Interactions of Troponin I and Its Inhibitory Fragment (Residues 104–115) with Troponin C and Calmodulin†

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Received December 21, 1988; Revised Manuscript Received April 4, 1989

ABSTRACT: Fluorescent probes have been used to study the interaction of troponin I and its inhibitory peptide TnIp with troponin C, calmodulin, and the proteolytic fragments of calmodulin. The probes used included the noncovalently bound ligand TNS and the covalently attached labels dansyl and AEDANS. The fluorescence intensity of TNS bound to troponin C, calmodulin, or the calmodulin fragments was greatly enhanced by the presence of TnIp. This effect was used to estimate the corresponding binding constants. It was found that TnIp is bound by the C-terminal half-molecule of calmodulin, TR2C, with an affinity comparable to that of intact calmodulin or troponin C, while the binding affinity of the N-terminal half-molecule, TR1C, was an order of magnitude less, suggesting that the TnIp-containing portion of troponin I combines with the C-terminal half of calmodulin or troponin C. The fluorescence properties of an AEDANS group linked to Cys-98 of troponin C were modified by interaction with troponin I or TnIp. The fluorescence properties of the same group linked to Cys-27 of wheat germ calmodulin were affected by TnI, but not TnIp. TnI had a small effect upon the fluorescence of a dansyl group linked to Met-25 of troponin C. TnIp also inhibited the tryptic hydrolysis of the midpoint of the central connecting strand of calmodulin and troponin C. It is concluded that the interaction of troponin I with troponin C or calmodulin involves both the N- and C-terminal halves, as well as the connecting strand, of the latter molecules, probably through contacts with the hydrophobic regions in the two halves and with the carboxyl group rich region in the connecting strand. TnIp binds troponin C or calmodulin near the C-terminal end of the connecting strand and extends into the hydrophobic site of the C-terminal half.

The two members of the calcium-binding protein family, troponin C (TnC)[1] and CaM, bind TnI with high affinity (Wang & Cheung, 1985; Keller et al., 1982). The TnC–TnI interaction is of central importance in the control of muscle contraction, while the CaM–TnI interaction has been used as a model in studies of CaM–target protein binding.

Troponin I inhibitory peptide (residues 104–115) is the minimum sequence necessary for the inhibition of actomyosin ATPase activity (Syska et al., 1976; Talbot & Hodges, 1981; Van Eyk & Hodges, 1988). It is interesting to compare the interactions TnIp–TnC and TnIp–CaM with those of TnI–TnC and TnI–CaM in detail. Since TnC and CaM resemble each other in amino acid sequence as well as in a dumbbell-shaped three-dimensional structure (Watterson et al., 1980; Kretsinger et al., 1986; Sundaralingam et al., 1985), studies on these two calcium-binding proteins may be mutually complementary.

In the present work, we studied those interactions mainly by monitoring the fluorescence of extrinsic and intrinsic probes of the proteins. It is the purpose of the present paper to identify the zones on the surfaces of TnC and CaM which are involved in the interaction with TnI and, in particular, the regions of TnC and CaM which combine with the inhibitory peptide of TnI. A secondary objective is to examine whether any structural change of the former two proteins accompanies the interaction.

Materials and Methods

Protein and Peptide Preparations. TnI and TnC were isolated and purified from rabbit skeletal muscle basically by the method of Potter (1982). CaM from bovine testes was isolated and purified by the method of Watterson et al. (1980). CaM from wheat germ was isolated and purified by the method of Strasburg et al. (1988). TnIp was synthesized on a model 430A Applied Biosystems peptide synthesizer and purified on HPLC. CaM fragments TR1C (residues 1–77), TR2C (residues 78–148), and TM1 (residues 1–106) were prepared from trypsin or thrombin digestion and purified on HPLC, using reverse-phase and ion-exchange separation (Newton et al., 1983; Guerini et al., 1984). The compositions of TnIp, TR1C, TR2C, and TM1 were verified by amino acid analysis.

Probes and Other Materials. DANZ, DNS, and TNS were purchased from Sigma. IAEDANS and DABMI were purchased from Molecular Probes.

Trypsin was purchased from Cooper (97% protein, 225 units/MGP); trypsin inhibitor was purchased from Sigma. All other chemicals were reagent grade or better.

Protein Labeling. AEDANS–Cys-98–TnC was prepared by the method of Cheung et al. (1982). The degree of labeling was 0.96 in this work, as determined spectrophotometrically.

To label TnC with DANZ, Cys-98 of TnC was first protected by PCMB, which was removed by treatment with DTT after reaction with DANZ; the procedures of Johnson et al. (1978) were subsequently followed. The ratio of DANZ/TnC

† This work was supported by NIH Grant DK 30322.

[1] Abbreviations: TnI, troponin I; TnIp, synthetic troponin I fragment, residues 104–115 (Ac-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-NH_2); TnC, troponin C; CaM, calmodulin; DANZ, *N*-dansylaziridine; PCMB, *p*-(chloromercuri)benzoic acid; IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonate; AEDANS, *N*-[(acetylamino)ethyl]-5-naphthylamine-1-sulfonate; TNS, 6-(*p*-toluidino)naphthalene-2-sulfonate; DABMI, *N*-[4-(dimethylamino)-phenyl-4′-azophenyl]maleimide; DAB, 4-[[4-(dimethylamino)phenyl]-azo]phenyl group; DNS, dansyl chloride; TAME, tosylarginine methyl ester; DTT, dithiothreitol.

0006-2960/89/0428-7380$01.50/0

was 0.31, determined spectrophotometrically by using the values $\epsilon_{350,DANZ} = 3980\ M^{-1}\ cm^{-1}$ and $\epsilon_{277,DANZ} = 0.463\epsilon_{peak}$ (Cheung et al., 1982).

The second label, DABMI, was conjugated to DANZ-TnC as described by Wang et al. (1987), except that a larger excess of DABMI was used. The degree of labeling was measured by using concentrations of protein determined by a comparison of the ellipticities at 220 nm of the conjugate and native TnC for identical conditions and by absorbance: DAB, $\epsilon_{460} = 24\,800\ M^{-1}\ cm^{-1}$ (Tao et al., 1983); TnC, $\epsilon_{277} = 4140\ M^{-1}\ cm^{-1}$ (Murray & Kay, 1972). Ellipticities for the Ca^{2+}-liganded species were determined with a Jasco circular dichroism apparatus. It was independently verified that substitution at Cys-98 does not alter the ellipticity at 220 nm. The degree of substitution was also determined spectrophotometrically, making a correction for the absorbance of the DAB groups at 277 nm ($\epsilon_{277,DAB} = 0.38\epsilon_{peak}$); the values obtained by the different methods were averaged. The DAB:TnC ratio was thus determined as 0.99.

AED–CaM was prepared by the method of Strasburg et al. (1988), in which the probe was attached at Cys-27 of wheat germ CaM. DNS–CaM labeling was a three-step procedure, in which bovine testes CaM was first nitrated at Tyr-99 (Richman & Klee, 1978) and then reduced to NH_2-Tyr-99 with sodium dithionite, followed by reaction with DNS at pH 5 (Lambooy et al., 1982).

Fluorescence Measurements and K_d Determination. Static fluorescence measurements were carried out on an SLM 8000 spectrofluorometer. Lifetimes of fluorescence were measured by using an Edinburgh nanosecond fluorometer in the laboratory of Dr. J. R. Lakowicz in the Department of Biological Chemistry, School of Medicine, University of Maryland at Baltimore. Data were analyzed by a least-squares fitting procedure, as described elsewhere (Steiner & Norris, 1987b). For each of the examples cited here, the goodness of fit, as monitored by χ^2, improved significantly as the assumed number of components increased from one to three.

By monitoring the intensity of TNS fluorescence, the dissociation constants, K_d, of TnIp with the proteins and the fragments were calculated from TnIp titration:

$$K_d = [([prot] - x)([TnIp] - x)]/x \quad (1)$$

where [prot] is the original concentration of protein or fragment, while [TnIp] is the concentration of total added TnIp; x is the concentration of protein–TnIp complex, which is assumed to be proportional to the TNS fluorescence intensity (F) enhancement produced by TnIp. The above equation can be rearranged into

$$1/x = K_d/\{[prot]([TnIp] - x)\} + 1/[prot] \quad (2)$$

which is proportional to $1/F$. Since $x \ll [TnIp]$, $[TnIp] - x \approx [TnIp]$ when [TnIp] is relatively large. By plotting $1/F$ vs $1/[TnIp]$, F_{max} can be obtained by extrapolating to $1/[TnIp] = 0$. The relation $x = [prot]F/F_{max}$ was then used in the next step of self-consistent calculation. Dilution and the contribution of the original TNS fluorescence were corrected for.

Radiationless Energy Transfer. (1) Donor Quenching. The efficiency of radiationless energy transfer between a fluorescent donor (DANZ) and acceptor (DAB) is given by

$$E = 1 - \tau_{d,a}/\tau_d \quad (3)$$

where $\tau_{d,a}$ and τ_d are the lifetimes of donor in the presence and absence of acceptor, respectively. E is related to R, the separation of donor and acceptor, by

$$E = R_0^6/(R_0^6 + R^6) \text{ or } R = R_0(E^{-1} - 1)^{1/6} \quad (4)$$

where R_0 is the critical distance at which $E = 0.5$. R_0 can be calculated by (Fairclough & Cantor, 1978)

$$R_0^6 = (8.79 \times 10^{-5})K^2n^{-4}Q_dJ\ (Å) \quad (5)$$

The orientation factor K^2 is usually assumed to be $^2/_3$ for random orientation; n is the refractive index of the medium and was taken to be 1.4 in this work; Q_d is the quantum yield of the donor; J is the overlap integral given by

$$J = \int F(\lambda)\epsilon(\lambda)\lambda^4\, d\lambda / \int F(\lambda)\, d\lambda \quad (6)$$

where $F(\lambda)$ is the donor's fluorescence intensity as a function of wavelength and $\epsilon(\lambda)$ is the acceptor's extinction coefficient as a function of wavelength.

(2) Sensitized Emission. The efficiency of radiationless energy transfer from Trp-158 of TnI in the binary complex to a AEDANS group on Cys-98 of TnC or on Cys-27 of CaM was determined by comparing the fluorescence intensity of the acceptor when excited at 290 nm and at 350 nm in the absence and presence of TnI. The change in intensity for excitation at 350 nm reflects the change in acceptor quantum yield upon complex formation, while the change for excitation at 290 nm includes a contribution from radiationless transfer from Trp-158 of TnI. The normalized intensity, I, arising from transfer is given by (Fairclough and Cantor, 1978)

$$I = [I(d,a)_{ex,290}/I(a)_{ex,290}]/[I(d,a)_{ex,350}/I(a)_{ex,350}] \quad (7)$$

where $I(d,a)$ and $I(a)$ are the intensities of acceptor fluorescence in the presence and absence of the donor, respectively. The subscript ex indicates excitation wavelength.

The efficiency of transfer is given by

$$E = (I - 1)[A(a)_{abs,290}/A(d,a)_{abs,290}] \quad (8)$$

where $A(a)_{abs,290}$ and $A(d,a)_{abs,290}$ are the absorbance at 290 nm of the acceptor in the absence of the donor and of the donor in the presence of the acceptor, respectively. The relation between E and R is still given by eq 4 (Fairclough & Cantor, 1978).

Tryptic Hydrolyses. Tryptic hydrolyses of the bonds between residues 84 and 89 of TnC and those between residues 77 and 78 of CaM in the absence and presence of TnIp were carried out in 50 mM MOPS, 100 mM KCl, and 1 mM Ca^{2+}, at pH 6.9, 25 °C. The TnIp:protein molar ratio was 4:1, and the protein:trypsin molar ratio was 100:1. To terminate the digestion, a 4-fold excess of soybean trypsin inhibitor was added to each aliquot at the desired incubation time. Electrophoresis was carried out in 12.5% acrylamide gel, containing 4 M urea and 2 mM EDTA at pH 8.0.

Results and Discussion

TNS Fluorescence as a Probe for the Binding Site for TnIp. The dye TNS is bound by Ca^{2+}-ligated CaM at two hydrophobic sites, one each of which is located on the N- and C-terminal half-molecules (Follenius & Gerard, 1984; Suko et al., 1985). TNS, which is almost nonfluorescent in water, acquires a moderate fluorescence when complexed with CaM or TnC (Figure 1). The intensity of fluorescence is dramatically enhanced in both cases by the addition of TnIp (Figure 1A,B). The implication is that complex formation with TnIp generates an altered binding site, perhaps comprising a hydrophobic cavity. A similar effect has been observed for a complex of the polypeptide melittin with CaM (Steiner & Norris, 1987a).

A major increase in fluorescence intensity also results from the addition of TnIp to TNS–TR2C (Figure 1). While an increase also occurs when TnIp is added to TNS–TR1C, the

Table I: Dissociation Constants (K_d) from TNS Fluorescence Titration[a]

complex	K_d (μM)	complex	K_d (μM)
CaM–TnIp	5–10	TnC–TnIp	10–14
TR1C–TnIP	~140	TR1C–TnI	~1[b]
TR2C–TnIp	~12	TR2C–TnI	~2[b]

[a] Experimental conditions are described in Figure 1A, inset. [b] Because of the low solubility of TnI which made it difficult to obtain high ambient concentrations (a 20 μM stock of TnI in 0.1 M NaCl, 0.5 mM DTT, and 50 mM MOPS was used in this work), K_d values of TnI–TR1C, and TnI–TR2C were estimated by using the fluoresence of TnI–TNS–CaM or TnIp(excess)–TNS–TR2C as the maximum intensity for TnI–TNS–TR1C and TnI–TNS–TR2C fluorescence, instead of TnI titrating TNS fluorescence.

Table II: Displacement by Calmodulin of Proteolytic Fragments of Calmodulin from Complexes with TnI, As Monitored by TNS Fluorescence

system	additive	I_{rel}[b]	λ_{max}[c]
TNS (6 μM)–TR1C (3 μM)		1.00	470
	TnI (3 μM)	4.86	437
	TnI (3 μM) + CaM (3 μM)	13.71	442
TNS (6 μM)–TR2C (3 μM)		1.00	475
	TnI (3 μM)	2.90	440
	TnI (3 μM) + CaM (3 μM)	11.00	442
TNS (6 μM)–TM1 (3 μM)		1.00	420
	TnIp (3 μM)	1.00	420
	TnI (3 μM)	1.63	429
	TnI (3 μM) + CaM (3 μM)	3.97	432

[a] The buffer is 50 mM MOPS and 0.1 M NaCl, pH 6.8, 25 °C. The excitation wavelength is 330 nm. [b] Relative fluorescence intensity at the wavelength of maximum intensity. [c] Wavelength of maximum intensity.

magnitude of the increase is much less than in the case of TNS–TR2C for equivalent levels of TnIp (Figure 1A).

The large magnitude of the enhancements observed makes TNS an attractive probe for the evaluation of the dissociation constants K_d. This was done, by using eq 2, for TR1C, TR2C, CaM, and TnC; the results are cited in Table I. The values of K_d are of similar magnitudes for TnIp and TnC, CaM, or TR2C; that for TnIp and TR1C is an order of magnitude greater. There is a strong implication that the preferred binding site of TnIp is on the C-terminal half of CaM or TnC. The N-terminal half-molecule of CaM shows only a weak interaction with TnIp.

Determinations were also made of the effect of the addition of TnI itself to TNS–TR1C and TNS–TR2C (Table I). The computed values of K_d are similar for the two and an order of magnitude smaller than for TnIp (Table I). It appears therefore that TnI interacts with both the N- and C-terminal

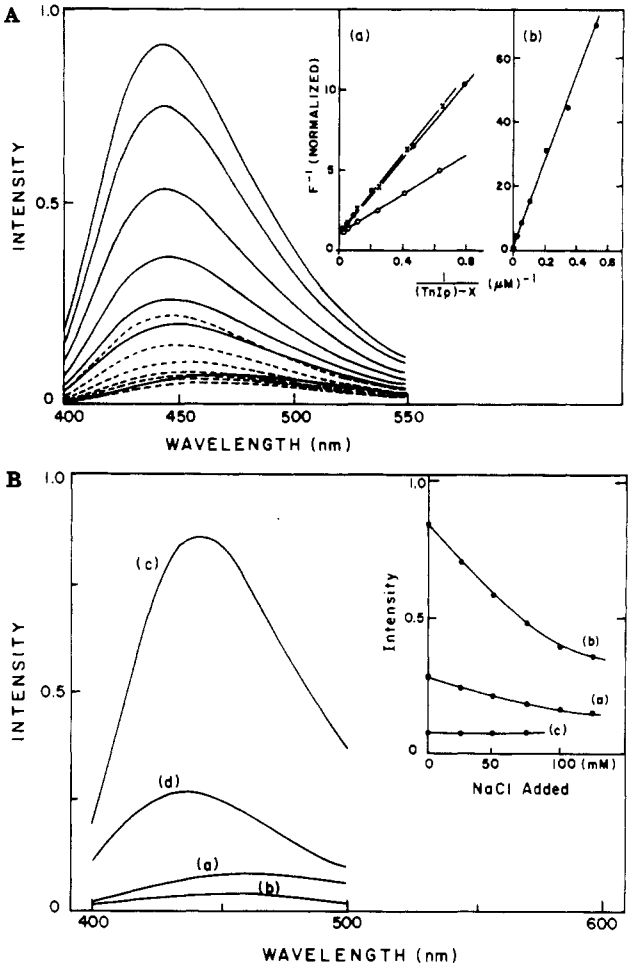

FIGURE 1: TNS fluorescence emission spectra. Excitation wavelength: 330 nm. Buffer: 50 mM MOPS, 0.1 M NaCl, and 2 mM Ca^{2+}, pH 6.8. T = 25 °C. (A) TnIp titration of the fluorescence of TNS–TR1C (---) and TNS–TR2C (—). Concentration used: TR1C or TR2C, 4 μM; TNS, 8 μM; TnIp (from bottom to top), 0, 2, 3, 5, 10, 20, 40 μM. (A, inset) TnIp titrations. (TNS fluorescence intensity)$^{-1}$ vs ([TnIp] − x)$^{-1}$; see Materials and Methods. (a) TNS (2 μM)–TnC (4 μM) (●); TNS (8 μM)–TR2C (4 μM) (×); TNS (1 μM)–CaM (2 μM) (○). (b) TNS (8 μM)–TR1C (4 μM) (●). (B) Fluorescence emission spectra of TNS (1 μM)–CaM (2 μM) (a) and TNS (2 μM)–TnC (4 μM) (b), and of those in the presence of 40 μM TnIp [TNS–CaM–TnIp (c) and TNS–TnC–TnIp (d)]. (B, inset) Fluorescence intensity of TNS–TnC–TnIp (a) or TNS–CaM–TnIp (b) as a function of the concentration of NaCl. (c) is the control buffer.

half-molecules of CaM and that the inhibitory peptide portion of TnI interacts preferentially with the C-terminal half. As expected from their weaker binding, both TR1C and TR2C are readily displaced by CaM from the complex with TNS–TnI (Table II).

Table III: Time Decay of Fluorescence Intensity

fluorophore	additive	α_1	τ_1	α_2	τ_2	α_3	τ_3	$\bar{\tau}$[a]	χ^2
TNS–CaM[b]		0.30	0.57	0.15	3.67	0.22	8.26	6.75	1.011
	TnIp	0.35	0.53	0.26	6.16	0.10	15.0	9.88	1.253
TNS–CaM[c]		3.66	0.24	0.11	3.92	0.14	8.24	4.61	3.7
	TnI	3.31	0.20	0.11	5.40	0.18	11.6	8.24	1.97
DNS–Tyr-99–CaM[d]		0.15	0.70	0.16	3.04	0.13	12.8	10.1	2.5
	TnI	0.28	0.82	0.26	3.56	0.28	13.1	10.7	3.4
AEDANS–Cys-27–CaM[e]		0.17	0.44	0.07	5.58	0.22	14.4	13.1	2.956
	TnI	0.18	0.42	0.09	5.75	0.26	14.9	13.6	3.005

[a] $\bar{\tau} = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$ (ns). The α_i values are the preexponentials in the equation describing the time dependence of fluorescence intensity $I(t)$: $I(t) = \sum \alpha_i e^{-t/\tau_i}$, where t is time and τ_i is the decay time corresponding to the ith decay mode. [b] Concentration used: TNS, 4 μM; CaM, 4 μM; TnIp, 4 μM. Buffer: 100 mM MOPS, 100 mM KCl, 0.5 mM EGTA, and 2 mM Ca^{2+}, pH 6.5. T = 25 °C. Excitation wavelength: 330 nm. [c] Concentration used: TNS, 4 μM; CaM, 2 μM; TnI, 2 μM. Buffer: 50 mM MOPS, 0.1 M NaCl, 2 mM Ca^{2+}, and 0.02 mM DTT, pH 6.8. T = 25 °C. Excitation wavelength: 310 nm. Emission: >372 nm. [d] Concentration used: DNS–Tyr-99–CaM, 2 μM; TnI, 3 μM. Other conditions were the same as in *c*. [e] Concentration used: AEDANS–Cys-27–CaM, 2 μM; TnI, 2 μM. Other conditions were the same as in *c*.

Table IV: Effects of Additives upon the Properties of Covalently Attached Fluorophores[a]

fluorophore	additive	I_{rel}[b]	λ_{max}[c]	K_{sv} (M^{-1})[d]
AEDANS-Cys-98-TnC (2 μM)		1.00	500	7.0
	TnI (2 μM)	1.30	493	
	TnIp (20 μM)	1.19	494	4.3
DANZ-Met-25-TnC (2 μM)		1.00	538	
	TnI (2 μM)	0.93	540	
	TnIp (10 μM)	1.05	540	
AEDANS-Cys-27-CaM (2 μM)		1.00	487	6.5
	TnI (2 μM)	1.08	487	6.1
	TnIp (10 μM)	1.00	487	
DNS-Tyr-99-CaM (2 μM)		1.00	547	
	TnI (2 μM)	1.06	537	
	TnIp (10 μM)	1.02	541	
Trp-158-TnI (2 μM)				16
	TnC (2 μM)			13
	CaM (2 μM)			13

[a] The buffer is 50 mM MOPS, 0.1 M NaCl, and 2 mM Ca^{2+}, pH 6.8. The excitation wavelengths are 350 nm for the conjugates and 290 nm for TnI. [b] Relative fluorescence intensity at the wavelength of maximum intensity. [c] The wavelength of maximum intensity. [d] The Stern-Volmer constant for quenching by acrylamide.

The dynamic fluorescence properties of TNS bound to CaM are also dramatically changed in the presence of TnIp or TnI, with a major increase in the average decay time of fluorescence (Table III) in both cases. The addition of TnI or TnIp appears therefore to cause an important change in the microenvironment of the TNS probe, presumably to one which is less polar and more shielded from solvent.

The TNS fluorescence which was enhanced by TnIp was sensitive to ionic strength (Figure 1B, inset). An increase in ionic strength results in a pronounced decrease in the fluorescence intensity of combined TNS. This in turn suggests that there is a significant electrostatic component in the free energy of interaction of TnIp with CaM or TnC. This is plausible in view of the positively charged nature of TnIp and the predominance of negative charges in CaM or TnC at neutral pH.

It is noteworthy that, while TNS-CaM fluorescence was enhanced by both TnIp and TnI (Figure 1), the fluorescence of TNS-TnC was enhanced by TnIp (Figure 1B), but not significantly by TnI; the observed change in the presence of TnI did not exceed the small intensity elicited by TnI alone, in the absence of other proteins (data not shown). A possible explanation is that the fit of hydrophobic contact regions on TnC and TnI is sufficiently close to leave no hydrophobic crevices for the binding of TNS.

The behavior of TM1 was somewhat anomalous. The peak of the TNS-TM1 emission was at 420 nm, which is shifted to a lower wavelength than those of the other species by 20 nm or more. While TnIp had no effect on TNS-TM1 fluorescence, TnI substantially enhanced the intensity and shifted its maximum to 430 nm (Table II). This raises the question of whether the conformation of TM1 is different from that of the corresponding part of native CaM, perhaps because of the folding back of the connecting strand, so that the bound TNS is in a significantly modified microenvironment, which is somewhat more shielded and hydrophobic. Combination with TnI may restore TM1 to a conformation closer to the native.

AEDANS-Cys-98-TnC Fluorescence. The fluorescence of an AEDANS group linked to Cys-98 at the C-terminal end of the connecting strand was enhanced and significantly shielded from acrylamide quenching in the presence of TnIp (Table IV). This is consistent with, and suggests, that the inhibitory TnIp region of TnI is in proximity to the junction of the C-terminal lobe and the connecting strand, in harmony with NMR (Cachia et al., 1983) and cross-linking (Leszyk et al., 1987) studies. This finding is consistent with the binding of TnIp by the C-terminal half of CaM.

AEDANS-Cys-27-CaM and DANZ-Met-25-TnC Fluorescence. The intensity of fluorescence of AEDANS-Cys-27-CaM, in which the AEDANS group is linked to Cys-27 of wheat germ CaM, increased slightly upon binding TnI, but not upon binding TnIp (Table IV). Neither the Stern-Volmer constant for acrylamide quenching nor the average lifetime of the fluorescence changed substantially upon binding TnI (Tables III and IV). The results showed that the TnIp binding site is separated from Cys-27 of CaM; the minor effect of TnI upon intensity may be indirect and arise from a conformational change, since TnI does not shield this group significantly from acrylamide quenching. The intensity of DANZ-Met-25-TnC fluorescence, on the other hand, decreased slightly upon binding TnI (Table IV). These results are consistent with those from fragment-binding studies which suggest that in the N-terminal half of TnC, only the C-helix (residues 50–60) may interact with TnI (Grabarek et al., 1981). TnIp increased very slightly the intensity of DANZ-Met-25-TnC fluorescence (Table IV). This may result from weak nonspecific binding of TnIp to the N-terminal half of TnC.

DNS-Tyr-99-CaM Fluorescence. The fluorescence intensity of a DNS group linked to Tyr-99 of CaM is almost unchanged upon binding TnI or TnIp, but a small blue-shift can be observed in both cases (Table IV). This effect may also be indirect. The DNS group is probably buried because acrylamide did not quench its fluorescence significantly (data not shown). The average decay time of DNS-Tyr-99-CaM fluorescence did not change significantly upon binding TnI (Table III).

Intrinsic Fluorescence. TnIp did not affect the tyrosine fluorescence of CaM and TnC to a perceptible degree (data not shown). The tryptophan fluorescence of TnI was only slightly affected by TnC or CaM (intensity increased 4–5%, data not shown); the acrylamide quenching results (Table IV) implied that Trp-158 of TnI was not in direct contact with TnC or CaM, since only a minor decrease in quenching efficiency occurred.

Radiationless Energy Transfer. TnC-DANZ-DAB has a shorter fluorescence decay time than TnC-DANZ because of radiationless energy transfer. Upon binding TnIp, the decay time of the former was recovered to a slight degree (Table V). Since TnIp did not influence the fluorescence lifetime (Table V) or the emission profile of TnC-DANZ, or the absorbance

Table V: Separation between DANZ–Met-25 and DAB–Cys-98, As Determined from Fluorescence Lifetimes

samples	additive	α_1	τ_1	α_2	τ_2	α_3	τ_3	$\bar{\tau}^a$	χ^2	R (Å)
TnC–DANZ (no Ca^{2+})		0.1412	2.06	0.2138	5.94	0.1041	16.80	11.3	1.612	36.9
TnC–DANZ–DAB (no Ca^{2+})		0.3087	1.03	0.1751	4.87	0.0485	16.31	8.9	1.429	
TnC–DANZ		0.1075	0.54	0.0832	7.21	0.0803	19.92	16.0	1.661	40.5
TnC–DANZ–DAB		0.1919	1.32	0.1188	6.30	0.0532	18.81	11.9	1.359	
TnC–DANZ	TnIp	0.0578	2.67	0.060	10.15	0.0665	20.24	16.0	3.378	43.9
TnC–DANZ–DAB	TnIp	0.1200	1.11	0.0789	6.20	0.0436	19.19	13.2	1.305	

[a] $\bar{\tau} = \sum_i \alpha_i \tau_i^2 / \sum_i \alpha_i \tau_i$ (ns). Concentrations used: TnC–DANZ, 22.5 μM; TnC–DANZ–DAB, 25.5 μM; TnIp, 51 μM. The buffer used: 50 mM MOPS, 100 mM KCl, 0.5 mM EGTA, and 5 mM Ca^{2+}, pH 6.5. The excitation wavelength was 330 nm. T = 25 °C. The spectra of absorbance of DAB and emission of DANZ were used to calculate the overlap intergrals: J (10^4 nm^4 cm^{-1} M^{-1}) = 3.14 (no Ca^{2+}); 3.71 (with Ca^{2+} or Ca^{2+} + TnIp). Q (no Ca^{2+}) = 0.144 (Cheung et al., 1982); Q = 0.270 for TnC–DANZ (with Ca^{2+}) was calculated from 0.144 times the ratio of the emission intensity integrals of TnC–DANZ (with Ca^{2+}/no Ca^{2+}). K^2 was assumed to be $^2/_3$. R_0 (Å) thus calculated: 29.7 (no Ca^{2+}); 33.9 (with Ca^{2+} or Ca^{2+} + TnIp). The cited values of R are apparent values (see text).

Table VI: Radiationless Energy Transfer from Tyr-158 of TnI to AEDANS Groups on TnC or CaM, As Determined by Sensitized Fluorescence

acceptor	I^a	J^b ($\times 10^{13}$ nm^4 cm^{-1} M^{-1})	R_0 (Å)	R (Å)
AEDANS–Cys-98 of TnC[c]	1.24	6.50	19.6	28.5
AEDANS–Cys-27 of CaM[c]	1.15	6.67	19.7	32.7

[a] See Materials and Methods. [b] Q = 0.057 was used (Wang & Cheung, 1986); the value of K^2 was assumed to be $^2/_3$. [c] Conditions were the same as in Table IV.

profile of TnC–DANZ–DAB, the same values of J and R_0 were used for the computation of the apparent separation of the DANZ and DAB groups in TnC–DANZ–DAB in the presence of both Ca^{2+} and Ca^{2+} plus TnIp (Table V). The value of K^2 was assumed to be $^2/_3$, corresponding to random orientation.

The apparent separations cited in Table V were computed without regard to the influence of any distribution of separations, as might arise from the presence of label mobility or internal flexibility of TnC. Since such a distribution has an important effect upon the efficiency of transfer (Gryczynski et al., 1988; Lakowicz et al., 1987) and because of the assumption that $K^2 = {}^2/_3$, the quantitative relationship of the distances cited in Table V to the true average separation of the DANZ and DAB groups is uncertain. However, it is probably safe to conclude that no major change in the separation occurs upon complex formation with TnIp.

Another two separations were also determined: from Trp-158 of TnI to AEDANS–Cys-98 of TnC, and from Trp-158 of TnI to AEDANS–Cys-27 of CaM in the binary complex. The results are shown in Table VI. Wang and Cheung did a series of measurements on the intersubunit distances in the TnC–TnI complex, including the distance between Trp-158 of TnI and AEDANS–Cys-98 of TnC, mainly by monitoring donor steady-state intensity quenching (Wang & Cheung, 1986). The distance between the same pair of the probes obtained in this work is close to their value and is therefore taken as the reference for the distance obtained between Trp-158 of TnI and AEDANS–Cys-27 of CaM, so that the relative position of the AEDANS group on CaM can be estimated. The value of R ($^2/_3$) computed here for the latter separation may be compared with the value (24 Å) obtained by Wang and Cheung (1986) for the distance between Trp-158 and DANZ–Met-25 in the TnI–TnC complex for otherwise similar conditions. Both separations, as well as the distance from Trp-158 to AED–Cys-98, are consistent with a location of Trp-158 in the vicinity of the N-terminal lobe, but near the opposite side of the complex from Met-25 (or Cys-27). The acrylamide quenching results would further indicate that Trp-158 is not located within the zone of contact of TnI and TnC or CaM.

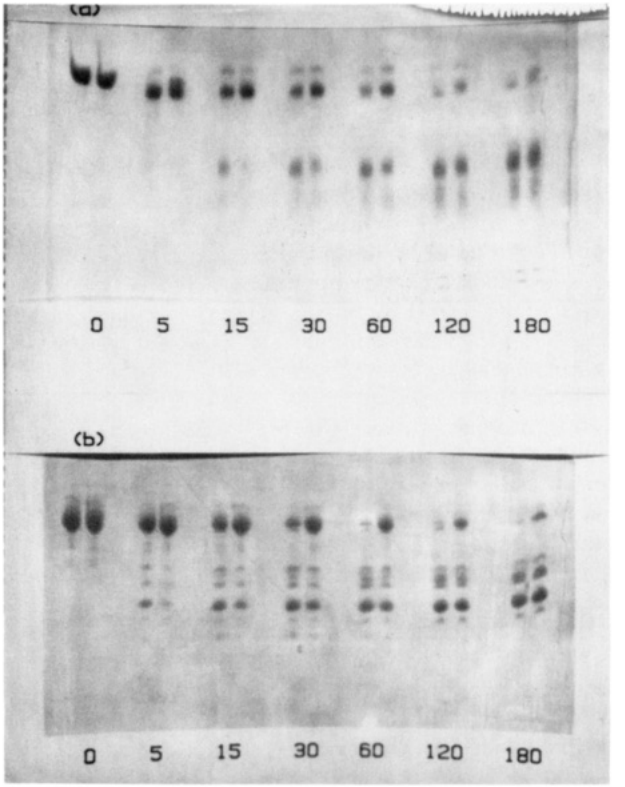

FIGURE 2: Tryptic digestions of TnC (a) and CaM (b) in the absence (left side of the pairs) and presence (right side of the pairs) of TnIp. Incubation times (minutes) are cited below each lane. The conditions of electrophoresis are indicated under Materials and Methods. For digestion, the starting protein concentrations were (a) 65 μM TnC and (b) 96 μM CaM.

Tryptic Hydrolyses. TnIp, although only 12 amino acid residues long, hindered the tryptic hydrolysis of CaM to a certain extent as has been found for some longer peptides (Caday & Steiner, 1986; Steiner & Norris, 1987a) (Figure 2). For TnC, the effect was less apparent. This is probably due to the extra α-helical turn (residues 88–90) in the C-terminal end of the connecting strand of TnC, which makes the distance between the tryptic cleavage site (84–85) and the Glu-rich region (92–97) longer than that in CaM (the distance between 77–78 and 82–87). This result implies that the TnIp-binding site on TnC and CaM is close to the tryptic hydrolysis site (middle of the connecting strand).

The assay of tryptic hydrolysis of TAME in the absence and presence of TnIp showed that TnIp itself was not a trypsin inhibitor (data not shown).

Conclusions

The above results showed that in the interaction with TnI, both N- and C-terminal halves and the connecting strands of

TnC or CaM are involved, although the fitting is not necessarily exactly the same for TnC and CaM. That TnIp binds TnC or CaM near the C-terminal end of the connecting strand agrees with other studies (Cachia et al., 1983; Leszyk et al., 1987). We further suggest that the binding of TnIp extends into the hydrophobic site of the C-terminal half of TnC and CaM. While a fluorescent label linked to Cys-98 of TnC appears to be imbedded in the zone of contact with TnI, Trp-158 of TnI and also labels attached to Met-25 of TnC and Cys-27 and Tyr-99 of CaM appear to be outside the zone of contact.

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