Fluorescence Lifetime and Acrylamide Quenching Studies of the Interactions between Troponin Subunits†

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ABSTRACT: Fluorescence lifetime and acrylamide quenching studies were carried out to characterize the interactions between the subunits of troponin under various conditions of metal ion binding. Troponin C was labeled at Cys-98 with *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine. In the presence of Ca^{2+}, the fluorescence decay of labeled troponin C (TnC*) was monoexponential, lifetime τ = 15.5 ns and quenching rate constant $k_q = 2.97 \times 10^8$ M^{-1} s^{-1}. In the absence of Ca^{2+}, the decay was resolvable into a major component with τ = 11.9 ns and a minor component with τ = 20.5 ns, with corresponding values of $k_q = 4.80 \times 10^8$ and 0.66×10^8 M^{-1} s^{-1}, respectively. Upon the binding of either troponin I (TnI) or troponin T (TnT) in the presence of Ca^{2+}, τ increased to ~18 ns, and k_q decreased to $\sim 0.8 \times 10^8$ M^{-1} s^{-1}. For the Ca^{2+} form of the TnC*–TnI–TnT ternary complex, values of τ = 17.6 ns and $k_q = 1.73 \times 10^8$ M^{-1} s^{-1} were obtained. These values did not vary significantly when Ca^{2+} was removed, or when Mg^{2+} replaced Ca^{2+}. These findings were interpreted as follows: the region around Cys-98 of TnC* adopts a looser conformation upon the removal of Ca^{2+} from the high-affinity sites. Both TnI and TnT bind to TnC* in the region containing Cys-98. The probe is shielded from the solvent to a greater extent in the binary complexes than in the ternary complex. The lack of metal-induced changes in the ternary complex indicates that conformational changes in this region of the molecule, if they do occur, do not result in changes in the environment of the probe.

Regulation of vertebrate skeletal muscle contraction is triggered by Ca^{2+} binding to the troponin C (TnC)[1] moiety of the troponin complex which, in turn, is part of the thin filament. Metal-induced changes in the structure of TnC are thought to be transferred to the other troponin subunits, TnI and TnT, and ultimately to tropomyosin and actin to effect a change in the actin–myosin interaction. Although the details of this process are as yet unknown, the regulatory mechanism is likely to involve changes in protein–protein interaction in the filament that result in stronger binding between some of the filament components, e.g., TnC and TnI (Margossian & Cohen, 1973; Head & Perry, 1974; Potter & Gergely, 1974; Hitchcock, 1975; Ohnishi et al., 1975), and dissociation of others, e.g., TnI and actin (Margossian & Cohen, 1973; Potter & Gergely, 1974; Hitchcock, 1975).

To further clarify the interactions between the three troponin subunits, we have used the fluorescence quenching technique (Lehrer & Leavis, 1978; Eftink & Ghiron, 1981) to probe the microenvironment of the interacting sites. Changes in quenching parameters are interpretable in terms of conformational changes and changes in interprotein interactions involving the labeled protein. In this work, TnC was labeled at its single cysteine residue, Cys-98, with the fluorescent probe 1,5-IAEDANS (Hudson & Weber, 1973), and the accessibility of this probe to the nonionic quencher acrylamide (Eftink & Ghiron, 1976) was determined in the absence and presence of Ca^{2+} and Mg^{2+} and the other troponin subunits. Our studies indicate that labeled TnC, TnC*, exhibits small but distinct metal-dependent changes in the fluorescence properties. Both TnI and TnT, when bound in binary complexes with TnC*, increase the lifetime and decrease the solvent accessibility of the probe. Our results suggest that both subunits bind to TnC* close to Cys-98, partially shielding the probe from the quencher molecules. A qualitatively similar decrease in quencher accessibility is also observed for the ternary TnC*–TnI–TnT complex although the shielding of the probe appears to be less in the ternary than in either binary complex. These studies support the view that the region of the TnC sequence containing Cys-98 is an important site of interaction for both TnI and TnT with TnC.

Theory

The theory of fluorescence quenching has been well documented (Lehrer & Leavis, 1978; Eftink & Ghiron, 1981), and only the main features are summarized here.

For a system containing a single emission component, the fluorescence lifetime, τ, decreases with the quencher concentration, [Q], according to the Stern–Volmer law:

$$1/\tau = 1/\tau_0 + k_q[Q] \quad (1)$$

where τ_0 is the lifetime in the absence of quencher and k_q is the bimolecular quenching rate constant, or, simply, the accessibility constant.

The steady-state fluorescence intensity, F, decreases with [Q] according to the relation:

$$F_0/F = (1 + K_{sv}[Q])e^{V[Q]} \quad (2)$$

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[1] Abbreviations: TnC, TnI, and TnT, calcium binding, inhibitory, and tropomyosin binding subunits of troponin, respectively; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; TnC*, TnC labeled at Cys-98 with 1,5-IAEDANS; Ca_4-TnC*, TnC* with all four metal binding sites saturated with Ca^{2+}; Ca_2-TnC* and Mg_2-TnC*, TnC* with the two high-affinity sites occupied by Ca^{2+} and Mg^{2+}, respectively; apo-TnC*, TnC* devoid of all divalent metal ions; Ca_4-TnC*–TnI and Ca_4-TnC*–TnT, Ca^{2+} forms of TnC* complexed with TnI and with TnT, respectively; Ca_4-TnC*–TnI–TnT, Mg_2-TnC*–TnI–TnT, and apo-TnC*–TnI–TnT, Ca^{2+}, Mg^{2+}, and apo forms of the TnC* + TnI + TnT ternary complex, respectively; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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where F_0 is the fluorescence intensity in the absence of quencher, V is the static quenching parameter that can be interpreted as the volume of a sphere within which the emission is quenched immediately after excitation (Eftink & Ghiron, 1976), and $K_{sv} = \tau_0 k_q$ is the Stern–Volmer quenching constant. In the limit of vanishing quencher concentration, we have

$$\lim_{[Q]\to 0} (F_0/F) = 1 + (K_{sv} + V)[Q] = 1 + K_{sv}'[Q] \quad (3)$$

where $K_{sv}' = K_{sv} + V$ is the apparent Stern–Volmer quenching constant that can be obtained from the initial slope of an F_0/F vs. [Q] plot.

For multicomponent systems, the lifetimes of each component still follow the Stern–Volmer law (eq 1). The apparent quenching constant becomes

$$K_{sv}' = \sum_{i=1}^{n} f_i(K_i + V_i) \quad (4)$$

where f_i is the fractional contribution from each component, n is the total number of components, and K_i and V_i are K_{sv} and the V parameter, respectively, for each component.

The second-order rate constant k_q serves as a quantitative measure of the emitter's accessibility to collisions with quenchers: a large k_q corresponds to high accessibility; a small k_q corresponds to low accessibility. Since K_{sv} is the product of k_q and τ_0, an observed decrease in K_{sv} can be interpreted to indicate a decrease in the accessibility of the emitter, provided that τ_0 does not decrease. It has also been found that the V parameter decreases when an emitter becomes inaccessible to quenchers (Eftink & Ghiron, 1976; Tao & Cho, 1979). Thus, with the same provision that τ_0 does not decrease, a decrease in K_{sv}' can also be taken as a qualitative indication of a decrease in the accessibility of the emitter.

Materials and Methods

Materials. Ultrapure acrylamide was obtained from Polysciences, Inc., Warrington, PA. 1,5-IAEDANS was obtained from Molecular Probes, Inc., Junction City, OR. All other common materials used for buffers and routine analyses were purchased from Sigma, St. Louis, MO.

Protein Preparations and Labeling. Troponin subunits were prepared from rabbit back and leg muscles by the method of Greaser & Gergely (1971). TnC was labeled with 1,5-IAEDANS at its single cysteine residue, Cys-98, following the method of Grabarek et al. (1983). Labeled TnC retains its metal binding properties and its ability to form ternary complexes with TnI and TnT. The reconstituted ternary complex is capable of conferring Ca^{2+} dependence on the actin-activated myosin ATPase (Grabarek et al., 1983) in both the presence and absence of 0.4 M acrylamide. The labeling ratio determined from the absorbance of the label at 337 nm, with ϵ_{337} = 6100 M^{-1} cm^{-1} (Hudson & Weber, 1973), was 0.8–1.0.

Sample Preparation for Quenching Studies. Ca_4-TnC* was prepared by adding 0.1 M $CaCl_2$ to a 2–5 μM solution of TnC* in 0.1 M KCl and 25 mM Hepes, pH 7.5. Apo-TnC* was prepared by adding 2 mM EGTA to a Ca_4-TnC* sample. Mg_2-TnC* was prepared by adding 2 mM $MgCl_2$ to an apo-TnC* sample. Ca_2-TnC* was obtained by using a Ca-EGTA buffer system to control the free Ca^{2+} concentration (Potter & Gergely, 1975). Ca_4-TnC*–TnI or Ca_4-TnC*–TnT was obtained by mixing TnI or TnT with equimolar TnC* in the presence of 8 M urea and 5 mM dithiothreitol and then dialyzing against 0.1 M KCl, 0.1 mM $CaCl_2$, 5 mM dithiothreitol, and 25 mM Hepes, pH 7.5. Ca_4-TnC*–TnI–TnT was obtained by mixing equimolar TnI, TnT, and TnC* in the presence of 8 M urea and 5 mM dithiothreitol and then dialyzing successively against buffers (0.1 mM $CaCl_2$, 5 mM dithiothreitol, and 25 mM Hepes, pH 7.5) containing 0.5 M KCl, 0.2 M KCl, and 0.1 M KCl. Apo-TnC*–TnI–TnT was obtained by adding 2 mM EGTA to Ca_4-TnC*–TnI–TnT. Mg_2-TnC*–TnI–TnT was obtained by adding 2 mM $MgCl_2$ to apo-TnC*–TnI–TnT.

Subunit–Subunit Binding Studies. Small increments of TnI (130 μM stock solution in 0.5 M KCl and 25 mM Hepes, pH 7.5) were added directly to a cuvette containing 2 mL of TnC* (10 μM) in 0.4 M acrylamide, 0.1 M KCl, and 25 mM Hepes, pH 7.5. The observed fluorescence increase during the titration was used to monitor complex formation. For studies in the presence of Ca^{2+}, 0.1 mM $CaCl_2$ was added. For studies in the absence of divalent cations, 2 mM EGTA was added. For studies in the presence of Mg^{2+}, 2 mM EGTA and 2 mM $MgCl_2$ were added. Titration of TnC* with TnT was carried out in the same manner, except that the concentration of the stock TnT solution was 140 μM. Assuming a binding ratio of 1 for both binary complexes, values of the association constant (K_B) and the maximal fluorescence increase (ΔF_{max}) were obtained from the titration data by applying an iterative nonlinear least-squares fitting procedure (Morris & Lehrer, 1984).

Spectroscopic Methods. Fluorescence quenching measurements were carried out according to published methods (Lehrer & Leavis, 1978; Tao & Cho, 1979). Steady-state fluorometry was carried out on a Perkin-Elmer MPF-4A spectrofluorometer equipped with a DCSU-2 correction device. Fluorescence lifetime measurements were carried out on a modified Ortec 9200 nanosecond fluorometer by using methods described previously (Tao & Cho, 1979). All measurements were made at 25 °C. Spectrophotometry was done on a Perkin-Elmer λ-3 spectrophotometer.

Data Analysis. Fluorescence decay analyses were carried out on a PDP-11/44 computer using the method of moments (Isenberg & Dyson, 1969). Because most of our decay curves were monoexponential, rapid convergence was obtained with or without the use of exponential depression (Isenberg et al., 1973). Results obtained from analyses that utilized moment index displacement (Small & Isenberg, 1976, 1977) of order 1 did not differ significantly from those obtained from analyses that did not utilize moment index displacement. This is because the lifetimes of our decay curves (10–20 ns) are significantly longer than any drift or shift that might occur in the zero-time point of the excitation. Also, due to the high quantum efficiency of the AEDANS label, scattering of the excitation source was insignificant. For decay curves reported as monoexponential, two-exponential analyses yielded second components of negligible amplitudes. For Ca_4-TnC*, for example, one-exponential analysis yielded τ = 15.44 ns, while two-exponential analysis yielded α_1 = 0.999, τ_1 = 15.42 ns, $\alpha_2 = 10^{-4}$, and τ_2 = 91 ns, where α's and τ's are fractional amplitudes and lifetimes, respectively, of the two components.

Results

Studies on Uncomplexed TnC.* Steady-state acrylamide quenching, fluorescence lifetime, and lifetime quenching studies were carried out on the various forms of uncomplexed TnC*: Ca_4-TnC*, Ca_2-TnC*, Mg_2-TnC*, and apo-TnC*. K_{sv}' values obtained from steady-state quenching measurements fall within the range of 6–7 M^{-1} for all four forms of TnC* (Figure 1; see Table I for a compendium of all quenching and lifetime results). The fluorescence decay curves for Ca_4-TnC* and Ca_2-TnC* are monoexponential for more than 4 decades (Figure 2A), with identical lifetimes of τ = 15.4 ns. The decay of Mg_2-TnC* is also monoexponential with a lifetime (14.1

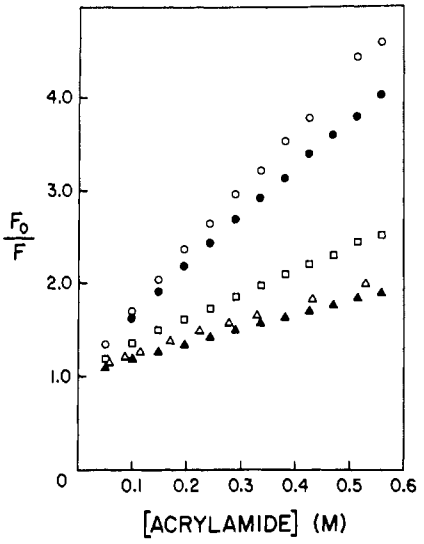

FIGURE 1: Steady-state acrylamide quenching plots for Ca_4-TnC* (○), apo-TnC* (●), Ca_4-TnC*–TnI–TnT (□), Ca_4-TnC*–TnT (△), and Ca_4-TnC*–TnI (▲). Excitation and emission wavelengths were 337 and 460 nm, respectively. See Materials and Methods for sample preparation and other experimental details.

Table I: Fluorescence Decay and Acrylamide Quenching Parameters for 1,5-IAEDANS-Labeled TnC (TnC*) and Its Complexes[a]

material	K_{sv}' (M^{-1})	τ (ns)	$k_q \times 10^{-8}$ (M^{-1} s^{-1})
Ca_4-TnC*	7.0	15.44	2.97
Ca_2-TnC*	6.8	15.32	3.37
Mg_2-TnC*	5.9	14.09	4.03
apo-TnC*	6.3	11.86 (0.84)[b]	4.80
		20.52 (0.16)[b]	0.66
Ca_4-TnC*–TnI	1.7	18.36	0.77
Ca_4-TnC*–TnT	2.4	18.65	0.91
Ca_4-TnC*–TnI–TnT	3.5	17.63	1.73
Mg_2-TnC*–TnI–TnT	3.2	17.58	1.92
apo-TnC*–TnI–TnT	3.0	17.74	1.31

[a] K_{sv}' is the apparent Stern–Volmer quenching constant, τ is the fluorescence lifetime, and k_q is the quenching rate constant. Estimated uncertainties are 0.5 M^{-1} for K_{sv}', 0.1 ns for τ, and 0.5 × 10^8 M^{-1} s^{-1} for k_q. All measurements were made at 25 °C. Definitions and preparation of samples are given in the abbreviations and footnote under Materials and Methods, respectively. [b] For apo-TnC*, two decay components were observed. The values in parentheses are fractional amplitudes of the two components.

ns) that is measurably shorter than that for the two Ca^{2+} forms. The decay for apo-TnC* is distinctly multiexponential and appears to be well described by two components (Figure 2B), a major component with τ = 11.9 ns and a minor component with τ = 20.5 ns.

The addition of acrylamide decreased the lifetime of Ca_4-TnC* according to the Stern–Volmer law, yielding a value of 2.97 × 10^8 M^{-1} s^{-1} for the quenching rate constant k_q (Figure 3, Table I). Similar results were obtained for Ca_2-TnC* and Mg_2-TnC*, with values of k_q that are similar to that for Ca_4-TnC*. For apo-TnC*, k_q for the short-lived major component (4.80 × 10^8 M^{-1} s^{-1}) is significantly higher than k_q for Ca_4-TnC*, while k_q for the minor long-lived component (0.66 × 10^8 M^{-1} s^{-1}) is 4.5-fold lower.

It can be seen from these results that although K_{sv}' for apo-TnC* is not significantly different from K_{sv}' for the other forms, the fluorescence decay and lifetime quenching characteristics are significantly different. It appears that the combination of the highly quenchable short-lived component with the virtually unquenchable long-lived component fortuitously yielded a K_{sv}' for apo-TnC* that falls in the range of 6–7 M^{-1}. This observation serves as a reminder of the pitfalls in interpreting fluorescence quenching results based on steady-state measurements alone.

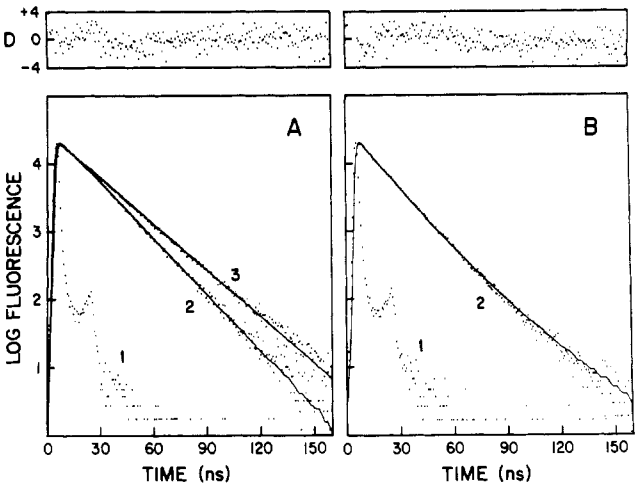

FIGURE 2: Fluorescence decay curves of Ca_4-TnC* (curve 2, panel A), Ca_4-TnC*–TnI (curve 3, panel A), and apo-TnC* (curve 2, panel B). Curves 1 in both panels are excitation curves. Dots are experimental points (F_e); solid lines are calculated curves (F_c) determined by using parameters obtained from the method of moments analysis. D is the deviation function, defined as $D = (F_c - F_e)/F_e^{1/2}$. In panel A, D for the decay of Ca_4-TnC* (curve 2) was plotted.

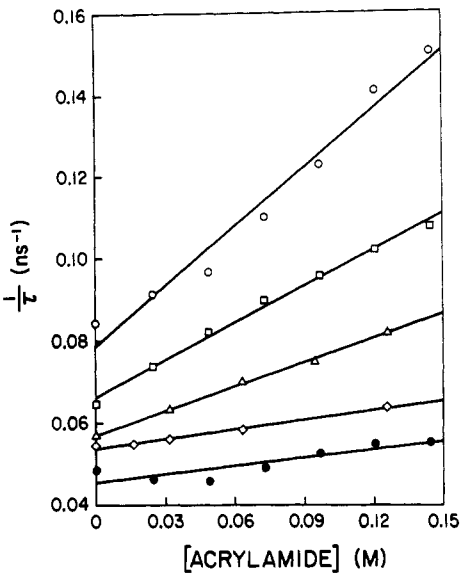

FIGURE 3: Stern–Volmer plots (eq 1) for the short-lived major component of apo-TnC* (○), Ca_4-TnC* (□), Ca_4-TnC*–TnI–TnT (△), and Ca_4-TnC*–TnI (◇) and the long-lived minor component (●) of apo-TnC*. τ is the fluorescence lifetime obtained by the method of moments analysis. Straight lines are obtained by linear regression. The slopes of the lines yield values of the quenching rate constant k_q (Table I).

Studies on the Binary Complexes. Values of K_{sv}' for the binary complexes Ca_4-TnI–TnC* and Ca_4-TnT–TnC* are ~3.5 times lower than K_{sv}' for Ca_4-TnC* (Figure 1, Table I). This suggests a substantial decrease in the accessibility of the probe upon the formation of the binary complexes provided that the fluorescence lifetimes do not decrease by the same factor. Lifetime measurements show that, in fact, τ increases from 15.4 ns for Ca_4-TnC* to ~18.5 ns for the two binary complexes (Figure 2A). Lifetime quenching measurements confirm that k_q decreases by a factor of ~3.7 upon the formation of either binary complex (Figure 3).

Titration Studies. Fluorescence titration curves in the presence of acrylamide were obtained for the three forms of each binary complex, and binding parameters were obtained

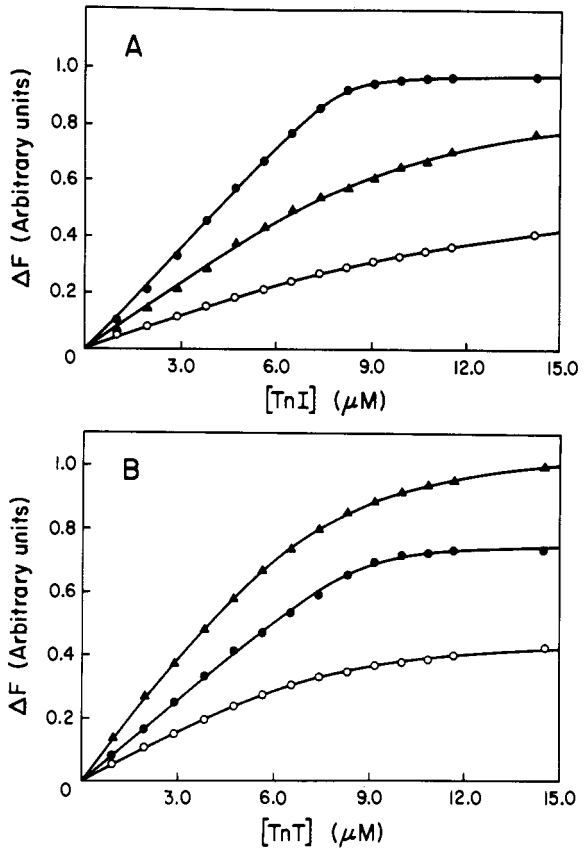

FIGURE 4: Fluorescence titration curves obtained in the presence of 0.4 M acrylamide. TnC* was titrated with TnI (A) or with TnT (B) in the presence of Ca^{2+} (●), the presence of Mg^{2+} (▲), or the absence of divalent metal ions (○). ΔF is the change in fluorescence upon the addition of either TnI or TnT. Solid lines are calculated binding curves determined by using parameters obtained from nonlinear least-squares fitting (Table II). See Materials and Methods for experimental details.

Table II: Binding Parameters Obtained from Fluorescence Titration Results[a]

complex formed	K_B (M^{-1})	ΔF_{max}
Ca_4-TnC*–TnI	2.24×10^7	97.8
Mg_2-TnC*–TnI	1.09×10^6	86.4
apo-TnC*–TnI	5.34×10^5	52.0
Ca_4-TnC*–TnT	5.87×10^6	75.8
Mg_2-TnC*–TnT	1.19×10^6	109.4
apo-TnC*–TnT	1.01×10^6	46.3

[a] K_B is the binding constant and ΔF_{max} is the maximal fluorescence increase (in arbitrary units). See Materials and Methods for experimental details.

by a least-squares fitting procedure (Figure 4, Table II). It appears that for both the TnC*–TnI and the TnC*–TnT complexes the stability decreases in the order Ca^{2+} forms > Mg^{2+} forms > apo forms.

Studies on the Ternary Complex. The values of K_{sv}', τ, and k_q do not vary significantly between the Ca^{2+}, Mg^{2+}, and apo forms of the TnC*–TnI–TnT complex (Table I). The values of all three parameters fall between the corresponding values for Ca_4-TnC* and for the binary complexes as is evident from Table I. The differences are well beyond the estimated uncertainties of the measurements.

Discussion

It has been shown that the fluorescence lifetime of the AEDANS moiety varies inversely with the polarity of the medium, ranging from 18.3 ns in ethanol to 9.4 ns in water (Hudson & Weber, 1973). For AEDANS-labeled proteins, one might expect the polarity around the labeling site to reflect the exposure of the site to the solvent. A compilation of values obtained from our previous and present studies in fact reveals an inverse relation between the fluorescence lifetime, τ, and the accessibility constant, k_q, for a variety of AEDANS-labeled proteins (Table III). It should be noted, however, that since τ varies by a factor of ~2 while k_q varies by a factor of ~10, the latter is a more sensitive indicator of changes in the environment.

Table III: Inverse Correlation between the Fluorescence Lifetime (τ) and the Quenching Rate Constant (k_q) for 1,5-IAEDANS-Labeled Proteins[a]

material	τ (ns)	$k_q \times 10^{-8}$ (M^{-1} s^{-1})
apo-TnC* (short-lived component)[b]	11.86	4.80
Tm*[c]	13.55	3.20
Ca_4-TnC*[b]	15.44	2.97
G-actin*[d]	17.25	2.31
F-actin*[d]	19.45	1.35
apo-TnC* (long-lived component)[b]	20.52	0.66

[a] Tm*, G-actin*, and F-actin* are 1.5-IAEDANS-labeled rabbit skeletal α-tropomyosin, G-actin, and F-actin, respectively. [b] Data are from this work. [c] From Lamkin et al. (1983). [d] From Tao & Cho (1979).

The metal binding properties of TnC have been well characterized (Potter & Gergely, 1975). Of the four metal binding sites, two (sites III and IV) bind Ca^{2+} with high affinity, and the remaining two sites (sites I and II) bind Ca^{2+} with low affinity. Mg^{2+} at millimolar levels binds only to the high-affinity sites. TnC contains a single cysteine residue at position 98 in the amino acid sequence proximal to Ca^{2+} binding site III on its N-terminal side. Our studies reveal small but characteristic changes in the environment around Cys-98 of TnC as a function of metal binding. For TnC* with all four metal binding sites saturated with Ca^{2+}, both τ and k_q indicate a moderately exposed environment at the Cys-98 labeling site (Table I). Removal of the two low-affinity Ca^{2+} sites produces no change in τ or k_q, indicating that Ca^{2+} binding to the low-affinity sites does not affect the environment at Cys-98. Replacement of Ca^{2+} by Mg^{2+} at the high-affinity sites caused a small but reproducible decrease in τ and an increase in k_q, indicating that whereas Mg^{2+} closely mimics the effects of Ca^{2+}, the effects are not exactly identical.

The fluorescence decay of apo-TnC* is clearly multiexponential, as previously reported by Cheung (1977). We found that the decay is well characterized by two components: a major component with values of τ and k_q that correspond to an exposed environment and a minor component with values of τ and k_q that correspond to a buried environment (Table I). The presence of the two components cannot be attributed to labeling sites other than Cys-98 because the amount of label incorporated as determined by its absorbance at 337 nm corresponded closely with the disappearance of the protein thiol group as determined by the 3-carboxy-4-nitrophenyl disulfide (Ellman's reagent) assay. Furthermore, the fact that the Ca^{2+} and Mg^{2+} forms of TnC* exhibit a monoexponential decay extending over 4 decades strongly suggests the presence of conformational heterogeneity. It is possible, for example, that in the absence of all metals, the protein possesses two conformational states in equilibrium with each other. Alternatively, it is possible that the protein exists in a single conformation but the label can equilibrate between an exposed polar environment and a buried hydrophobic environment. A priori, we cannot distinguish between these two possibilities. Either interpretation, however, indicates that the region around Cys-98 is more open in apo-TnC* than in Ca_4-TnC*. This

is consistent with structural studies showing that upon removal of Ca^{2+}, the Cys-98 region of TnC undergoes a helix to coil transition (Nagy et al., 1978) and loses its interaction with a C-terminal helix of the protein (Drabikowski et al., 1983). Our findings are also consistent with studies by Potter et al. (1976), who found that the thiol group at Cys-98 is more reactive and that a spin-label attached at Cys-98 exhibits higher mobility in the apo form of TnC than in the Ca^{2+} or Mg^{2+} forms. Although the conformations of the Mg^{2+} and Ca^{2+} forms of TnC are similar in many respects (Kawasaki & van Eerd, 1972; Seamon et al., 1977; Levine et al., 1978), we find small differences in conformation between the two forms.

Both our quenching and our lifetime studies indicate that upon the binding of either TnI or TnT to TnC*, the label becomes less exposed to the aqueous medium. The simplest interpretation of these findings is that both TnI and TnT bind to TnC at a region near the Cys-98 labeling site and serve to directly shield the label from collisions with quenchers in the medium. This view is consistent with earlier reports showing that both TnI and TnT decrease the mobility of a spin probe attached to Cys-98 (Potter et al., 1976) and both decrease the reactivity of Cys-98 of TnC (Potter et al., 1976; Chong & Hodges, 1982). Also, proteolytic fragments of TnC containing Cys-98 (more exactly, residues 89–100) exhibit Ca^{2+}-dependent complex formation with TnI or TnT (Leavis et al., 1978; Weeks & Perry, 1978; Grabarek et al., 1981). In contrast, fragments not containing residues 89–100 exhibit only weak binding to TnI or TnT (Grabarek et al., 1981). Finally, magnetic resonance studies have shown that TnC spin-labeled at Cys-98 affects several resonances that are attributable to TnI residues (Dalgarno et al., 1982), and in turn, a fluorine-labeled TnI fragment affects resonances arising from TnC residues in the 89–100 region (Cachia et al., 1983).

The most direct evidence that TnI interacts with TnC at the Cys-98 region has come from cross-linking studies, which show that sulfhydryl-specific photoactivatable cross-linking reagents attached at Cys-98 of TnC are capable of forming cross-links with TnI (Chong & Hodges, 1981a,b; Lamkin & Tao, 1982). Similarly, our recent finding that TnT can also be photo-cross-linked to TnC labeled at Cys-98 with the cross-linker 4-maleimidobenzophenone (Tao et al., 1984) suggests that TnT as well as TnI interacts with TnC in the vicinity of Cys-98.

The affinity constant of TnT for TnC* in the presence of Ca^{2+} is similar to that of TnI for TnC*, and, like that of TnI, the affinity is significantly reduced in the absence of calcium. This is consistent with the view that Ca^{2+} serves to stabilize the binary complexes (Potter et al., 1976; Zot & Potter, 1982). The affinity constants for both complexes are reduced when Mg^{2+} replaces Ca^{2+} at the high-affinity sites, indicating once again that the properties of the Mg^{2+} form of TnC are somewhat different from those of the Ca^{2+} form. We noted that the binding constants for the formation of complexes between labeled TnC and TnI or TnT are considerably lower than those for the unlabeled complexes, suggesting that the presence of the probe may modify the interactions between the proteins [cf. Grabarek et al. (1984)]. It should be pointed out, however, that (i) TnC* binds Ca^{2+} with the same characteristics as unlabeled TnC (Grabarek et al., 1983), (ii) like the binary TnC–TnI and TnC–TnT complexes, the binary TnC*–TnI and TnC*–TnT complexes are stabilized by Ca^{2+} and Mg^{2+}, and (iii) the ternary TnC*–TnI–TnT complex behaves identically with the TnC–TnI–TnT complex in conferring Ca^{2+}-sensitive control on actomyosin ATPase activity. Thus, we believe that any perturbations arising from the presence of the probe do not seriously interfere with the mode of intersubunit binding or regulatory function of the complex.

Our lifetime and quenching results indicate that the probe is considerably less exposed in the ternary TnC*–TnI–TnT complex than in uncomplexed TnC*. It is somewhat surprising, however, that the probe seems to be slightly more exposed in the ternary complex than in either the TnC*–TnI or the TnC*–TnT binary complexes (cf. Table I). One may infer from these findings that although both TnI and TnT bind to TnC near the Cys-98 site in the ternary complex, steric interference between TnI and TnT leads to greater exposure of the probe in the ternary complex than in either binary complex.

Because of its greater stability, we were able to carry out lifetime and quenching measurements for the apo and Mg^{2+} forms as well as for the Ca^{2+} form of the ternary complexes. The fact that none of these values changes with metal ion occupancy indicates that changes in the conformation of TnC* in the ternary complex, if they do occur, do not result in changes in the immediate environment of the probe. The possibility that the presence of the label or of acrylamide in the medium interferes with metal-induced conformational changes in the complex cannot be entirely ruled out although we have been able to demonstrate essentially normal Ca^{2+}-dependent actomyosin ATPase activity under these conditions.

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Registry No. Ca, 7440-70-2; Mg, 7439-95-4.

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Effect of Lipid Composition upon Fusion of Liposomes with Sendai Virus Membranes†

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ABSTRACT: How the lipid composition of liposomes determines their ability to fuse with Sendai virus membranes was tested. Liposomes were made of compositions designed to test postulated mechanisms of membrane fusion that require specific lipids. Fusion does not require the presence of lipids that can form micelles such as gangliosides or lipids that can undergo lamellar to hexagonal phase transitions such as phosphatidylethanolamine (PE), nor is a phosphatidylinositol (PI) to phosphatidic acid (PA) conversion required, since fusion occurs with liposomes containing phosphatidylcholine (PC) and any one of many different negatively charged lipids such as gangliosides, phosphatidylserine (PS), phosphatidylglycerol, dicetyl phosphate, PI, or PA. A negatively charged lipid is required since fusion does not occur with neutral liposomes containing PC and a neutral lipid such as globoside, sphingomyelin, or PE. Fusion of Sendai virus membranes with liposomes that contain PC and PS does not require Ca^{2+}, so an anhydrous complex with Ca^{2+} or a Ca^{2+}-induced lateral phase separation is not required although the possibility remains that viral binding causes a lateral phase separation. Sendai virus membranes can fuse with liposomes containing only PS, so a packing defect between domains of two different lipids is not required. The concentration of PS required for fusion to occur is approximately 10-fold higher than that required for ganglioside G_{D1a}, which has been shown to act as a Sendai virus receptor. When cholesterol is added as a third lipid to liposomes containing PC and G_{D1a}, the amount of fusion decreases if the G_{D1a} concentration is low. This suggests cholesterol may decrease the availability of G_{D1a} for multivalent binding. These data are consistent with the postulate that viral binding and the activities of the viral proteins play the major role in the fusion of viral and liposomal membranes.

Many hypotheses about the mechanism of membrane fusion involve properties of the membrane that result from specific lipids present in the membrane. These hypotheses include local micellization (Lucy, 1970) which could result from aggregation of gangliosides (Haywood, 1974a), lamellar to hexagonal phase transitions or formation of inverted micelles (Cullis & de Kruijff, 1979), packing defects due to transitions between domains of individual lipids with different packing packing properties or to lateral phase separations such as that due to the binding of Ca^{2+} to negatively charged lipids (Papahadjopoulos, 1978). These hypotheses also include the metabolic conversion of phosphatidylinositol (PI) to phosphatidic acid (PA) or changes in membrane hydration due to anhydrous Ca^{2+} complexes (Portis et al., 1979) or to the varying affinities of different lipids for water (Jendrasiak & Hasty, 1974).

Most of these hypotheses have been tested by examining fusion between two model membranes and may not reflect the mechanism of fusion of biologic membranes where membrane

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