NON-COOPERATIVE RESPONSE OF ACTIN-CYSTEIN 373 IN COOPERATIVELY BEHAVING REGULATED ACTIN FILAMENTS

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1. Introduction

Contraction in vertebrate skeletal muscle is regulated by modifying the behavior of the thin filaments which consist of actin complexed with tropomyosin and troponin. Both tropomyosin and troponin are needed to switch from contraction to relaxation [1,2].

Tropomyosin alone can make the actin–myosin interaction cooperative [3]. Actin–tropomyosin filaments containing some rigor complexes (complexes between actin and nucleotide-free myosin) are better cofactors for myosin ATPase activity than actin tropomyosin filaments free of rigor complexes [3,4], a behavior we have called potentiation. It is not clear how potentiation is achieved; the most likely explanation is a propagated conformation change in the actin molecules [5]. Therefore, we decided several years ago to investigate whether actin filaments have the capacity to respond to myosin binding by propagated conformational changes. Choosing pure actin filaments as the simplest system, we probed the protein domain between Cys 373 and the high affinity divalent ion site looking for changes in the dipole–dipole interaction between a cysteine-bound nitroxide label and manganous ion bound to that divalent ion binding site. We now retract these findings [6,7] because extensive studies (A. W., G. Reed, unpublished) have shown that dipole–dipole interaction is not sensitive to myosin binding in either a linear or cooperative fashion.

We present here a study probing the domain around Cys 373 with two different dansyl labels that are known to be sensitive to myosin binding, dansyl aziridine (J. Eccleston, personal communication) and IAEDANS (E. W. Taylor, personal communication). We observed that the response of these probes to S-1 binding is not cooperative in regulated actin whose cofactor activity towards myosin behaves in a highly cooperative fashion.

2. Methods

S-1, actin and regulated actin were prepared as in [4]. Labeling of G- or F-actin with dansyl aziridine took place at 15°C and pH 8.0, using 0.1–0.2 mM dansyl aziridine excess over dithiothreitol (DTT) for 6 h (in the course of 6 h the dansyl aziridine precipitates). G-actin was incubated with 1,5-IAEDANS [5-(iodoacetamidoethyl) aminonaphthalene-1-sulfonic acid (0.1 mM excess over DTT and actin)] and salt, polymerized for 15 min at 15°C, and transferred to 4°C for 6 h. Actin was blocked at Cys 373 by incubating F-actin with tritiated *N*-ethyl maleimide ([^{3}H]NEM) in 0.3 mM excess over DTT (usually 0.1 mM) for 3 h at 4°C and pH 8.0. All reactions were stopped with 1 mM DTT, and excess reagent was removed with a polymerization and depolymerization cycle. ATPase activity was measured as in [4]. Fluorescence was measured in a temperature-controlled Perkin-Elmer fluorescence spectrophotometer no. MPF-3L.

3. Results and discussion

The fluorescence intensity of dansyl aziridine or IAEDANS attached to actin changes when actin binds

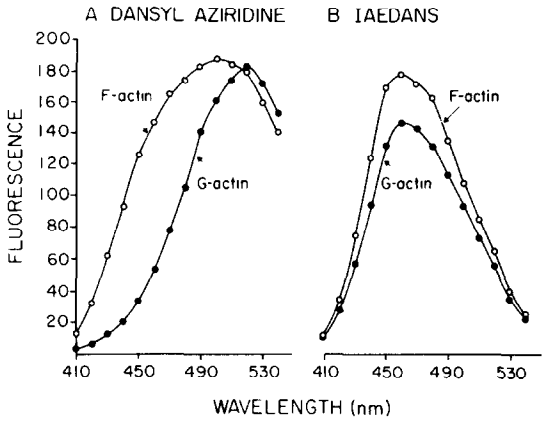

Fig.1. Effect of polymerization on fluorescence intensity of dansyl-labeled actins. Fluorescence units have been adjusted to the same standard (1 μM quinine in H_2SO_4) for both experiments. 15–16°C; 5 μM actin 10 mM Tris (pH 8.0), 0.5 mM ATP, 0.2 mM Ca before polymerization was induced by the addition of KCl and $MgCl_2$ to 0.1 M and 1.0–2.0 mM, respectively. Ordinate: 100 units = emission intensity of 1 μM quinine sulfate at 450 nm, excited at 350 nm.

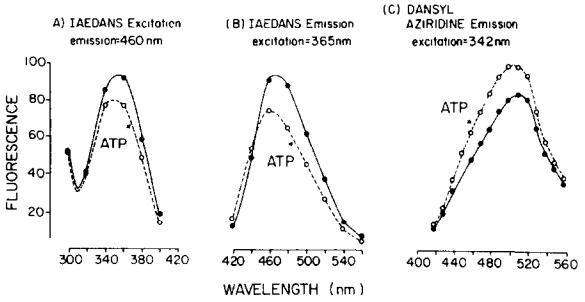

Fig.2. Comparison of emission and excitation spectra of F-actin in the presence of S-1 before and after the dissociation of the S-1–actin complex with ATP. Salts as above, except that initially ATP was omitted. (A, B) 5 μM actin, 5 μM S-1; (C) 4.7 μM actin, 6 μM S-1. Spectra of actin before S-1 addition were nearly identical to those after ATP addition. Units arbitrary.

myosin. The response to myosin binding apparently originates from dansyl attached to Cys 373. Blocking of Cys 373 with *N*-ethyl maleimide (NEM) (16 and 17) reduced labeling with IAEDANS to 5% and with dansyl aziridine to ~30%. The dansyl aziridine bound to residues other than Cys 373 did not respond to S-1 binding. Cys 373–dansyl was also sensitive to polymerization (fig.1A,B), which caused an increase in fluorescence intensity and the development of a blue shoulder for IAEDANS and dansyl aziridine–actin respectively. All observations with dansyl aziridine disagree with those reported [8] for reasons that are not clear. Both F-actin spectra were corrected for the fraction of unpolymerizable actin present, i.e., the fluorescence that remained in the supernatant after a 2 h centrifugation at 100 000 × *g* was subtracted from that of the total F-actin.

Both probes responded to myosin binding by a red shift. This caused quenching of dansyl aziridine fluorescence at wavelengths <530 nm in agreement with the observations by J. Eccleston, E. W. Taylor and S. Marston (personal communications) and an increase in IAEDANS fluorescence at wavelengths >445 nm (fig.2B,C) in agreement with the findings of E. W. Taylor (personal communication). After dissociation of the S-1 from actin by ATP these changes in the emission spectra were reversed.

The red shift and the increase in intensity of IAEDANS fluorescence at 500 nm seemed to be restricted to the actin monomer to which S-1 is complexed since the total fluorescence change was directly proportional to the bound S-1 (fig.3A–C). We titrated IAEDANS actin with S-1 by preparing separate samples of increasing S-1–actin ratios so that reversal of the fluorescence increase after S-1 dissociation by ATP could be checked, thereby differentiating fluorescence changes due to an increase in actin polymerization from those due to S-1 binding. (Slightly unstable actin preparations frequently contain monomers that will not polymerize under optimal salt conditions, 0.1 M KCl and 1 mM $MgCl_2$, but will be induced to polymerize by S-1. If that happens the response to myosin binding will be artificially large for IAEDANS actin, while the quenching response of dansyl aziridine actin will be reduced or even reversed by the simultaneous intensity increase due to polymerization.) In the experiment of fig.3 all actins were very stable and changes in polymerization did not occur since ATP completely reversed the effects of S-1 binding. We allowed the ATP to be hydrolyzed over 15–18 h at 4°C so that we could subtract the fluorescence and the fraction of denatured actin (15–18%) and S-1 (5%) remaining in the supernatant after centrifugation for 2 h at 100 000 × *g*. Such cor-

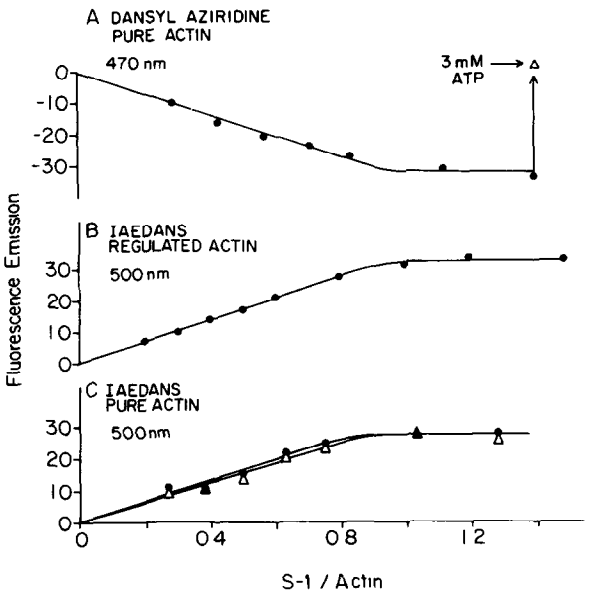

Fig.3. Titration of dansyl actins with S-1. Emission at 500 nm for IAEDANS and 470 nm for dansyl aziridine. In arbitrary units difference between acto–S-1 and (A) actin alone; (B) acto–S-1 + 3 mM ATP (within 4% the same as actin alone); (C) acto–S-1 + ATP, (△) as well as actin alone (●). Salt conditions for dansyl aziridine as in fig.2; for IAEDANS 25 mM KCl, 10 mM Tris (pH 7.0) 2 mM Mg.

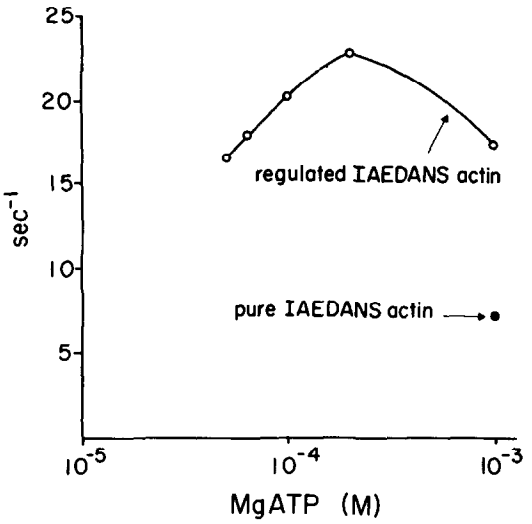

Fig.4. Potentiation of ATPase activity of regulated IAEDANS acto–S-1. Actin, 2 μM (regulated as well as pure), 5 μM S-1; 1 mM CaEGTA, 0.05 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM imidazole (pH 7.0), 0.1 mM MgEDTA, 1 mg/ml creatine kinase, 5 mM creatine phosphate; 25°C.

rections were not made for the titration of dansyl aziridine-labeled actin (fig.3C) which also showed a linear fluorescence quenching with increasing S-1 in agreement with the observations by S. Marston (personal communication) and, on ATP addition, a return of fluorescence to the level before S-1 addition. The presence of regulatory proteins did not convert the linear response of dansyl cysteine to myosin binding into a cooperative one, although the activation of S-1 ATPase activity by regulated IAEDANS actin was biphasic in the fashion typical of potentiation (fig.4). (The ATPase activity of regulated IAEDANS actin remained potentiated, i.e., higher than that of pure IAEDANS actin even at 1.0 mM ATP because of contamination of the S-1 with a fraction whose actin binding was ATP insensitive [9].)

In conclusion, it can definitely be stated that dansyl attached to Cys 373, does not see the cooperative changes of the actin molecule in response to myosin binding that result in potentiation of ATPase activity by the regulated filament. While there is a report on cooperative conformation changes in regulated actin filaments [10] these changes do not seem to be associated with potentiation. First, the changes were reversed at increasing saturation with rigor complexes and potentiation is not. Secondly, no difference in behavior was found between pure and regulated actin although pure actin filaments do not give rise to potentiation or any other indication of cooperative behavior during ATPase activation. None of the cooperative conformational changes reported for pure actin [11–14] are reflected in its cofactor activity.

The fluorescence change could have been linear with increasing S-1 saturation because it was due to a displacement of the dansyl group onto a myosin site of high affinity for the dansyl. If the increase in fluorescence reflects a conformational change one may have expected it to be cooperative in regulated actin since Cys 373 is located on one of the actin–actin interfaces [15]. However, we do not know whether it is on the surface through which conformational changes can be communicated to the next actin. Other probes and methods are needed to decide whether potentiation is the result of propagated conformational changes.

Acknowledgements

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