Fluorescence Probe Studies 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

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ABSTRACT: The monomer fluorescence of *N*-(1-pyrenyl)maleimide-labeled tropomyosin bound to F-actin (PTm–actin) increases when myosin subfragment 1 (S1) binds to actin and is half complete when only ~1 S1 is bound to 7 actin subunits [Ishii, Y., & Lehrer, S. S. (1985) *Biochemistry 24*, 6631–6638]. Similar studies of the binding of S1 and S1–ADP to fully reconstituted thin filaments [PTm–actin–troponin (Tn)] are now reported. The pyrene monomer fluorescence change was half complete when ~0.5 S1/7 actin subunits and ~1.5 S1/7 actin subunits were bound in the presence and absence of Ca^{2+}, respectively. In the presence of Mg^{2+}-ADP, when S1 binding is weakened, the S1 binding profiles and fluorescence changes were sigmoidal, with the cooperative transitions occurring at lower [S1] in the presence of Ca^{2+} as first shown by Greene and Eisenberg for S1 binding [Greene, L., & Eisenberg, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2616–2620]. It was possible to fit both the binding and fluorescence data with the same parameters of a two-state (weak and strong S1 binding) cooperative binding model [Hill, T., Eisenberg, E., & Greene, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3186–3190] for each Ca^{2+} situation if the fluorescence change is interpreted as the fraction of tropomyosin (Tm) units in the strong S1 binding state. These data indicate that the fluorescence change is a direct measure of the S1-induced change of state of Tm in the fully reconstituted thin filament. In the absence of S1, Ca^{2+} did not change the fluorescence of PTm–Tn–actin, suggesting that Ca^{2+}, per se, does not affect the state of Tm.

The binding of myosin subfragment 1 (S1)[1] and the S1–ADP complex to F-actin shows cooperativity when troponin–tropomyosin (Tn–Tm) (Greene & Eisenberg, 1980) or Tm (Williams & Greene, 1983) is bound to F-actin. To explain the cooperativity, an allosteric binding model was developed in which Tm equilibrates between two states on the actin filament: a state in which S1 binds weakly and a state in which S1 binds strongly to the F-actin filament (Hill et al., 1980). In this model, Ca^{2+} acts on troponin by facilitating the shift in equilibrium of Tm to the strong binding state. Cooperative effects have also been observed on the acto–S1 ATPase activity, in both the presence and absence of Tn (Bremel et al., 1972; Eaton et al., 1975; Lehrer & Morris, 1982) and in the kinetics of S1 binding (Trybus & Taylor, 1980). With the parameters obtained from fits of the theoretical to the experimental S1-binding profile, the fraction of Tm–Tn units in the strong binding state, P_2, can be calculated, but no direct measurement of P_2 was available. The fluorescence of a probe on one of the components of Tn (Trybus & Taylor, 1980) appeared to be a measure of P_2 in the presence of Ca^{2+} but not in its absence (Greene, 1986).

We have previously reported that the monomer fluorescence of pyrene–Tm bound to the F-actin complex is sensitive to the binding of S1, and the fluorescence change is complete at low binding ratios, in agreement with the cooperative binding model (Ishii & Lehrer, 1985). In the following we extend this approach to the reconstituted thin filament in the absence and presence of Ca^{2+}. We show that the thin filament cooperativity parameters that were used to fit the S1 binding profiles could be used to fit the fluorescence change, indicating that the pyrene monomer fluorescence provides a direct measure of P_2.

Experimental Procedures

Rabbit skeletal myosin, its chymotryptic fragment (S1), actin, Tn, and Tm were prepared as previously outlined (Lehrer & Morris, 1982). Tropomyosin was labeled at its cysteine residues with *N*-(1-pyrenyl)maleimide, and the product with cleaved succinimido rings was prepared by alkaline hydrolysis at pH 8.5 (Ishii & Lehrer, 1985). The degree of labeling was 1.8 pyrene/Tm. The fluorescence measurements were carried out on a Spex Fluorolog 2/2/2 photon counting fluorometer (Edison, NJ). The 90° light scattering and fluorescence data were obtained in the same cuvette after each addition of S1 by cycling the emission monochromometer wavelengths between the pyrene monomer fluorescence (at 382 nm) and the light scattering (at 348 nm) and averaging each measurement over 20 s. The data were corrected for dilution effects and fitted to the equations of Hill et al. (1980) using a nonlinear least-squares computer fitting program written by Dr. Edward P. Morris.

Results

The fluorescence spectrum of highly labeled pyrenyl-Tm (PTm) exhibits a structured monomer band with a peak at

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* Address correspondence to this author at the Boston Biomedical Research Institute.

[1] Abbreviations: PTm, *N*-(1-pyrenyl)maleimide-labeled tropomyosin; S1, myosin subfragment 1; Tm, tropomyosin; Tn, troponin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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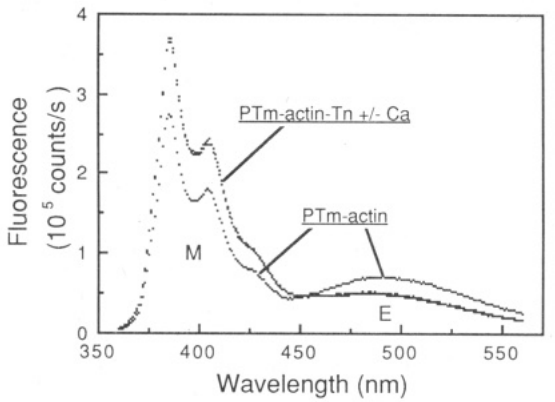

FIGURE 1: Pyrene fluorescence change due to troponin (Tn) binding to PTm–actin in the presence and absence of Ca^{2+} as indicated. [PTm] = 1 μM, [actin] = 7 μM, and [Tn] = 1.5 μM in 0.03 M NaCl, 5 mM $MgCl_2$, and 10 mM Hepes buffer, pH 7.5, with 1 mM EGTA ($-Ca^{2+}$) or 0.1 mM Ca^{2+} ($+Ca^{2+}$). λ_{ex} = 340 nm at 25 °C. M = monomer band; E = excimer band.

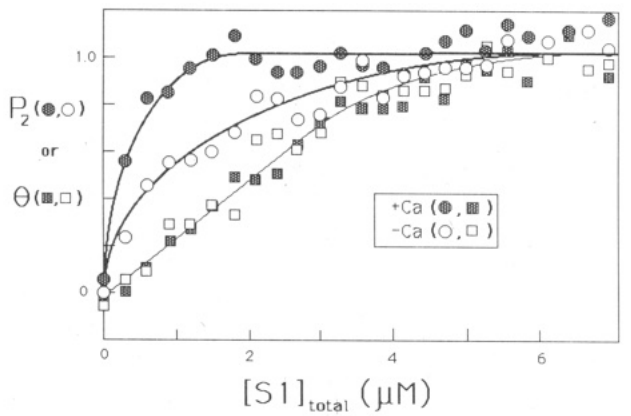

FIGURE 2: Binding of S1 to PTm–actin–Tn in the absence of nucleotide. θ is the fractional saturation as measured by the light scattering increase at 348 nm; P_2 is the fraction of Tm units in the strong binding state as measured by the monomer fluorescence increase at 382 nm. $-Ca^{2+}$, open symbols; $+Ca^{2+}$, filled symbols. [Actin] = 4.8 μM, [Tm] = 0.66 μM, and [Tn] = 1 μM in 0.1 M NaCl at 20 °C. Other conditions as in Figure 1.

385 nm and a broad excimer band centered at 480 nm, which is due to an excited-state interaction between the neighboring Cys-190's of each chain (Betcher-Lange & Lehrer, 1978). As previously reported, the pyrene monomer fluorescence of lightly labeled PTm (little or no excimer present) was not affected by the binding of PTm to F-actin, indicating a similar pyrene environment (Ishii & Lehrer, 1985). The binding of Tn to PTm–actin caused an immediate increase in monomer fluorescence and a decrease in excimer fluorescence, which were independent of Ca^{2+} (Figure 1). The binding of S1 to the fully reconstituted thin filament (PTm–actin–Tn) resulted in a further immediate increase in pyrene monomer fluorescence and a very slow decrease in excimer fluorescence as seen earlier for the PTm–actin system (Ishii & Lehrer, 1985). S1 titrations were performed by monitoring both the 90° light scattering and the monomer fluorescence intensity after each addition of S1 under conditions where the excimer fluorescence did not appreciably change. In the absence of nucleotide, where the binding is known to be quite strong, the light scattering was essentially linear up to a stoichiometry of 1:1 (S1:actin) in both the presence and absence of Ca^{2+} (Figure 2), indicating that the light scattering is a quantitative measure of the fraction bound, θ, as was found in previous similar studies of S1 binding to Tm–actin (Ishii & Lehrer, 1985). Also, as previously found, the fluorescence changes, interpreted

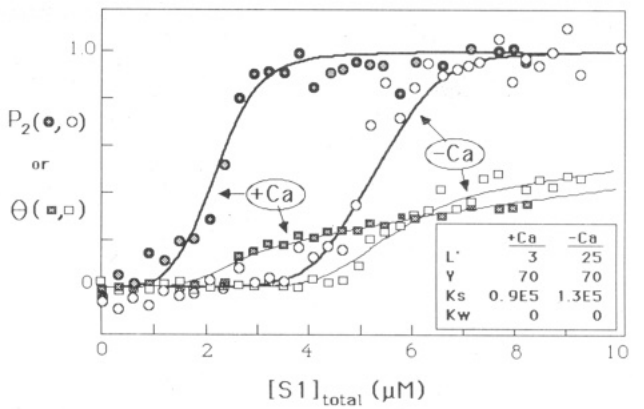

FIGURE 3: Binding of S1 to PTm–actin–Tn in the presence of ADP. Conditions as in Figure 2 except that 1 mM ADP was present. The curves represent fits to the data obtained with the parameters indicated (inset) from the Hill et al. (1980) cooperative binding theory.

as P_2, the fraction of Tm units in the strong binding state, were completed at low degrees of binding of S1 to actin. In the absence of Ca^{2+}, half of the fluorescence change was complete at about 20% saturation; in the presence of Ca^{2+}, half of the fluorescence change was complete at about 7% saturation. These can be compared to the change in the absence of troponin, which was half complete at about 15% (Ishii & Lehrer, 1985). Both the fluorescence and light scattering changes were completely reversed when ATP was added to dissociate the S1. The initial and final fluorescence values were independent of Ca^{2+} within experimental error, and the S1-induced fluorescence change was 17 ± 2%, independent of the presence of ADP.

In the presence of ADP where S1 binding is weakened, the cooperative attachment of S1 was indicated by sigmoid binding curves (Figure 3). With the fractional light scattering increase as a quantitative measure of binding, it can be seen that the fluorescence change was half complete at about 7% and 20% saturation, in the absence and presence of Ca^{2+}, respectively, in agreement with the data in the absence of nucleotide. The curves of Figure 3, which represent best fits of the equations of Hill et al. (1980) to the data, allowed values to be obtained for L', the equilibrium constant between the two states of Tm, Y, the cooperativity parameters, and K_S, the S1–actin binding constant for the strong binding state. By assuming that the light scattering and fluorescence changes were measures of the binding and fraction of Tm units in the strong binding state, respectively, it was possible to fit both sets of data at each Ca^{2+}, using the same values of L', Y, and K_S. The best fit values of L' and Y were in reasonable agreement with values obtained from binding profiles (Greene, 1982). These results therefore indicate that the monomer fluorescence of *N*-(1-pyrenyl)maleimide-labeled Tm is a probe of the state of Tm on the thin filament. It should be mentioned that the data in the absence of ADP (Figure 2) could only be fitted at low degrees of saturation (θ <0.5) with curves generated with the same values of L' and Y as in the presence of ADP and with suitably high values of K_s. The apparent increase in fluorescence in the absence of Ca^{2+} for θ >0.5, where it should be saturated according to the data in Figure 3, appeared to be due to time-dependent artifacts operating under these conditions.

Discussion

The studies of Greene and Eisenberg (1980) showed that the binding of S1–ADP to reconstituted thin filaments was strongly cooperative in the absence, and less so in the presence,

of Ca^{2+}. The binding profiles could be fitted with curves obtained from an allosteric model involving four parameters: L', Y, K_w, and K_s (Hill et al., 1980). The model assumes that the reconstituted thin filament can exist in two states, a weak and a strong S1-binding state, with the respective S1 binding constants K_w and K_s. The equilibrium constant for the transition of all of the units from the weak to the strong states, $1/L'$, is independent of the binding of S1. The greater the L' value, the more units initially in the weak binding state and the greater the [S1] necessary to switch Tm units to the strong binding state. The Y parameter was introduced to increase the cooperativity beyond the 7 actin unit, in agreement with the known end-to-end interactions of Tm. The principal difference between the high and low Ca^{2+} cases, independent of nucleotide, was the value of L' (2 and 33, respectively) (Greene, 1982). These binding studies showed that, in the absence of S1, the regulated thin filament was largely in the weak S1-binding state in both the absence and presence of Ca^{2+}. In all of these studies the binding profile was obtained by centrifugation of the S1–F-actin complex and a measurement of the free S1 remaining in the supernatant, using radioactively labeled S1. The fraction of Tm's in the strong binding state on the thin filament, P_2, could then be indirectly obtained from the binding profile and the model.

The data presented here indicate that the pyrene monomer fluorescence is sensitive to the state of Tm on the thin filament as defined by the model of Hill et al. (1980). The main evidence for this is that the same values of L', Y, and K_s, which produced a best fit for the binding profiles as measured by the light scattering, generated P_2 profiles that also fit the fluorescence data. The values of L' and Y estimated from the fits in the absence and presence of Ca^{2+} are in reasonable agreement with the values obtained in the previous binding studies (Greene & Eisenberg, 1980; Greene, 1982). The observation that the initial fluorescence and the S1-induced fluorescence change are independent of the presence of Ca^{2+} shows that Ca^{2+} does not appreciably affect the state of Tm in the absence of S1 and that Ca^{2+} acts indirectly as an allosteric effector (Hill et al., 1980). It is possible that Ca^{2+}, which produces changes in the Tn–Tm interaction (Leavis & Gergely, 1984), affects a part of the Tm molecule undetectable by *N*-(1-pyrenyl)maleimide fluorescence at Cys-190, but if there is such an effect, it appears to be independent of the S1-induced change in pyrene fluorescence.

The pyrene fluorescence increase produced by Tn binding to PTm–actin is in agreement with earlier studies, which showed proximity of Tn to the Cys-190 region of Tm in the thin filament (Lamkin et al., 1983; Morris & Lehrer, 1984). The environmental changes caused by binding S1 to PTm–actin and to PTm–Tn–actin are not due to S1–probe interaction (Ishii & Lehrer, 1985), but it is impossible to discriminate between a changed probe–actin or probe–Tm environment. Another environmentally sensitive probe, acrylodan, attached to Cys-190 of $\alpha\alpha$Tm (ACTm), behaved similarly to PTm, i.e., no change in acrylodan fluorescence on binding to actin and Ca^{2+}-independent changes of fluorescence on binding Tn to ACTm–actin. Although an enhancement of fluorescence was produced by the binding of S1 to ACTm–actin with similar P_2 binding profiles, no enhancement was observed for S1 binding to ACTm–Tn–actin (Ishii & Lehrer, 1987). Thus, it appears that although there is a Tn–probe interaction in the case of both PTm–Tn–actin and ACTm–Tn–actin, the environment of the probe in the latter system appears to not further change on binding S1.

It has been shown that Tm equilibrates between two conformational states at physiological temperature: a chain-closed state and a locally unfolded chain-open state (Graceffa & Lehrer, 1980, 1984; Ueno, 1984). Although it appears that probes at Cys-190 indicate a supression of the chain-open state in the reconstituted thin filament (Ishii & Lehrer, 1985), further studies will be necessary to determine whether other regions of Tm are unfolded and whether there is a relationship between the conformation of Tm, S1 binding, and Ca^{2+}-dependent Tn states of the reconstituted thin filament.

Structural evidence has indicated that Tm moves on the thin filament in response to the binding of Ca^{2+} (Huxley, 1972; Haselgrove, 1972; Parry & Squire, 1973; Wakabayashi et al., 1975; O'Brien et al., 1975), but evidence for movement has, as yet, not been obtained with in vitro studies (Tao et al., 1983; Lin & Dowben, 1983). S1-induced Tm movement has been incorporated into the model of Hill et al. (1980) (Lehrer & Morris, 1982; Hill et al., 1983; El-Saleh et al., 1986; Phillips et al., 1986), yet Ca^{2+}-induced Tm movement appears to be essentially complete before cross-bridge attachment (Kress et al., 1986). Further structural and biochemical studies will therefore be necessary to clarify the nature of the two S1-induced states of Tm and their relationship to the two Ca^{2+} positions.

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Registry No. ADP, 58-64-0; MgADP, 7384-99-8; Ca, 7440-70-2.

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Articles

Carboxylic Ionophore (Lasalocid A and A23187) Mediated Lanthanide Ion Transport across Phospholipid Vesicles†

Bhamidipati P. Shastri,‡ Mantripragada B. Sankaram,§ and Kalpathy R. K. Easwaran*
Molecular Biophysics Unit, Indian Institute of Science, Bangalore-560012, India
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ABSTRACT: The transport kinetics of three lanthanide ions (viz., Pr^{3+}, Nd^{3+}, and Eu^{3+}) across dimyristoylphosphatidylcholine and dipalmitoylphosphatidylcholine unilamellar vesicles mediated by the two carboxylic ionophores lasalocid A and A23187 have been studied by proton nuclear magnetic resonance spectroscopy. Time-dependent changes in the chemical shifts of head group choline signals have been measured to calculate apparent rate constants of transport. These experiments have been done at different ionophore concentrations to determine the stoichiometry of the transporting species. The rates of transport have been found to be faster in the absence of intravesicular La^{3+} compared to those observed in its presence. The stoichiometry of the transporting species has been found to be 2:1 (ionophore:cation) for both lasalocid A and A23187 in dimyristoylphosphatidylcholine vesicles. However, stoichiometries of greater than 2 have been obtained for lasalocid A mediated lanthanide ion transport across dipalmitoylphosphatidylcholine vesicles. Possible reasons for the observations of such noninteger stoichiometries are discussed. Our results also indicated that A23187 is a more efficient carrier ionophore than lasalocid A.

Ever since the discovery that paramagnetic lanthanide ions (Pr^{3+} and Eu^{3+}) can distinguish between the nuclear magnetic resonance (NMR)[1] signals of choline protons from the internal and external surfaces of egg yolk lecithin (EYL) liposomes (Bystrov et al., 1971), their use to study the kinetics of mediated cation transport across model membranes has been steadily growing (Ting et al., 1981). A variety of both natural and synthetic ionophores have been shown by NMR (Fernandez et al., 1973; Hunt, 1975; Hunt et al., 1978; Degani et al., 1981; Donis et al., 1981; Grandjean & Laszlo, 1982, 1984) to transport paramagnetic cations (Pr^{3+}, Eu^{3+}, and Mn^{2+}) across unilamellar vesicles (ULVs) prepared by sonication of aqueous dispersions of synthetic lipids. It has also been suggested that this method can distinguish between the diffusive carrier and the pore mechanisms (Ting et al., 1981).

Extensive conformational studies in solution and in solid state have been carried out to demonstrate the diverse capabilities of the carboxylic polyether antibiotic ionophores (lasalocid A and A23187) to complex with physiologically important mono- and divalent cations and their abilities to form both the equimolar 1:1 (ionophore:cation) and the "ion sandwich" 2:1 complexes (Johnson et al., 1970; Reed & Lardy, 1972; Alpha & Brady, 1973; Degani et al., 1973; Degani & Friedman, 1974, 1975; Haynes & Pressman, 1974; Pfeiffer et al., 1974; Schmidt et al., 1974; Puskin & Gunter, 1975; Anteunis, 1976; Patel & Shen, 1976; Pfeiffer & Lardy, 1976; Shen & Patel, 1976; Chiang & Paul, 1977; Young & Gomperts, 1977; Chen & Springer, 1978; Vishwanath & Easwaran, 1983, 1985; Shastri & Easwaran, 1984). Our studies on the interaction of lanthanide ions with the carboxylic ionophore lasalocid A in acetonitrile and methanol have shown that it forms both 1:1 and 2:1 complexes with lanthanides and that their binding constants depend upon the size of the cation and the solvent polarity (Shastri et al., 1987). In spite of the observation of such nonequimolar complexes (both "ion sandwich" and "ionophore sandwich") for many ionophores in solution (Devarajan & Easwaran, 1981; Vishwanath & Easwaran, 1982, 1983, 1985; Shastri & Easwaran, 1984; Sankaram & Easwaran, 1985; Easwaran, 1985), experimental studies on their relevance to cation transport are limited

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‡ Present address: Biophysics Institute, Boston University School of Medicine, Boston, MA 02118.
§ Present address: Abteilung Spektroskopie, M P I fur biophysikalische Chemie, D-3400 Gottingen, FRG.

[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; EYL, egg yolk lecithin; NMR, nuclear magnetic resonance; ULVs, unilamellar vesicles; MLVs, multilamellar vesicles.

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