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Tropomyosin–Troponin and Tropomyosin–Actin Interactions: A Fluorescence Quenching Study†

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ABSTRACT: Rabbit skeletal $\alpha\alpha$-tropomyosin was specifically labeled at Cys-190 with the fluorescent probe *N*-(iodoacetyl)-*N'*-(1-naphthyl-5-sulfo)ethylenediamine (1,5-IAEDANS). The fluorescence decay of the resultant AEDANS-labeled $\alpha\alpha$-tropomyosin (Tm*) was monoexponential with a lifetime of 13.55 ns. When acrylamide was used as the quencher, the apparent Stern–Volmer quenching constant K_{sv}' for Tm* was measured to be 5.78 M^{-1} and the quenching rate constant k_q to be 3.20×10^8 M^{-1} s^{-1}. The presence of troponin reduced the magnitude of K_{sv}' to 4.14 M^{-1} and induced the appearance of a second decay component. This second component had an amplitude of ~20% of the total intensity, a lifetime of ~20 ns, and a k_q of 4.5×10^{-7} M^{-1} s^{-1}. Similarly, the presence of F-actin induced the appearance of a minor longer lived decay component with a decreased k_q. On the basis of the increase in the lifetime and the decrease in k_q, the appearance of the long-lived decay component was interpreted to be due to troponin or actin interacting with Tm* near the Cys-190 site in both cases. Our results further suggest that the label was capable of equilibrating between an exposed hydrophilic environment on the surface of Tm* and a buried hydrophobic environment at the troponin–Tm* or actin–Tm* interaction interfaces.

It is now well established that tropomyosin (Tm)[1] in conjunction with troponin (Tn) mediates the regulation of mammalian skeletal muscle contraction by calcium ions (Ebashi & Endo, 1968). The molecular mechanism of this regulatory process is as yet not fully understood. It is, therefore, important to obtain information on the interactions between Tm and the other contractile proteins. It has been inferred that Tn binds to Tm near the Cys-190 site of Tm from electron microscopy studies (Cohen et al., 1972; Ohtsuki, 1974; Stewart, 1975) and from sequence analysis studies (McLachlan & Stewart, 1976a). More recently, evidence suggesting that the Tn binding site in Tm is more extensive was reported (Mak & Smillie, 1981). Repeated regions of sequence similarity in

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[1] Abbreviations: Tm, tropomyosin; Tn, troponin; S1, myosin subfragment 1; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(1-naphthyl-5-sulfo)-ethylenediamine; Tm*, 1,5-IAEDANS-labeled $\alpha\alpha$Tm; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Nbs_2, 5,5'-dithiobis-(2-nitrobenzoate); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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Tm have been suggested as actin interaction sites (McLachlan & Stewart, 1976b), and a low-resolution three-dimensional model of Tm–F-actin complex has been obtained by image reconstruction electron microscopy (Wakabayashi et al., 1975).

The fluorescence quenching technique (Lehrer & Leavis, 1978; Eftink & Ghiron, 1981) has been used successfully to study macromolecular interactions (Tao & Cho, 1979). In this work, we used this technique to study the interaction between a fluorescently labeled Tm with Tn, and with actin. Rabbit skeletal $\alpha\alpha$Tm was specifically labeled at Cys-190 with the fluorescent probe 1,5-IAEDANS (Hudson & Weber, 1973), and the accessibility of the bound probe to the nonionic quencher acrylamide (Eftink & Ghiron, 1976) was measured in the absence or presence of other proteins. We found that the presence of Tn decreased the probe's accessibility. The most straightforward interpretation of this observation is that the bound Tn can shield the label from quenchers in the aqueous medium, suggesting that Tn binds near the Cys-190 site of Tm. We found that the presence of F-actin also decreased the accessibility of the probe, suggesting that actin subunits also interact directly with the label at Cys-190. Finally, we found that only a small proportion (10–20%) of the labels was affected by the presence of Tn and/or F-actin. We postulate that the label is capable of equilibrating between two environments: an exposed hydrophilic one on the surface of the Tm* molecule and a buried hydrophobic one at the Tn–Tm* or actin–Tm* interaction interfaces.

Theory of Fluorescence Quenching. For a single component system, the quenching of the fluorescence by the collisional process is described by the Stern–Volmer law:

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

where τ_0 and τ are the fluorescence lifetimes in the absence and presence of quencher, respectively, [Q] is the quencher concentration, and k_q is the bimolecular quenching rate constant, which can be taken as a quantitative measure of the accessibility of the emitter. If the extent of quenching was monitored by steady-state fluorometry, then

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

where F_0 and F are fluorescence intensities in the absence and presence of quencher, respectively, and $K_{sv} = \tau_0 k_q$ is the Stern–Volmer quenching constant. Note that in the limit of low quencher concentration, eq 2 approaches

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

where K_{sv}', the apparent Stern–Volmer constant is given by

$$K_{sv}' = K_{sv} + V \quad (4)$$

and can be obtained experimentally from a plot of F_0/F vs. [Q] by taking the slope of the curve in the limit of zero quencher concentration.

For multicomponent emission systems, the lifetimes of each component still follow eq 1. The corresponding equations for steady-state quenching are given by

$$F_0/F = \left[\sum_{i=1}^{n} \frac{f_i}{(1 + K_i[Q])e^{V_i[Q]}}\right]^{-1} \quad (5)$$

and

$$K_{sv}' = \sum_{i=1}^{n} f_i(K_i + V_i) \quad (6)$$

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

It had been found that both k_q and V decrease when the emitter becomes inaccessible to quenchers (Eftink & Ghiron, 1976; Tao & Cho, 1979). Therefore, subject to the condition that the lifetime τ_0 does not decrease significantly, a decrease in K_{sv} and also K_{sv}' can be interpreted to indicate a decrease in the accessibility of the emitter.

Experimental Procedures

Materials. 1,5-IAEDANS was from Aldrich. Other common materials used for buffers and routine analyses were from Sigma.

Protein Preparations. Skeletal Tm and Tn were prepared from the back and hind leg muscles of rabbits according to Greaser & Gergely (1971) and Ebashi et al. (1971), respectively. $\alpha\alpha$Tm was fractionated from rabbit skeletal Tm according to Cummins & Perry (1973). Actin was prepared from rabbit skeletal acetone powder according to Spudich & Watt (1971). Myosin was prepared according to Balint et al. (1975). Chymotryptic S1 was prepared from myosin according to Weeds & Pope (1977).

Protein Labeling. Tm* was prepared as follows: $\alpha\alpha$Tm was first reduced in 10 mM dithiothreitol for 2 h at 37 °C and isolated by isoelectric precipitation. The reduced $\alpha\alpha$Tm (2 mg/mL) was reacted with a 20-fold molar excess of 1,5-IAEDANS in the dark for 24 h at 37 °C in 20 mM Tris, 1 M NaCl, and 1 mM EDTA, pH 8.0. The reaction was quenched by the addition of excess dithiothreitol, and the excess reagents were removed by dialysis against 2 mM Hepes and 0.1 M NaCl, pH 7.5.

The labeling ratio for Tm* was determined as follows: the label concentration was determined from the absorbance at 337 nm by using ϵ_{337}(AEDANS) = 6100 M^{-1} cm^{-1} (Hudson & Weber, 1973). The absorbance due to the label at 277 nm was then calculated by using ϵ_{277}(AEDANS) = 1060 M^{-1} cm^{-1} (Hudson & Weber, 1973). The absorbance due to Tm was obtained from the total absorbance minus the absorbance due to the label at 277 nm. The concentration of Tm was calculated by using a specific absorbance of E_{277}(Tm) = 0.24 $(mg/mL)^{-1}$ cm^{-1} (Lehrer, 1975) and molecular weight [M_r(Tm)] of 66000 (Stone et al., 1975). Most of the experiments were performed on Tm* with a labeling ratio of 1.2 mol of AEDANS/mol of Tm. The number of unmodified sulfhydryl groups in Tm* was determined by treating sodium dodecyl sulfate denatured Tm* with Nbs_2 (Ellman, 1959).

Other constants used for protein concentration determinations include the following: E_{290}(actin) = 0.93 $(mg/mL)^{-1}$ cm^{-1} (Lehrer & Kerwar, 1972); M_r(actin) 42300 (Elzinga et al., 1973); E_{280}(Tn) = 0.45 $(mg/mL)^{-1}$ cm^{-1} (Hartshorne & Mueller, 1969); M_r(Tn) 69000 (taken from the amino acid sequences of the components: Pearlstone et al., 1976; Wilkinson & Grand, 1975; Collins et al., 1973); E_{280}(S1) = 0.77 $(mg/mL)^{-1}$ cm^{-1} (Young et al., 1965); M_r(S1) 115000 (Lowey et al., 1969).

Binding Assays. Samples containing labeled or unlabeled Tm, F-actin, and, when indicated, Tn as well were sedimented at 80000*g* and 25 °C for 3 h. The pellets were resuspended in low ionic strength buffer (2 mM Hepes, 0.2 mM $CaCl_2$, and 0.2 mM ATP, pH 7.5). The amount of actin-bound labeled Tm was determined by comparing the fluorescence intensity of the supernatant with that of a Tm* solution at the same concentration as the original unsedimented sample. The amount of bound unlabeled Tm or Tn was determined by scanning the Coomassie stained electrophoretic gels and comparing the amounts of materials in the unsedimented samples, the resuspended pellets, and the supernatants.

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). Under our experimental conditions, the extent of light scattering in the presence of F-actin amounts to 2% of the total fluorescence (Tao & Cho, 1979). Method of moments fluorescence decay analysis (Isenberg & Dyson, 1969) was carried out on a PDP-11/03 microcomputer. Spectrophotometry was carried out on a Perkin-Elmer λ-3 spectrophotometer. Circular dichroism measurements were carried out on a Cary 60 spectropolarimeter.

Results

Specific Labeling of αα Tm. Because of the relatively harsh conditions used to label ααTm with 1,5-IAEDANS, we considered the possibility that the label might be attached nonspecifically at a site(s) other than Cys-190. Several observations argue against the presence of substantial nonspecific labeling: (1) The fluorescence decay of Tm* could be fitted satisfactorily by a single exponential (Figure 4, curve 1). A two-exponential analysis yielded a second component of negligible relative amplitude. This is suggestive, although does not prove, that the label is attached primarily at a single site.

(2) ααTm contains only two equivalent sulfhydryls, one on each chain at Cys-190. If labels were incorporated nonspecifically at sites other than these sulfhydryls, then the sum of n_L (number of labels per molecule of Tm*) and n_S (number of unmodified sulfhydryls per molecule of Tm*) would exceed 2. For two separate batches of Tm*, we found $n_L + n_S = 1.2 + 0.8 = 2.0$, and $n_L + n_S = 1.7 + 0.5 = 2.2$. In so far as the determinations of n_L and n_S are accurate to about 10%, we concluded that the extent of nonspecific labeling cannot be more than 10%.

(3) Since there are two sulfhydryls per molecule of ααTm available to labeling, a given batch of Tm* would contain a statistical distribution of three species: species A with both sulfhydryls labeled, species B with one of the two sulfhydryls labeled, and species C with neither sulfhydryls labeled. Of the three species, only species C can be cross-linked via the formation of a disulfide bridge upon treatment with Nbs_2 (Lehrer, 1975). If nonspecific labeling were present, this cross-linked product would be fluorescent. A sample of Tm* with a labeling ratio of $n_L = 1.2$ was treated with Nbs_2 and subjected to polyacrylamide gel electrophoresis. The dimer band corresponding to cross-linked species C showed virtually no fluorescence when less than 4 μg of protein was loaded (Figure 1). When larger amounts of protein was loaded, some fluorescence associated with the dimer band was detected, indicating that a small amount of nonspecific labeling was present. From the relative amounts of fluorescence associated with the monomer and dimer band, we estimated that the extent of nonspecific labeling to be not more than 8% of the total.

The effects that 1,5-IAEDANS labeling might have on the properties of Tm were investigated to a certain extent. The melting profile (monitored by circular dichroism as a function of temperature) of Tm* was found to be similar to that of unlabeled Tm; at 1 M NaCl, pH 7.5, 50% of the ellipticity change at 222 nm occurred at a temperature of 45 °C for labeled Tm, compared to 44 °C for unlabeled Tm (Lehrer, 1978). Tm* binds to actin with the same capacity as unlabeled

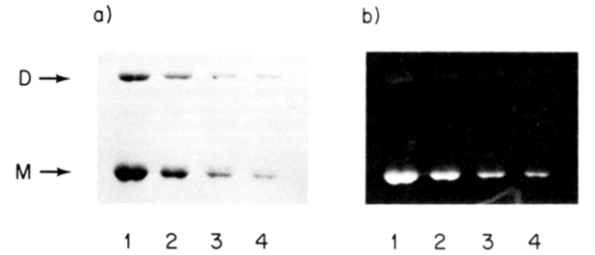

FIGURE 1: (a) Coomassie stained sodium dodecyl sulfate–polyacrylamide (9%) gel electrophoretogram of a sample of Tm* that was treated with Nbs_2 in order to cross-link the two chains via disulfide bridge formation. M and D designate bands corresponding to monomeric (M_r 33 000) and dimeric (M_r 66 000) species. Lanes 1, 2, 3, and 4 designate successively lower amounts of protein loaded, viz., 20, 8, 4, and 2 μg. (b) The same gel shown in (a) was photographed while illuminated with ultraviolet light in order to record the amount of fluorescence associated with the monomer and dimer bands. Note that very little fluorescence is associated with the dimer band even when 20 μg of protein was loaded.

Table I: Comparison of ATPase Activities for Reconstituted Systems Containing Labeled and Unlabeled Tm[a]

samples	ATPase [mol of ATP (mol of S1)$^{-1}$ s^{-1}]
S1	0.031
S1 + F-actin	0.154
S1 + Tm–F-actin	0.077
S1 + Tn–Tm–F-actin + Ca^{2+}	0.096
S1 + Tn–Tm–F-actin + EGTA	0.032
S1	0.035
S1 + F-actin	0.170
S1 + Tm*–F-actin	0.094
S1 + Tn–Tm*–F-actin + Ca^{2+}	0.182
S1 + Tn–Tm*–F-actin + EGTA	0.007

[a] Tm and Tm* are unlabeled and labeled Tm, respectively. [S1] = 2.2 μM, [F-actin] = 1.3 μM, [Tm] = 0.27 μM, [Tn] = 0.3 μM, [Ca^{2+}] = 50 μM, and [EGTA] = 1.3 mM, in 0.03 M NaCl and 5 mM $MgCl_2$, 23 °C, pH 7.9. ATP hydrolysis rates were obtained from pH-stat traces.

ααTm, viz., 75–80% at 25 °C, at a molar ratio of 1:7 Tm:actin. Finally, Tm* inhibited acto-S1 ATPase to similar extents as unlabeled Tm and was capable of regulating acto-S1 ATPase in conjunction with troponin and Ca^{2+} (Table I). Taken together, these observations suggest that labeling with 1,5-IAEDANS does not greatly affect the structure and function of ααTm.

Steady-State Acrylamide Quenching. Using acrylamide as the quencher, we found that the apparent Stern–Volmer quenching constant K_{sv}' for Tm* decreased by 25% when Tn and Ca^{2+} was present (Figure 2 and Table II). This suggests that the probe became less accessible to quenchers in the medium when Tn was bound to Tm*. A sample of Tm* was titrated with Tn in the presence of 0.29 M acrylamide, and the resultant increase in fluorescence was used to construct a titration curve (Figure 3). Analysis of the curve yielded a stoichiometry of 0.9 Tn/Tm* and a binding constant of 2.67×10^6 M^{-1}.

Lifetime Quenching Studies. The fluorescence decay of Tm* alone was monoexponential at acrylamide concentrations up to 0.4 M. The quenching rate constant k_q for Tm* alone was obtained from the slope of the Stern–Volmer plot (eq 1; Figure 5 and Table II). The fluorescence decay of Tm* in the presence of Tn and Ca^{2+} was characterized by a sum of two exponentials (Figure 4, curve 2, and Table II), of which the lifetime of the major component (12.83 ns) was nearly the same as that of Tm* alone (13.55 ns), and the lifetime of the

Table II: Fluorescence Decay and Acrylamide Quenching Parameters for Tm* Complexed with Tn or F-Actin[a]

material	A_1	τ_1 (ns)	A_2	τ_2 (ns)	K_{sv}' (M^{-1})	$k_{q_1} \times 10^{-8}$ (M^{-1} s^{-1})	$k_{q_2} \times 10^{-8}$ (M^{-1} s^{-1})
Tm*	1.0	13.55[b]			5.78	3.20	
Tn-Tm* (1:1)[c]	0.814	12.83	0.186	19.62	4.14	3.53	0.45
Tm*-F-actin	0.828	12.45	0.172	19.80	4.55	2.95	0.43
Tn-Tm* (4:1)[d]	0.819	14.11	0.181	21.52	ND[e]	ND	ND
Tn-Tm*-F-actin	0.786	13.39	0.214	20.83	ND	ND	ND

[a] A's are amplitudes; τ's are lifetimes in the absence of acrylamide; K_{sv}' is the apparent Stern-Volmer quenching constant; k_q's are quenching rate constants; the subscripts 1 and 2 refer to decay components 1 and 2. Typically, [Tm*] = 2 μM, [Tn] = 2 μM, and [actin] = 19 μM, in 10 mM Hepes, 50 mM NaCl, 2 mM $MgCl_2$, and 0.1 mM $CaCl_2$, pH 7.5, 25 °C. From repeated runs, the uncertainty in τ is estimated to be 0.5 ns and in k_q to be 0.3 × 10^8 M^{-1} s^{-1}. [b] The mean of four determinations on four different preparations of Tm*, with a standard deviation of 0.08 ns. [c] Contains 1 mol of Tn/mol of Tm*. [d] Contains 4 mol of Tn/mol of Tm*. [e] Not determined.

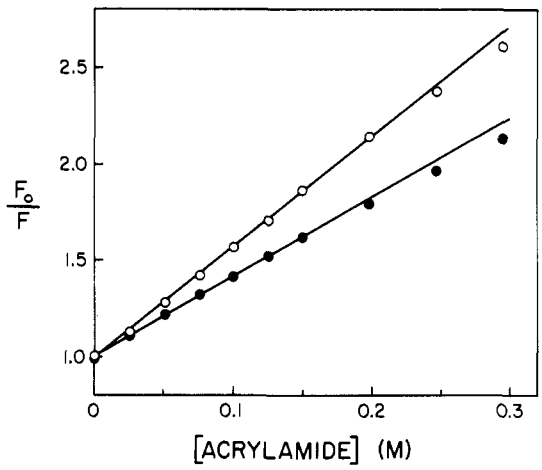

FIGURE 2: Steady-state acrylamide quenching of Tm* (2 μM) in the absence (○) and presence (●) of Tn (2 μM). F_0 and F are intensities in the absence and presence of acrylamide, respectively. In 10 mM Hepes, 50 mM NaCl, 2 mM $MgCl_2$, and 0.1 mM $CaCl_2$, pH 7.5, 25 °C. Initial slopes yielded K_{sv}' values of 5.78 M^{-1} and 4.14 M^{-1} in the absence and presence of Tn, respectively.

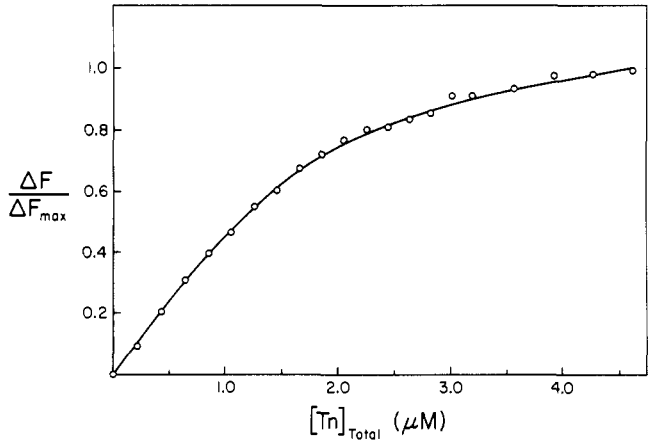

FIGURE 3: Titration curve obtained from the increase in fluorescence intensity when Tn was added to Tm* (1.65 μM) in the presence of 0.29 M acrylamide. ΔF_{max} is the fluorescence increase when 4.5 μM Tn was added. ΔF is the fluorescence increase when 0–4.5 μM Tn was added. Open circles are experimental points. Solid line is the calculated best fit (by minimizing the sum of the square of the residuals), which yielded a stoichiometry of 0.9 mol of Tn/mol of Tm* and a binding constant of 2.67 × 10^6 M^{-1}.

minor component was significantly longer (19.62 ns). From the slopes of the Stern–Volmer plots (Figure 5 and Table II), we found that k_q for the major component (3.53 × 10^8 M^{-1} s^{-1}) is nearly the same as k_q for Tm* alone (3.20 × 10^8 M^{-1} s^{-1}), while k_q for the minor component is significantly smaller (0.45 × 10^8 M^{-1} s^{-1}).

Interaction between Tm and F-Actin.* Steady-state and lifetime quenching studies on Tm* in the presence of F-actin

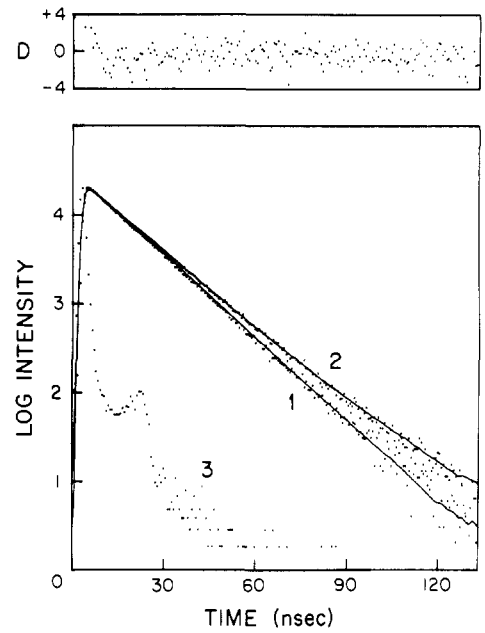

FIGURE 4: Fluorescence decay of Tm* (2 μM) in the absence (curve 1) and presence (curve 2) of Tn (2 μM). Dots are experimental points (F_e), and solid lines are calculated curves (F_c) by using decay parameters shown in Table II. Top panel shows the deviation function for Tm* alone, defined as $D = (F_c - F_e)/F_e^{1/2}$. Curve 3 is the excitation curve. Note that the presence of Tn induces the appearance of a long-lived component in the fluorescence decay of Tm*.

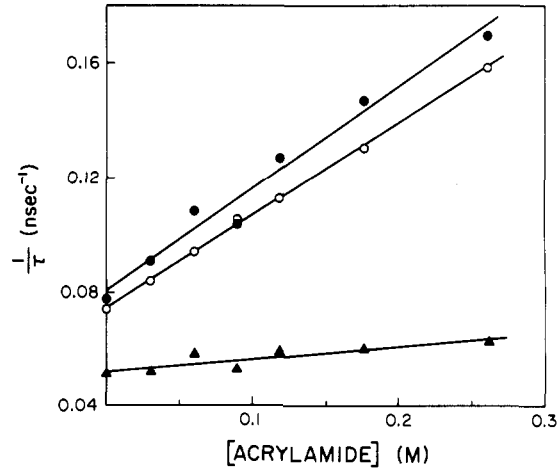

FIGURE 5: Reciprocal lifetime Stern–Volmer plots for Tm* (2 μM) in the absence of Tn (○), and the presence of 2 μM Tn [(●) for the short-lived component and (▲) for the long-lived component]. Slopes of the plots yield quenching rate constants k_q. Note that k_q for the short-lived component of Tn–Tm* is nearly the same as Tm* alone, while k_q for the long-lived component of Tn–Tm* is considerably smaller.

yielded results that were very similar to those in the presence of Tn: the presence of F-actin reduced the magnitude of K_{sv}'

by 21% (Table II) and induced the appearance of a minor long-lived component in the fluorescence decay. As in the case of Tn, k_q for this minor component is significantly smaller than k_q for Tm* alone (Table II).

Excess Tn (4 mol of Tn/mol of Tm*) was added to ensure saturation of the Tm*, and fluorescence lifetime measurements were carried out. There was no significant change in the lifetimes of the major and minor components. Neither was the amplitude of the minor component significantly increased (Table II). The same measurements were carried out on Tm* in the presence of both Tn and F-actin. Sedimentation assay showed that nearly all the Tm* (95%) was complexed as Tn–Tm*–F-actin. Although a slight increase in the amplitude of the minor component was found, it was still only 20% of the total (Table II).

Discussion

Previous studies have shown that the fluorescence lifetime of the AEDANS moiety decreases with the polarity of the medium (Hudson & Weber, 1973). For ethanol–water mixtures, the lifetime ranges from 18.3 ns in ethanol to 9.4 ns in water. On this scale, the lifetime of Tm* (13.55 ns) corresponds roughly to that for a ethanol–water mixture containing 25% ethanol, suggesting that the environment of the AEDANS label in Tm* is on the polar end of the scale. The lifetime of Tm* is also distinctly shorter than that for a number of other AEDANS-labeled proteins such as AEDANS-G-actin (17.3 ns; Tao & Cho, 1979), AEDANS-F-actin (19.5 ns; Tao & Cho, 1979), and AEDANS-S1 (20.6 ns; Mendelson et al., 1973; Tao & Lamkin, 1981), indicating that the environment of the Cys-190 label attachment site in Tm* is relatively polar.

We have noted that the lifetime of the label in AEDANS-labeled proteins is inversely correlated with its accessibility; the shorter the lifetime, the larger the accessibility constant k_q (Tao & Cho, 1979). In accordance with this correlation, we found the accessibility of the label in Tm* to be relatively high ($k_q = 3.20 \times 10^8\ M^{-1}\ s^{-1}$) when compared to AEDANS-G-actin ($k_q = 2.31 \times 10^8\ M^{-1}\ s^{-1}$; Tao & Cho, 1979) and AEDANS-F-actin ($k_q = 1.35 \times 10^8\ M^{-1}\ s^{-1}$; Tao & Cho, 1979). Thus, both the relatively short lifetime and the relatively high accessibility indicate that the AEDANS label in Tm* lies on the surface of the protein and is considerably exposed to both water molecules and quenchers in the medium.

In order to label the sulfhydryls in $\alpha\alpha$Tm, we had used relatively large excess of labeling reagent, high temperature, and long reaction time (see Experimental Procedures). This suggests that the sulfhydryls in $\alpha\alpha$Tm are relatively unreactive, in apparent contradiction with the aforementioned exposed character of the label in Tm*. A molecular model of the $\alpha\alpha$Tm molecule in the Cys-190 region reveals that before labeling, the two sulfhydryls [being class "d" residues in the classification according to McLachlan & Stewart (1975)] are buried in the hydrophobic ridge of the coiled-coil structure. After label attachment, the bulky AEDANS moiety cannot occupy the hydrophobic core without seriously distorting the native coiled-coil conformation. In view of the hydrophilic nature of the AEDANS moiety, it is not surprising that the label prefers the exposed outer surface of the Tm molecule. These observations serve to remind us that sulfhydryl reactivity studies do not necessarily yield the same information as fluorescence quenching studies.

Our studies show that the binding of Tn causes K_{sv}' of Tm* to decrease. This is indicative of a decrease in the accessibility of the probe, provided that the fluorescence lifetime does not decrease. Our lifetime measurements verify that there is in fact no lifetime decrease. Further, our lifetime quenching studies show that Tn induces the appearance of a long-lifetime (20 ns) component with much reduced accessibility ($k_q = 0.45 \times 10^8\ M^{-1}\ s^{-1}$) in the fluorescence decay of Tm*. The most straightforward interpretation of these observations is that Tn binds sufficiently near the label as to shield the label from quenching either by water molecules or by quenchers. Since the label is attached primarily at Cys-190, our results therefore indicate that Tn (or some part of Tn) binds to Tm at or near the Cys-190 site. This conclusion is in agreement with that derived from structural studies (Cohen et al., 1972; Ohtsuki, 1974; Stewart, 1975) and fluorescence studies (Morris & Lehrer, 1982).

It is possible that the long-lived component arises from Tn interacting with labels attached at a nonspecific site(s). However, since we determined that the extent of nonspecific labeling is less than 10% and the amplitude of the long-lived component is ~20%, we consider this possibility to be an unlikely one.

It should be noted that our findings by themselves cannot rule out the possibility that Tn binds to Tm at a site remote from Cys-190 but exerts an effect on the label attached at Cys-190 via an induced conformational effect. However, recent reports by Lamkin & Tao (1982) and by Chong & Hodges (1982) showed that it is possible to form a cross-link between Tn and photoaffinity labels attached at Cys-190 of Tm. These observations provide further evidence for Tn directly interacting with Tm at or near Cys-190.

It is surprising that the amplitude of the Tn-induced long-lived component is only ~20% of the total intensity, suggesting that only a small proportion of the labels interacts with Tn directly. To ensure full saturation of the Tm*, Tn was added to 4-fold molar excess over Tm*, or Tn was added to Tm* in the presence of F-actin. For the latter, cosedimentation experiments show conclusively that every Tm* contains one bound Tn. Yet, neither treatment appreciably increased the amplitude of the long-lived component (Table II). Since both the lifetime and k_q for the short decay component of Tn–Tm* are similar to those of Tm* alone, it is reasonable to associate this component with labels that lie on the exposed hydrophilic surface of Tm* even though Tn is present. On the basis of its increased lifetime and its decreased k_q, it is also reasonable to associate the long-lived component with labels that occupy a buried hydrophobic environment at the Tn–Tm* interaction interface.

Anisotropy decay studies (Wahl et al., 1978) suggested that the AEDANS label in Tm* possesses considerable rotational freedom, such that it might be able to equilibrate between the two environments. Given the hydrophilic nature of the AEDANS moiety, we might expect the equilibrium to favor the hydrophilic environment, accounting for the fact that the amplitude of the long-lived component is relatively small. It is also possible that the two proteins are not rigidly attached to each other in this region, and the label samples the two environments as the proteins fluctuate between an attached and a locally detached state. It is interesting that other fluorescent labels attached at the same Cys-190 site of Tm have been shown to be capable of sampling multiple environments in the Tm molecule (Lehrer et al., 1981).

We found the effect of F-actin on the fluorescence decay and the accessibility of the label in Tm* to be similar to those of Tn. The interpretation is therefore also similar: F-actin directly interacts with the AEDANS label attached at Cys-190 of Tm*. The interaction is such, however, that only a fraction of the labels is affected. This finding can be rationalized by postulating that the AEDANS label alternatively samples an

open hydrophilic environment and the buried hydrophobic environment at the interaction interface, as the Cys-190 region of Tm* transiently makes and breaks contact with F-actin subunits. This picture is consistent with a somewhat flexible Tm strand (Lehrer, 1978; Phillips et al., 1980; Lehrer et al., 1981) that is "loosely" attached to F-actin. An image reconstruction model of the Tm–F-actin complex showed that certain regions of the Tm strand make little or no contact with the subunits of the actin filament (Wakabayashi et al., 1975). It is possible that the Cys-190 site is within one of these regions and that the label attached at Cys-190 samples the actin–Tm* interface only a small proportion of the time.

In conclusion, our studies provide evidence that Tn binds to Tm at or near the Cys-190 site of Tm. In addition, we found that the fluorescence quenching technique is capable of providing some details regarding the dynamics of the interactions between Tm and Tn and between Tm and actin. Work is in progress to characterize the calcium dependence of these interactions by using this technique.

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