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Apocalmodulin Binds to the Myosin Light Chain Kinase Calmodulin Target Site*

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The interaction of a 20-residue-long peptide derived from the calmodulin-binding domain of the smooth muscle myosin light chain kinase with calcium-free calmodulin (apocalmodulin) was studied using a combination of isothermal titration calorimetry and differential scanning calorimetry. We showed that: (i) a significant binding between apocalmodulin and the target peptide (RS20) exists in the absence of salt ($K_a = 10^6$ M^{-1}), (ii) the peptide interacts with the C-terminal lobe of calmodulin and adopts a partly helical conformation, and (iii) the presence of salt weakens the affinity of the peptide for apocalmodulin, emphasizing the importance of electrostatic interactions in the complex. Based on these results and taking into account the work of Bayley *et al.* (Bayley, P. M., Findlay, W.A., and Martin, S. R. (1996) *Protein Sci.* 5, 1215–1228), we suggest a physiological role for apocalmodulin.

Calcium is considered as one of the most important cellular second messengers. Variations of calcium concentration are monitored by calcium detectors in the cell (1). Among them, calmodulin (CaM)[1] is the most ubiquitous, being present in all eukaryotic cells where it interacts with and activates numerous proteins, some of them being antagonists. To explain such specificity and diversity of actions is a challenge for structural and cell biologists.

Numerous studies have addressed the mechanism of calcium binding to CaM, its interaction with target proteins, and finally activation of such proteins. Although the mechanism of calcium or enzyme binding to CaM appears to be qualitatively similar, small variations in ionic concentrations or changes in primary structure modify quantitative parameters of binding mechanisms.

In addition, to analyze the interaction between CaM and a CaM-binding protein, the CaM-binding peptides were obtained from the CaM-binding domain of specific enzymes. For example, RS20 peptide, the 20-residue peptide corresponding to the calmodulin-binding domain of the smooth muscle myosin light chain kinase, is derived from the smooth muscle myosin light chain kinase (2), and M13 is derived from the skeletal muscle myosin light chain kinase (3). Numerous other peptides able to bind to CaM are used for such studies. Although these peptides fold as an amphiphilic α-helix, their primary structures are highly diverse (4). An accepted hypothesis is that the mechanism of binding is qualitatively the same for different peptides but the quantitative parameters of the binding mechanism rely on the primary structure of the peptide and CaM. Although most published works were done in the presence of saturating calcium concentrations, some target proteins, such as melittin and seminal plasmin (5), significantly interacted also with the apoform of calmodulin or only with the apoform as neuromodulin (6). However, in all cases, when the thermodynamic parameters were measured, the binding was mainly entropically driven because the enthalpy of the process was about zero.

In the cell, CaM oscillates between the apoform and the calcium saturated form. The characterization of this overall system in thermodynamic terms requires investigation of the apoCaM thermodynamic state in the absence or presence of an interacting peptide. Concerning CaM, we have shown that a combination of isothermal titration calorimetry and differential scanning calorimetry allows one to pinpoint the thermodynamic properties of this exquisitely tuned system (7, 8).

In this report, we analyze the thermodynamic parameters of the RS20-apoSynCaM complex. Our results suggest a physiological role for apocalmodulin that contributes both diversity and specificity to the calmodulin interaction and activation processes.

EXPERIMENTAL PROCEDURES

Materials—Synthetic calmodulin (SynCaM) was produced and purified as described previously (9). Protein purity was checked by SDS gel electrophoresis, high performance capillary electrophoresis, and ESI mass spectrometry. Protein concentration was measured spectrophotometrically with molar extinction of 1560 M^{-1} cm^{-1} at 280 nm (10). For all experiments, ultrapure water (milli-Q apparatus, Millipore Inc.) and plasticware washed in 1 N HCl were used to minimize calcium contamination. Calcium was removed from protein with trichloroacetic acid (11). A 20-residue peptide (RS20) corresponding to the calmodulin-binding domain of the smooth muscle myosin light chain kinase was synthesized and purified as described previously (2, 12). Peptide concentration was determined spectrophotometrically, assuming molar extinction of 5600 M^{-1} cm^{-1} at 280 nm as determined by comparing UV spectrum and amino acid analysis. Other commercially available chemicals were of the highest grade. Protein solutions were prepared in 10 mM sodium cacodylate (some samples also in 50 mM Hepes to compare with the standard medium) buffer, pH 7.5, containing 10 mM EGTA or 1 mM $CaCl_2$. The SynCaM-RS20 complex used in DSC and CD experiments was prepared by mixing the protein with approximately 2-fold molar excess of RS20.

Isothermal Titration Calorimetry—The RS20 peptide binding to SynCaM was analyzed by ITC using a MicroCal MCS ITC instrument. Experiments were carried out at 25 °C. SynCaM concentration (in the 1.34-ml calorimetric cell) ranged from 0.02 to 0.06 mM, whereas RS20 concentration (in the syringe) varied from 0.2 to 0.6 mM. SynCaM was titrated up to the peptide/protein concentration ratio of about 2. The heat of dilution was measured by injecting the peptide into the protein-free buffer solution or by additional injections of peptide after satura-

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[1] The abbreviations used are: CaM, calmodulin; SynCaM, synthetic calmodulin; apocalmodulin, calcium-free calmodulin; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry.

tion; the value obtained was subtracted from the heat of reaction to obtain effective heat of binding. Data were analyzed using the MicroCal Origin software and were fitted with a "one set of sites" model to obtain values for the stoichiometry of binding and the thermodynamic parameters of interaction: enthalpy of binding (ΔH) and association constant (K_a), and, consequently, free energy change (ΔG) and entropy change (ΔS).

Differential Scanning Calorimetry—Microcalorimetric measurements of SynCaM and its complex with RS20 peptide were carried out on a MicroCal VP-DSC instrument in 0.51-ml cells at a heating rate of 1 K/min. Protein concentration varied from 0.6 to 1.9 mg/ml. The heating curves were corrected for an instrumental baseline obtained by heating the solvent used for protein solution. The reversibility of unfolding was checked routinely by sample reheating after cooling in the calorimetric cell. The calorimetric denaturation enthalpy (ΔH_{cal}) and the partial molar heat capacity of the protein (C_p) were determined as described elsewhere (13), assuming that the molecular mass of SynCaM is 16628 Da and the partial specific volume is 0.72 $cm^3\ g^{-1}$ (7). To analyze functions of excess heat capacity, the MicroCal Origin software was used. The calorimetric denaturation enthalpy values are accurate within ±8%, and C_p values at 20 °C are accurate within ±1.5 kJ $K^{-1}\ mol^{-1}$. The errors in the parameters of individual transitions obtained by deconvolution of complex endotherms did not exceed ±10% for transition enthalpy and ±0.6 °C for transition temperature.

Circular Dichroism—CD spectra were recorded on a Jasco J-715 spectropolarimeter. The cell had a light path of 0.02 cm, and protein concentration was 0.4–0.5 mg/ml. The results were expressed as molar ellipticity, [Θ] (deg $cm^2\ dmol^{-1}$), based on a mean amino acid residue weight of 112.4 for calmodulin, 112.6 for calmodulin-RS20 complex and 114.7 for RS20 peptide. The molar ellipticity was determined as $[\Theta] = (\theta \times 100 \text{ mean amino acid residue weight})/(cl)$, where c is the protein concentration in mg/ml, l is the light pathlength in centimeters, and θ is the measured ellipticity in degrees.

RESULTS AND DISCUSSION

RS20 Binds to ApoSynCaM—Isothermal titration calorimetry was used to monitor the RS20 binding to apoSynCaM at pH 7.5 in the presence of 10 mM EGTA (Fig. 1). There was no ITC signal upon apoSynCaM titration with EGTA. Cacodylate buffer (10 mM) was used because we have shown that this buffer concentration does not induce a major change in the secondary structure of apoprotein (7). Some experiments were done in 50 mM Hepes. Despite the different heats of ionization for these buffers (−2 kJ/mol for cacodylate and 20.5 kJ/mol for Hepes), the measured enthalpy of binding has no buffer dependence, as was also shown for Ca^{2+}-CaM (14). Hence, the effect of protonation/deprotonation events is absent, and we obtain true intrinsic enthalpy of binding. Thermodynamic data show that there is a one-to-one complex between RS20 and apocalmodulin (Fig. 1). The RS20 binding to apoCaM is an enthalpically driven process and is entropically unfavorable (Table I). The same was concluded for the peptide binding to Ca^{2+}-CaM (14). Salt increases ordering of the bound state (Table I). In the absence of salt, the association constant was of the order of $10^6\ M^{-1}$ with the interaction enthalpy of −38.9 kJ/mol and interaction entropy of −16 J/K mol. It should be noted that this is the first thermodynamic characterization of a significant binding between apocalmodulin and RS20 in the absence of salt.

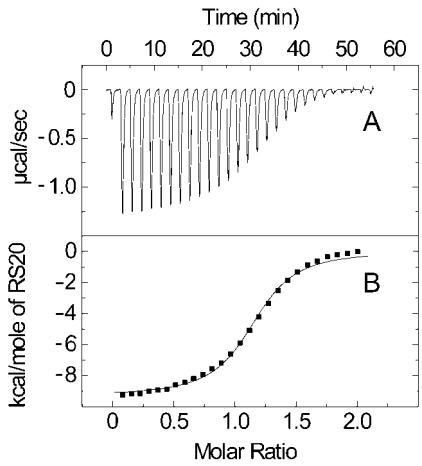

FIG. 1. **Typical ITC data curves of apoSynCaM-RS20 interaction at 25 °C in 10 mM cacodylate buffer at pH 7.5 with 10 mM EGTA.** *A*, titration of 1.34 ml of apoSynCaM ($4.6\ 10^{-5}$ M) by RS20 peptide ($5.5\ 10^{-4}$ M) with 27 injections of 10 μL each. *B*, binding isotherm derived from *A*.

We used 150 mM KCl and 5 mM Mg to approach intracellular conditions. Salt strongly decreases the peptide affinity for apoSynCaM and increases enthalpy and entropy of interaction (Table I). That is probably why previous studies did not report any interaction between RS20 and apoCaM (14). This result demonstrates the importance of electrostatic interactions in the RS20-apoCaM complex. Addition of magnesium ions in the presence of salt has little effect on the peptide binding thermodynamic parameters. In the presence of calcium and the absence of salt, the interaction enthalpy is equal to −84 kJ/mol, which is twice higher than in the absence of calcium.

Modification of Thermodynamic Parameters of Heat Denaturation of ApoSynCaM upon Complex Formation with RS20 Proves That the Peptide Binds to the C-terminal Lobe of the Protein—Temperature dependence of the partial molar heat capacity of apoSynCaM-RS20 complex considerably differs from that of free apoSynCaM (Fig. 2*A*); although the asymmetry of the curve is retained, the pronounced shoulder at lower temperatures is absent. Reheating the samples after cooling from 90 to 5 °C demonstrates that denaturation is highly reversible (90–95%). As seen in Fig. 2*A*, partial molar heat capacities of apoSynCaM and its complex with RS20 at 20 °C are the same, *i.e.* the apoSynCaM flexibility does not change upon complex formation. Free and complexed apoSynCaM do not differ in calorimetric denaturation enthalpies (Table II). The differences appear when we deconvolute both thermograms to two-state transitions; although the number of deconvoluted peaks and the second transition melting temperature do not change, the temperature of the first transition in the complex is 15 °C higher than in the free protein (Fig. 2, *B* and *C*, and Table II). The effect was also observed in Hepes buffer, although the change in the first transition temperature upon complex formation was not so pronounced (Table II). Evidently, RS20 does not contribute to the melting curve. Indeed, as was demonstrated by CD, we were not able to observe any cooperative transition for peptide melting in trifluoroethanol where it assumes the α-helical conformation (data not shown). The first deconvoluted peak corresponds to the C-terminal lobe melting,

TABLE I
Thermodynamic parameters of RS20 peptide binding to ApoSynCaM at pH 7.5 (10 mM cacodylate buffer, 10 mM EGTA)

Sample	Added salt	K_a	ΔH	ΔG	ΔS
		M^{-1}	*kJ/mol*	*kJ/mol*	*J/K mol*
ApoSynCaM	None	1.0×10^6	−38.9	−34.2	−16
ApoSynCaM	100 mM NaCl	4.2×10^3	−106.6	−20.7	−288
ApoSynCaM	150 mM KCl	1.2×10^3	−142.1	−17.6	−418
ApoSynCaM	150 mM KCl 5 mM $MgCl_2$	3.6×10^3	−154.7	−20.3	−451

and the second one is assigned to N-terminal lobe unfolding (7, 15). Thus, the peptide influences only the stability of C-terminal lobe. Consequently, RS20 binds to the C-terminal lobe of apoSynCaM. The twice lower enthalpy of binding for the apoCaM complex with RS20 than for Ca^{2+}-CaM-peptide complex (see above), where both lobes interact with the peptide, supports this conclusion.

Temperatures of apoSynCaM denaturation are increased in the presence of salts, and the highest stabilization is achieved with the combination of KCl and $MgCl_2$ (Table II). In parallel with the ITC results indicating an abrupt drop of the RS20 affinity to apoSynCaM, the DSC data demonstrate that salts abolish the selectivity of RS20 effect on the temperature of the first calorimetric peak (Table II), although ITC suggests the existence of a weak complex. A possible explanation is that the dissociation of the complex takes place in the temperature region before the first transition temperature.

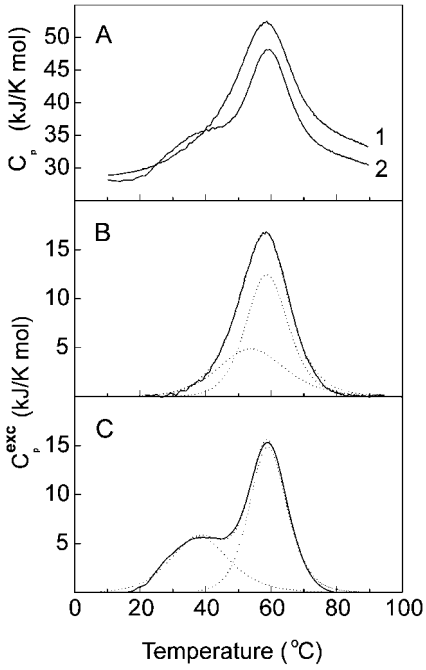

FIG. 2. *A*, temperature dependence of the partial molar heat capacity of apoSynCaM-RS20 complex (*curve 1*) and apoSynCaM (*curve 2*) at pH 7.5 with 10 mM EGTA. Computer deconvolution of the transition excess heat capacity of apoSynCaM-RS20 complex (*B*) and apoSynCaM (*C*). *Solid lines*, experimental results; *dotted lines*, deconvoluted peaks and their sum.

DSC Confirms That in the Presence of Calcium RS20 Interacts with Both Lobes of SynCaM—In the Ca^{2+}-CaM-peptide ternary complex the two domains of Ca^{2+}-CaM interact simultaneously with opposite ends of the peptide (16). Fig. 3 demonstrates melting of this complex and free Ca^{2+}-SynCaM in 150 mM KCl. The presence of salt allows recording much better thermograms of Ca^{2+}-SynCaM and its complex with RS20, which are easily deconvoluted to two two-state transitions (Table II). The peptide binding to Ca^{2+}-SynCaM strongly decreases the partial molar heat capacity at 20 °C (Fig. 3). Hence, Ca^{2+}-SynCaM bound to RS20 is more compact than the unbound form, in accordance with Ref. 16. In contrast to the apoprotein, RS20 in the presence of calcium influences thermodynamic parameters of both transitions. For the first transition, temperature is increased by 20.2 °C and its enthalpy by 30%. For the second transition, temperature is increased by 1.8 °C and its enthalpy is almost doubled. The significant increase of the second peak enthalpy confirms the tight interaction of RS20 with the N-terminal lobe of calmodulin. Binding of RS20 to SynCaM in the presence of calcium is too strong to allow the association constant to be determined by ITC (12, 14).

CD Spectroscopy of ApoSynCaM-RS20 Complex Suggests That the Peptide Acquires a Partly Helical Conformation—The far-UV CD spectra of apoSynCaM, RS20, and their complex at pH 7.5 are shown in Fig. 4. CD spectrum of free peptide (*curve 1*) is typical of an unordered conformation. In general, the spectra of SynCaM and its complex with RS20 are similar to each other and to the α-helical type with characteristic minima near 208 and 222 nm. The peptide addition has no effect on the CD spectrum of Ca^{2+}-saturated SynCaM (data not shown) but results in a noticeable increase of CD minima of apoSynCaM (Fig. 4, *curves 2* and *3*), raising the helicity from 36% (apoSynCaM) to 42% (apoSynCaM-RS20). The α-helical content was calculated from the ellipticity of the samples at 222 nm according to Ref. 17.

As is known, the two domains of Ca^{2+}-CaM remain essentially unchanged upon complex formation with RS20, whereas the central helix of the native calmodulin unwinds, and the bound peptide acquires an α-helical conformation from resi-

TABLE II

Scanning calorimetry thermodynamic parameters of temperature-induced denaturation of ApoSynCaM, SynCaM, and their complexes with RS20 peptide at pH 7.5 (10 mM cacodylate buffer)

ApoSynCaM is in the presence of 10 mM EGTA, and SynCaM is in the presence of 1 mM $CaCl_2$.

Sample	Added salt	ΔH_{cal}[a]	First transition[b]		Second transition[b]	
			T_t^1	ΔH_t^1	T_t^2	ΔH_t^2
		kJ/mol	*°C*	*kJ/mol*	*°C*	*kJ/mol*
ApoSynCaM	None	348	38.9	124	59.6	224
ApoSynCaM-RS20	None	335	53.9	118	58.7	217
ApoSynCaM	100 mM NaCl	358	50.1	134	65.4	224
ApoSynCaM-RS20	100 mM NaCl	405	49.7	173	64.4	232
ApoSynCaM	150 mM KCl	361	47.4	147	65.0	214
ApoSynCaM-RS20	150 mM KCl	353	47.8	138	64.8	215
ApoSynCaM	150 mM KCl 5 mM $MgCl_2$	348	65.6	131	80.7	217
ApoSynCaM-RS20	150 mM KCl 5 mM $MgCl_2$	369	64.5	143	79.7	226
ApoSynCaM[c]	None	297	41.4	110	60.8	210
ApoSynCaM-RS20[c]	None	341	47.1	126	60.9	222
SynCaM	150 mM KCl	336	80.2	129	103.4	250
SynCaM-RS20	150 mM KCl	557	100.4	170	105.2	486

[a] Total calorimetric enthalpy of denaturation.
[b] Transitions were determined by deconvolution analysis of the heat absorption curves to two-state transitions.
[c] In 50 mM Hepes buffer.

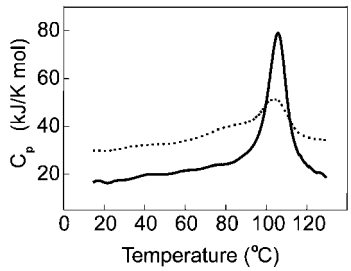

FIG. 3. **Temperature dependence of the partial molar heat capacity of Ca^{2+}-SynCaM (*dotted line*) and Ca^{2+}-SynCaM-RS20 complex (*solid line*) at pH 7.5 with 1 mM $CaCl_2$ and 150 mM KCl.**

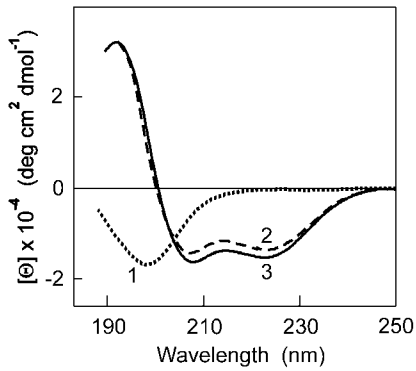

FIG. 4. **CD spectra of RS20 peptide (*curve 1*), apoSynCaM (*curve 2*) and apoSynCaM-RS20 complex (*curve 3*) at pH 7.5 with 10 mM EGTA.** The curve width determines the accuracy of the ellipticity values.

dues 4–18 (18). The identity of CD spectra of Ca^{2+}-SynCaM and its complex with RS20 demonstrates that the increase in CD resulting from the secondary structure formation in the peptide is compensated by the decrease of CD effect because of partial destruction of the central helix of calmodulin.

As is shown above, in the apoSynCaM-RS20 complex the peptide is bound only to the C-terminal lobe of the protein. This type of binding does not require large conformational changes as for Ca^{2+}-SynCaM. Hence, the 6% increase in helicity for the apoSynCaM-RS20 complex in comparison to apoSynCaM is caused by a conformational change in the peptide from a random coil state to about 50% helical conformation upon binding to apoSynCaM.

Physiological Implications of RS20-Apocalmodulin Complex—The interaction and activation of a set of target enzymes by calmodulin has raised the question of selectivity of these processes. It has been proposed that different CaM-enzyme complexes may exist depending on the extent of calcium saturation of calmodulin (11, 19, 20). To analyze such structures, model peptides are used such as those derived either from the smooth muscle myosin light chain kinase (RS20) or from the skeletal myosin light chain kinase (M13). X-ray crystallography (18) and NMR studies (21) have allowed description of the structure of a peptide-$CaMCa_4$ complex. In the recent and stimulating work, Bayley *et al.* (20) have described the existence of a peptide-CaM complex when two calcium ions are bound to CaM. This complex involves contacts between the C-terminal lobe of CaM and the N-terminal part of the peptide. The results presented in this report show the existence of the RS20-apoCaM complex that is mainly driven by electrostatic interactions. Therefore, besides the existence of the peptide-$CaMCa_2$ described in Ref. 20 and the RS20-$CaMCa_4$ described in Ref. 18, we propose the third physiological relevant complex, namely the RS20-apocalmodulin. The sensitivity of these complexes to ionic strength and magnesium ion is probably different and allows a fine tuning in the interaction with the diverse CaM-binding domains under different ionic conditions. Such tuning is due to an exquisite equilibrium between electrostatic and hydrophobic interactions in the CaM-peptide complexes (22). Such equilibrium is modulated by calcium binding, sequence diversity, and ionic conditions of the medium. The apoSynCaM-RS20 complex is mainly driven by electrostatic interaction, whereas RS20-SynCaM-Ca_4 is driven by hydrophobic forces.

Similar results concerning the calmodulin-binding domain of cyclic phosphodiesterase appeared in the paper of Yuan *et al.* (23) when our work was almost finished. This reinforces our conclusion that the target proteins interact with apoCaM, but the affinity is quantitatively modulated.

In conclusion, the existence of a complex between apoCaM and target proteins allows us to propose that such preformed complexes may exist in the cell and that calmodulin is not free to diffuse in the cytoplasm. Therefore, the role of cell architecture in the function of a given protein is as important as that of kinetic and thermodynamic mechanisms.

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