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Determination of the Free-Energy Coupling for Binding of Calcium Ions and Troponin I to Calmodulin†

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ABSTRACT: Regulation of a wide variety of biological systems by Ca^{2+} is now known to be mediated through calmodulin, a Ca^{2+}-binding protein. Calmodulin forms Ca^{2+}-dependent complexes with several proteins, including troponin I. We have determined the free-energy coupling ($\Delta G°_{CT}$) for binding of Ca^{2+} and troponin I to calmodulin by measuring Ca^{2+} binding to calmodulin and to the 1:1 calmodulin·troponin I complex by equilibrium dialysis. The dissociation constant for the $Ca^{2+}{}_4$·calmodulin and troponin I complex was also determined directly by monitoring fluorescence intensity changes accompanying complex formation between $Ca^{2+}{}_4$·CaM and *N*-dansylaziridine–troponin I, a fluorescent derivative of troponin I. Calmodulin displayed four Ca^{2+}-binding sites of similar affinity with a geometric mean dissociation constant of 14 μM. In the presence of saturating troponin I, the geometric mean dissociation constant for the four Ca^{2+}-binding sites was shifted to 1.7 μM. $\Delta G°_{CT}$ was therefore −1.25 kcal/mol of Ca^{2+}. Saturation of calmodulin with Ca^{2+} would therefore be expected to increase its affinity for troponin I about 4500-fold. A dissociation constant of 20 nM was determined for the $Ca^{2+}{}_4$·calmodulin·*N*-dansylaziridine–troponin I complex. The dissociation constant of the calmodulin·*N*-dansylaziridine–troponin I complex in the absence of Ca^{2+} should therefore be about 90 μM. A prediction of this data is that binding of Ca^{2+} to calmodulin will show strong positive cooperativity when Ca^{2+} binding to a substoichiometric number of sites is sufficient to promote calmodulin·troponin I complex formation.

Calmodulin (CaM)[1] was discovered by Cheung as an activator of the Ca^{2+}-sensitive 3′:5′-cyclic-nucleotide phosphodiesterase (Cheung, 1970). This regulatory protein mediates Ca^{2+} stimulation of several enzymes including an isozyme of 3′:5′-cyclic-nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi et al., 1970), brain adenylate cyclase (Brostrom et al., 1975), myosin light chain kinase (Dabrowska et al., 1978), phosphorylase kinase (Cohen et al., 1978), (Ca^{2+} + Mg^{2+})-ATPase (Gopinath & Vincenzi, 1977; Jarrett & Penniston, 1977), NAD kinase (Anderson & Cormier, 1978), and phospholipase A_2 (Wong & Cheung, 1979). CaM also mediates the Ca^{2+} sensitivity of microtubule disassembly and contraction during mitosis (Welsh et al., 1978) and binds in a Ca^{2+}-dependent manner to troponin I (Amphlett et al., 1976) and to calcineurin (Wang & Desai, 1977; Klee & Krinks, 1978). Ca^{2+}-dependent complex formation between CaM and several of these proteins has been demonstrated by a number of techniques including gel filtration (Teshima & Kakiuchi, 1974), electrophoresis on nondenaturing gels (Amphlett et al., 1976; LaPorte & Storm, 1978), CaM–Sepharose affinity chromatography (Watterson & Vanaman, 1976; Klee & Krinks, 1978; Westcott et al., 1979), fluorescence techniques (LaPorte et al., 1981), and cross-linking of [^{125}I]CaM to CaM-binding proteins LaPorte et al., 1979; Richman & Klee, 1978; Andreasen et al., 1981).

Although it is generally agreed that CaM binds 4 mol of Ca^{2+}, there are significant differences in the reported affinities for Ca^{2+} (Teo & Wang, 1973; Lin et al., 1974; Watterson et al., 1976; Wolff et al., 1977; Dedman et al., 1977; Jarrett & Kyte, 1979; Crouch & Klee, 1980). Some of these discrepancies in affinity of CaM for Ca^{2+} have been attributed to differences in ionic strength (Dedman et al., 1977). Binding of Ca^{2+} to CaM in the presence of CaM-binding proteins has not been well-characterized. A widely accepted model for interaction of CaM and the Ca^{2+}-sensitive phosphodiesterase is based on results from several laboratories [reviewed in Wang (1977)]:

$$n\mathrm{Ca}^{2+} + \mathrm{CaM} \rightleftarrows \mathrm{Ca}^{2+}{}_n\cdot\mathrm{CaM}^*$$

$$(\mathrm{PDE})_2 + 2(\mathrm{Ca}^{2+}{}_n\mathrm{CaM}^*) \rightleftarrows (\mathrm{Ca}^{2+}{}_n\cdot\mathrm{CaM}^*)_2(\mathrm{PDE})_2 \rightleftarrows (\mathrm{Ca}^{2+}{}_n\cdot\mathrm{CaM}^*)_2(\mathrm{PDE}^*)_2$$

where PDE represents one subunit of the dimeric phospho-

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[1] Abbreviations: AEDANS-CaM, 5-[[(acetylamino)ethyl]amino]-1-naphthylaminesulfonic acid labeled calmodulin; CaM, calmodulin; DANZ-TnI, *N*-dansylaziridine-labeled troponin I; DNP-Gly, dinitrophenylglycine; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; [^{125}I]CaM, ^{125}I-labeled calmodulin; $\bar{K}_d$, geometric mean dissociation constant; Mops, 3-(*N*-morpholino)propanesulfonic acid; PDE, Ca^{2+}-sensitive 3′:5′-cyclic-nucleotide phosphodiesterase; TnI, troponin I; EPPS, 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

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diesterase and CaM* and PDE* are the activated forms of the respective proteins. According to this model, Ca^{2+} binds to the inactive, unliganded CaM, causing it to associate with the PDE in the second step. Ca^{2+} is assumed to be obligatory for CaM·PDE complex formation. However, this description does not adequately describe the Ca^{2+}-independent affinity of CaM and CaM-binding proteins predicted by thermodynamic considerations and does not provide a basis for quantitative prediction of induced positive cooperativity in CaM Ca^{2+} binding in the presence of CaM-binding proteins. Quantitative models based on kinetic data for Ca^{2+}·CaM interactions with myosin light chain kinase (Blumenthal & Stull, 1980) and with the PDE (Wang et al., 1980; Huang et al., 1981; Cox et al., 1981) have only recently been published. We have investigated the interactions of CaM, Ca^{2+}, and one CaM-binding protein, troponin I (TnI), by use of equilibrium binding techniques (Keller et al., 1980). Ca^{2+} binding to CaM was measured in the presence and absence of troponin I (TnI), and the data were analyzed by the method proposed by Weber (1975) to determine the free-energy coupling between binding of Ca^{2+} and TnI to CaM. This thermodynamic parameter is required to develop quantitative models for CaM interactions with various protein systems. TnI was used in these studies because it can be readily purified in high yield and does not bind Ca^{2+}. The behavior of the CaM·TnI complex is typical of other CaM·protein complexes in its relatively low K_d (~60 nM) in the presence of Ca^{2+} (LaPorte et al., 1981) and in its competitive antagonism of CaM stimulation of PDE (LaPorte et al., 1980).

Materials and Methods

Materials. $^{45}CaCl_2$ and [*acetic*-2-^{14}C]EDTA were purchased from ICN. ^3H_2O was from NEN. *N*-Dansylaziridine was obtained from Molecular Probes. Dialysis membrane, M_r 6000–8000 cutoff, was a product of Spectrum Medical Industries. All other chemicals were of reagent grade or better.

Protein Preparations. CaM was prepared as previously described (LaPorte et al., 1979). Parvalbumin was obtained from Sigma. TnI was purified by the method of Wilkinson (Wilkinson, 1974). Preparation of [^{125}I]CaM was as described previously (LaPorte & Storm, 1978). The *N*-dansylaziridine derivative of TnI (DANZ-TnI) was prepared by using methods similar to Scouten et al. (1974) for modification of sulfhydryl residues. The labeling of TnI was performed as the TnI·$Ca^{2+}{}_4$·CaM complex consisting of 2 mg of TnI and 1.7 mg of CaM in 0.1 M EPPS, pH 8.2, 150 mM KCl, 0.25 mM $CaCl_2$, and 20 mg of *N*-dansylaziridine absorbed to 30% (w/w) Celite in a volume of 2 mL. The resultant slurry was rotated for 6 h at room temperature and spun at low *g* values to sediment the Celite. The supernatant was applied to a Sephadex G-25 column (1 × 15 cm) equilibrated in 100 mM sodium acetate, pH 5.0, 8 M deionized urea, 50 mM KCl, 2 mM DTT, and 1 mM EGTA. DANZ-TnI was separated from CaM by passing the sample over Whatman CM-52 resin preequilibrated in 100 mM sodium acetate, pH 5.0, 50 mM KCl, 2 mM DTT, 8 M deionized urea, and 1 mM EGTA and washed with the same buffer to elute CaM. Bound DANZ-TnI was eluted with 0.1 N HCl, dialyzed against 0.01 N HCl, and stored in aliquots at −70 °C. The extent of *N*-dansylaziridine incorporation into TnI was determined by absorbance at 350 nm with the molar extinction coefficient taken to be 3980 M^{-1} cm^{-1} (Johnson et al., 1978) in 50 mM Mops, pH 7.2, 8 M deionized urea, 150 mM KCl, and 2 mM DTT. CaM contamination of DANZ-TnI was not detectable by $NaDodSO_4$–polyacrylamide gel electrophoresis following separation of CaM from DANZ-TnI on CM-52 resin. The extent of incorporation was typically ~0.8 mol of probe/mol of TnI.

Protein concentrations were determined either by the method of Lowry (Lowry et al., 1951) or by spectrophotometry using $E_{1\%}$ = 1.8 at 277 nM for purified CaM (Watterson et al., 1976) and $E_{1\%}$ = 3.97 at 280 nm for purified TnI (Wilkinson, 1974). Where molar concentrations are reported for CaM or TnI, molecular weight values of 16723 (Vanaman et al., 1977) or 23000 (Wilkinson, 1974) were used, respectively.

Gel Filtration. A Sephadex G-150 column (1 × 95 cm) was equilibrated with 20 mM Mops (pH 7.0), 150 mM KCl, 1 mM $MgCl_2$, 0.1 mM DTT, and 0.1 mM $CaCl_2$. Protein samples in the same buffer were applied in a 1-mL volume and eluted at a flow rate of 5 mL/h.

Ca^{2+} Binding to CaM. Binding of $^{45}CaCl_2$ to CaM was quantitated by equilibrium dialysis in microdialysis cells (Riverside Scientific). All reagents were prepared and stored in polypropylene vessels previously washed in 0.25 N HCl and distilled H_2O. The microdialysis cells were washed successively with 0.1% Lubrol PX, distilled water, 95% ethanol, 0.25 N HCl, and distilled water before use. Dialysis membranes were washed with EDTA and H_2O before use, with [^{14}C]EDTA being used to monitor removal of the chelator. CaM was dialyzed at 6 °C against 10 mM Mops (pH 7.2) and 150 mM KCl. The CaM was passed through a 1.4 × 4 cm parvalbumin–Sepharose column (5 mg of parvalbumin/mL of packed resin) to ensure adequate removal of Ca^{2+}. This column was regenerated before use as described by Lehky et al. (1977) except that [^{14}C]EDTA was included to monitor removal of chelator. $^{45}CaCl_2$ was added to the dialyzed CaM in order to monitor removal of Ca^{2+} by the parvalbumin–Sepharose column. The calcium concentrations of the protein solutions and reagents were determined by atomic absorption using a Perkin-Elmer Model 305B atomic absorption spectrophotometer with a graphite furnace. Total calcium contamination from protein solutions and buffer components ranged from 1.1 to 2.8 μM in different experiments. Equilibrium dialysis in microdialysis cells (100 μL/side) was at 25 °C for 4 h, with the equilibration time for Ca^{2+} being 1.5 h. Unless otherwise indicated, dialysis was in 10 mM Mops (pH 7.2), 150 mM KCl, 2 mM DTT, and 1 mM $MgCl_2$ containing $^{45}CaCl_2$ (1.2×10^5 cpm/cell) and varying concentrations of $CaCl_2$. $MgCl_2$ and KCl were included to approximate physiological ionic strength and Mg^{2+} concentration, while DTT was included to prevent oxidation of TnI sulfhydryl groups. Recovery of $^{45}Ca^{2+}$ from the dialysis cells was quantitative, indicating that the cells did not bind significant amounts of Ca^{2+}. Recovery of protein was also quantitative, as determined by protein assay or by recovery of [^{125}I]CaM in control experiments. No evidence of proteolytic degradation was observed when CaM and TnI samples were incubated under equilibrium dialysis conditions (with NaCl replacing KCl) and then submitted to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Experimental errors from all sources in calcium-binding determinations were about 10%.

Fluorescence Measurements. Fluorescence measurements were made with an SLM 4800 S spectrofluorometer. Sample temperatures were maintained at 25 ± 0.1 °C. The buffer used for the titrations was 10 mM Mops, pH 7.2, 150 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, and 0.1 mM $CaCl_2$ with other additions as indicated. Excitation was at 350 nm, and resolution of the excitation monochromator was set at 4 nm.

Protein–protein complex formation between Danz-TnI and $Ca^{2+}{}_4$·CaM was monitored by measuring fluorescence intensity changes. Saturation of Danz-TnI with $Ca^{2+}{}_4$·CaM resulted in a 1.8-fold increase in fluorescence intensity. The observed

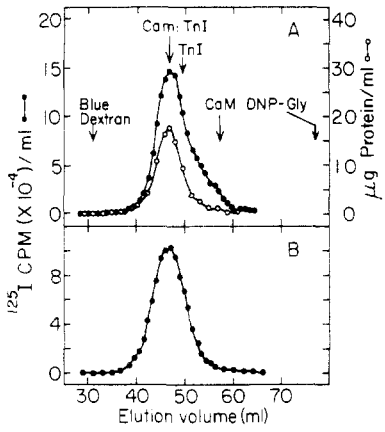


FIGURE 1: Sephadex G-150 elution profile for the CaM·TnI complex. Gel filtration was carried out as described under Materials and Methods. (A) Elution profile for a sample containing 73 μg of CaM (4.4 μM), 1.5×10^{-6} cpm of [^{125}I]CaM, and 100 μg of TnI (4.4 μM). (B) elution profile for a sample containing CaM (0.52 μM), 1.0×10^{-6} cpm of [^{125}I]CaM, and 100 μg of TnI (4.4 μM). Protein mixtures were incubated for 1 h at 6 °C before application to the column. The elution volumes for Blue Dextran, TnI, CaM, and DNP-Gly were detemined in independent experiments. The column was calibrated by using catalase (M_r 250 000), alcohol dehydrogenase (M_r 140 000), BSA (M_r 67 000), soybean trypsin inhibitor (M_r 22 500), myoglobin M_r 17 300), and cytochrome (M_r 13 300). The column buffer contained 20 mM Mops, pH 7.0, 150 mM KCl, 1 mM $MgCl_2$, 0.1 mM DTT, and 0.1 mM $CaCl_2$.

fluorescence intensity in a mixture of species is the weighted average of the individual fluorescence intensities. For a binary mixture, the solution is

$$I = f_1 I_1 + f_2 I_2 \quad (1)$$

where f_1 and f_2 are the mole fractions of the first and second species, respectively, and I_1 and I_2 are the intensities. Equation 1 yields the following relationship for the calculation of α, the fraction of DANZ-TnI bound;

$$\alpha = f_B = \frac{I - f_F I_F}{I_B} \quad (2)$$

where I_F and I_B are the limiting intensities for the free and bound species, respectively, and f_F and f_B are the mole fractions of the free and bound species. Samples consisting of 4.0×10^{-8} M DANZ-TnI in 1.5 mL of buffer were titrated with $Ca^{2+}{}_4$·CaM in parallel with a buffer blank.

Total fluorescence intensity was determined without polarizers, and background fluorescence intensity was subtracted from the total fluorescence intensity measured as above. The blank-corrected sample intensity was corrected for dilution for each titration point. Dilution correction factors did not exceed 7% of the total measured fluorescence intensity. I_F and I_B were determined from the limits of titration, and α was calculated by using eq 2 and the corrected intensity.

Results

Stoichiometry of the CaM·Troponin I Complex. The stoichiometry of the CaM·TnI complex was examined by Sephadex G-150 gel filtration. In independent experiments, CaM and TnI were eluted as single symmetrical peaks of apparent molecular weights 24 000 and 46 000, respectively. The apparent molecular weight observed for the free TnI might suggest that the TnI exists as a dimer. However, on titration of 0.1 μM DANZ-TnI with native TnI to 10 μM, no significant change in either fluorescence anisotropy or intensity was detected (data not shown). This would suggest that the anomalously high Stokes' radius of the free TnI is attributable to its shape or degree of hydration, rather than dimerization.

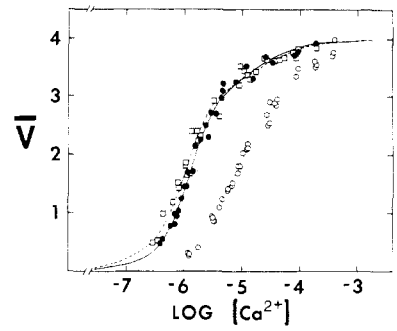


FIGURE 2: Ca^{2+} binding to CaM in the absence (○) and in the presence of stoichometric ● and higher (□) concentrations of TnI. Binding of Ca^{2+} to CaM was determined by equilibrium dialysis in 10 mM Mops (pH 7.2), 150 mM KCl, 1 mM $MgCl_2$, and 2 mM DTT as described under Materials and Methods and that data plotted as a function of the log of free molar Ca^{2+} concentration, where $\bar{V}$ represents moles of Ca^{2+} bound per mole of CaM. The experimental points are duplicate or triplicate determinations for five independent experiments. The curves drawn were generated by fitting the data to the Adair equation (Adair, 1925) by an iterative procedure. CaM was 10 μM in all cases, while TnI, where present, was either 10 or 30 μM.

When stoichiometric concentrations of CaM and TnI were run in the presence of 100 μM $CaCl_2$, a complex of apparent molecular weight 66 000 was observed (Figure 1A). The CaM·TnI complex was not observed when 100 μM EGTA was substituted for 100 μM $CaCl_2$ in the column buffer. When the ratio of TnI to CaM was 0.5, two peaks were observed at molecular weights of 24 000 and 66 000, corresponding to free CaM and CaM·TnI complex (data not shown). No higher molecular weight species were detected when the ratio of TnI to CaM was increased 8-fold (Figure 1B). The invariance of the apparent molecular weight of the CaM·TnI complex detected at TnI to CaM molar ratios of 0.5–8 suggests that the stoichiometry of the CaM·TnI complex is 1:1. This conclusion is consistent with other published data concerning the stoichometry of the CaM·TnI complex (LaPorte et al., 1980; Andreasen et al., 1981).

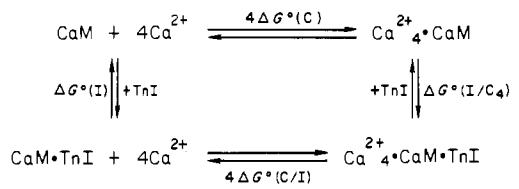
Binding of Ca^{2+} to Calmodulin and the Calmodulin·Troponin I Complex. Ca^{2+} binding to CaM was quantitated in the absence of TnI and in the presence of stoichiometric and higher TnI concentrations (Figure 2). The binding experiments were at pH 7.2 in 10 mM Mops, 150 mM KCl, 1 mM $MgCl_2$, and 2 mM DTT, conditions chosen to approximate physiological ionic strength and Mg^{2+} concentration. CaM exhibited four Ca^{2+}-binding sites, both in the presence and in the absence of TnI. No Ca^{2+} binding to TnI was detected (data not shown). Half-maximal Ca^{2+} saturation was at 11.2 μM for CaM and 1.58 and 1.41 μM for CaM with stoichiometric and higher TnI concentrations, respectively. Addition of a stoichiometric concentration of TnI resulted in a severalfold increase in affinity for Ca^{2+}, while a further increase in the TnI–CaM ratio of up to 3:1 had only a slight additional effect on the affinity for Ca^{2+}. Identical data were obtained at 5 μM CaM and similar molar ratios of TnI (not shown). For an ideal system of equivalent noninteracting binding sites, the logarithmic interval of the free Ca^{2+} concentration from 0.1 to 0.9 fractional saturation is 1.908 (Weber, 1975), with lower values indicative of positive cooperativity and higher values indicative of either negative cooperativity or heterogeneity in the affinities of the sites. For 10 μM CaM, this interval was 2.0, 1.9, and 2.1 in the presence of 0, 10, and 30 μM TnI, respectively. Scatchard plots (Scatchard, 1949) of the data in Figure 2 showed evidence of both positive cooperativity and site heterogeneity for Ca^{2+} binding to CaM alone (Hill coefficient 1.29) and to CaM in the presence of a

Table I: Macroscopic Ca^{2+} Dissociation Constants ($M \times 10^6$) [a]

	CaM	CaM-TnI (1:1)	CaM-TnI (1:3)
K_1	5.3	0.71	0.38
K_2	3.6	3.0	6.3
K_3	22.0	0.53	0.19
K_4	83.0	16.0	20.0
$\bar{K}_d$	14	2.1	1.7

[a] The constants shown are for Ca^{2+} binding to 10 μM CaM in 10 mM Mops (pH 7.2), 150 mM KCl, 1 mM $MgCl_2$, and 2 mM DTT with a relative TnI concentration as indicated.

Scheme I



stoichiometric TnI concentration (Hill coefficient 1.45) or with TnI 3-fold in excess of CaM (Hill coefficient 1.36). Cooperativity for Ca^{2+} binding to CaM has been reported by Crouch & Klee (1980). Since these data were not strictly compatible with a system displaying four noninteracting binding sites of equal affinity, they were fit to the Adair equation (Adair, 1925) by an iterative procedure. The individual macroscopic dissociation constants which describe the data are shown in Table I.

Calculation of the Free Energy of Coupling for Binding of Ca^{2+} and Troponin I to CaM. The data shown in Figure 2 can be analyzed by the method proposed by Weber (Weber, 1975). Although the data are complex, showing some evidence of both positive cooperativity and heterogeneity (or negative cooperativity), the total free-energy change on saturating the CaM or CaM·TnI complex with Ca^{2+} can be most simply described as

$$\Delta G^\circ = 4RT \ln [Ca^{2+}]_m = 4RT \ln \bar{K}_d$$

where $[Ca^{2+}]_m$ is the median ligand concentration (Wyman, 1967), equivalent to the geometric mean dissociation constant, $\bar{K}_d$. This is equal to $(K_1K_2K_3K_4)^{1/4}$, the fourth root of the product of the macroscopic dissociation constants. As shown in Table I, these values are 14 μM for CaM and 2.1 and 1.7 μM for CaM at stoichiometric and saturating TnI concentrations, respectively. The following standard free energy changes can be identified for formation of the Ca^{2+}_4·CaM·TnI complex: $\Delta G^\circ(C)$ is the free-energy change upon binding of 1 mol of Ca^{2+} to CaM, $\Delta G^\circ(I)$ is the free-energy change upon binding of TnI to CaM, $\Delta G^\circ(C/I)$ is the free-energy change upon binding of 1 mol of Ca^{2+} to CaM·TnI complex, and $\Delta G^\circ(I/C_4)$ is the free-energy change upon binding of TnI to Ca^{2+}_4·CaM. These free-energy changes are shown in Figure 3 where it is assumed for simplicity that each of the individual Ca^{2+}-binding sites have the same $\Delta G^\circ(C)$ and $\Delta G^\circ(C/I)$ values. The relationship of these free-energy changes is also described by the thermodynamic cycle shown in Scheme I. As can be seen in Figure 3 and Scheme I, conservation of standard free energy requires that

$$4[\Delta G^\circ(C/I) - \Delta G^\circ(C)] = \Delta G^\circ(I/C_4) - \Delta G^\circ(I) = 4\Delta G^\circ_{IC}$$

where ΔG°_{IC} is the average free energy of coupling for binding of 1 mol of Ca^{2+} and TnI to CaM. The geometric mean dissociation constants ($\bar{K}_d$'s) determined for CaM and CaM saturated with TnI (Table I) correspond to $\Delta G^\circ(C)$ and

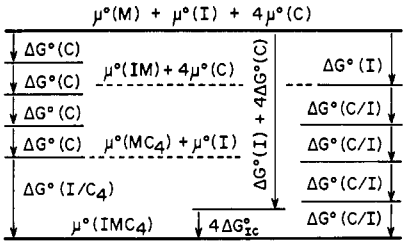


FIGURE 3: Standard free energy changes associated with formation of the Ca^{2+}_4·CaM·TnI Complex (IMC_4) from Ca^{2+} (C), CaM (M), and TnI (I). $\Delta G^\circ(C)$ is the free-energy change upon binding of 1 mol of Ca^{2+} to CaM, $\Delta G^\circ(I)$ is the free-energy change upon binding of TnI to CaM, $\Delta G^\circ(C/I)$ is the free-energy change upon binding of 1 mol of Ca^{2+} to CaM·TnI, and $\Delta G^\circ(I/C_4)$ is the free-energy change upon binding of TnI to Ca^{2+}_4·CaM. ΔG°_{IC} is the free-energy coupling/mole of Ca^{2+}.

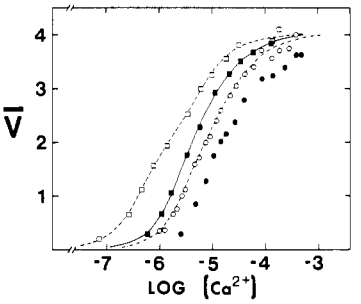


FIGURE 4: Ca^{2+} binding to CaM at 20 (□), 100 (■), 150 (○), and 300 mM KCl (●). CaM was at 10 μM in 10 mM Mops (pH 7.2), 2 mM DTT, and KCl at the indicated concentrations. The data are expressed as a function of the log of the free molar Ca^{2+} concentration, where $\bar{V}$ represents moles of Ca^{2+} bound per mole CaM. The experimental points are duplicate or triplicate determinations for two independent experiments. Ca^{2+}-binding determinations and treatment of data were as described under Materials and Methods.

$\Delta G^\circ(C/I)$, respectively. Calculation of ΔG°_{IC} from the $\bar{K}_d$'s determined for CaM and CaM saturated with TnI (Table I) yielded an average free-energy coupling of −1.25 kcal/mol of Ca^{2+}. Thus, for $4\Delta G^\circ_{IC} = -5.0$ kcal, the affinity of TnI for CaM should be increased about 4500-fold when CaM is saturated with Ca^{2+}, since $4\Delta G^\circ_{IC} = RT \ln K(I/C_4) - RT \ln K(I)$.

Effect of Experimental Conditions on Ca^{2+} Binding to CaM. Ca^{2+} binding to CaM was examined over a range of KCl and $MgCl_2$ concentrations. The affinity of CaM for Ca^{2+} in the absence of Mg^{2+} varied 14-fold within a KCl concentration range of 20–300 mM (Figure 4). The $\bar{K}_d$'s for Ca^{2+} binding to CaM at 20, 100, 150, and 300 mM KCl were 1.6, 4.9, 9, and 23 μM, respectively. The corresponding log intervals of the free Ca^{2+} concentrations between 0.1 and 0.9 fractional saturation were 2.1, 1.8, 1.9, and 2.1. As with the Ca^{2+} binding to CaM shown in Figure 2, this data showed some evidence of both positive cooperativity and heterogeneity at all KCl concentrations. The explanation for the nonmonotonic behavior of these log intervals with increasing ionic strength is not known, but the conformation of CaM is dependent upon ionic strength (Richman & Klee, 1979; Crouch & Klee, 1980). Such alterations in the conformation of CaM may differentially effect the extent of cooperativity of the higher affinity binding sites and the degree of heterogeneity of the four sites. The individual macroscopic dissociation constants determined for CaM Ca^{2+} binding in 100 mM KCl differed by less than 2-fold from those determined at the same KCl concentration by Crouch & Klee (1980). CaM Ca^{2+} binding was also examined over a pH range of 6.8–7.8, but only slight differences in affinity were detected over this range when binding was examined at 150 mM KCl.

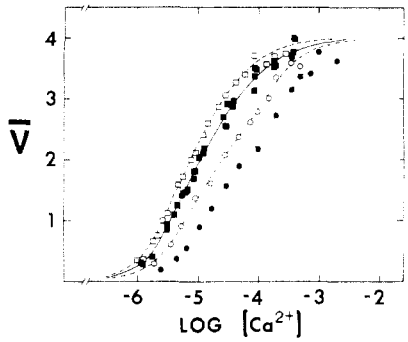


FIGURE 5: Ca^{2+} binding to CaM at 0 (□), 1 (■), 3 (○), and 10 mM $MgCl_2$ (●). CaM was at 10 μM in 10 mM Mops (pH 7.2), 150 mM KCl, 2 mM DTT, and $MgCl_2$ at the indicated concentrations. The experimental points are duplicate or triplicate determinations for two independent experiments. Ca^{2+}-binding determinations and treatment and expression of the data are as described under Materials and Methods and in Figure 2.

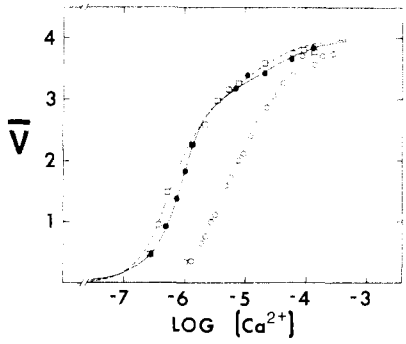


FIGURE 6: Ca^{2+} binding to CaM in the absence (○) and in the presence of stoichiometric (●) and higher (□) concentrations of TnI. CaM was at 10 μM in all cases, in 10 mM Mops (pH 7.2), 150 mM KCl, and 2 mM DTT. TnI, when present, was at 10 or 30 μM. Ca^{2+}-binding determinations were described under Materials and Methods and treatment and expression of the data as in Figure 2.

The affinity of CaM for Ca^{2+} with 150 mM KCl present was influenced by Mg^{2+} (Figure 5), varying 7-fold within a range of 0–10 mM $MgCl_2$. The $\bar{K}_d$'s at 0, 1, 3, and 10 mM $MgCl_2$ were 9, 14, 29, and 63 μM, respectively, with corresponding log intervals from 0.1 to 0.9 fractional saturation of 1.9, 2.0, 2.2, and 2.3. Four Ca^{2+}-binding sites were detected over this range of Mg^{2+} concentrations. Mg^{2+} (3 mM) increased the $\bar{K}_d$ for Ca^{2+} binding about 3-fold, again in approximate agreement with data of Crouch & Klee (1980), although they were unable to distinguish whether 3 or 4 mol of Ca^{2+} was bound at saturation. The results shown in Figure 4 differ from those of Wolff et al. (1977) in the affinities of the CaM Ca^{2+} sites and the magnitude of the inhibition of CaM Ca^{2+} binding by Mg^{2+}, but a reduction in affinity for Ca^{2+} and a decrease in Mg^{2+} inhibition of Ca^{2+} binding with increasing ionic strength may be anticipated from a comparison of the observations of Wolff et al. (1977) and Dedman et al. (1977).

Since the affinity of CaM for Ca^{2+} showed some dependence on Mg^{2+} with the given assay conditions, Ca^{2+} binding to CaM·TnI complex was also examined in the absence of Mg^{2+} (Figure 6). The effect of saturation of CaM with TnI on its affinity for Ca^{2+} was similar to that observed with 1 mM $MgCl_2$ present. The observed $\bar{K}_d$'s for Ca^{2+} binding to CaM and to CaM with stoichiometric and excess TnI present were 9, 1.7, and 1.3 μM, respectively. The average free energy of coupling for binding of 1 mol of Ca^{2+} and TnI to CaM in the absence of Mg^{2+} was therefore −1.15 kcal/mol of Ca^{2+}, a value similar to that observed with 1 mM $MgCl_2$ present. The lower value (−0.95 kcal/mol of Ca^{2+}) determined in a preliminary

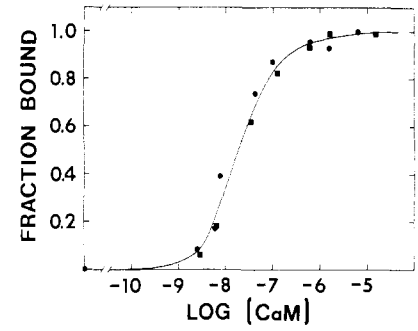


FIGURE 7: Titration of DANZ-TnI with Ca^{2+}_4·CaM. The fraction of DANZ-TnI bound to CaM is plotted as a function of the free molar CaM concentration. Blank, dilution corrections, and fraction bound were calculated as described under Materials and Methods. The sample consisted of 60 pmol of DANZ-TnI in 10 mM Mops, pH 7.2, 150 mM KCl, 1 mM $MgCl_2$, 2 mM DTT, 25 μg/mL BSA, and 0.10 mM $CaCl_2$ in an initial volume of 1.5 mL. Addition of EGTA to CaM·Ca^{2+}·TnI complexes caused the fluorescent intensity to return to ~10% of initial values seen without addition of CaM. The sample was titrated in parallel with a buffer blank, allowing 5 min after each addition for equilibration before measurement of fluorescence intensity as described under Materials and Methods. Excitation was at 350 nm, and emitted light was isolated with Schott KV 470 filters. The temperature was 25 ± 0.1 °C. The data shown represent two independent experiments.

experiment (Keller et al., 1980) may be attributable to differences in experimental conditions. Once again some degree of induced cooperativity in Ca^{2+} binding was apparent in the relative steepness of the data at a stoichiometric TnI concentration. Ca^{2+} binding to the CaM·TnI complex was not examined at lower KCl concentrations because of the relatively poor solubility of TnI at lower ionic strength (Greaser & Gergely, 1973).

Titration of DANZ-TnI with Ca^{2+}_4·CaM. It is evident in Figure 3 and Scheme I that knowledge of $\Delta G°_{IC}$ and of the K_d for complex formation between Ca^{2+}_4·CaM and TnI is sufficient to calculate the K_d for the CaM·TnI complex in the absence of Ca^{2+}. A K_d of 60 nM has been determined for complex formation from Ca^{2+}_4·AEDANS-CaM and TnI (LaPorte et al., 1981), similar to the apparent K_i of 30 nM for inhibition of CaM stimulation of PDE by TnI (LaPorte et al., 1980). We have also determined the K_d for Ca^{2+}_4·CaM complex formation with DANZ-TnI, a fluorescent derivative of TnI, by monitoring fluorescence intensity changes accompanying complex formation (Figure 7). A K_d of 20 nM was determined.

Thus, determinations of the K_d for complex formation of TnI with Ca^{2+}_4·CaM yielded values in the range 20–60 nM. From the determined $\Delta G°_{IC}$ of −1.25 kcal/mol of Ca^{2+} (or −5 kcal/mol of TnI), the K_d for CaM·TnI complex in the absence of Ca^{2+} can be estimated at about 90–270 μM.

Discussion

The interaction of Ca^{2+} and TnI with CaM can be described by a unique free-energy coupling, $\Delta G°_{IC}$, as can be seen from Scheme I and the free-energy diagram in Figure 3. Knowledge of this thermodynamic parameter is necessary to develop a quantitative description of the interaction of Ca^{2+}, CaM, and CaM-binding proteins. The free-energy coupling can be determined either from the relative affinities of the Ca^{2+}-binding sites of CaM and of the CaM·TnI complex or from the affinities of TnI for CaM and for Ca^{2+}_4·CaM, provided the stoichiometry of the Ca^{2+}_4·CaM·TnI complex is known. The free-energy coupling for the individual Ca^{2+}-binding sites can be best determined by equilibrium binding techniques, since the Ca^{2+} dependence determined for CaM-stimulated enzymes

is not necessarily a true measure of the CaM association with these proteins. Since the stoichiometry of troponin C and TnI in rabbit skeletal muscle troponin has been variously reported as 1:1 (Ebashi et al., 1973) or 1:2 (Sperling et al., 1979), we have examined the stoichometry of the CaM·TnI complex by gel filtration and found a 1:1 stoichiometry, consistent with the stoichiometry of TnI inhibition of the fluorescence intensity associated with binding of 9-anthroylcholine to CaM (LaPorte et al., 1980) and with crossl-linking studies utilizing a photoreactive derivative of CaM (Andreasen et al., 1981).

$\Delta G°_{IC}$ was determined through investigation of the binding of Ca^{2+} to CaM and to the CaM·TnI complex. CaM displayed four Ca^{2+}-binding sites, the affinities of which were dependent upon ionic strength and Mg^{2+} concentration. Some degree of positive cooperativity and heterogeneity (or negative cooperativity) was evident, as reported by Crouch & Klee (1980). Each set of Ca^{2+}-binding data was characterized by a geometric mean dissociation constant, or $\bar{K}_d$, equivalent to the fourth root of the combined macroscopic dissociation constants. It should be noted that the macroscopic dissociation constants determined for CaM differ by less than 3-fold from those predicted for a system of four noninteracting sites of intrinsic affinity equal to the $\bar{K}_d$, indicative of a relatively minor degree of cooperativity and site heterogeneity. This is also evident in the observed log intervals from 0.1 to 0.9 fractional saturation for Ca^{2+}. In the absence of Mg^{2+}, the $\bar{K}_d$'s determined for CaM varied from 1.6 to 23 μM over a range of 20–300 mM KCl, consistent with the proposed dependence of Ca^{2+} affinity on ionic strength (Dedman et al., 1977) and with the dependence of the activity coefficient of free Ca^{2+} on ionic strength. It has also been suggested that monovalent cations bind to CaM (Crouch & Klee, 1980; Delville et al., 1980). At 150 mM KCl the $\bar{K}_d$'s determined over a range of 0–10 mM $MgCl_2$ varied from 9 to 63 μM, consistent with competitive binding of Mg^{2+} at the Ca^{2+} sites (Wolff et al., 1977). At this ionic strength, only minor increases in heterogeneity of Ca^{2+} affinity were observed with increasing Mg^{2+} concentration. It is evident from these data thay any comparison of CaM binding data or prediction of CaM Ca^{2+}-binding behavior must take these variables into account.

The observed $\bar{K}_d$ for Ca^{2+} binding to CaM in the presence of TnI was 7–8–fold lower than that for CaM alone. This is comparable to the 10-fold difference in affinity for Ca^{2+} of free troponin C and troponin C in the whole troponin complex (Potter & Gergely, 1975). The increased affinity observed at excess TnI concentrations was only slightly greater than that seen at stoichiometric CaM and TnI concentrations. Although the affinity of CaM for Ca^{2+} was quite dependent on experimental conditions, the effect of TnI on CaM Ca^{2+} affinity was similar with and without Mg^{2+} present; 8-fold and 7-fold increases in affinity were observed, respectively. It is apparent that calculated distributions of the various Ca^{2+}_n·CaM species based on the dissociation constants determined for free CaM *cannot* be used to predict the behavior of CaM-stimulated enzymes, since these constants are not valid for CaM·protein complexes. The free-energy coupling determined for Ca^{2+} and TnI binding to CaM with 1 mM Mg^{2+} present was −5 kcal/mol of TnI or an average of −1.25 kcal/mol of Ca^{2+}. The free-energy coupling of the individual sites can be calculated from the macroscopic dissociation constants, though the errors in determining the individual dissociation constants are greater than in determination of the geometric mean dissociation constant.

The affinity of TnI for CaM should be increased approximately 4500-fold when CaM binds 4 mol of Ca^{2+}. A K_d of 20 nM was determined for binding of Ca^{2+}_4·CaM to DANZ-TnI by monitoring the fluorescence intensity of this TnI derivative. This is similar to an apparent K_i of 30 nM for TnI inhibition of CaM stimulation of phosphodiesterase (LaPorte et al., 1980) and a K_d of 60 nM determined by monitoring the fluorescence anisotropy change accompanying AEDANS-CaM and TnI complex formation (LaPorte et al., 1981). A K_d of about 90–270 μM would then be predicted for the CaM·TnI complex formation in the absence of Ca^{2+}.

In addition to the known CaM-binding proteins, a number of small molecules of relatively hydrophobic nature are known to bind to CaM in a Ca^{2+}-dependent manner, such as trifluoperazine (Levin & Weiss, 1979) and 8-anilino-1-naphthalenesulfonate (LaPorte et al., 1980). These molecules bind competitively with CaM-binding proteins (Weiss & Levin, 1977; LaPorte et al., 1980). It might be anticipated that an effect on Ca^{2+} binding similar to that observed for TnI might be elicited by these molecules. We have observed a comparable increase in affinity of CaM for Ca^{2+} in the presence of trifluoperazine and 8-anilino-1-naphthalene-sulfonate, but the free-energy coupling for binding of Ca^{2+} and these molecules to CaM could not readily be quantitated since the stoichiometry of binding of these compounds is unclear; Ca^{2+}-independent binding sites also exist.

The relative affinity of other CaM-binding proteins for CaM in the presence and absence of Ca^{2+} may vary considerably from that determined for TnI. For example, Huang et al. have predicted a 10 000-fold or greater increase in the affinity of CaM for PDE on saturation with Ca^{2+}, on the basis of kinetic evidence (Huang et al., 1981). Since CaM concentrations in many tissues are thought to be in the micromolar range, the magnitude of the free-energy couplings must be determined to predict the extent of Ca^{2+}-independent complex formation of CaM and CaM-binding proteins. The free-energy coupling determined in this study is relatively small compared to the free energies for binding of Ca^{2+} to CaM and CaM to TnI. These are typical, however, of the free-energy coupling values found for other systems, which are generally 1–1.5 kcal/mol (Weber, 1975). The limited free-energy coupling associated with each site may explain the necessity for evolution of multiple Ca^{2+}-binding sites, each of which may be energetically coupled to CaM interactions with CaM-binding proteins.

Another implication of these results is that induced positive cooperativity for Ca^{2+} binding would be predicted when Ca^{2+} binding to a substoichiometric number of sites is sufficient to promote CaM·Protein complex formation. Activation of several CaM-sensitive enzymes by Ca^{2+}, including PDE, has been shown to be highly cooperative (Brostrom & Wolff, 1976; Dedman et al., 1977). Only a limited degree of induced positive cooperativity was observed in this study at a 1:1 CaM–TnI ratio because of the CaM and TnI concentrations were within an order of magnitude of the K_d for Ca^{2+}-independent CaM·TnI complex formation. Prediction of the cooperative Ca^{2+}-binding behavior of these systems will require knowledge of the free-energy coupling for the individual Ca^{2+}-binding sites. The techniques described in this study will be generally useful in similar thermodynamic characteriztaions of other CaM·protein complexes, which will be of importance in defining the interactions between the components of each system.

Since submission of this paper, an analysis of the effect of K^+ and Mg^{2+} on Ca^{2+} binding to calmodulin has been published by Haiech et al. (1981). Although their buffers and source of calmodulin were different than ours, the qualitative effects of K^+ and Mg^{2+} on the affinity of CaM for Ca^{2+} were

comparable. They have interpreted their data as evidence of competitive binding of K^+ and Mg^{2+} at each of the four Ca^{2+}-binding sites of calmodulin.

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