Interaction of a Fluorescent *N*-Dansylaziridine Derivative of Troponin I with Calmodulin in the Absence and Presence of Calcium†

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ABSTRACT: Rabbit skeletal muscle troponin I was covalently labeled with *N*-dansylaziridine, resulting in a fluorescent labeled protein. This derivative (DANZTnI) and native troponin I (TnI) inhibited calmodulin (CaM) stimulation of bovine heart Ca^{2+}-sensitive cyclic nucleotide phosphodiesterase with identical inhibition constants. Association of DANZTnI with calmodulin was monitored directly by changes in fluorescence intensity in the presence of Ca^{2+} and by changes in fluorescence anisotropy in the absence of Ca^{2+}. Quantitation of the affinity of calmodulin for calmodulin-binding proteins in both the presence and absence of Ca^{2+} is necessary for prediction of the extent of interaction of both Ca^{2+} and calmodulin-binding proteins with calmodulin in vivo. The dissociation constants for the DANZTnI·calmodulin·$4Ca^{2+}$ and DANZTnI·calmodulin complexes were 20 nM and 70 μM, respectively. These dissociation constants define a free energy coupling of −4.84 kcal/mol of troponin I for binding of Ca^{2+} and troponin I to calmodulin. The Ca^{2+} dependence for troponin I·calmodulin complex formation predicted from these experimentally determined parameters was closely approximated by the Ca^{2+} dependence for complex formation between troponin I and fluorescent 5-[[[(iodoacetyl)amino]ethyl]-amino]-1-naphthalenesulfonic acid derivatized calmodulin as determined by fluorescence anisotropy. Complex formation occurred over a relatively narrow range of Ca^{2+} concentration, indicative of positive heterotropic cooperativity for Ca^{2+} and troponin I binding to calmodulin.

Calmodulin (CaM),[1] discovered by Cheung (1970) as an activator of the Ca^{2+}-sensitive isozyme of the nucleotide cyclic 3′,5′-phosphodiesterase, has since been shown to activate several other enzymes in the presence of Ca^{2+} [reviewed in Klee et al. (1980), Cheung (1980), and Wang & Waisman (1979)]. CaM complex formation in the presence of Ca^{2+} has been demonstrated for several CaM-binding proteins by a variety 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 anisotropy (LaPorte et al., 1981), fluorescence intensity (Johnson et al., 1981), and cross-linking of ^{125}I-labeled CaM (LaPorte & Storm, 1978; Richman & Klee, 1978; Andreason et al., 1981).

Since these techniques are in general not well suited for detection of the weaker interactions of CaM and CaM-binding proteins in the absence of Ca^{2+}, the Ca^{2+}-independent binding has been largely ignored in proposed models for Ca^{2+} activation of CaM-stimulated enzymes. Only recently have more quantitative kinetic models for Ca^{2+} and CaM interactions with myosin light chain kinase (Blumenthal & Stull, 1980) and the Ca^{2+}-sensitive phosphodiesterase (Wang et al., 1980; Huang et al., 1981; Cox et al., 1981) been proposed. We have investigated the interaction of CaM with troponin I (TnI), a CaM-binding protein, by equilibrium binding techniques (Keller et al., 1980, 1982; LaPorte et al., 1981). TnI forms a complex with CaM in the presence of Ca^{2+} (Amphlett et al., 1976). We have previously reported the free energy coupling for binding of Ca^{2+} and TnI to CaM, where TnI and CaM interact on a 1:1 stoichiometric basis (Keller et al., 1982). These free energy coupling values and the dissociation constants for Ca^{2+} interaction with CaM and for CaM interaction with its binding proteins in the absence of Ca^{2+} are necessary for quantitative prediction of the extent of association of Ca^{2+}, CaM, and CaM-binding proteins. The equilibrium dissociation constants for the AEDANSCaM·$4Ca^{2+}$·TnI and DANZTnI·CaM·$4Ca^{2+}$ complexes were determined by fluorescence techniques (LaPorte et al., 1981; Keller et al., 1982). In this study, we describe in detail the properties of DANZTnI, a fluorescent derivative of TnI. DANZTnI was developed to determined the dissociation constant for Ca^{2+}-independent complex formation between TnI and CaM, because the low solubility of TnI (Greaser & Gergeley, 1973) precluded use of AEDANSCaM for this purpose. The dissociation constants determined for both the DANZTnI·CaM·$4Ca^{2+}$ complex and the DANZTnI·CaM complex independently confirm the free energy coupling for Ca^{2+} and TnI binding to CaM determined previously by equilibrium Ca^{2+} binding (Keller et al., 1980, 1982).

A thermodynamic scheme and equations describing the interactions between CaM, Ca^{2+}, and TnI are reported. A prediction of these equations is that positive heterotropic cooperativity for binding of Ca^{2+} and TnI to CaM should be evident at concentrations where binding of Ca^{2+} to a substoichiometric number of binding sites is sufficient to promote CaM·TnI complex formation. The Ca^{2+} dependence for

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[1] Abbreviations: CaM, calmodulin; DANZTnI, *N*-dansylaziridine derivative of troponin I; TnI, troponin I; AEDANSCaM, 5-[[[(iodoacetyl)amino]ethyl]amino]-1-naphthalenesulfonic acid derivative of calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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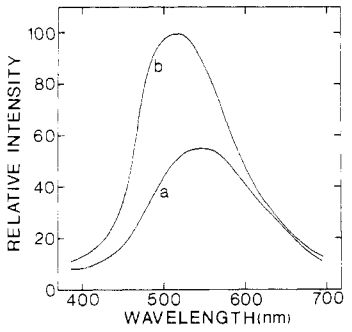


FIGURE 1: Corrected emission spectra for DANZTnI (a) and DANZTnI·CaM·4Ca^{2+} (b). Sample a consisted of 5 μM DANZTnI in 1.5 mL of buffer A and 0.1 mM $CaCl_2$. For spectrum b, 0.080 mL of CaM in buffer A + 0.1 mM $CaCl_2$ was added directly to sample a, resulting in a final CaM concentration of 20 μM. Background relative intensity was 5% and featureless across the emission spectrum. Spectra were obtained on a Turner Model 210 spectrofluorometer as described under Materials and Methods.

Table I: Fluorescence Properties of DANZTnI, DANZTnI·CaM·4Ca^{2+}, and DANZTnI·CaM

parameter[a]	DANZTnI	DANZTnI·CaM·4Ca^{2+}	DANZTnI·CaM
emission max (nm)	550	500	ND[b]
av fluorescence lifetime (ns)[c]	14.1 (0.4)[d]	18.8 (0.1)	ND
rel fluorescence intensity (%)[e]	55	100	53
anisotropy	0.150	0.162	0.190

[a] Parameters determined in 1.5 mL of buffer A containing 0.1 mM $CaCl_2$ + 5 μM DANZTnI (a), 0.1 mM $CaCl_2$ + 20 μM CaM + 5 μM DANZTnI (b), or 0.5 mM EGTA + 1.7 mM CaM + 0.1 μM DANZTnI (c). [b] Not determined. [c] Fluorescence lifetimes are the average values reported for phase shift and demodulation determined by the method of Spencer & Weber (1969), as described under Materials and Methods. [d] Standard error given in parentheses. [e] Relative intensity normalized to a maximum of 100 with exciting light at a wavelength of 350 nm.

saturation of CaM by TnI predicted from the experimentally determined dissociation constants and free energy coupling values was closely approximated by that observed from the fluorescence anisotropy of AEDANSCaM on titration with Ca^{2+} in the presence of TnI. In addition, the fluorescence anisotropy of DANZTnI has provided information concerning the hydrodynamic properties of DANZTnI and DANZTnI·CaM·4Ca^{2+}.

Materials and Methods

N-Dansylaziridine and 5-[[[(iodoacetyl)amino]ethyl]-amino]-1-naphthalenesulfonic acid were obtained from Molecular Probes, Inc. Beckman ultrapure grade sucrose was used for Perrin plots. All other chemicals were reagent grade or better.

Protein Preparations. CaM was prepared as previously described (LaPorte et al., 1979). TnI was prepared from rabbit skeletal muscle by the method of Wilkinson (1974). DANZTnI was prepared as previously described (Keller et al., 1982). AEDANSCaM was prepared as previously described (LaPorte et al., 1981). Protein concentrations were determined by the method of Lowry et al. (1951) or spectrophotometrically by using $E^{1\%}_{280nm}$ = 3.97 for purified TnI (Wilkinson, 1974) and $E^{1\%}_{277nm}$ = 1.8 for purified CaM (Watterson et al., 1976). Where molar concentrations are reported for CaM or TnI, molecular weight values of 16 723 (Vanaman et al., 1977) or 23 000 (Wilkinson, 1974) were used, respectively. For Ca^{2+}-independent titrations with CaM, the CaM was dialyzed against 10 mM Mops, pH 7.2, 150 mM KCl, and 5 mM EGTA, followed by dialysis against 10 mM Mops, pH 7.2, 150 mM KCl, and 0.5 mM EGTA. The CaM was then concentrated in the same buffer by negative-pressure dialysis in a Micro-Pro-Dicon apparatus. For Ca^{2+} titrations, calcium contaminating the proteins and reagents was removed as described previously (Keller et al., 1982). Total calcium contamination in samples was determined by atomic absorption using a Perkin-Elmer Model 305 atomic absorption spectrophotometer with a graphite furnace.

Fluoresence Measurements. The buffer used in all fluorescence experiments was 10 mM Mops, pH 7.2, 150 mM KCl, 1 mM $MgCl_2$, and 2 mM dithiothreitol (buffer A) with additions as indicated. Fluorescence emission spectra of DANZTnI and DANZTnI·CaM·4Ca^{2+} were obtained by using a Turner Model 210 spectrofluorometer. Excitation was at 340 nm, and both monochromators were set at a 10-nm band-pass. Emission spectra were corrected for instrument response as described by the manufacturer. All other fluorescence measurements were made with an SLM 4800S spectrofluorometer with sample temperature maintained at 25 ± 0.1 °C. Fluorescence intensity measurements were corrected for background intensity and the fraction of DANZTnI bound as previously described (Keller et al., 1982).

Anisotropy measurements were corrected and plotted as the fraction of fluorescent protein bound as previously described (LaPorte et al., 1981). Fluorescence lifetimes were determined by the method of Spencer & Weber (1969). Exciting light was polarized 35° from the vertical to correct for the influence of Brownian rotation on the lifetimes (Spencer & Weber, 1970). the excitation light at each wavelength was modulated at 18 MHz, and emitted light was isolated by using Schott KV470 filters. The hydrodynamic properties of DANZTnI were investigated as previously described (LaPorte et al., 1981).

Results

Spectra of DANZTnI and DANZTnI·CaM·4Ca^{2+}. DANZTnI exhibited a featureless emission spectrum with a maximum at 550 nm (Figure 1a, Table I). Saturation of DANZTnI with CaM·4Ca^{2+} resulted in a 1.8-fold increase in fluorescence intensity with the emission spectrum maximum at 500 nm (Figure 1b). The average fluorescence lifetime also increased from 14.1 to 18.8 ns (Table I). Addition of EGTA in excess over $CaCl_2$ had no effect on the spectrum of DANZTnI; however, a comparable addition of EGTA to the DANZTnI·CaM·4Ca^{2+} complex resulted in a spectrum identical with that of DANZTnI (data not shown).

Titration of CaM-Stimulated Cyclic Nucleotide Phosphodiesterase with DANZTnI and Native TnI. DANZTnI and native TnI were identical in ability to inhibit CaM stimulated of bovine heart cyclic nucleotide phosphodiesterase at subsaturating CaM concentrations (Figure 2). The concentration of DANZTnI required for half-maximal inhibition of CaM stimulation of the phosphodiesterase was identical with the dissociation constant (K_d) determined for DANZTnI binding to CaM·4Ca^{2+}.

Titration of DANZTnI with CaM in the Presence and Absence of Ca^{2+}. Determination of the dissociation constant

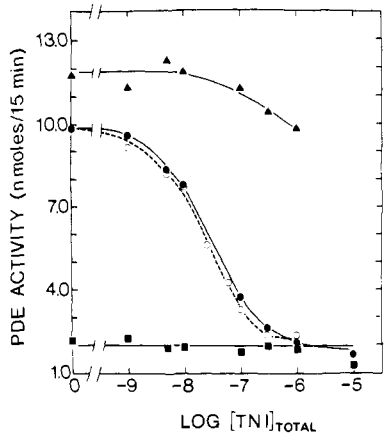


FIGURE 2: DANZTnI inhibition of CaM stimulation of bovine heart Ca^{2+}-sensitive cyclic nucleotide phosphodiesterase (PDE). PDE activity is shown as a function of the log of the total molar troponin I (TnI) concentration. The PDE was purified as described previously (LaPorte et al., 1979) and assayed by a modification of the method of LaPorte & Storm (1978). 4 milliunits of PDE was assayed for 15 min at 30 °C in a total volume of 0.5 mL. Assays contained 10 mM Mops, pH 7.2, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM KCl, 0.1 mg/mL bovine serum albumin, 2 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 0.05 mM [^{3}H]cAMP (60 000 cpm), 5-[^{14}C]AMP (6000 cpm), and the indicated concentrations of CaM, TnI, and DANZTnI. Basal PDE activity (■) was determined in the absence of CaM, at the indicated total concentrations of native TnI. CaM, where present, was at 9.6×10^{-10} M (●, ○) or 6.0×10^{-8} M (▲). Native TnI (▲, ●, ■) and DANZTnI (○) were at the indicated total concentrations. One unit of PDE activity is that hydrolyzing 1 μmol of cAMP/min in the presence of 20 mM imidazole and saturating CaM and cAMP concentrations (LaPorte et al., 1979).

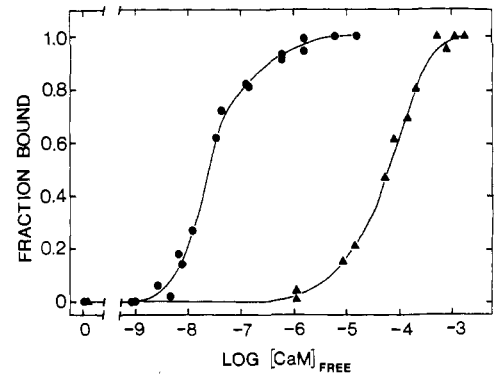


FIGURE 3: Titration of DANZTnI with CaM in the presence (●) and absence (▲) of Ca^{2+}. The fraction of DANZTnI bound to CaM in the presence of either 0.1 mM $CaCl_2$ or 0.5 mM EGTA is shown as a function of the log of the molar free CaM concentration. Titration of DANZTnI with CaM in the presence of Ca^{2+} was with 30 pmol of DANZTnI in 1.5 mL of buffer A + 0.1 mM $CaCl_2$. The sample was titrated in parallel with a buffer blank. Final sample volume was 1.74 mL. The observed fluorescence intensity was subtracted from the background fluorescence intensity, corrected for sample dilution, and the fraction of DANZTnI bound was determined as described under Materials and Methods. Titration of DANZTnI with Ca^{2+}-free CaM in the absence of Ca^{2+} was with 150 pmol of DANZTnI in 1.5 mL of buffer A + 0.5 mM EGTA. (Ca^{2+}-free CaM was prepared as described under Materials and Methods.) The sample was titrated in parallel with a buffer blank. Final sample volume was 1.95 mL. Fluorescence anisotropy was corrected for background, and the fraction of DANZTnI bound to CaM was calculated as described under Materials and Methods.

for the binding of DANZTnI to CaM·4Ca^{2+} was by fluorescence intensity as reported previously (Keller et al., 1982), while complex formation between DANZTnI and CaM in the absence of Ca^{2+} was by fluorescence anisotropy. Fluorescence intensity was used for monitoring complex formation in the prescence of Ca^{2+} because the large fluorescence lifetime increase prevented quantitation of measurable fluorescence anisotropy changes over the titration curve (Table I). The results of titrations of DANZTnI with CaM in the presence and absence of Ca^{2+} are shown in Figure 3, where the fractional saturation of DANZTnI with CaM was plotted by the method of Bjerrum (1923). No attempt was made to compete the complexed DANZTnI with native TnI due to the low solubility of TnI above approximately 1.0×10^{-5} M (Greaser & Gergely, 1973). Data shown in Figure 3 for titration of DANZTnI with CaM·4Ca^{2+} have appeared previously and are described in detail (Keller et al., 1982). Complex formation of DANZTnI·CaM in the absence of Ca^{2+} was monitored by fluorescence anisotropy. From the limits of the titration, values of 0.1501 and 0.1903 were determined for the anisotropy of DANZTnI and DANZTnI·CaM, respectively. The ratio of the fluorescence intensity of the DANZTnI·CaM species over that of the DANZTnI species was 0.95. The fraction of DANZTnI bound to CaM was calculated for each data point as described under Materials and Methods. Total calcium contamination in the sample was ≤7 μM at the conclusion of the titration.

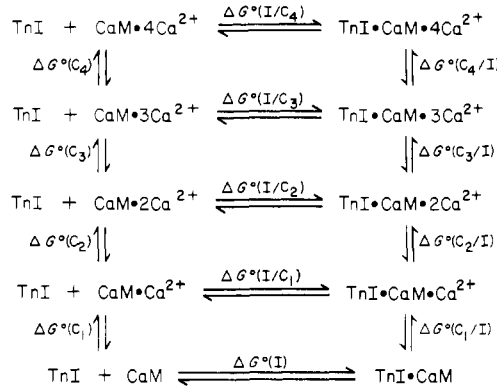
From the midpoints of the titration curves shown in Figure 3, dissociation constants of 20 nM and 70 μM were determined for interaction of DANZTnI with CaM·4Ca^{2+} and CaM, respectively. Both titration curves shown in figure 3 include data from two independent experiments in which different preparations of DANZTnI were used. Points plotted are results of individual experiments and have approximate errors of 10% in the determination of dissociation constants for the DANZTnI·CaM·4Ca^{2+} and DANZTnI·CaM complexes. The logarithmic interval of the free CaM concentration between 0.1 and 0.9 fractional saturation was 2.0 for CaM·4Ca^{2+} titration of DANZTnI and 1.9 for titration with CaM in the absence of Ca^{2+}. The predicted logarithmic interval for a single class of noninteracting binding sites is 1.908. Any significant deviation from 1.908 is an indication of the extent of cooperativity or heterogeneity of the experimental system (Weber, 1975). The observed logarithmic intervals of 2.0 and 1.9 indicate that the extent of heterogeneity in the affinity of the DANZTnI species preseent for CaM is minor. Values of the anisotropy of the DANZTnI·CaM species and the ratio of fluorescence intensity of the DANZTnI·CaM species over that of the DANZTnI species may be slightly underestimated for the titration of DANZTnI with CaM in the absence of Ca^{2+}, since saturation of the DANZTnI with CaM was not complete. However, from the shape of the curve, it is evident that saturation was closely approached. Complete saturation was not achieved because of the very high concentrations of CaM required.

Calculation of the Free Energy Coupling for Ca^{2+} and TnI Binding to CaM. The free energy coupling for binding of Ca^{2+} and CaM-binding proteins to CaM can be analyzed by the method proposed by Weber (1975), with the free energy changes for formation of the 4Ca^{2+}·CaM·TnI complex shown in Scheme I. From the thermodynamic analysis presented previously (Keller et al., 1982), it is evident that the total free energy coupling for the system is given by

$$\Delta G^\circ(I/C_4) - \Delta G^\circ(I) = \sum_{n=1}^{4} [\Delta G^\circ(C_n/I) - \Delta G^\circ(C_n)] = 4\Delta G^\circ_{IC} \quad (1)$$

where ΔG°_{IC} is the average free energy coupling per mole of

Scheme I: Thermodynamic Scheme for TnI·CaM·4Ca^{2+} Complex Formation[a]



[a] ΔG° (I) is the free energy change for binding of TnI to CaM in the absence of Ca^{2+}, ΔG° (C_n) is the free energy change for Ca^{2+} binding to the nth CaM Ca^{2+}-binding site, ΔG° (C_n/I) is the free energy change for Ca^{2+} binding to the nth CaM Ca^{2+}-binding site when TnI is already bound, and ΔG° (I/C_n) is the free energy change for TnI binding to CaM with n moles of Ca^{2+} already bound.

Ca^{2+} bound. The free energy coupling for the system can therefore by experimentally determined both from the K_d's for TnI binding to CaM and to CaM·4Ca^{2+} and from the K_d's for Ca^{2+} binding to CaM and to CaM·TnI. We have previously determined an average free energy coupling of −1.25 kcal/mol of Ca^{2+} from the K_d's for Ca^{2+} binding to CaM and to the CaM·TnI complex by equilibrium Ca^{2+} binding (Keller et al., 1982). This result was verified by the data shown in Figure 3; the respective K_d's for DANZTnI binding to CaM·Ca^{2+} and to CaM in the absence of Ca^{2+} differed by 3500-fold. Since the respective K_d's of 20 nM and 70 μM for DANZTnI binding to CaM·4Ca^{2+} and to CaM correspond to values of −10.50 kcal/mole of DANZTnI for ΔG°(I/Ca_4) and −5.66 kcal/mol of DANZTnI for ΔG°(I), an average free energy coupling of −1.21 kcal/mol of Ca^{2+} can be calculated from eq 1. This result supports both the validity of the free energy coupling value determined by equilibrium Ca^{2+}-binding determinations and the utility of the DANZTnI in characterization of TnI-binding interactions.

From these free energy coupling relationships and Scheme I, it can be predicted that strong positive heterotropic cooperativity will be evident in Ca^{2+} and TnI binding to CaM if Ca^{2+} binding to a substoichiometric number of CaM Ca^{2+}-binding sites is sufficient to promote complex formation. Functions describing the fractional saturation of CaM for Ca^{2+} (S_c) and for TnI (S_I) were derived as described by Weber (1975). These saturation functions are shown in eq 2 and 3,

$$S_c = \left\{\frac{[C]}{K(C)}\left(\frac{1+\epsilon\beta^{-1}}{1+\epsilon}\right) + \frac{3[C]^2}{K(C)^2}\left(\frac{1+\epsilon\beta^{-2}}{1+\epsilon}\right) + \frac{3[C]^3}{K(C)^3}\left(\frac{1+\epsilon\beta^{-3}}{1+\epsilon}\right) + \frac{[C]^4}{K(C)^4}\left(\frac{1+\epsilon\beta^{-4}}{1+\epsilon}\right)\right\} \Big/ \left\{1 + \frac{4[C]}{K(C)}\left(\frac{1+\epsilon\beta^{-1}}{1+\epsilon}\right) + \frac{6[C]^2}{K(C)^2}\left(\frac{1+\epsilon\beta^{-2}}{1+\epsilon}\right) + \frac{4[C]^3}{K(C)^3}\left(\frac{1+\epsilon\beta^{-3}}{1+\epsilon}\right) + \frac{[C]^4}{K(C)^4}\left(\frac{1+\epsilon\beta^{-4}}{1+\epsilon}\right)\right\} \quad (2)$$

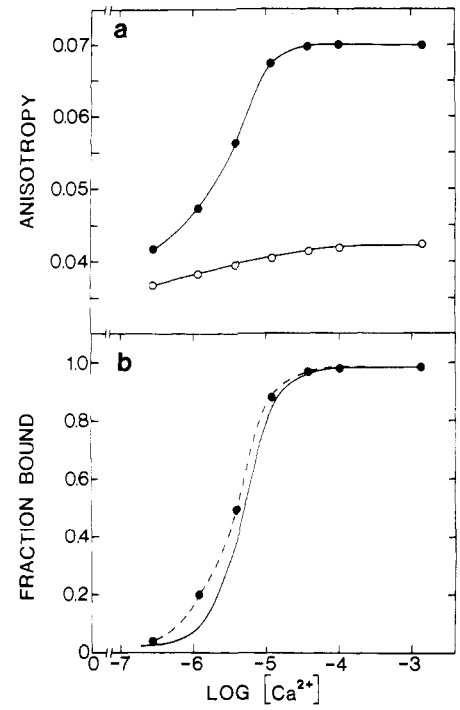


FIGURE 4: Ca^{2+} dependence for complex formation between AEDANSCaM and TnI. Fluorescence anisotropy of AEDANSCaM in the presence (●) and absence (○) of TnI as a function of the log of the free Ca^{2+} concentration (a). AEDANSCaM (60 nM) in 1.5 mL of buffer A was titrated with $CaCl_2$ in the presence and absence of 1 μM TnI. Ca^{2+} was first removed from the buffer and AEDANSCaM as described under Materials and Methods. Final sample volume was 1.69 mL. Anisotropy was corrected for background as described under Materials and Methods. Free Ca^{2+} and TnI were assumed to be equivalent to total concentrations as discussed under Results. The fraction of AEDANSCaM bound to TnI is shown as a function of the log of the molar free Ca^{2+} concentrations (●) (b). The unbroken line shows the calculated fractional saturation of CaM for TnI (eq 3, Results), assuming values of 70 μM for the dissociation constant for Ca^{2+}-independent binding of CaM to TnI, free energy coupling of −1.25 kcal/mol of Ca^{2+}, and an intrinsic dissociation constant of 14 μM for Ca^{2+} binding to CaM. The assumptions made for the calculation of the fraction of AEDANSCaM bound to TnI are discussed under Results.

$$S_I = [I]/\{[I] + K(I)\{[1 + [C]/[K(C)]]/[1 + [C]/[K(C)\beta]]\}^4\} \quad (3)$$

where it is assumed for simplicity that each of the Ca^{2+}-binding sites of CaM has an equal intrinsic affinity and free energy coupling with TnI binding. In eq 2 and 3, [C] and [I] are the respective free concentrations of Ca^{2+} and TnI, K(I) is the K_d for TnI·CaM in the absence of Ca^{2+}, K(C) is the intrinsic K_d for Ca^{2+} binding to CaM in the absence of TnI, β is a function of the free energy coupling equal to $e^{-[\Delta G^\circ_{IC}/(RT)]}$, and ϵ is equal to [I]/[K(I)]. Similar equations can be derived without the assumption of equal intrinsic affinities and free energy coupling values for each of the Ca^{2+} binding sites of CaM. It should be noted that at [C] such that [C] >> K(C) or [C] << K(C), eq 3 reduces to a simple saturation function with $K_d = K(I)\beta^4$ or K(I), respectively. Similarly, at [I] such that [I] << K(I) or [I] >> K(I), eq 2 reduces to a more familiar form with K_d = K(C) or K(C)β, respectively. The system will display maximum cooperativity when $\epsilon^2 = \beta^5$, where the difference in the first and fourth macroscopic dissociation constants for Ca^{2+} is greatest (Weber, 1975).

Predicted and Observed Cooperativity in TnI Binding to AEDANSCaM. AEDANSCaM (60 nM) was titrated with $CaCl_2$ in the presence of 1 μM TnI and the fluorescence anisotropy monitored (Figure 4a). The saturation of CaM for TnI predicted dfor these conditions by eq 3 is shown in Figure 4b, where $K(I)$ = 70 μM, [I] = 1 μM, $K(C)$ = 14 μM, and β = 0.121. $K(I)$ was determined from the fluorescence titration data shown in Figure 3, $K(C)$ from the geometric mean dissociation constant for Ca^{2+} binding to CaM (Keller et al., 1982), and β from the assumption of an average free energy coupling of −1.25 kcal/mol of Ca^{2+} (Keller et al., 1982). A saturation curve of CaM for TnI generated by using the individual K_d's and β values for each CaM Ca^{2+}-binding site determined previously was quite similar (not shown). At the AEDANSCaM and TnI concentrations of the titration, the free TnI concentration was equivalent to the total TnI concentration, with a maximum error of 6% on saturation of the AEDANSCaM with TnI. The free Ca^{2+} concentration was also assumed to be equivalent to the total calcium concentration present, since the fraction of the total calcium present which is bound to the AEDANSCaM can be shown from eq 2 and 3 to be <10% of the total calcium concentration over the range of the Ca^{2+} titration. This assumes comparable Ca^{2+}-binding constants for AEDANSCaM and native CaM. It also assumes that the respective affinities of AEDANSCaM for TnI and DANZTnI for CaM are identical.

Correction of the anisotropy data shown in Figure 4a to the fraction bound requires consideration of the individual anisotropies of multiple AEDANSCaM·$n$$Ca^{2+}$ and TnI·AEDANSCaM·$n$$Ca^{2+}$ species. However, an approximate correction of these data to the fraction bound was made (Figure 4b) by using some simplifying assumptions. The curve shown was corrected from the anisotropy data (Figure 4a) as described under Materials and Methods. The anisotropies of the saturated AEDANS·4Ca^{2+}·TnI species and the free AEDANSCaM·$n$$Ca^{2+}$ species were equal to 0.0705 and 0.0404, respectively. The value of the ratio of the fluorescence intensity of the saturated species over that of the free species was 1.16. The anisotropy of AEDANSCaM increases with increasing additions of Ca^{2+} as shown in Figure 4a. Therefore, the simplifying assumption of a median anisotropy of 0.0404 for AEDANSCaM·$n$$Ca^{2+}$ was used to correct the raw anisotropy data, because the individual anisotropies of the AEDANSCaM·$n$$Ca^{2+}$ and AEDANSCaM·$n$$Ca^{2+}$·TnI species cannot be determined. This simplifying assumption results in an error of less than 5% for determination of the fraction of AEDANSCaM·$n$$Ca^{2+}$ bound to TnI, with the greatest error at low AEDANSCaM·$n$$Ca^{2+}$·TnI complex concentrations. The number of assumptions made to correct the raw anisotropy data for the fraction of AEDANSCaM bound is indicative of the complex nature of the interaction. Nevertheless, the experimental curve follows the predicted curve closely.

Hydrodynamic Properties of DANZTnI and DANZTnI·CaM·4Ca^{2+}. Fluorescence anisotropy was used to determine the hydrodynamic properties of DANZTnI and DANZTnI·CaM·4Ca^{2+} according to the method of Perrin (1929) and Weber (1953) as described under Materials and Methods. Anisotropy was determined with exciting light at wavelengths of 320, 350, and 380 nm. The temperature was maintained at 25 ± 0.1 °C. The viscosity (η) was varied by additions of sucrose. The limiting anisotropies (A_0) determined for DANZTnI and DANZTnI·CaM·4Ca^{2+} were not significantly different at the same wavelengths of exciting light, and the respective fluorescence lifetimes were constant for all wavelengths of exciting light (data not shown). The apparent Stokes radii (R_{app}) for DANZTnI were 28.9, 29.1, and 28.1 Å for wavelengths of exciting light at 320, 350, and 380 nm, respectively, with corresponding rotational relaxation times, $\langle\rho\rangle$, of 66.4, 67.8, and 62.8 ns. The DANZTnI·CaM·4Ca^{2+} complex exhibited respective Stokes radii of 31.1, 31.1, and 29.1 Å for wavelengths of exciting light at 320, 350, and 380 nm, respectively, with corresponding rotational relaxation times, $\langle\rho\rangle$, of 82.8, 82.5, and 70.4 ns. The small change in $\langle\rho\rangle$ for DANZTnI upon binding to CaM·4Ca^{2+} results in a relatively small change in anisotropy, assuming the fluorescence lifetime remains constant.

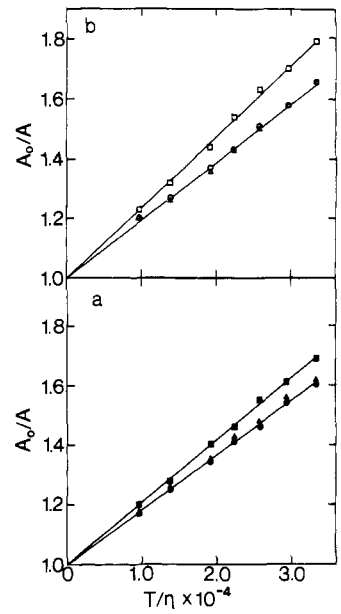


FIGURE 5: Normalized Perrin plots for DANZTnI and DANZTnI·CaM·4Ca^{2+}. (a) Normalized Perrin plot for DANZTnI. The sample consisted of 5 μM DANZTnI in 1.5 mL of buffer A + 0.1 mM $CaCl_2$. (b) Normalized Perrin plot for DANZTnI·CaM·4Ca^{2+}. The sample consisted of 5 μM DANZTnI + 20 μM CaM in 1.5 mL of buffer A + 0.1 mM $CaCl_2$. Anisotropy was monitored at excitation wavelengths of 320 (○, ●), 350 (△, ▲), and 380 nm (□, ■), respectively for DANZTnI (○, △, □) and DANZTnI·CaM·4Ca^{2+} (●, ▲, ■). The sample was titrated in parallel with a buffer blank and corrected for background as described under Materials and Methods. This correction did not exceed 8%. Alteration of the viscosity (η) was by increasing gravimetrically determined additions of dry sucrose. Sample dilution at each concentration of sucrose was calculated by using the specific molar volume of sucrose given by Lee & Timasheff (1981). Final sample volume was 1.87 mL. Perrin plots were used to determine A_0 and the data replotted as normalized Perrin plots (Wiltholt & Brand, 1970).

Discussion

We have developed a fluorescent derivative of troponin I (DANZTnI) that retains activity comparable to native TnI when assayed for inhibition of CaM stimulation of the CaM-sensitive cyclic nucleotide phosphodiesterase. DANZTnI was synthesized to eliminated TnI solubility problems encountered in determination of the Ca^{2+}-independent K_d for interaction of TnI with AEDANSCaM (LaPorte et al., 1981). The interaction of DANZTnI with CaM has been investigated in the presence and absence of Ca^{2+}, allowing a determination of the unique free energy coupling, $\Delta G°_{IC}$, required for a quantitative thermodynamic description of Ca^{2+} and TnI interactions with CaM.

Complex formation between CaM·4Ca^{2+} and DANZTnI resulted in a 1.8-fold increase in fluorescence intensity and a shift in the spectrum maximum from 550 to 500 nm. These

spectral changes were accompanied by an increase of 4.7 ns in the average fluorescence lifetime upon complex formation. These data suggest the DANZ probe underwent an extreme reduction in solvent exposure on interaction with CaM·4Ca^{2+}. In the absence of Ca^{2+}, complex formation between DANZTnI and CaM does not result in a change in fluorescence intensity. The anisotropy does increase and was saturable, and thus demonstrated complex formation. These data suggest that the interaction occurring between DANZTnI and CaM may be different than the interaction of DANZTnI with CaM·4Ca^{2+}.

Hydrodynamic parameters for DANZTnI and DANZTnI·CaM·4Ca^{2+} were investigated by using fluorescence anisotropy to construct "normalized Perrin plots" (Wiltholt & Brand, 1970) (Figure 5). The apparent rotational relaxation, $\langle\rho\rangle$, for DANZTnI is increased from 68 to 83 ns upon interaction with CaM·4Ca^{2+}. The relatively small change in $\langle\rho\rangle$ for complex formation would result in a small anisotropy increase, assuming the fluorescence lifetime and limiting anisotropy remain constant. In the presence of Ca^{2+}, complex formation shows an extremely small change in anisotropy due to the compensatory increase in the fluorescence lifetime. However, in the absence of Ca^{2+}, complex formation of DANZTnI·CaM shows nearly the expected increase in anisotropy if the fluorescence lifetime and limiting anisotropy are assumed to remain constant. These data for the interaction of DANZTnI with CaM in the presence and absence of Ca^{2+} support evidence previously reported for a 1:1 interaction of TnI with CaM·4Ca^{2+} (LaPorte et al., 1980; Andreason et al., 1981; Keller et al., 1982).

Calmodulin complex formation with DANZTnI was investigated by using fluorescence intensity in the presence of Ca^{2+} and fluorescence anisotropy in the absence of Ca^{2+}. Respective dissociation constants determined from the titration curves for DANZTnI interaction with CaM in the presence and absence of Ca^{2+} were 20 nM and 70 μM. The observed K_d of 20 nM for DANZTnI interaction with CaM·4Ca^{2+} is similar to the K_d of 60 nM for the binding of AEDANSCaM·4Ca^{2+} to TnI (LaPorte et al., 1981). The K_d for DANZTnI binding to CaM in the absence of Ca^{2+} is 70 μM, similar to the K_d of 90 μM predicted from Ca^{2+} equilibrium binding studies by Keller et al. (1982).

It is evident from Scheme I that the K_d's for Ca^{2+}-dependent and -independent binding of CaM to DANZTnI are sufficient to calculate the standard free energy coupling, $\Delta G°_{IC}$, for the binding of 1 mol of Ca^{2+} and DANZTnI to CaM. Application of eq 3 yields an average $\Delta G°_{IC}$ of −1.21 kcal/mol of Ca^{2+}, with $4\Delta G°_{IC}$ equal to −4.84 kcal for binding of 4 mol of Ca^{2+} and 1 mol of DANZTnI to CaM. These data demonstrate that the affinity of CaM for DANZTnI is increased 3500-fold when CaM binds 4 mol of Ca^{2+}. The affinities of other CaM-binding proteins for CaM and their respective free energy coupling values may vary considerably from the values determined for DANZTnI. The concentration of CaM in some tissues, such as bovine cerebral cortex, is 10 μM or higher (Watterson et al., 1980). Therefore, complexes between CaM and CaM-binding proteins may exist at significant levels even when the concentration of free Ca^{2+} is quite low ($\leq$0.1 μM). The rate of activation of calmodulin-activated enzymes may be dependent on the diffusion of free Ca^{2+} ions to a preformed Ca^{2+}-independent calmodulin–enzyme complex.

The free energy coupling and the Ca^{2+}-independent K_d for the DANZTnI·CaM complex predict apparent induced positive cooperativity for DANZTnI·CaM complex formation when Ca^{2+} is bound to a substoichiometric number of sites of CaM. Application of eq 3 results in a theoretical binding curve (Figure 4b) which displays positive cooperativity for CaM·TnI complex formation as a function of the free Ca^{2+} concentration. Complex formation for DANZTnI binding to CaM·nCa^{2+} exhibits apparent positive cooperativity even though a 1:1 protein complex is formed, with the degree of apparent positive cooperativity dependent on the free energy coupling between the Ca^{2+}-binding sites and TnI. Application of Scheme I and eq 2 and 3 yields a predicted curve describing the positive coopertivity occurring for CaM·TnI complex formation when Ca^{2+} is bound to a substoichiometric number of sites on CaM (Figure 4b). Because of the complex distribution of TnI·AEDANSCaM·nCa^{2+} and AEDANSCaM·nCa^{2+} species occurring over the Ca^{2+} titration range, correction of the experimental curve for the fraction of AEDANSCaM bound requires some simplifying assumptions. However, the experimental and theoretical curves agree closely.

Activation of CaM-stimulated enzymes by Ca^{2+} shows strong positive cooperativity (Brostrom & Wolff, 1976; Dedman et al., 1977; Cox et al., 1981; Johnson et al., 1981). The thermodynamic analysis presented here describes CaM binding rather than CaM activation; however, we have not observed CaM-stimulated phosphodiesterase activity in the absence of Ca^{2+} at CaM concentrations of 350 μM, suggesting that Ca^{2+} binding is obligatory for activation of the phosphodiesterase.

The experimental techniques and thermodynamic analysis reported here for the CaM–TnI system are applicable to all CaM-binding protein systems and should provide important information about the interactions of Ca^{2+} and these CaM-binding proteins with CaM.

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High-Affinity Uptake and Degradation of Apolipoprotein E Free High-Density Lipoprotein and Low-Density Lipoprotein in Cultured Porcine Hepatocytes†

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ABSTRACT: Isolated pig liver membranes contain a specific "lipoprotein binding site" that recognizes low-density lipoprotein (LDL) and apolipoprotein E (apoE) free high-density lipoprotein (HDL) [Bachorik, P. S., Kwiterovich, P. O., & Cooke, J. (1978) *Biochemistry 17*, 5287–5299]. We report here that a similar site exists in cultured porcine hepatocytes and that it mediates the uptake and degradation of apoE-free HDL. The binding of ^{125}I-labeled HDL and ^{125}I-labeled LDL (^{125}I-HDL and ^{125}I-LDL, respectively) at 4 °C and the uptake and degradation of the lipoproteins at 37 °C were time dependent and saturable and were not inhibited by unrelated proteins. Chloroquine (6×10^{-5} M) inhibited the degradation of ^{125}I-HDL by 76% and of ^{125}I-LDL by >99%; leupeptin inhibited the degradation of both lipoproteins by about 25%. ^{125}I-HDL binding (4 °C), uptake, and degradation (37 °C) were inhibited by LDL, methyl-LDL, and methyl-HDL about as well as by unlabeled HDL but were unaltered in Pronase-treated cells or in cells that were cultured for 24 h in either lipoprotein-free medium or medium containing HDL or LDL (200 μg/mL). In contrast, these conditions affected the uptake and degradation of ^{125}I-LDL disproportionately. HDL and methyl-LDL inhibited ^{125}I-LDL uptake by 50% or more but had little effect on degradation. ^{125}I-LDL binding was reduced by 12% and degradation by 57% in Pronase-treated cells. Preincubation of the cells with LDL (200 μg/mL) reduced uptake by 35% and degradation by 68%. Similar preincubation with HDL (200 μg/mL) increased ^{125}I-LDL degradation by 60% but did not affect ^{125}I-LDL uptake. The findings indicated the presence in porcine hepatocytes of at least two distinct sites for lipoproteins. One site resembled the LDL receptor and mediated ^{125}I-LDL degradation. A second, Pronase-insensitive site recognized both HDL and LDL. This site mediated almost all of the degradation of ^{125}I-HDL but little if any degradation of ^{125}I-LDL.

Plasma low density (β) lipoprotein (LDL)[1] is an end product of the catabolism of VLDL (Havel, 1980) and constitutes an important source of cholesterol for many tissues. The metabolism of LDL has been studied in various types of cultured cells. Studies in human fibroblasts elucidated a receptor-mediated pathway in which LDL is bound to a specific cell surface receptor, internalized, transported to lysosomes, and degraded (Goldstein & Brown, 1977; Brown et al., 1979). During this process, cholesterol released from the lipoprotein

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[1] Abbreviations: VLDL, very low density lipoprotein(s); LDL, low-density lipoprotein(s); HDL, high-density lipoprotein(s); ^{125}I-LDL and ^{125}I-HDL, iodine-125-labeled LDL and HDL; LPDS, lipoprotein-deficient serum; FCS, fetal calf serum; EDTA, disodium ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; Cl_3CCOOH, trichloroacetic acid; PBS, phosphate-buffered saline; apoE, apolipoprotein E; $NaDodSO_4$, sodium dodecyl sulfate.

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