions is difficult with 1D NMR methods, especially if indirect effects result from the formation of the peptide–calmodulin complex (Klevit et al., 1985).

The fluorescence experiments have established the affinity of the interaction between calmodulin and the peptides and the effects of the tryptophan substitutions. The constants obtained from the titration experiments with dansylcalmodulin are comparable to those obtained by following the inhibitory effect of the peptides on the activation of the intact ATPase by calmodulin (Enyedi et al., 1989). Thus, the dansylation of calmodulin did not influence the binding properties of the peptides.

The results of the experiments with dansylated calmodulin summarized in Table IV have shown that peptide C15, corresponding to the N-terminal half of the calmodulin binding domain, bound to calmodulin with inadequate affinity, almost 3 orders of magnitude lower than in the native enzyme. The affinity of peptide C20W approached that of the intact enzyme, indicating that the first 20 residues would in principle be sufficient to confer to the domain the appropriate affinity for calmodulin, i.e., a K_d of 4 nM (Graf & Penniston, 1981). Moreover, the affinity was increased further by the eight C-terminal residues of the domain, i.e., in peptide C28W. Interestingly, the binding of a second peptide molecule to calmodulin was detected by fluorescence quenching in the experiments with the latter peptide. Replacement of the Trp residue by Ala or Tyr in peptide C28W significantly decreased the affinity for calmodulin: the binding of a second peptide molecule in this case caused strong fluorescence enhancement rather than quenching. The affinity of peptide C20A was also decreased as compared to that of peptide C20W, confirming that tryptophan 1107 plays a role in the interaction with calmodulin. The results with the analogue C20AA have shown a further affinity decrease, indicating that the adjoining phenylalanine residue (1108) is also important in the binding process: The affinity for calmodulin decreased 3- and 15-fold for peptides C20A and C20AA, respectively, to values clearly out of the range prevailing in the intact enzyme. Although the work on the analogues does not indicate by which mechanism the tryptophan plays its role in the binding of calmodulin, the experiments nicely rationalize the preservation of the tryptophan residue in most calmodulin binding domains. Interestingly, peptide C28W but not peptide C28Y formed

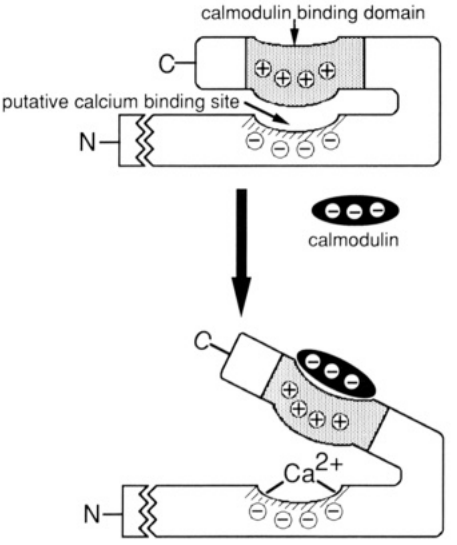

FIGURE 10: Hypothetical scheme of the function of the calmodulin binding domain in the plasma membrane Ca^{2+} pump.

a dimer in 25% TFE buffer solution, again indicating the importance of the Trp in this sequence.

Since peptide C28Y had a higher percentage of secondary structure than peptide C28W in all sovlent systems, it could be suggested that the bulky tryptophanyl residue hinders the formation of a stable secondary structure, thus mediating the flexibility of the domain that is required for optimal packing and hence high-affinity binding. Additional experiments are necessary to determine the exact site of the interaction between calmodulin and its binding domain, and the role played by the α-helix plus β-structure arrangement in the complex. Work currently in progress on other analogues will hopefully help determine the importance of other residues in the domain, e.g., the positively charged amino acids.

Following a suggestion originally made by Klee (1980), work on a number of calmodulin-regulated enzymes (Benaim et al., 1984; Kennelly et al., 1987; Pearson et al., 1988; Foster & Gaeta, 1988) has shown that the calmodulin binding domain, or a portion of it (Benaim et al., 1984), may function as a natural inhibitor of the enzymes, limiting access to the active site. In the case of the plasma membrane Ca^{2+} pump the domain defined here as the active site could either be the site of aspartyl phosphate formation and ATP binding or a Ca^{2+} binding site. The most obvious way to rationalize the inhibition, i.e., the limitation of active site(s) accessibility, is by postulating that the C-terminal calmodulin binding domain, protruding out of the membrane into the cytosol, interacts with the main body of the pump in the absence of calmodulin. A plausible candidate for the interaction would be the negatively charged sequence immediately N-terminal to the calmodulin binding domain (peptide A18). The very high proportion of negatively charged residues favors this sequence as a candidate for a Ca^{2+} binding site (Verma et al., 1988). The experiment shown in Figure 9 has demonstrated that the positively charged calmodulin binding domain and peptide A18 indeed interacted. The experiment presented in Figure 9 leaves the question of the specificity of the interaction between the acidic peptides A18 and the calmodulin binding domain open: i.e., under the experimental conditions other unrelated peptides could also have interacted with the calmodulin binding domain. Peptide A18, however, *is* contained in the pump structure. Work currently under way attempts to determine whether the acidic peptide A18 indeed binds Ca^{2+} as postulated and investigates in detail the role of Ca^{2+} in the interaction of peptide A18 with

the calmodulin binding domain. The scheme shown in Figure 10, while highly simplified, offers a pictorial view of the hypothesis discussed here.

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SUPPLEMENTARY MATERIAL AVAILABLE

Synthesis, deprotection, and purification procedures for the peptides used in this work and various calmodulin CD spectra (15 pages). Ordering information is given on any current masthead page.

Registry No. ATPase, 9000-83-3; peptide A18, 124042-29-1; peptide C15, 123065-58-7; peptide C14, 124069-33-6; peptide C20W, 123045-86-3; peptide C28W, 120057-55-8; peptide C20A, 124042-30-4; peptide C20AA, 124042-31-5; peptide C28A, 123045-88-5; peptide C28Y, 123045-87-4; Ca, 7440-70-2; L-tryptophan, 73-22-3.

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