Smith, T. F., & Waterman, M. S. (1981) *Adv. Appl. Math. 2*, 482-489.
Stephens, R. E. (1975) *Anal. Biochem. 65*, 369-379.
Titani, K., Sasagawa, T., Ericsson, L. H., Kumar, S., Smith, S. B., Krebs, E. G., & Walsh, K. A. (1984) *Biochemistry 23*, 4193-4199.
von Heijne, G. (1986) *EMBO J.* 1335-1342.
Wilbur, W. J., & Lipman, D. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 726-730.
Yeaman, S. J. (1989) *Biochem. J. 257*, 625-632.
Zhang, B., Kuntz, M. J., Goodwin, G. W., Harris, R. A., & Crabb, D. W. (1987) *J. Biol. Chem. 262*, 15220-15224.
Zhang, B., Crabb, D. W., & Harris, R. A. (1988) *Gene 69*, 159-164.

Excimer Fluorescence of Pyrenyliodoacetamide-Labeled Tropomyosin: A Probe of the State of Tropomyosin in Reconstituted Muscle Thin Filaments†

Yoshiharu Ishii and Sherwin S. Lehrer*

Department of Muscle Research, Boston Biomedical Research Institute, Boston, Massachusetts 02114, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Received July 18, 1989; Revised Manuscript Received October 2, 1989

ABSTRACT: Rabbit skeletal tropomyosin (Tm) specifically labeled at cysteine groups with *N*-(1-pyrenyl)-iodoacetamide (PIA) exhibits excimer fluorescence. The excimer fluorescence was sensitive to the local conformation of Tm, to actin binding, and, in reconstituted thin filaments, to the Tm state change induced by binding of myosin subfragment 1 (S1). The properties of PIATm were similar to previously studied pyrenylmaleimide-labeled Tm (PMTm) [Ishii, Y., & Lehrer, S. S. (1985) *Biochemistry 24*, 6631] except that S1 binding to actin–Tm increased the excimer fluorescence in contrast to the time-dependent decrease seen for PMTm. The fluorescence properties of PIATm are sensitive to the Tm chain–chain interaction via equilibria among pyrene configurations and nonfluorescent dimer as well as the monomer and excimer-forming configurations. The effect of bound troponin (Tn) on the excimer fluorescence of PIATm in the reconstituted systems was dependent on ionic strength with a slight Ca^{2+} dependence. S1 titrations in the absence and presence of Tn and Ca^{2+} indicated that the excimer fluorescence probes the state change of Tm from the weak S1 binding state to the strong S1 binding state which is facilitated by Ca^{2+} [Hill et al. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3186]. Binding of MgADP–S1 and MgAMPPNP–S1 produced the same total excimer fluorescence change as for nucleotide–free S1, showing that the strong S1 binding state of Tm–actin is independent of nucleotide. TnT1, the N-terminal fragment of troponin T, did not affect the S1-induced Tm state change profile although it strengthened the binding of Tm to actin due to enhanced end-to-end interaction. Thus, changes in Tm chain–chain interactions are associated with the Tm state change important in regulation.

Tropomyosin (Tm),[1] a two-chain coiled-coil α-helical molecule, is an essential component of Ca^{2+}-dependent thin filament regulation of striated muscle contraction (Leavis & Gergely, 1984). Biochemical studies have shown that reconstituted thin filaments of actin–Tm and actin–troponin (Tn)–Tm equilibrate between two states, a weak myosin binding state or an inhibited state and a strong myosin binding state or an activated state, which results in cooperative myosin subfragment 1 (S1) binding (Greene & Eisenberg, 1980) and cooperative S1 dependence of ATPase activity (Lehrer & Morris, 1982; Nagashima & Asakura, 1982). The state change induced by the S1 binding has been directly monitored with fluorescence probes on Cys-190 of Tm sensitive to environmental changes (Ishii & Lehrer, 1985, 1987; Lehrer & Ishii, 1988) and to changes in the geometrical relationship between Tm and actin (Lehrer & Ishii, 1988).

Previous studies with pyrenylmaleimide-labeled Tm (PMTm) showed excited-state dimer (excimer) fluorescence from pyrenes attached to Cys-190 on each chain (Betcher-Lange & Lehrer, 1978). The excimer fluorescence from PMTm is sensitive to localized conformational changes (Graceffa & Lehrer, 1980), to binding to actin, and to binding of S1 to the PMTm–actin complex (Ishii & Lehrer, 1985). Some problems were noted with the PM probe on Tm. Although the monomer fluorescence could be used to study the S1 binding profile, the excimer fluorescence could not be used due to time-dependent changes, and the PM probe caused an appreciable perturbation of the Tm conformation.

In this study, we used *N*-(1-pyrenyl)iodoacetamide-labeled Tm (PIATm) which contains the pyrene fluorophore covalently

† This work was supported by grants from the National Institutes of Health (HL-22461) and the National Science Foundation (DMB-8417368). A preliminary report has been presented (Ishii & Lehrer, 1989).

* Address correspondence to this author at the Department of Muscle Research, Boston Biomedical Research Institute, 20 Staniford St., Boston, MA 02114.

[1] Abbreviations: Tm, tropomyosin; S1, myosin subfragment 1; Tn troponin; TnT1, N-terminal half of the chymotryptic fragment of the troponin T component; PIATm, Tm labeled with *N*-(1-pyrenyl)iodoacetamide; PMTm, Tm labeled with *N*-(1-pyrenyl)maleimide; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; GuHCl, guanidine hydrochloride; DTT, dithiothreitol; AMPPNP, 5′-adenylyl imidodiphosphate.

0006-2960/90/0429-1160$02.50/0

attached to Cys groups via an acetamido linkage rather than maleimido. Although the properties of PIATm were found to be very similar to PMTm, the PIA probe did not perturb the conformation as much as the PM probe and did not exhibit the time-dependent changes in excimer fluorescence observed for PMTm. In addition, a large change in the excimer fluorescence intensity of PIATm was observed when S1 was bound to the PIATm–actin complex. Previous studies with PMTm and PM-labeled dithiothreitol (Lehrer & Ishii, 1989) have indicated that, in contrast to organic solvents where there is no ground-state interaction (Birks, 1970), in aqueous solutions the intramolecular excimer forms rapidly from pyrenes that interact hydrophobically in the ground state. For PIATm, in contrast to PMTm, the contribution of a class of interacting pyrenes that are nonfluorescent needs to be considered.

With PIATm, it is shown that the excimer fluorescence directly monitors the S1-induced cooperative change in state of Tm in Tm–actin and Tm–Tn–actin reconstituted thin filament complexes in both the presence and the absence of Ca^{2+}. Effects on the cooperative parameters due to Tn and Ca^{2+}, due to different nucleotides and due to TnT1, the fragment of troponin T which enhances the end-to-end interaction of Tm (Jackson et al., 1975; Brisson et al., 1986), have been obtained.

Experimental Procedures

All proteins used in this study were prepared from rabbit skeletal muscle. Actin was prepared by the method of Spudich and Watt (1971), myosin by that of Balint et al. (1975), and myosin S1 by chymotryptic cleavage as described by Weeds and Pope (1977). Tn was purified by the methods of Ebashi et al. (1971) and van-Eerd and Kawasaki (1973), and TnT1, the chymotryptic fragment of troponin T component, was purified as described in Morris and Lehrer (1984). Tm was prepared and reduced with dithiothreitol (DTT) as described previously (Lehrer & Morris, 1982). Tm at ~2 mg/mL in 5 M GuHCl and 10 mM Hepes (pH 7.0) was labeled with a 5× molar excess of *N*-(1-pyrenyl)iodoacetamide (PIA) (Molecular Probe) dissolved in dimethylformamide for 4 h at room temperature. The reaction was quenched with excess dithiothreitol (DTT). The sample was filtered to remove some undissolved PIA, dialyzed vs 5 M GuHCl and 1 mM DTT at pH 7.0 to remove unreacted PIA and renatured by dialysis vs 0.5 M NaCl/10 mM Hepes (pH 7.0) followed by exhaustive dialysis vs 2 mM Hepes and 1 mM EDTA. The concentration of PIATm was determined by the BCA–protein assay (Pierce) using unlabeled Tm as a standard, and the concentration of PIA bound to Tm was determined by using $\epsilon_{344} = 2.2 \times 10^4$ (Kouyama & Mihashi, 1981). The labeling ratio of pyrene to Tm was 1.8. Skeletal Tm consists of a mixture of $\alpha\alpha$ and $\alpha\beta$ molecules where α contains a single Cys at residue 190 and β contains Cys at residues 190 and 36. The excimer fluorescence only can originate from pyrenes at Cys-190. In agreement with this, similar results were obtained with labeled cardiac Tm which is $\alpha\alpha$.

Steady-state fluorescence measurements were obtained with a Spex Fluorolog 2/2/2 photon-counting fluorometer (Edison, NJ) in the ratio mode with a 2.25-nm band-pass for both excitation and emission with sample in the thermostated housing. S1 titrations were carried out by programming the Datamate computer to cycle the emission monochromator wavelengths to monitor the PIA excimer fluorescence (at 485 nm) and the light scattering (at 350 nm) obtained in the same cuvette after each addition of S1 (Ishii & Lehrer, 1987). Before titration, the sample was clarified by centrifugation at low speed for 10 min. For S1 titrations in the presence of ADP, a small amount of contaminating ATP was converted to ADP by incubating the solution with a small amount of S1 for 1 h. The fluorescence and light-scattering titrations were fitted to the equations of Hill et al. (1980) using a nonlinear least-squares computer-fitting program written by Dr. Edward P. Morris. The binding of S1 (light scattering), θ, and the fraction of Tm in state 2 (fluorescence change), P_2, are expressed as a function of concentration of free S1, c, as

$$\theta = (1 - P_2)K_1c/(1 + K_1c) + P_2K_2/(1 + K_2c) \quad (1)$$

$$P_2 = 2aY^{-1}/[\nu(1 - a + \nu)] \quad (2)$$

$$\nu \equiv [(1 - a)^2 + 4aY^{-1}]^{1/2} \quad (3)$$

$$a \equiv (1 + K_2c)^7/[L'(1 + K_1c)^7] \quad (4)$$

L' is the equilibrium constant between the two actin–Tm states, state 1 (weak S1 binding state) and state 2 (strong S1 binding state), Y is the end-to-end cooperativity parameter, and K_1 and K_2 are the binding constants of S1 to states 1 and 2, respectively.

The binding of PIATm to actin was measured by sedimentation and fluorescence measurements. The sedimentation experiments were carried out in a Beckman airfuge, and the concentration of PIATm in the supernatant was determined by the monomer fluorescence in the presence of SDS.

Fluorescence lifetime measurements were made on a modified Ortec 9200 nanosecond fluorometer. Excitation was selected with a 340 interference filter and emission with a 380 interference filter for monomer and with KV 470 filter for excimer. The circular dichroism data were obtained with an Aviv Associates 60 DS instrument with a Hewlett Packard thermoelectric temperature controller as previously described (Lehrer & Ishii, 1988).

Results

Properties of PIATm. The fluorescence spectrum of PIATm is composed of a broad excimer band centered at 485 nm as well as a structured monomer band with peaks at 385 and 405 nm (Figure 1A). The excitation spectrum of the excimer fluorescence was broadened compared to the monomer fluorescence, indicating pyrene–pyrene ground-state interaction. The fluorescence decay showed the presence of multiple lifetimes for both monomer and excimer with longer excimer lifetimes than the monomer (Figure 1B). The time $t_{1/e}$ when the fluorescence intensity decreased to $1/e$ was 4.0 and 38.7 ns for monomer and excimer, respectively, in agreement with previous results; i.e., $t_{1/e}$ was estimated to be 4.5 and 39.2 ns for monomer and excimer, respectively, using the data of Lin (1982). There was little time lag between excimer and monomer peaks (<2–3 ns), indicating a rapid reorientation to the excimer configuration during the monomer excited-state lifetime. These fluorescence properties are similar to those of PMTm in which the pyrene succinimido ring attached to Cys is cleaved by alkaline hydrolysis (Graceffa & Lehrer, 1980; Ishii & Lehrer, 1986). The perturbation by the probe on the conformation of Tm was minor and much less than for PMTm (Ishii & Lehrer, 1985a) as seen by a comparison of the thermal helix unfolding profile of PIATm with unlabeled Tm (Figure 2). The main effect of the probe was a small increase in the magnitude of a helix unfolding pretransition in the range of 25–40 °C.

The excimer fluorescence increased in the pretransition due to local unfolding in the region of Cys-190 and then decreased in the main transition due to further chain separation and subunit dissociation (Figure 2). The excimer fluorescence intensity increase was associated with an excitation spectrum

Table I: Effects of Nucleotide, Ionic Strength, and Tn Components on Parameters Obtained from S1 Titrations of PIATm–Actin

system (actin +)	[NaCl][a] (mM)	nucleotide	L'^b	Y^b	$K_1{}^b$ (M^{-1})	$K_2{}^b$ (M^{-1})	$\Delta E/E_0{}^b$ (%)
Tm	30	–	16	6	1×10^4	1×10^8	128
Tm	30	AMPPNP	16	6	1×10^4	1.2×10^6	115
Tm	100	ADP	8	2	1×10^4	5×10^6	120
Tm + TnT1	30	–	16	10	1×10^4	1×10^8	116
Tm + Tn ($+Ca^{2+}$)	30	–	18	10	1×10^4	1×10^8	38

[a] Other solvent conditions are 5 mM $MgCl_2$/20 mM Hepes (pH 7.5) at 25 °C. [b] L', state 1/state 2 equilibrium constant; Y, end-to-end cooperative parameter; K_1, binding constant of S1 to state 1; K_2, binding constant of S1 to state 2 (Hill et al., 1980); $\Delta E/E_0$, total fluorescence change. The same values are obtained by fitting both excimer fluorescence and light scattering.

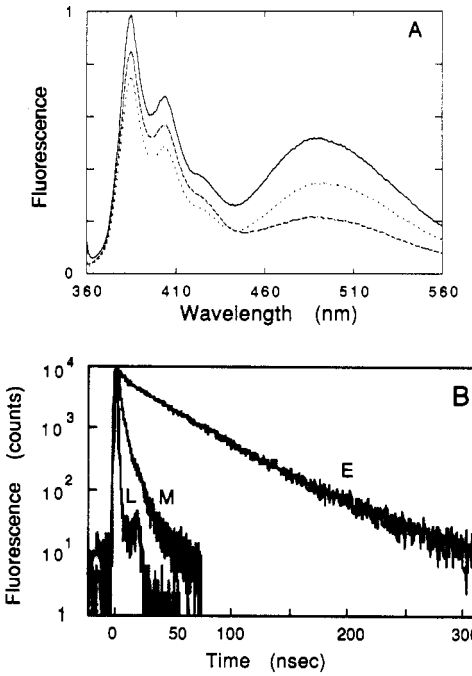

FIGURE 1: (A) Fluorescence emission spectra of PIATm (···), PIATm–actin (- - -), and PIATm–actin–S1 (—). Excitation wavelength was 340 nm. Solvent background was subtracted, and the spectrum was corrected for the variation in sensitivity. (B) Effects of the binding of S1 to PIATm–actin on the fluorescence decay of the excimer (E) and monomer (M). L is the lamp pulse. The curves in the absence and presence of S1 are identical for both excimer and monomer. Conditions: 0.70 μM PIATm, 5.0 μM actin, and 5.5 μM S1 in 20 mM Hepes (pH 7.5), 5 mM $MgCl_2$, and 30 mM NaCl at 25 °C.

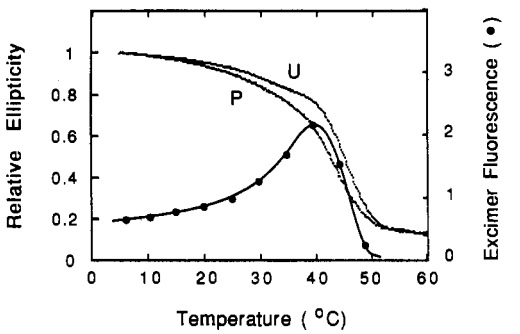

FIGURE 2: Comparison of the helix unfolding of PIATm (P) and unlabeled Tm (U) with the temperature dependence of the excimer fluorescence of PIATm (●). The ellipticity was monitored at 222 nm. The excimer fluorescence was monitored at 485 nm with the excitation wavelength at 340 nm. Concentrations of PIATm and unlabeled Tm were 0.75 and 0.77 μM, respectively, for CD and 0.65 μM for fluorescence. The solvent conditions for both measurements were 0.5 M NaCl, 20 mM Hepes (pH 7.5), 1 mM EDTA, and 1 mM DTT.

shape change; the shoulder at 333 nm relative to the peak intensity at 343 nm increased from 0.8 at 25 °C to 0.9 at 40 °C, indicating a change in the ground-state environment of excimer-forming pyrenes as Tm locally unfolds. The temperature of maximum excimer fluorescence was 40 °C, compared to 30 °C for PMTm, indicating that the PIA probe did not perturb the Tm conformation as much as the PM probe.

When PIATm was bound to actin, the excimer fluorescence decreased, and the monomer fluorescence slightly increased (Figure 1). In the complex, the excimer fluorescence did not increase with temperature up to 40 °C, indicating that actin suppressed the unfolding pretransition of Tm as previously noted with PMTm (Ishii & Lehrer, 1985). The excimer fluorescence increased above 40 °C toward the values of PIATm alone, because PIATm starts to dissociate from actin. The dissociation temperature of ~44 °C for PIATm was higher than that for PMTm (~33 °C) in further agreement with a decreased conformational perturbation.

Effects of Myosin S1 Binding to PIATm–Actin. S1 binding to the actin–PIATm complex caused a 2.2× increase in the excimer fluorescence and a slight increase in monomer fluorescence (Figure 1A). Despite these changes in steady-state fluorescence, the fluorescence decay profiles of the excimer and monomer fluorescence did not change (Figure 1B). This indicates that the S1-induced increase in the excimer fluorescence is due to a greater fraction of pyrenes contributing to excimer fluorescence rather than a change in the excited-state properties of the excimer. The effects of S1 were reversed by the addition of ATP, in agreement with the known ATP-induced S1 dissociation, and recovered after all ATP was hydrolyzed by the acto–S1 ATPase.

The excimer fluorescence and light-scattering intensity were measured during titrations with S1 in the absence and presence of the nucleotides, MgADP and MgAMPPNP. The light scattering is a measure of the binding of S1 to actin–PIATm. The profile of the excimer fluorescence change with S1 binding was similar to the fluorescence profiles monitored by other probes at the same site interpreted as an S1-induced change in state of Tm on actin (Ishii & Lehrer, 1985; Lehrer & Ishii, 1988). Whether or not nucleotide was present, the excimer fluorescence change was half-complete when an average of one S1 was bound to seven actin subunits, and was almost complete when three S1's were bound (Figure 3). The data were fit to the Hill et al. cooperative S1 binding model (1980), and reasonable agreement was obtained as seen by the fit of the theoretical curves to the experimental data. The parameters obtained from the fits are listed in Table I. L' and Y were found to be independent of nucleotide under the same ionic conditions, even though binding affinities of S1 (K_2) are different (compare the data in the absence of nucleotide and presence of MgAMPPNP). The smaller values of L' and Y obtained in the presence of MgADP were explained by the salt concentration dependence of these parameters (Greene, 1982); e.g., the excimer fluorescence profile in the presence of MgADP was the same as in the absence of MgADP at 0.03 M NaCl and 5 mM $MgCl_2$ where the S1 binding was so strong that the binding profiles were indistinguishable in the presence or absence of MgADP. K_2 was found to be nucleotide-de-

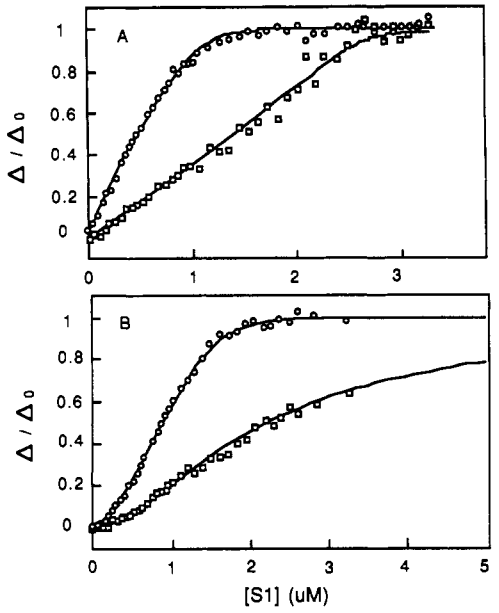

FIGURE 3: Effects of the binding of myosin S1 to PIATm–actin on excimer fluorescence (O) and light scattering (□). (A) No nucleotide present, 5 mM $MgCl_2$ and 30 mM NaCl. (B) 4 mM AMPPNP, 7 mM $MgCl_2$, and 30 mM NaCl. Other conditions: [PIATm] = 0.41 μM, [actin] = 2.88 μM, 20 mM Hepes (pH 7.5) at 25 °C. Theoretical curves are drawn with the parameters listed in Table I. The total light-scattering change was ~10×.

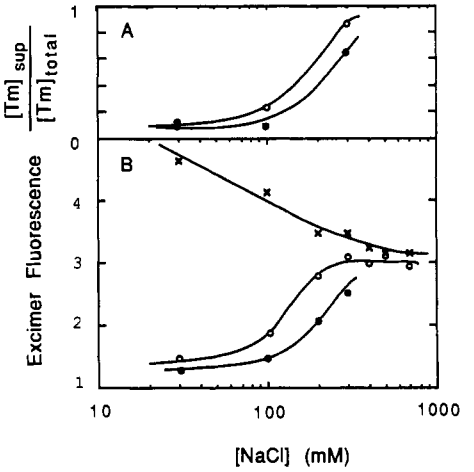

FIGURE 4: Effects of TnT1 on the binding of PIATm to actin. (A) Salt dependence of the fraction of free PIATm determined by sedimentation. (B) Salt dependence of the binding of PIATm to actin determined by excimer fluorescence. The experiment was done in the absence (O) and presence (●) of 0.55 μM TnT1. [PIATm] = 0.5 μM and [actin] = 3.5 μM. (×) is the excimer fluorescence of PIATm alone as a control. Solvent conditions: 20 mM Hepes (pH 7.5)/5 mM $MgCl_2$ and 25 °C.

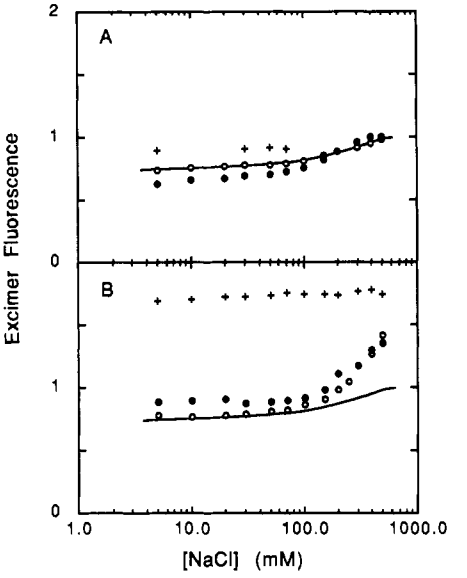

FIGURE 5: Salt dependence of the excimer fluorescence of PIATm–Tn–actin in the absence (A) and presence (B) of S1. The fluorescence was corrected for dissociation of Tm–Tn from actin. (+) PIATm–actin; (●) PIATm–Tn–actin in the presence of 0.1 mM Ca^{2+}; (O) PIATm–Tn–actin in the presence of 0.1 mM EGTA. The line in (B) is the same as the line in (A) for PIATm–actin–Tn in the absence of Ca^{2+} to more easily see S1 effects in the absence of Ca^{2+}. Conditions: [PIATm] = 0.6 μM, [actin] = 4.2 μM, [Tn] = 1.0 μm, and [S1] = 4.2 μM in 20 mM Hepes (pH 7.5) and 5 mM $MgCl_2$ at 25 °C.

pendent as expected. To assess the significance of these parameters, the range of acceptable parameters was estimated from χ^2, the sum of squared differences between calculated and experimental values, around the minimum, χ^2_0, when one parameter varied from the best fit with others held constant. For example, in the absence of nucleotide, $L' = 15$–18, $Y = 4$–10, $K_1 < 1 \times 10^6\ M^{-1}$, and $K_2 > 5 \times 10^7\ M^{-1}$ could be used to fit the data to give similar deviations of χ^2 from χ^2_0.

The total fluorescence change was independent of the presence and type of nucleotide within experimental error, indicating that the resulting state of Tm is independent of these nucleotides. This result was confirmed by direct comparison; i.e., the excimer fluorescence did not change upon the addition of MgADP or MgAMPPNP in the presence of excess S1.

Control experiments showed that the label did not affect the S1 binding, state change of Tm, and ATPase activity. S1 binding was the same for PIATm–actin or unlabeled Tm–actin in the presence of MgADP, S1-induced excimer fluorescence changes were similar in mixtures of different ratios of PIATm and unlabeled Tm (1:0 and 1:3), and PIATm inhibited acto-S1 ATPase to a similar extent as unlabeled Tm.

Effects of TnT1. TnT1, which is known to enhance the end-to-end polymerization of Tm, does not appear to bind close to Cys groups, because the environment of the probes attached to Cys groups did not change (Morris & Lehrer, 1984; Ishii & Lehrer, 1986b). TnT1 was, therefore, used to study the relationship of end-to-end interactions of Tm to the strength of binding to actin and to the S1-induced Tm-state change.

The strength of the interaction between Tm and actin was measured by determining the salt concentration at which Tm dissociates from actin. It was found that in the presence of TnT1, a 0.1 M greater concentration of salt was required to dissociate Tm from actin, indicating that TnT1 increased the binding strength of Tm to actin (Figure 4), in agreement with previous results (Morris & Lehrer, 1984). A small decrease (15%) of the excimer fluorescence of PIATm–actin caused by TnT1 at low salt (0.03 M NaCl) appears to be due to a small conformational change of PIATm transmitted to Cys-190 induced by TnT1 binding (Tishii & Lehrer, 1986b).

The S1-induced final fluorescence and the state change were not affected by the presence of TnT1 (Table I).

Effects of Tn on PIATm–Actin. The binding of Tn to PIATm–actin in low-salt solutions (<0.1 M NaCl in the presence of 5 mM $MgCl_2$) increased the monomer fluorescence (74% and 66% in the presence and absence of Ca^{2+}, respectively) and decreased the excimer fluorescence (19% and 8%, respectively). The excimer fluorescence of the reconstituted thin filament was thus slightly Ca^{2+} concentration dependent. When the NaCl concentration was increased above 0.1 M NaCl, the excimer fluorescence of PIATm–Tn–Actin increased toward the value of PIATm alone independent of the presence

Table II: Effects on Ca^{2+} of Parameters Obtained from S1 Titrations of Reconstituted Thin Filament, PIATm–Tn–Actin

$[Ca^{2+}]^a$	nucleotide	L'	Y	K_1 (M^{-1})	K_2 (M^{-1})	$\Delta E/E_0$ (%)
+	–	4	10	1×10^4	1×10^8	28
+	ADP	4	10	1×10^4	2.5×10^5	29
–	–	40	5	1×10^4	1×10^8	27
–	ADP	40	5	1×10^4	2.5×10^5	31

[a] Experimental conditions are 0.25 M NaCl, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.5), 0.1 mM $CaCl_2$, or 0.1 mM EGTA at 25 °C.

or absence of Ca^{2+} (Figure 5A). These data were corrected for dissociation of Tm–Tn from actin which took place in 0.4–0.5 M NaCl (e.g., 20% and 50% PIATm were dissociated from actin in 0.4 and 0.5 M NaCl in the presence of Ca^{2+}, respectively). The excimer fluorescence increase with salt was associated with a monomer fluorescence decrease.

In the presence of bound S1, the binding of Tn to PIATm–actin in low-salt solutions decreased the excimer fluorescence (42% and 48% in the presence and absence of Ca^{2+}, respectively), and as in the absence of S1, the excimer fluorescence increased with salt concentration above 0.1 M NaCl toward the value of PIATm–actin–S1 which was independent of salt concentration (Figure 5B).

Effects of S1 Binding to PIATm–Tn–Actin. The excimer fluorescence change induced by S1 binding to PIATm–actin–Tn was small (~30%) in the presence of Ca^{2+} in low-salt solutions. The profile of the S1-induced state change of PIATm–actin–Tn in the presence of Ca^{2+} was similar to that of PIATm–actin (Table I). In the absence of Ca^{2+} in low-salt solutions, the excimer fluorescence change induced by the S1 binding was too small to compare with the presence of Ca^{2+}. Effects of Ca^{2+} on S1 titrations, however, could be observed at high salt (0.25 M NaCl) (Figure 6). In the absence of nucleotide, where the S1 binding is strong and stoichiometric both in the presence and in the absence of Ca^{2+}, the fluorescence change could distinguish effects of Ca^{2+}; i.e., more S1 binding was required to switch the Tm state in the absence of Ca^{2+}. In the presence of MgADP, where the S1 binding was weakened and the light-scattering change therefore appeared sigmoidal, the profiles of both the binding and the fluorescence change were Ca^{2+} concentration dependent. Whether or not MgADP was present, however, the fluorescence change was half-complete when ~0.5 S1 and ~1.5 S1 were bound to 7 actin subunits, in the presence and absence of Ca^{2+}, respectively. These data were fitted to the Hill et al. model and gave L' and Y values that are Ca^{2+} concentration dependent and a K_2 value which is nucleotide concentration dependent (Table II). The ratio of L' in the absence to presence of Ca^{2+} was 40/4 = 10, in reasonable agreement with the value obtained in a previous study with PMTm, 8.3 (Ishii & Lehrer, 1987), and the values obtained from the S1 binding studies, 6–16 (Greene et al., 1982).

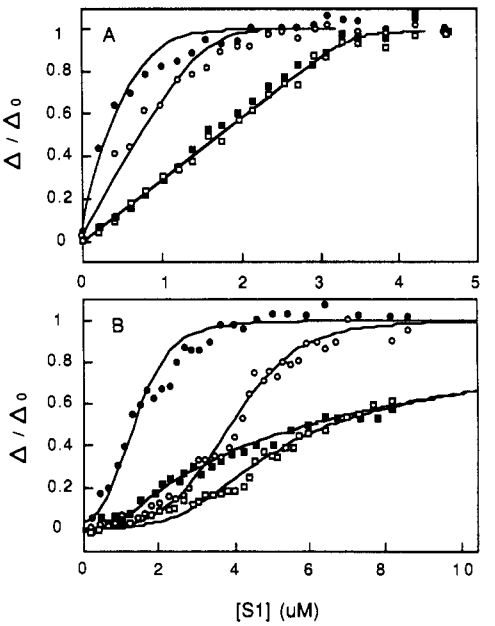

FIGURE 6: Effects of the binding of S1 to PIATm–Tn–actin on excimer fluorescence. The excimer fluorescence (○, ●) and light scattering (□, ■) were measured in the absence (A) and presence (B) of 2 mM ADP. Theoretical curves are drawn by using the parameters listed in Table II. [PIATm] = 0.5 μM, [Tn] = 0.55 μM, and [actin] = 3.5 μM in 0.25 M NaCl, 5 mM $MgCl_2$, 20 mM Hepes (pH 7.5), and 0.1 mM $CaCl_2$ (●, ■) or 0.1 mM EGTA (○, □).

Discussion

Fluorescence Properties of PIATm. The properties of excimer fluorescence of PIATm in solutions and the effects of the binding to actin are similar to those obtained with PMTm (Graceffa & Lehrer, 1980; Ishii & Lehrer, 1985) which were interpreted by a model in which the probes equilibrate between two sets of ground-state configurations, interacting pyrene–pyrene configurations leading rapidly to excimer and noninteracting configurations resulting in monomer. With this model, when excimer fluorescence decreases due to temperature or actin binding, monomer fluorescence increases and vice versa.

The data obtained for S1 binding to actin–PIATm, however, indicate that nonfluorescent probe configurations should also be considered. The lack of change in the fluorescence decay profile with S1 binding despite the large intensity change indicates changes in ground-state pyrene–pyrene interactions. However, two sets of ground-state configurations cannot explain a fluorescence increase of both excimer and monomer. Nonfluorescent dimer configurations, in which interacting pyrenes are incapable of forming excimer, must be considered to equilibrate with monomer and excimer configurations. It is reasonable that pyrenes will interact hydrophobically in the ground state in a variety of configurations in these aqueous solutions and that certain configurations may result in static quenching analogous to quenching in concentrated solutions. Further evidence for nonfluorescent dimer configurations was obtained for PIATm under certain conditions: (1) When the pH was lowered from neutral pH (>6.5) to 5.7 at low temperature, which did not change the helix content, the excimer fluorescence increased 50% without a monomer fluorescence change, suggesting a shift from nonfluorescent dimer configurations to excimer configurations. (2) On introduction of low concentrations of GuHCl (1–1.5 M) at low temperature, which did not cause helix unfolding, the excimer fluorescence decreased 30–40%, and monomer fluorescence decreased 10–15%, suggesting a shift from both excimer and monomer configurations to nonfluorescent dimer configurations. Thus, the distribution between these three classes of probe configurations is influenced by small conformational changes.

By increasing both monomer and excimer fluorescence, S1 binding to PIATm–actin affects Tm chain–chain interaction, causing a redistribution of pyrene probes away from nonfluorescent dimer configurations toward excimer and monomer configurations. The conformation change of Tm involved does

not appear to be the same as the local unfolding of Tm that occurs in its unfolding pretransition in the absence of actin, in view of the somewhat different fluorescence changes observed; e.g., the local unfolding produced a small change in the excitation spectrum of excimer fluorescence which was not observed when S1 was bound.

For PMTm, no corresponding evidence for nonfluorescent dimer configurations has been obtained. If nonfluorescent configurations are present, their contribution does not appreciably change. The main difference between the PM and PIA probes is an extra charge from a carboxylate group produced by the cleavage of the succinimido ring attached to Cys in PMTm (Ishii & Lehrer, 1986a). Absorption and fluorescence spectra of the model compound, PM–dithiothreitol (Lehrer & Ishii, 1989), have indicated that electrostatic repulsion inhibits the formation of nonfluorescence dimer configurations. When the carboxylates are protonated at acid pH values, the nonfluorescent dimer configurations are no longer inhibited, and both excimer and monomer fluorescence is quenched. Such electrostatic interactions as well as conformational effects may be operating in the case of PMTm to minimize pyrene dimer formation.

States of Tm in Actin–Tm and Actin–Tm–Tn Thin Filaments. S1 titrations showed that the excimer fluorescence change was sensitive to the S1-induced state change of Tm for both PIATm–actin and PIATm–Tn–actin thin filaments. The ability of the excimer fluorescence to probe the Tm state change thereby provides a direct test of the cooperative binding theory in which the fraction of Tm in state 2 originally could only be inferred from the S1 binding profile to reconstituted thin filaments (Greene & Eisenberg, 1980). Another advantage of direct measurement of the state change is that cooperativity parameters can be obtained even under stoichiometric binding conditions; i.e., even when S1 binding does not appear cooperative, the fluorescence profile shows cooperativity. To date, changes in several different properties of probes at Cys-190 correlated with the S1-induced Tm state change: a change in the Tm chain–chain interaction (this work), a change in the environment at Cys-190 (Ishii & Lehrer, 1985; Lehrer & Ishii, 1988), and a change in the geometrical relationship between actin and Tm (Lehrer & Ishii, 1988).

These data with PIATm can distinguish between two proposed alternative Tm state models which are not distiguishable by S1 binding profiles alone (Hill et al., 1980, 1983). In the original model or "two conformation" model (Hill et al., 1980), Tm occupies two states, while in an alternative model or "induced fit" model Tm is allowed to exist in a continuum of nucleotide-dependent states (Hill et al., 1983). Our data support the former model because the total fluorescence change was independent of nucleotide. The same conclusion has been suggested from the ATPase activity studies with S1 chemically modified with *N,N'-p*-phenylenedimaleimide (Greene et al., 1987).

TnT1 did not affect the profile of the S1-induced Tm state change although it strengthened the binding of Tm to actin, most probably by increasing the end-to-end interactions of Tm on the actin filament. The importance of the end-to-end interactions in the binding of Tm to actin has been demonstrated by analyzing binding profiles (Wegner, 1979) and by using Tm whose polymerization is inhibited, via carboxypeptidase A treatment (Ueno et al., 1971; Mak & Smillie, 1981) or PM labeling (Graceffa & Lehrer, 1980). One form of cooperativity in the S1-induced state change of Tm on actin is believed to result from the difference in strength of end-to-end interactions when neighboring Tm's are the same state compared to different states through the end-to-end cooperative parameter Y (Hill et al., 1980). The lack of effect of TnT1 on Y of the S1-induced Tm state change indicates that the end-to-end interactions are increased to a similar extent independent of the state of neighboring Tm's. The profile of the state change in the presence of Tn and Ca^{2+} is very similar to that in the absence of Tn, in agreement with the S1 binding experiments (Williams & Greene, 1983). The difference in actin-activated S1 ATPase of the two systems is probably explained by different kinetic parameters or slightly different equilibrium constants (Lehrer & Morris, 1982; Williams et al., 1988).

In contrast to our results with probes on Tm, fluorescence probes on TnI appear to monitor the thin filament state change induced by S1 binding in the presence of Ca^{2+} but not in the absence of Ca^{2+} (Trybus & Taylor, 1980; Greene, 1986). This is probably because a probe on TnI may be sensitive not only to the state change induced by S1 binding but also to other concomitant changes, e.g., changes in TnI–actin interactions. Thus, probes on Tm appear to monitor the thin filament state change more accurately.

Salt-Dependent Tn–Tm Interactions. Previous studies have shown changes in the environment of probes at Cys-190 of Tm caused by Tn binding, suggesting close interaction of regions of Tn with the Cys-190 region of Tm (Morris & Lehrer, 1984; Ingraham & Swenson, 1985; Lamkin et al., 1983). The decrease in excimer fluorescence of PMTm and PIATm on binding of Tn also indicated direct or indirect effects on the probe environment (Ishii & Lehrer, 1986b). The studies with PIATm–Tn–actin reported here indicated that the Tn–Tm interaction on the thin filament is ionic strength dependent. Similar ionic strength effects on the Tm–Tn interaction were noted in the absence of actin (Ishii and Lehrer, unpublished results). Both of these probe studies show that at high salt the Tn–Tm interaction is weakened near the Cys-190 region of Tm. Tn binds to Tm via an extended region of TnT (Ohtsuki, 1979). Because this interaction is weakened at high salt when Tn is still bound to Tm, it appears that the part of TnT that binds near Cys-190 is flexible and interacts with Tm with a strong electrostatic component. The observation that the increase in excimer fluorescence produced by S1 binding to PIATm–Tn–actin, which was small at low salt and approached the value for PIATm–actin at high salt, is consistent with a weakened Tm–Tn interaction. Thus, at low salt, a strong Tn–Tm interaction appears to mask the S1-induced conformational change of Tm. At high salt, the Tm–Tn interaction is weakened, and a greater effect of S1 is produced.

Ca^{2+}- vs S1-Induced Change. The major effect to change the state of Tm on the thin filament appears to be due to S1 binding. A small Ca^{2+} effect on the excimer fluorescence of PIATm–Tn–actin was observed at low salt which disappears at [NaCl] >0.2 M. This does not appear to be related to the S1-induced state change of Tm. It is estimated that only about 2.5% of Tm is in state 2 ($P_2 \sim 1/L'Y$) even in the presence of Ca^{2+}. The small fluorescence change caused by Ca^{2+} can be understood in terms of Ca^{2+}-induced changes of subunit interactions in the thin filament affecting the environment or the conformation near Cys-190 of Tm. In agreement with this, it has been observed that the TnT–Tm interaction is influenced by the interaction of TnT with the other Tn components, TnI and TnC (van-Eerd & Kawasaki, 1973; Morris & Lehrer, 1984; Ishii & Lehrer, 1986b). The TnI–actin interaction which is weakened in the presence of Ca^{2+} (Hitchcock, 1975; Potter & Gergely, 1974; Tao et al., 1987), and other Tn

subunit interactions are strengthened (Leavis & Gergely, 1984), may change the TnT–Tm interaction.

It appears that the effect of activation of muscle on X-ray diffraction changes prior to crossbridge attachment (Kress et al., 1986) could be due to Ca^{2+}-induced Tn subunit reorganization rather than a Ca^{2+}-induced shift of Tm to the "on" state. The effect of Ca^{2+} on the fluorescence profiles, therefore, suggests that the role of Ca^{2+} is to facilitate the S1-induced shift of Tm from state 1 to state 2. With this model, a change in geometry between Tm and actin (Lehrer & Ishii, 1988) would be correlated with attachment of myosin heads to the thin filament in state 2.

Registry No. Ca, 7440-70-2.

References

Balint, M., Sreter, F. A., Wolf, I., Nagy, B., & Gergely, J. (1975) *J. Biol. Chem. 250*, 6168–6177.

Betcher-Lange, S., & Lehrer, S. S. (1978) *J. Biol. Chem. 258*, 3757–3760.

Birks, J. B. (1970) in *Photophysics of aromatic molecules*, pp 301–370, Wiley-Interscience, London.

Brisson, J., Golosinska, K., Smillie, L. B., & Sykes, B. D. (1986) *Biochemistry 25*, 4548–4555.

Ebashi, S., Wakabayashi, T., & Ebashi, F. (1971) *J. Biochem. (Tokyo) 69*, 441–445.

Graceffa, P., & Lehrer, S. S. (1980) *J. Biol. Chem. 255*, 11296–11300.

Greene, L. E. (1982) *J. Biol. Chem. 257*, 13993–13999.

Greene, L. E. (1986) *J. Biol. Chem. 261*, 1279–1285.

Greene, L. E., & Eisenberg, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2616–2620.

Greene, L. E., Williams, D. L., & Eisenberg, E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3102–3106.

Hill, T. L., Eisenberg, E., & Greene L. E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3186–3190.

Hill, T. L., Eisenberg, E., & Greene, L. E. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 60–64.

Hitchcock, S. E. (1975) *Eur. J. Biochem. 52*, 255–263.

Ingraham, R. H., & Swenson, C. A. (1985) *Biochemistry 24*, 5221–5225.

Ishii, Y., & Lehrer, S. S. (1985) *Biochemistry 24*, 6631–6638.

Ishii, Y., & Lehrer, S. S. (1986a) *Biophys. J. 50*, 75–80.

Ishii, Y., & Lehrer, S. S. (1986b) *Biophys. J. 49*, 259a.

Ishii, Y., & Lehrer, S. S. (1987) *Biochemistry 26*, 4922–4925.

Ishii, Y., & Lehrer, S. S. (1989) *Biophys. J. 55*, 202a.

Jackson, P., Amphlett, G., & Perry, S. V. (1975) *Biochem. J. 151*, 85–97.

Kouyama, T., & Mihashi, K. (1981) *Eur. J. Biochem. 114*, 33–38.

Kress, M., Huxley, H. E., Faruqi, A. R., & Hendrix, J. (1986) *J. Mol. Biol. 22*, 3053–3058.

Lamkin, M., Tao, T., & Lehrer, S. S. (1983) *Biochemistry 22*, 3053–3058.

Leavis, P. C., & Gergely, J. (1984) *CRC Crit. Rev. Biochem. 16*, 235–305.

Lehrer, S. S., & Morris, E. P. (1982) *J. Biol. Chem. 257*, 8073–8080.

Lehrer, S. S., & Ishii, Y. (1988) *Biochemistry 27*, 5899–5906.

Lehrer S. S., & Ishii, Y. (1989) in *Fluorescence Biomolecules* (Jameson, D. N., & Reinhart, G. D., Eds.) pp 423–425, Plenum Press, New York.

Lin, T.-I. (1982) *Biophys. Chem. 15*, 277–288.

Mak, A., & Smillie, L. B. (1981) *Biochem. Biophys. Res. Commun. 101*, 208–214.

Morris, E. P., & Lehrer, S. S. (1984) *Biochemistry 23*, 2214–2220.

Nagashima, H., & Asakura, S. (1982) *J. Mol. Biol. 155*, 407–428.

Ohtsuki, I. (1979) *J. Biochem. (Tokyo) 86*, 491–497.

Potter, J. D., & Gergely, J. (1974) *Biochemistry 13*, 2697–2703.

Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866–4871.

Tao, T., Gong, B.-J., & Leavis, P. C. (1987) *Biophys. J. 51*, 27a.

Trybus, K. M., & Taylor E. W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 7209–7213.

Ueno, H., Tawada, Y., & Ooi, T. (1971) *J. Biochem. (Tokyo) 80*, 283–290.

van-Eerd, J.-P., & Kawasaki, Y. (1973) *Biochemistry 12*, 4972–4980.

Weeds, A. G., & Pope, B. (1977) *J. Mol. Biol. 111*, 129–157.

Wegner, A. (1979) *J. Mol. Biol. 131*, 839–853.

Williams, D. L., & Greene, L. E. (1983) *Biochemistry 22*, 2770–2774.

Williams, D. L., Greene, L. E., & Eisenberg, E. (1988) *Biochemistry 27*, 6987–6993.