Fluorescence Studies of the Conformation of Pyrene-Labeled Tropomyosin: Effects of F-Actin and Myosin Subfragment 1†

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ABSTRACT: The fluorescence of pyrene-TM [rabbit skeletal tropomyosin (TM) labeled at Cys with *N*-(1-pyrenyl)maleimide] consists of monomer and excimer bands [Betcher-Lange, S., & Lehrer, S. S. (1978) *J. Biol. Chem. 253*, 3757–3760]; an increase in excimer fluorescence with temperature is due to a shift in equilibrium from a chain-closed state (N) to a chain-open state (X) associated with a helix pretransition [Graceffa, P., & Lehrer, S. S. (1980) *J. Biol. Chem. 255*, 11296–11300]. In this study, we show that the presence of appreciable excimer fluorescence at temperatures below the N → X pretransition (initial excimer) is due to perturbation of the TM chain–chain interaction by the pyrenes at Cys-190. Fluorescence and ATPase titrations indicated that the label caused a decrease in TM binding to F-actin primarily due to reduced end to end TM interactions on the actin filament. Under conditions where pyrene-TM was bound to F-actin, however, the excimer fluorescence did not increase with temperature, indicating that F-actin stabilizes tropomyosin by inhibiting the N → X transition. The binding of myosin subfragment 1 (S1) to pyrene-TM–F-actin at low ratios to actin caused time-dependent changes in fluorescence. After equilibrium was reached, the initial excimer fluorescence was markedly reduced and remained constant over the pretransition temperature range. Further stabilization of tropomyosin conformation on F-actin is therefore associated with S1 binding. Effects of the binding of S1 to the F-actin–tropomyosin thin filament on the state of tropomyosin were studied by monitoring the monomer fluorescence of pyrene-TM. Samples with low and high labeling ratios (0.17 and 1.7, respectively) both gave an 18% increase in pyrene monomer fluorescence with the same S1 titration profile, indicating a lack of preferential interaction of S1 with the probe. The fluorescence change, which saturated much before the 1:1 stoichiometric binding to actin was complete, followed a curve described by the random binding of between one (or more) and two (or more) S1's to seven actin subunits, showing that the state (or position) of tropomyosin is changed by the strong binding of a small number of myosin heads to a tropomyosin–actin unit. The low [S1]/[actin] ratios that were sufficient for the changes in monomer and excimer fluorescence indicate that myosin can produce long-range effects on tropomyosin in the thin filament.

Tropomyosin (TM) is involved in the Ca^{2+}-dependent thin filament regulation of contraction in conjunction with troponin (Ebashi & Endo, 1968; Smillie, 1979; Leavis & Gergely, 1984). Recent studies have shown that the striated muscle thin filament exists in two states: a weak myosin subfragment 1 (S1) binding state and a strong S1 binding state which results in cooperative S1–ADP binding because the tropomyosin units shift to another state (or tropomyosin position), making available seven strong S1 binding sites per actin subunit on the actin filament (Greene & Eisenberg, 1980; Hill et al., 1980). The relationship between the states of the thin filament, the movement of TM on the thin filament (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973; Lowy & Vibert, 1972), and the regulation of ATPase activity has been the subject of considerable recent experimental and theoretical activity (Chalovich & Eisenberg, 1982; Hill et al., 1981, 1983; Lehrer & Morris, 1982; Nagashima & Asakura, 1982; Murray et al., 1982; Lin & Dowben, 1983; Tao et al., 1983). Since cooperative effects of S1 binding and of S1 on acto-S1 ATPase have been observed in the absence of troponin (Bremel et al., 1972; Eaton et al., 1975; Lehrer & Morris, 1982; Williams & Greene, 1983), information about the two thin filament states can be obtained by studying the simpler tropomyosin–actin system.

Using pyrene-labeled TM, we have obtained evidence that striated tropomyosin exists in two states in solution under physiological conditions: a more stable high α-helical "chain-closed" state (N) and a somewhat less stable "chain-open" state (X), in which the α-helical chains are locally unfolded and separated near Cys-190 (Lehrer et al., 1981; Graceffa & Lehrer, 1980). Pyrene-TM shows a considerable increase of excimer fluorescence at the expense of monomer fluorescence, associated with the N → X transition. Evidence for selective instability in the region of the molecule which contains Cys-190 has also been obtained for TM alone in solution by other techniques (Woods, 1977; Chao & Holtzer, 1975; Pato & Smillie, 1978; Krishnan et al., 1978; Wahl et al., 1978; Lehrer, 1978; Protekhin & Privalov, 1978, 1982; Phillips et al., 1980; Edwards & Sykes, 1980; Williams & Swenson, 1981; Graceffa & Lehrer, 1984; Ueno, 1984).

In the work reported below, we studied the effect of the pyrene probe on the conformation of TM and found that it decreased the helix content of the N state by about 10% and also increased the unfolding associated with the N → X pretransition as a result of hydrophobic interaction of the neighboring pyrenes which locally perturbed the chain–chain interaction of the coiled-coil structure. These effects explain the observation of appreciable excimer fluorescence in the N

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state of TM (initial excimer). Perturbation by the pyrene, which appears to be responsible for the inhibition of the end to end polymerization of TM (Graceffa & Lehrer, 1980), is also shown to weaken the binding of TM to F-actin.

Studies of the temperature dependence of the excimer fluorescence of pyrene-TM bound to F-actin indicated that F-actin stabilized TM by preventing the increase in excimer associated with the N → X transition. The initial excimer fluorescence was not greatly affected, showing that the pyrene perturbation in the N state was still largely present in the complex of pyrene-TM with F-actin. S1 binding to the pyrene-TM–F-actin filament markedly reduced the initial excimer fluorescence which remained constant over the pretransition temperature range. Thus, S1 further stabilized the conformation of TM on actin.

Information about the S1-induced change in state of TM on the TM–actin filament was obtained by comparing the pyrene monomer fluorescence increase with the light-scattering increase. Under conditions where S1 was tightly bound with a 1:1 stoichiometry, the fluorescence change was only half complete at an S1/actin ratio of 1/7. A random-binding model indicated that the binding of between one and two S1's to a tropomyosin–actin unit was sufficient to produce the change in state. These studies show that the binding of S1 to the TM–actin thin filament produces long-range effects on the state and stability of tropomyosin.

Experimental Procedures

Materials

All proteins used in this study were prepared from rabbit skeletal muscle. Actin was prepared by the method of Spudich & Watt (1971), myosin by the method of Balint et al. (1975), and S1 by chymotryptic cleavage as described by Weeds & Pope (1977).

Tropomyosin (TM) was prepared and reduced with dithiothreitol as described previously (Lehrer & Morris, 1982), and excess dithiothreitol was removed by Bio-Gel P-60G column chromatography. TM was labeled with *N*-(1-pyrenyl)maleimide at pH 6 in the denatured state [5 M guanidinium chloride (GdmCl)] and quenched with excess dithiothreitol, and the succinimido ring of the cysteine adduct was opened to form the type II product by incubation at pH 8.4 as described earlier (Graceffa & Lehrer, 1980). The sample was dialyzed vs. 5 M GdmCl, 10 mM dithiothreitol, and 10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer (pH 7.5) to remove unreacted reagent, and GdmCl was removed by dialysis vs. 0.5 M NaCl, 10 mM Hepes (pH 7.5), and 1 mM ethylenediaminetetraacetic acid (EDTA) followed by exhaustive dialysis vs. 2 mM Hepes (pH 7.5) and 1 mM EDTA. Pyrene-TM was kept at 0 °C at low salt in the presence of 1 mM EDTA. The concentration of TM in salt-containing solutions ([NaCl] >0.1 M, to reduce polymerization and resulting light scattering) was determined from the difference in absorbance between 277 and 320 nm, $\Delta A = 0.24$ (mg/mL)$^{-1}$ (Lehrer, 1978), and from the molecular weight, 66 000. The concentration of pyrene-TM was determined by the Lowry method using unlabeled TM as a standard, and the concentration of pyrene linked to TM was determined from the absorbance at 343 nm with $\epsilon_{343nm} = 2.3 \times 10^4$ M^{-1} (Graceffa & Lehrer, 1980). The degree of labeling determined from the protein and label concentrations was 1.7, indicating that a large fraction of the molecules was doubly labeled. Singly labeled pyrene-TM was prepared by mixing doubly labeled pyrene-TM with unlabeled TM in a 1/10 ratio in the presence of 5 M GdmCl and 5 mM dithiothreitol to unfold and separate the chains; the chains were recombined by removing the GdmCl with exhaustive dialysis. The excimer to monomer peak fluorescence ratio (Betcher-Lange & Lehrer, 1978) decreased from 0.55 to 0.078 as a result of the decrease of doubly labeled molecules.

Methods

The concentrations of the other proteins were determined from absorbance measurements by using the following extinction coefficients and molecular weights: $A_{280nm} = 0.77$ (mg/mL)$^{-1}$ and $M_r = 120\,000$ for S1; $A_{290nm} = 0.63$ (mg/mL)$^{-1}$ and $M_r = 43\,000$ for G-actin.

The fluorescence measurements were carried out by using the thermostated sample housing of the Spex Fluorolog 2,2,2 spectrofluorometer (Edison, NJ) in the ratio mode with slit widths of 2.25 nm for both excitation and emission. All spectra were corrected for spectral variations in sensitivity. The circular dichroism measurements were obtained with an updated Cary 60 CD spectrometer (Aviv Associates, Lakewood, NJ) using 1-mm thermostated cuvettes. Solution temperatures were measured with a calibrated thermocouple (Omega Engineering, Stamford, CT) inserted in the cuvette. ATPase activity was determined by the pH-stat method as recently described (Lehrer & Morris, 1982).

Calculations

The fluorescence binding data of S1 to pyrene-TM–F-actin were compared to calculated curves assuming random binding of one (or more) or two (or more) S1's to a 1 TM–7 actin unit.

The probabilities of one or more and two or more S1's, $P(>1)$ and $P(>2)$, respectively, are given by

$$P(>1) = \sum_{i=1}^{7} P(i) = 1 - P(0)$$

$$P(>2) = \sum_{i=2}^{7} P(i) = 1 - P(0) - P(1)$$

where

$$P(i) = {}_7C_i(x)^i(1 - x)^{7-i}$$

and x is the molar ratio of bound S1 to actin and ${}_7C_i$ is the number of ways that i S1's can bind to a seven-actin unit.

The fluorescence binding data of pyrene-TM to F-actin were fitted to the McGhee–von Hippel equation (McGhee & von Hippel, 1974) by using a nonlinear least-squares computer-fitting program written by Dr. E. P. Morris.

Results

Properties of Pyrene-Tropomyosin. (A) Unfolding Properties. The thermal helix unfolding profiles of pyrene-TM and unlabeled TM at high salt concentration are compared in Figure 1. The unlabeled sample showed a weak pretransition near 30 °C as well as a major transition at 47 °C as described earlier (Woods, 1976; Lehrer, 1978). As can be seen, one effect of the label is to increase the unfolding associated with the pretransition and to shift it to lower temperatures without appreciably affecting the main transition. In addition, at low temperatures, where unlabeled TM is largely α-helical, the presence of the pyrene label causes about a 10% loss of initial α-helix. At low salt, the label also caused a 10% loss of initial helix (P. Graceffa, Y. Ishii, and S. S. Lehrer, unpublished results).

(B) Effects of F-Actin. The fluorescence of doubly labeled pyrene-TM consists of a structured band with peaks at 385

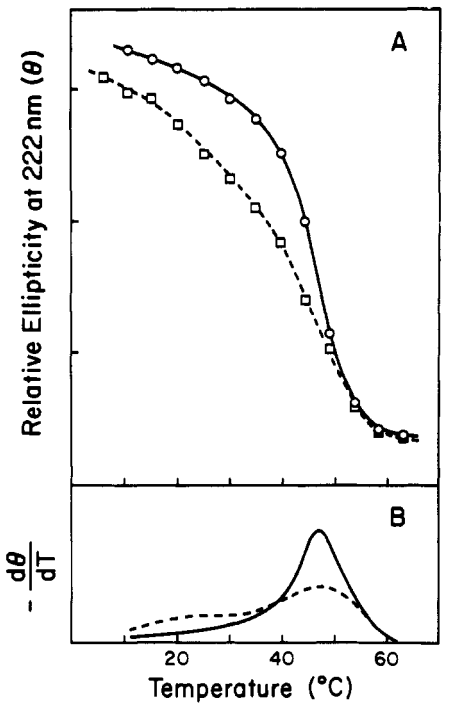

FIGURE 1: (A) Helix thermal unfolding profile of pyrene-TM (□) and unlabeled TM (○). (B) Slopes of the curves in (A). Conditions: 10 μM protein in 5 mM Hepes buffer, pH 7.5, 0.5 M NaCl, and 1 mM EDTA.

and 405 nm and a shoulder at 430 nm, originating from excited monomer pyrenes, as well as a broad band centered at 490 nm, originating from excited dimer pyrenes (excimer) (Figure 2). In the presence of F-actin at 25 °C under conditions of optimum binding (Eaton et al., 1975), the excimer (E) fluorescence was decreased by about 40%, and the monomer (M) fluorescence was increased by about 20% with an isoemissive point at 442 nm, without any spectral shape change (Figure 2A). Similar decreases in the E/M ratio with the same isoemissive point were observed for pyrene-TM when the temperature was lowered below 30 °C (Graceffa & Lehrer, 1980), when a few percent ethanol was added, and when the pH was decreased from 8.5 to 7.5 (Ishii & Lehrer, 1984). All of these perturbations are known to increase the stability of the helical conformation (Tanaka, 1972). Studies with singly labeled pyrene-TM, which mainly showed monomer fluorescence due to the low degree of labeling, provided further information about the actin effect. In the presence of F-actin, only a slight increase in monomer fluorescence (<5%) was observed (Figure 2B). This increase was undoubtedly due to the presence of a small fraction of doubly labeled molecules since the small amount of excimer fluorescence was seen to decrease upon the addition of F-actin. These observations indicate that the actin-induced spectral changes observed for doubly labeled pyrene-TM can be most readily explained by a shift in equilibrium toward the more stable chain-closed or N state (Graceffa & Lehrer, 1980); i.e., a direct interaction between pyrene on TM and the actin surface does not appear to occur.

(*C*) *Binding Studies.* The 40% decrease in excimer fluorescence caused by the presence of F-actin was used to study the binding of pyrene-TM to F-actin at 23 and 35 °C under optimum salt conditions. At 23 °C, the binding of pyrene-TM was quite tight as indicated by the fluorescence titration which saturated close to the molar ratio of TM/actin (=1/7) (Figure 3). A similar tight binding was observed for pyrene-TM when the TM-induced inhibition of acto-S1 ATPase was monitored (Lehrer & Morris, 1982) under essentially identical conditions as the fluorescence titrations (Figure 3). A mixture of pyrene-TM and unlabeled TM in a 1/4 molar ratio, respectively, was also used in the fluorescence and AT-

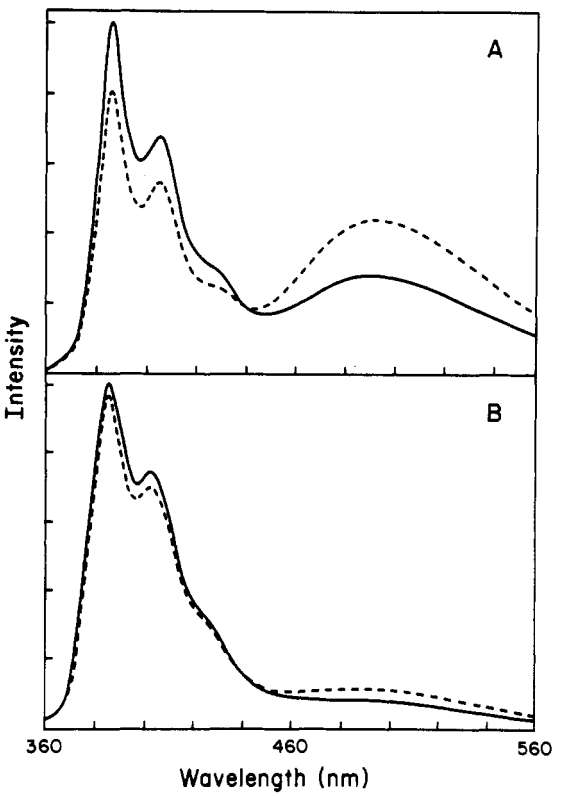

FIGURE 2: Fluorescence spectra of doubly labeled (A) and singly labeled (B) pyrene-TM in the absence (---) and presence (—) of F-actin. Conditions: 1.75 μM doubly labeled pyrene-TM + 12.25 μM F-actin, or 2.6 μM singly labeled pyrene-TM + 24.5 μM F-actin; in 10 mM Hepes buffer, pH 7.5, 5 mM $MgCl_2$, 30 mM NaCl, and 0.2 mM ATP, 25 °C. Excitation wavelength, 340 nm; solvent background subtracted.

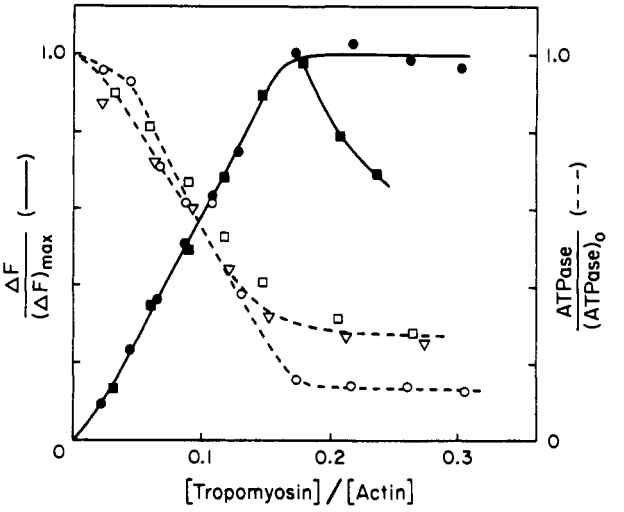

FIGURE 3: Binding of TM to F-actin at 23 °C monitored by the excimer fluorescence change (—) and inhibition of acto-S1 ATPase (---). Pyrene-TM (●, ○); pyrene-TM–TM mixture ([pyrene-TM]/[unlabeled TM] = 1/4) (■, □); unlabeled TM (▽). Conditions: [F-actin] = 7.3 or 8.2 μM in 0.03 M NaCl, 5 mM $MgCl_2$, and 20 mM Hepes buffer, pH 7.9. For ATPase measurements, 1 mM ATP, 1.0 μM S1 (▽, □), or 0.7 μM S1 (○) was included, and a pH stat was used to maintain the pH in an essentially unbuffered system. The drop in fluorescence change for the pyrene-TM–TM mixture (■) after saturation is due to exchange. Excitation wavelength, 340 nm; emission wavelength, 480 nm.

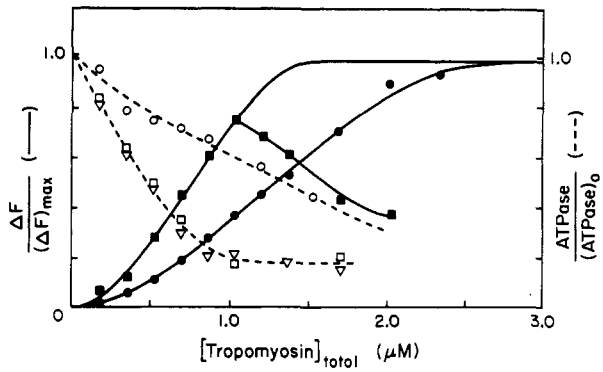

FIGURE 4: Binding of TM to F-actin at 35 °C monitored by the excimer fluorescence change (—) and inhibition of acto-S1 ATPase (---). All symbols and solution conditions same as in Figure 3. [F-Actin] = 8.24 μM except for the fluorescence titration with the pyrene-TM–TM mixture where [F-actin] = 7.24 μM. For ATPase measurements, 1 mM ATP and 0.5 μM S1 were included. The fluorescence curves for pyrene-TM (●) and the pyrene-TM–TM mixture (■) were obtained by fitting to the McGhee–von Hippel equation. For the pyrene-TM–TM mixture, it was assumed that exchange did not take place; i.e., points above 1 μM were not used (see the text).

Pase titrations. Below saturation, the fluorescence titration curve of the mixture was identical with the pyrene-TM system with the same final fluorescence change, indicating similar tight binding. Above saturation, the fluorescence signal decreased due to exchange of bound pyrene-TM with free unlabeled TM, showing that unlabeled TM binds more strongly than pyrene-TM. These observations were confirmed by centrifugation of solutions containing F-actin and increasing amounts of pyrene-TM or the pyrene-TM–unlabeled TM mixture at 23 °C under similar conditions and measuring the pyrene fluorescence in the pellet and in the supernatant in the presence of 0.5% sodium dodecyl sulfate. It was found that 80% ± 5% of the pyrene-TM was bound to F-actin near saturation in both cases. In the presence of excess TM (2× saturation), there was no decrease in fluorescence in the pellet fraction for the pure pyrene-TM but about a 50% decrease for the TM mixture, indicating exchange of unlabeled TM for pyrene-TM. No essential difference in the ATPase titration was observed between the labeled–unlabeled mixture, the pyrene-TM system, and the unlabeled system, indicating that bound unlabeled TM inhibits to the same extent as pyrene-TM under these conditions.

Differences between the binding properties of unlabeled and pyrene-TM to F-actin were observed at 35 °C (Figure 4). Unlabeled TM bound relatively tightly as shown by the definite end point near the stoichiometric binding ratio in the ATPase titrations. In contrast, pyrene-TM bound much more weakly to F-actin as measured both by the ATPase inhibition and by the excimer fluorescence change. The McGhee–von Hippel equation (McGhee & von Hippel, 1974) was computer fitted to the fluorescence data for pyrene-TM binding, yielding a value for the intrinsic binding constant $K = 7 \times 10^3$ M^{-1} and the end to end interaction parameter $w = 3 \times 10^2$. The fluorescence titrations showed that pyrene-TM in the unlabeled–labeled TM mixture bound much more strongly than pyrene-TM alone. As in the case of the 23 °C fluorescence titration with the mixture, exchange took place when there was an appreciable amount of free TM which resulted in a decreased signal. It was possible, however, to computer fit the fluorescence titration of the mixture to the McGhee–von Hippel equation by using the data at low saturation and the same final value of fluorescence since this was the case at 23 °C. This yielded values of K and w which were consistent with

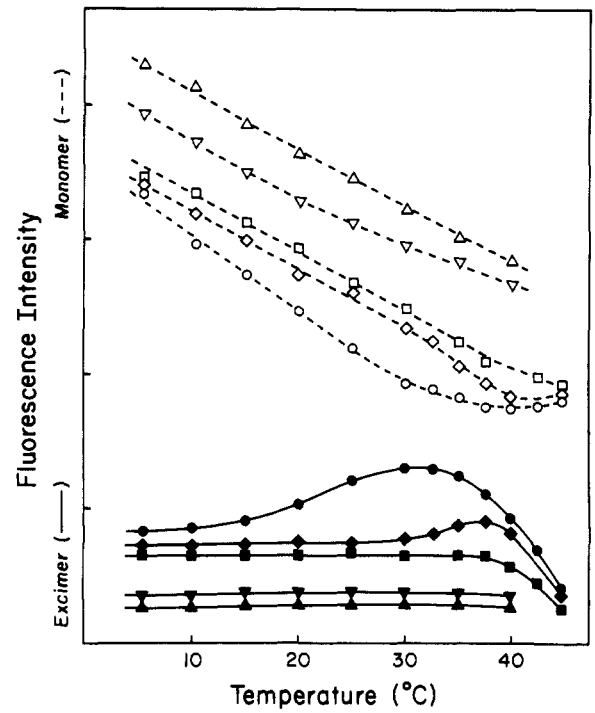

FIGURE 5: Temperature dependence of the excimer (—) and monomer (---) fluorescence of pyrene-TM systems. 0.58 μM pyrene-TM alone (○, ●); 0.58 μM pyrene-TM + 12.25 μM F-actin (◇, ◆); 1.75 μM pyrene-TM–TM mixture + 12.25 μM F-actin (□, ■) ([pyrene-TM]/[TM] = 1/2); 1.75 μM pyrene-TM–TM mixture + 12.25 μM F-actin + 1.75 μM S1 (▽, ▼) ([pyrene-TM]/[TM] = 4/1); 1.75 μM pyrene-TM–TM mixture + 12.25 μM F-actin + 12.25 μM S1 (△, ▲), ([pyrene-TM]/[TM] = 4/1). Solvent conditions as in Figure 2 except without ATP when S1 is present. Excitation wavelength, 340 nm; monomer emission at 385 nm; excimer emission at 480 nm.

a doubling of w without much change in K. It should be pointed out that the exchange of bound pyrene-TM with unlabeled TM which was clearly seen in the fluorescence titration was not detected in the ATPase titration. This indicates that the ATPase inhibition by bound pyrene-TM was not much different from that of bound unlabeled TM in this mixture, as was also seen in the 23 °C titrations.

Temperature Dependence of the Excimer Fluorescence of Pyrene-TM Bound to F-Actin. For pyrene-TM alone in solution, the excimer fluorescence increases from an initial value at low temperatures to a maximum at about 30 °C, showing a transition midpoint at about 25 °C (Figure 5) which correlates with the helix pretransition shown in Figure 1. In the presence of excess F-actin, the initial excimer was only slightly decreased and did not change when the temperature was raised to about 30 °C. The increase in excimer fluorescence above 30 °C is due to pyrene-TM dissociation from F-actin since no increase was observed when unlabeled TM was also present, in agreement with the observations described above in which the presence of unlabeled TM aided the binding of pyrene-TM to F-actin at 35 °C. Thus, under conditions that pyrene-TM remains bound to F-actin, the chain-closed state is stabilized, preventing a shift in equilibrium to the chain-open state. The decrease in excimer fluorescence above 40 °C is most probably due to dissociation from F-actin with subsequent chain dissociation of the free TM. It should be noted that the fluorescence changes observed in the presence of F-actin were reversible at least to 45 °C and that they occurred within the mixing time of the pyrene-TM and actin solutions.

Although the excimer fluorescence arises only from molecules doubly labeled at Cys-190, the monomer fluorescence is more heterogeneous since it arises from some singly labeled

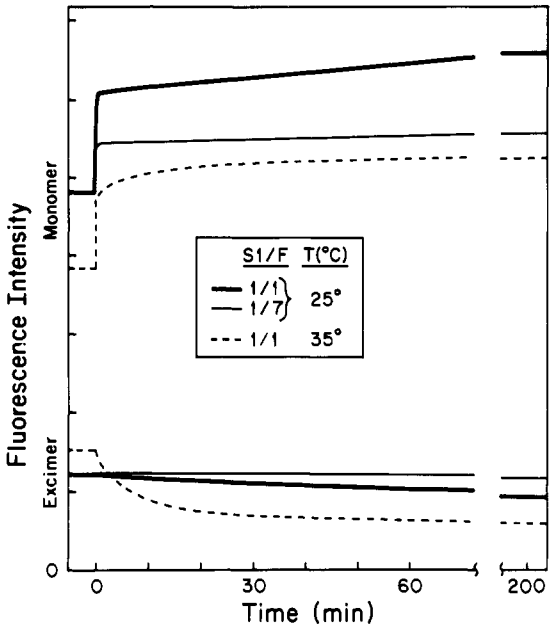

FIGURE 6: Time dependence of monomer and excimer fluorescence of doubly labeled pyrene-TM bound to F-actin after S1 addition. Upper curves, monomer fluorescence; lower curves, excimer fluorescence. Solid lines, 25 °C; dashed lines, 35 °C. S1 was added at $t = 0$ to 12.25 μM F-actin + 1.75 μM pyrene-TM–TM mixture ([pyrene-TM]/[TM] = 4/1). [S1]/[F-actin] ratios are indicated in the inset. Solvent conditions same as in Figure 2 except without ATP. Fluorescence conditions as in Figure 5.

molecules, from a small fraction of labels at Cys-36 in $\alpha\beta$-TM, and also from doubly labeled molecules that equilibrate between monomer- and excimer-forming states (Graceffa & Lehrer, 1980). The monomer temperature dependence should be due to a superposition of the thermally activated generalized solvent quenching expected with the changes in the monomer contribution caused by the thermal dependence of the chain-closed = chain-open equilibrium which changes the excimer/monomer ratio. The monomer fluorescence monotonically decreased with temperature for model compounds, for singly labeled TM (data not shown), and for pyrene-TM systems that did not show much excimer variation (e.g., for pyrene-TM bound to F-actin and to F-actin–S1; see below). When appreciable excimer fluorescence was present, the monomer fluorescence decreased more rapidly with temperature in the temperature range that the excimer fluorescence increased, i.e., between 20 and 30 °C for pyrene-TM alone and between 30 and 40 °C for pyrene-TM + excess F-actin (Figure 5). At higher temperatures where the excimer decreased due to chain dissociation, the monomer fluorescence decreased less rapidly or slightly increased with temperature. These data indicate that although the solvent quenching mechanism dominates there is a correlation between thermally induced changes in excimer and monomer fluorescence.

Binding of Myosin Subfragment 1 (S1) to the F-Actin–Pyrene-TM Complex. (A) Time-Dependent Effects. When S1 was added to the doubly labeled pyrene-TM–F-actin complex, time-dependent fluorescence changes of both monomer and excimer occurred. In general, two kinetic phases were seen: a fast phase that took place during the mixing time (a few seconds) in which the monomer fluorescence increased about 18% without affecting the excimer and a slow phase in which the monomer fluorescence increased somewhat further and the excimer fluorescence correspondingly decreased with about the same rate (Figure 6). When ATP was added to dissociate the S1 from the TM-actin at 20 °C, the monomer and excimer fluorescence reversed to the initial values; after

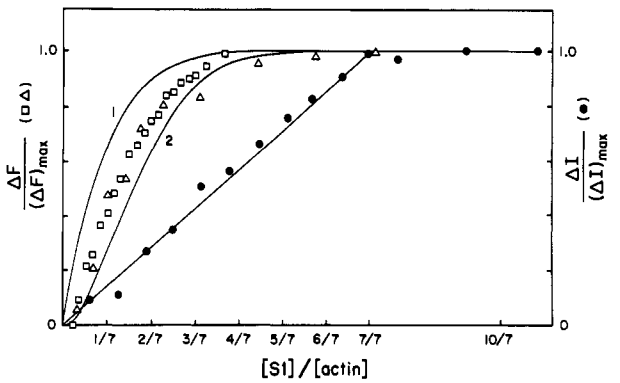

FIGURE 7: Effect of S1 binding on monomer fluorescence of doubly labeled and singly labeled pyrene-TM bound to F-actin. (●) S1 binding as monitored by the light-scattering increase at 340 nm for the doubly labeled system. (□, △) Monomer fluorescence increase at 385 nm excited at 340 nm: (□) doubly labeled system (1.75 μM total TM + 12.25 μM F-actin, [pyrene-TM]/[unlabeled TM] = 4); (△) singly labeled system (3.5 μM total TM + 24.5 μM F-actin). Experimental conditions: 10 mM Hepes buffer, pH 7.5, 5 mM $MgCl_2$, and 30 mM NaCl at 25 °C. The smooth curves labeled 1 and 2 were calculated by assuming that the total fluorescence change was due to the binding of 1 (or more) and 2 (or more) S1's to a 7 actin/1 TM unit, respectively.

the ATP was exhausted due to the acto-S1 ATPase, the monomer fluoresence quickly returned. Addition of S1 to a singly labeled pyrene-TM–F-actin complex only produced fast changes in monomer fluorescence. At 25 °C at an [S1]/[actin] ratio of 1, the slow process took a few hours to reach the final value; at an [S1]/[actin] ratio of 1/7, much longer incubation times were necessary to reach equilibrium. At 35 °C and an [S1]/[actin] ratio of 1, the slow process took 5–10 min.

The fast monomer fluorescence increase appears to be due to a changed environment of pyrenes that do not form excimer. The slow fluorescence phase, however, appears to be due to an indirect effect of S1 binding to actin on the pyrene-TM structure to change the distribution of excimer- and monomer-forming species, since the rate is much too slow to be related to the actual S1 binding process (Trybus & Taylor, 1980). Also, we measured the light-scattering changes associated with S1 binding to this system and found the changes to take place within the mixing time.

(B) Effects of S1 Binding on the State of Pyrene-TM Bound to F-Actin. The [S1] dependence of the monomer fluorescence increase was studied at 25 °C. Three S1 titrations were performed by monitoring (i) the monomer fluorescence of doubly labeled pyrene-TM–F-actin, (ii) the monomer fluorescence of singly labeled pyrene-TM–F actin, and (iii) the light-scattering increase produced by the binding of S1 to the doubly labeled system under identical conditions as the fluorescence measurement (Figure 7). The light-scattering titration indicated that the binding was strong (little or no free S1 present throughout the titration) with a 1:1 stoichiometry, within experimental error. By comparing the light-scattering titration with the fluorescence titrations, it can be seen that the pyrene monomer fluorescence change was completed much before the actin filament was saturated with S1; i.e., the fluorescence change was almost half complete when about one S1 per seven actin subunits was bound. The similar results obtained with the singly labeled TM system verified that this monomer fluorescence change was independent of the presence of excimer and also independent of the degree of labeling. The latter observation indicates that there is no preferential binding of S1 to pyrene-TM or unlabeled TM or preferential interaction of the bound S1 with the pyrene label at Cys-190 or

with a subpopulation of probes, e.g., with Cys-36 of the β chain on $\alpha\beta$-TM ($\sim$13% of total SH's). To ensure that the fluorescence increase is not due to S1 inducing the binding of pyrene-TM, a control study was made in which the F-actin–tropomysin was pelleted in the absence and presence of S1 ([S1]/[actin] = 2/7) and the fluorescence in the sodium dodecyl sulfate (SDS)-solubilized pellet and supernatant was compared. It was found that 85% ± 5% of the fluorescence was in the pellet in both cases.

Theoretical curves were generated for the cases where the random binding of one or more and two or more S1's to an actin–TM unit (consisting of one TM and seven actin subunits) was necessary to produce the fluorescence change. It can be seen that the experimental curve lies between the one or more and two or more theoretical random-binding curves. Thus, it appears that the random binding of between one and two myosin heads to an actin–TM unit can affect the state of the entire TM molecule.

(C) Temperature Dependence of the Fluorescence of S1–Pyrene-TM–F-Actin Complexes. The temperature dependence of the fluorescence of the S1–pyrene-TM–F-actin system was studied after equilibrium was reached, by cycling the system between 5 and 35 °C until no further change took place. The initial excimer was greatly reduced by the presence of bound S1 at an [S1]/[actin] ratio of 1/7 and decreased somewhat further at the higher [S1]/[actin] ratio of 1 (Figure 5). In both cases, however, there was no further change in excimer with temperature to 40 °C.

Discussion

Properties of Pyrene-TM. These data show that the pyrene label attached to Cys-190 affects the conformation of TM in two ways. It causes a 10% loss of helix at low temperatures below any thermal transition. The label also increases the magnitude of the localized unfolding (Woods, 1976; Lehrer, 1978) and decreases its pretransition temperature. At low salt, where TM is much less stable (Woods, 1969) and where it unfolds without exhibiting a pretransition (Betteridge & Lehrer, 1983), the pyrene label also causes a 10% initial helix loss without the introduction of a pretransition (P. Graceffa, Y. Ishii, and S. S. Lehrer, unpublished results). These studies and the previous studies of the consequences of modification at Cys-190 (Lehrer, 1975; Betteridge & Lehrer, 1983) suggest that this region of the molecule is particularly sensitive to perturbation.

Previous studies of pyrene-TM associated the increase and subsequent decrease of the excimer fluorescence with temperature with the unfolding pretransition and main transition, respectively, of TM (Graceffa & Lehrer, 1980). The studies reported here confirm this interpretation by showing that the increase in excimer fluorescence occurred over the same temperature range as the helix unfolding pretransition of pyrene-TM, about 5 °C lower than the helix pretransition of unlabeled TM. The decrease in excimer fluorescence associated with the main transition (the transition temperature of which was not appreciably affected by the probe) appears to be due to complete chain dissociation (Pont & Woods, 1971; Holtzer et al., 1983). Although the excimer is an excited-state species, evidence has been presented that indicates that excimers form rapidly upon excitation of pyrenes that interact hydrophobically in the ground state (Betcher-Lange & Lehrer, 1978; Graceffa & Lehrer, 1980). Model building of the coiled-coil α-helix of TM has indicated that the neighboring pyrenes on each chain can interact in the ground or excited state only if there is some localized chain separation or unfolding (Lehrer et al., 1981); i.e., excimer fluorescence would not be observed in the *unperturbed* chain-closed state (N state) since the pyrenes can not sterically interact around the outside of the double helix. Studies that we have performed at pH 2, where the molecule is much more stable (Woods, 1977), showed the initial excimer was absent, in agreement with these concepts. The observation of appreciable initial excimer fluorescence at neutral pH and the associated 10% loss of helix (at temperatures below any transitions) therefore shows that the N state of the unlabeled TM is somewhat perturbed by the pyrenes, leading to some localized unfolding caused by the hydrophobic pyrene labels interacting with the hydrophobic residues in the ridge between the two chains. The increased unfolding in the X state appears to be due to further perturbation by the pyrene labels facilitated by the local chain separation associated with the pretransition. Thus, the region around Cys-190 which is preferentially unstable in unlabeled TM is further destabilized by the pyrene-TM interaction. The resulting localized unfolding is probed by the excimer fluorescence arising from the interaction between the two adjacent pyrenes in doubly labeled TM.

In general, it is difficult to determine whether multiple probe configurations are related to multiple protein conformations since the probe can equilibrate between different environments presented by the protein surface if the probe has sufficient degrees of freedom to sample the different environments. In previous studies of TM labeled with different probes at the same site (Cys-190), multiple probe configurations were detected; a dansyl probe gave two fluorescence lifetimes (Betteridge & Lehrer, 1983), and a nitroxide spin-label showed two spectral components (Graceffa & Lehrer, 1984). In both cases, the temperature dependence of the relative contribution of the two components correlated with the conformational pretransition, showing that the relative contributions of the two protein conformations, N and X, were being probed. The dansyl probe perturbed the TM conformation less than the pyrene probe probably because it was less hydrophobic. When a more polar fluorescence probe was used [*N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine (AEDANS)], only one lifetime was detected (Tao et al., 1983), and no other component appeared at temperatures in the pretransition (T. Tao and S. S. Lehrer, unpublished results). These results indicate that the more hydrophobic the probe, the better it can interact with the hydrophobic region of TM exposed in the X state, and the greater is the resulting perturbation.

In view of these new observations, a more general schematic model than the one originally proposed (Graceffa & Lehrer, 1980) is now presented which allows the probes to equilibrate between two configurations; stacked (resulting in excimer, E) and unstacked (resulting in monomer, M) in each protein state, chain closed (N) and chain open (X) (Figure 8). Due to the perturbation by the pyrene moieties, the stacked configuration of the pyrenes is indicated to have less helical content than the corresponding unstacked configuration. It is difficult to obtain the distribution of the various species as a function of temperature because of the heterogeneity of the monomer fluorescence (discussed above) and the lack of knowledge of the respective quantum yields.

The pyrene label also weakens the binding of TM to F-actin. By studying the binding of mixtures of pyrene-TM and unlabeled TM to F-actin, it was shown that the decreased binding of the pyrene-TM was consistent with a decreased end to end interaction parameter, w, without much change in the intrinsic binding constant, K (McGhee & von Hippel, 1974). This observation, that a pyrene label at Cys-190 affects the end to end interactions of TM on the actin filament, correlates with

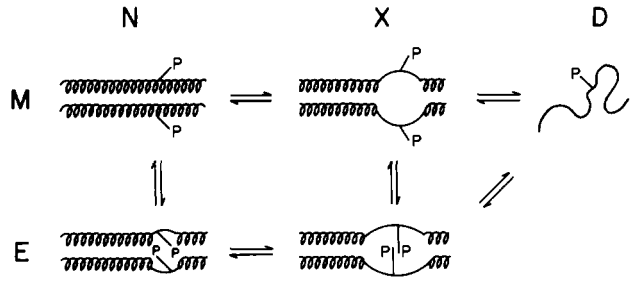

FIGURE 8: Schematic model which assumes two probe configurations, M and E, in the principal TM conformations on the unfolding pathway N = X = D. N is the native, chain-closed, fully helical state observed at low temperatures; X is the chain-open, partially unfolded intermediate; D is the fully unfolded state. Excimer fluorescence, E, originates from a stacked configuration of adjacent pyrenes; monomer fluorescence, M, from an unstacked configuration. The results indicate that although excimers form more readily in the chain-open state, X, the stacked configuration perturbs both the N and X states, decreasing the helix content and resulting in some excimer fluorescence in the N state. Binding of pyrene-TM to F-actin shifts the N = X conformational equilibrium to the N state without changing the distribution between the two probe configurations. Binding of S1 to the pyrene-TM–F-actin complex further stabilizes pyrene-TM by decreasing the contribution of the E probe configuration in the N state.

the early observation that the end to end polymerization of pyrene-TM in solution is greatly inhibited (Graceffa & Lehrer, 1980). It appears that the weakened binding is related to the initial 10% loss of helix associated with the perturbed N state rather than the pyrene-induced increase in localized unfolding associated with the X state since there is no pretransition when TM is bound to F-actin but there is appreciable initial excimer fluorescence (see below).

Although the details of the relationship between the 10% loss of helix due to the pyrene–pyrene interaction at Cys-190 and the way the end to end molecular interactions are affected in solution and on the actin filament are not known, it appears that perturbations can be transmitted over large distances in coiled-coil molecules (for TM, Cys-190 is located about 130 Å from the carboxyl end and 270 Å from the amino end). In agreement with this general concept, it has recently been shown that the introduction of disulfide bonds between Cys in TM (Ueno, 1984) and myosin (Lu & Lehrer, 1984) has resulted in changes in trypsin cleavage points located at considerable distances away from the disulfide bonds. We have previously suggested that the end to end interactions of TM on the actin filament could be modified by troponin perturbation of TM near Cys-190 (Lehrer et al., 1981) in view of our earlier results (Graceffa & Lehrer, 1980) and the knowledge that the bulk of the troponin complex binds near Cys-190 (McLachlan & Stewart, 1977; Stewart, 1975; Chong & Hodges, 1982; Tao et al., 1983; Morris & Lehrer, 1984). The results presented here further reinforce this model. Although an alternate model, involving a direct effect of troponin in the end to end TM overlap region, has been presented by Pearlstone & Smillie (1982), more recent results from their laboratory have provided evidence for indirect effects (Pearlstone & Smillie, 1983).

Effects of F-Actin and S1 on the Conformation of Tropomyosin. The excimer fluorescence changes produced by the binding of pyrene-TM to F-actin at room temperature can be explained by a shift in the N = X equilibrium to the N state; i.e., actin stabilizes TM. This is clearly shown by the lack of excimer change over the complete temperature range, indicating the lack of an N → X pretransition for pyrene-TM bound to F-actin. Appreciable pyrene–pyrene interaction is maintained in the complex along with the accompanying localized distortion discussed above since only about a 10–20% decrease in initial excimer fluorescence resulted from the binding of pyrene-TM to F-actin. Thus, although F-actin stabilizes the structure of TM, the stabilization is not sufficient to overcome the pyrene–pyrene-induced destabilization in the N state.

The excimer fluorescence changes produced by S1 binding to the pyrene-TM–F-actin complex indicate a further stabilizing conformational change of Tm since the value of the initial excimer intensity was markedly reduced. Thus, the pyrene–pyrene interaction in the N state which was not appreciably changed by F-actin was greatly reduced by the subsequent binding of S1; i.e., there is a shift in *probe* configuration in the N state from E to M (see Figure 8). The binding of low ratios of S1/actin was sufficient to produce most of the excimer reduction, indicating long-range stabilization of the TM structure.

These studies have provided information about the conformation of TM in the thin filament. This information was obtained by first ascertaining the correspondence between the thermally induced conformation changes and the fluorescence changes for unlabeled TM and pyrene-TM alone in solution and also by defining the perturbation that the probe produced. It is interesting to note that in the absence of the perturbation no effect of S1 binding would be observed since the initial excimer would be zero and would not vary with temperature either in the absence or in the presence of S1. Thus, this work does show that even though spectroscopic probes inevitably perturb conformation (Weber, 1972), a study of the resulting perturbation as well as the spectroscopic properties of the probe can yield useful conformational information.

Effects of S1 on the State of TM on the F-Actin Filament. In these studies, the monomer fluorescence of pyrene-TM bound to F-actin was monitored by using either singly labeled TM for which there is no excimer or doubly labeled TM under conditions where the excimer fluorescence change was too slow to contribute. It should be noted that the lack of change of monomer fluorescence when singly labeled TM binds to F-actin indicates that the environment around the pyrene label has not changed in the complex. In contrast, binding of small ratios of S1 produced a 20% increase in monomer fluorescence. Studies that we have performed with pyrene model compounds having spectra identical with those of pyrene-TM indicated that small intensity increases without spectral change can occur as a result of small decreases in the solvent polarity. Decreased accessibility to solvent caused by an increased hydrophobic interaction of pyrene on TM with a protein subunit surface would explain an increase in fluorescence. It was not possible to unambiguously decide if the environmental change is the result of pyrene interacting with an actin surface in the presence of S1, which it did not interact with in the absence of S1 since the spectral change could also be the result of a changed pyrene interaction with TM. In any case, this appears to be the first report of a specific TM probe which is sensitive to the binding of low S1/actin ratios. Previous studies under weaker S1 binding conditions showed that a fluorescence probe on troponin was also sensitive to the binding of low ratios of S1–ADP to the complete thin filament (Trybus & Taylor, 1980).

Comparison of the experimental data with theoretical curves indicates that the monomer fluorescence change can be explained by the random binding of between one and two S1's per actin–TM unit. Thus, these data provide evidence for one of the two cooperative effects of myosin head binding—that the binding of a small fraction of myosin heads can affect the state of the entire TM molecule (Hill et al., 1980, 1983). To obtain information about the other effect, the cooperativity

between TM molecules, weaker S1 binding conditions would be required. The data indicate that the fluorescence change requires about 1.5 S1's per actin–TM unit rather than 1.0 S1 per actin–TM unit expected for a rigid TM; i.e., the binding of 1 S1 appears to affect 5–6 actin subunits rather than 7. It is possible that this indicates that TM exhibits some flexibility when bound to actin although in view of the excimer results discussed above, the flexibility of TM on actin is less than that when alone in solution. Further studies will be required to clarify this and to distinguish between the two cooperative S1 binding models of Hill et al. (1980, 1983).

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