Biochemistry (Moscow), Vol. 70, No. 10, 2005, pp. 1136-1139. Translated from Biokhimiya, Vol. 70, No. 10, 2005, pp. 1376-1381.

Orientation and Mobility of Actin in Different Intermediate States of the ATP Hydrolysis Cycle

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Received May 17, 2004
Revision received July 13, 2004

Abstract—Using polarization fluorimetry, we have investigated conformational changes of FITC-phalloidin-labeled F-actin in ghost muscle fibers. These changes were induced by myosin subfragment-1 (S1) in the absence and presence of MgADP, MgAMP-PNP, MgATPγS, or MgATP. Modeling of various intermediate states was accompanied by discrete changes in actomyosin orientation and mobility of fluorescent dye dipoles. This suggests multistep changes of orientation and mobility of actin monomers during the ATPase cycle. The most pronounced differences in orientation (~4°) and in mobility (~43%) of actin were found between the actomyosin states induced by MgADP and MgATP.

Key words: muscle contraction, ATP analogs, intermediate states of actomyosin, conformational changes of actin, fluorescence polarization

The interaction of two contractile proteins, actin and myosin, accompanied by ATP hydrolysis, is the molecular basis of muscle contraction. However, structural changes of actomyosin (AM) associated with force generation still require detailed investigation. Results of kinetic studies have provided some evidence that depending on the nucleotide bound and the interaction with actin, the myosin head, also known as myosin subfragment-1 (S1), undergoes several conformational changes during the ATPase cycle [1-4]. Structural states of actomyosin could be assigned to two groups typical for weak and strong binding of myosin with actin. The group of weak binding states is characterized by low affinity of myosin for actin, high rate of attachment/detachment of myosin heads to actin filaments, and myosin heads being insensitive to cooperative activation by regulated actin [1, 5-7]. The group of strong binding states is characterized by myosin high affinity for actin, and myosin heads are sensitive to cooperative activation by regulated thin filaments [8-13]. It is widely accepted that heads of myosin molecules (myosin cross-bridges) undergo structural changes during transition of the weak binding form to their strong binding with actin [10, 14]. Involvement of structural changes of myosin cross-bridges in the mechanisms of force generation was firmly recognized in recent studies [15, 16]. Evidence also exists that structural changes of actin also play an important role in mechanisms of muscle contraction [17-19].

Polarization fluorimetry is a highly sensitive method employed for study of the conformational changes of actin that occur during the muscle contraction process [20-24]. In this study we have used this method for investigation of structural changes of F-actin modified with the fluorescent probe FITC-phalloidin, induced in ghost muscle fiber, by modeling of various intermediate states of the ATPase cycle. The fluorescent probe was covalently attached to actin monomers making excitation and emission dipoles sensitive to changes in orientation and mobility of actin monomers in thin filaments of the muscle fiber. The intermediate states of actomyosin were modeled by decorating thin filaments with S1 in the absence or in the presence of MgADP, MgAMP-PNP, MgATPγS, and MgATP. Results of this study indicate that the interaction of S1 with F-actin causes nucleotide-dependent conformational changes of actin in thin filaments.

Abbreviations: AM) actomyosin; DTT) dithiothreitol; S1) myosin subfragment-1; FITC) fluorescin isothiocyanate; AMP-PNP) 5′-adenylylimidodiphosphate; ATPγS) adenosine 5′-O-(3-thiotriphosphate); pPDM) N,N′-*p*-phenylenedimaleimide; NEM) N-ethylmaleimide.

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MATERIALS AND METHODS

Myosin was isolated from rabbit skeletal muscles [25]. S1 was obtained by treatment of myosin with α-chy-

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motrypsin at 25°C for 10 min [26]. In some experiments S1 was modified with N-ethylmaleimide (NEM-S1) or N,N'-*p*-phenylenedimaleimide (pPDM-S1). Such S1 lacks ATPase activity but exhibits actin-binding capacity [27]. Tropomyosin was obtained as described earlier [28].

Glycerinated fibers were obtained from rabbit skeletal muscles by the method of Szent-Gyorgyi [29]. Thick filament proteins, troponin, and tropomyosin were removed by incubating a single glycerinated muscle fiber in solution containing 0.8 M KCl, 1 mM $MgCl_2$, 10 mM ATP, 67 mM phosphate buffer, pH 7.0, for 1.5 h. Such fibers (so-called ghost fibers) contain more than 80% actin [30]. S1 and tropomyosin were attached to thin filaments by their incubation with the ghost fiber in solution containing 20 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 mM Tris-HCl buffer, pH 6.8, and 1-2.5 mg/ml protein [31]. Unbound protein was removed with the same solution but without protein. The protein composition of muscle fiber and S1/actin molar ratio were determined by SDS-PAGE [32] followed by subsequent gel densitometry (UltroScan XL, Pharmacia LKB, Sweden). The molar ratios S1/actin and tropomyosin/actin were 1 : 5 (±2) and 1 : 6.5 (±1), respectively.

Polarized fluorescence of FITC-phalloidin–actin emission was recorded at 550-650 nm after excitation at 479 ± 5 nm. Measurements were carried out in solution containing 10 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, 6.7 mM phosphate buffer, pH 7.0, in the absence of nucleotides and in the presence of 2.5 mM ADP, 25 mM AMP-PNP, 10 mM ATPγS, or 5 mM ATP. The absence of nucleotide modeled the AM state of the actomyosin complex. MgADP, MgAMP-PNP, MgATPγS, and MgATP were used for modeling of the following intermediate states, AM^·ADP, AM**·ADP·P_i, and AM*·ATP, respectively [33, 34], where A is actin and M, M*, M**, and M^ are conformational states of the myosin head. Four intensities of polarized fluorescence of muscle fiber were registered by fiber orientation parallel ($_{\parallel}I_{\parallel}$, $_{\parallel}I_{\perp}$) and perpendicular ($_{\perp}I_{\perp}$, $_{\perp}I_{\parallel}$) to the polarization plane of the exciting light. The ratio of polarized fluorescence was defined as:

$$P_{\parallel} = (_{\parallel}I_{\parallel} - _{\parallel}I_{\perp})/(_{\parallel}I_{\parallel} + _{\parallel}I_{\perp}),\ P_{\perp} = (_{\perp}I_{\perp} - _{\perp}I_{\parallel})/(_{\perp}I_{\perp} + _{\perp}I_{\parallel}).$$

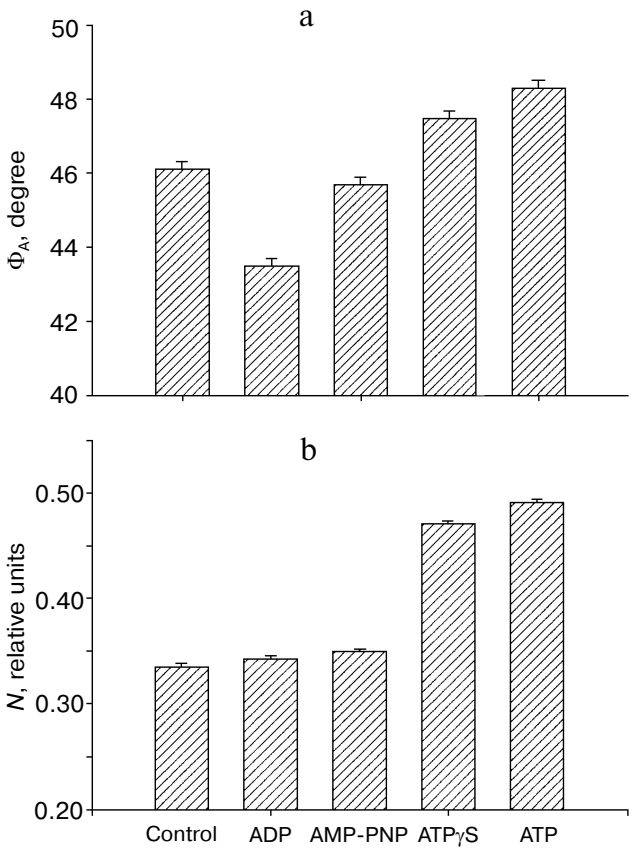
Experimental data were analyzed using a model-dependent method [35] and assuming that in the muscle fiber there is a fraction of helically located fluorophores (characterized by the orientation angles of oscillator absorbance and emission of Φ_A and Φ_E, respectively), and also a fraction of disordered fluorophores (N). A thin filament was considered as a rigid structure and the angle between the fiber axis and the thin filament axis was defined as zero. Changes in Φ_A, Φ_E, and N were considered as evidence of altered orientation and mobility of actin monomers, respectively [21]. Since in all experiments changes in Φ_A correspond to Φ_E changes, only Φ_A values are shown. Statistical significance of changes was evaluated by Student's criterion.

RESULTS AND DISCUSSION

It is believed that attachment of FITC-phalloidin to F-actin does not influence ATPase activity of S1 and tension developed by the glycerinated muscle fiber [24]. Ghost fibers containing tropomyosin, S1, and F-actin modified by FITC-phalloidin are characterized by high anisotropy of polarized fluorescence. In accordance with previously published data [24], the polarized fluorescence degree was high during parallel ($P_{\parallel}$) orientation of the fiber to the light polarization plane ($P_{\parallel} = 0.361 \pm 0.002$) and low during perpendicular ($P_{\perp}$) fiber orientation to the light polarization plane ($P_{\perp} = 0.063 \pm 0.002$). This suggests that dye absorption and emission dipoles are preferentially oriented along the muscle fiber axis. Analysis of polarized fluorescence revealed that in the absence of nucleotide in the muscle fiber the emission dipoles are positioned at the angle $\Phi_A = 46.1 \pm 0.1°$ (figure). (Number of disordered dye molecules changed insignificantly, $N = 0.335 \pm 0.003$.)

Figure shows that MgADP, MgAMP-PNP, MgATPγS, and MgATP significantly influenced Φ_A and N values. These changes in polarization parameters were statistically significant ($p < 0.05$) and reversible. Thus, replacement of a solution containing MgATP for the same solution but without this nucleotide almost completely restored the initial values of the polarization parameters.

The interdomain region of actin monomer contains ADP; under certain conditions, this ADP can be exchanged for ATP or its analogs available in solution. Such exchange occurs very slowly. For example, in the case of fluorescent ADP analog, ε-ADP, exchange of actin ADP for this analog requires several hours (see, e.g., [23]). The duration of our experiments was limited to several seconds. This suggests the possibility for exchange of only a small proportion of nucleotides. Substitution of nucleotide free solution for the solutions containing ATP or its nonhydrolyzing analogs did not influence polarized fluorescence of non-decorated S1 thin filaments and also polarized fluorescence of thin filaments decorated with myosin heads imitating strong or weak binding forms (myosin heads modified with pPDM or NEM as described in "Materials and Methods"). The nucleotide effect was evident only when myosin heads were able to change their conformation in the nucleotide-dependent manner. Consequently, nucleotide exchange (provided that it really occurs) causes an insignificant influence on orientation and mobility of the fluorescent probe and therefore on the structural state of actin. Thus, changes in polarized fluorescence of thin filaments were induced only by binding of myosin heads to actin and they depend on structural states of myosin heads.



Effects of MgADP, MgAMP-PNP, MgATPγS, and MgATP on orientation (a) and mobility (b) of actin monomers in a ghost muscle fiber; Φ_A is the mean of the angle between the fiber axis and emission oscillator FITC-phalloidin; *N* is the relative number of chaotically positioned fluorophores. Data represent the mean of 80 determinations obtained using 16 fibers. Standard errors for Φ_A and *N* did not exceed 0.2° and 0.01, respectively. There were statistically significant changes in Φ_E and *N* values ($p < 0.05$) between complexes actin–S1, actin–S1–ADP, actin–S1–AMP-PNP, actin–S1–ATPγS, and actin–S1–ATP

Previously published data (e.g. [36]) and results of the present study (figure) indicate that weak and strong binding of myosin to actin is accompanied by different changes in orientation and mobility of the fluorescent probe. Change in number of myosin molecules influenced only differences between the parameters studied under weak and strong binding. Increase in molar ratio S1/actin in the range from 1 : 20 to 1 : 5 increased differences between fluorophore orientation and its mobility under weak and strong binding. Subsequent increase in myosin head concentration (the ratio ranged from 1 : 5 to 1 : 3) did not cause any effect on the polarization parameters. All measurements were carried out at S1/actin molar ratio close to 1 : 5; under these conditions the polarization parameters did not depend on myosin head concentrations. Consequently, differences in orientation and mobility of the fluorescent probe found under weak and strong binding can be attributed to different modes of interaction between actin and myosin.

In the presence of MgATP as well as under muscle fiber relaxation [24] or modeling of weak binding between myosin and actin the values of Φ_A and *N* increased by $2.2 \pm 0.1°$ and 46.5%, respectively (figure).

It is well documented that FITC-phalloidin binds with four neighboring actin monomers, where it is positioned in the groove formed by the actin monomers; this means that the fluorescent probe is located far from the site of actin–myosin interaction [37]. It is possible that changes of polarized fluorescence of FITC-phalloidin initiated by ATP reflect conformational changes of actin–actin sites [17, 21, 23].

FITC-phalloidin is covalently bound to actin and so changes in the fluorescent probe orientation can be considered as changes in orientation of the whole protein molecule or its larger part [24, 36, 38]. The data obtained may be more easily interpreted by changes in azimuthal orientation of actin in thin filaments (e.g., by changes of actin monomer slope). This interpretation of spatial organization of actin in thin filaments is consistent with similar interpretation of FITC-phalloidin orientation found during muscle contraction [24, 38].

In accordance with this interpretation, the data shown in the figure indicate that weak and strong forms of actomyosin binding differ in slope of actin monomers and their mobility in thin filaments. In the strong binding state actin is "switched on" (state A), whereas in the weak binding state this protein is "switched off" (state A*). Our results could be well explained assuming that actin monomer "switch-off" results in rotation of actin monomers from the muscle fiber axis accompanied by increase in their mobility in thin filaments.

Marked increase in the polarization parameters Φ_A and *N* was also found in the presence of MgATPγS. Values of Φ_A and *N* increased by $1.4 \pm 0.1°$ and 40%, respectively (figure). This means that in the presence of MgATPγS as well as in the presence of MgATP actin monomers rotate from the muscle fiber axis, and this is accompanied by an increase in their mobility in thin filaments. In the presence of this nucleotide weak binding between myosin heads and actin is formed [41, 42]. Interestingly, in the presence of MgATPγS the values of Φ_A and *N* were significantly lower than in the presence of MgATP (figure). Consequently, in the actin–S1–ATPγS complex the structural state of actin (state A**) differs from its state (A*) in the actin–S1–ATP complex.

In contrast to the effect of MgATP (MgATPγS), MgADP caused reduction in Φ_A by $2.6 \pm 0.1°$ and increase in *N* by 2% (figure). This indicates that mobility of actin monomers increases and actin monomers rotate to the axis of thin filament in the direction opposite to that observed during weak binding (in the presence of MgATPγS and MgATP). The presence of MgADP induces strong binding between actin and myosin [34]. In the presence of

MgADP the polarization parameters, Φ_A and N, were markedly lower than the corresponding values obtained in the absence of this nucleotide. This means that in the actin–S1 complex the structural state of actin (state A) differs from its state A^ in the actin–S1–MgADP complex.

A similar mode of actin monomer rotation to the fiber axis was also observed in the presence of MgAMP-PNP. However, these changes were about two times smaller than in the presence of MgADP. In the presence of MgAMP-PNP there was reduction in Φ_A by $0.4 \pm 0.1°$ and increase in N by 4.4% (figure). These results indicate that in the actin–S1–MgADP complex the structural state of actin state (A^) differs from its state (A^*) in the actin–S1–MgAMP-PNP complex. It is possible that MgAMP-PNP causes rotation of actin monomers to the axis of the thin filaments accompanied by their mobility in thin filaments.

Thus, the data obtained in this study suggest that the structural states of actin in the ternary actin–S1–nucleotide complex significantly differ in weak and strong binding of myosin and actin. For each of these groups of binding, actomyosin complex exists as a series of intermediate states differing in mobility and orientation of monomers in thin filaments. Very recently we demonstrated that intermediate states of actomyosin also differed in orientation and mobility of myosin heads, and the direction of changes in myosin head orientation coincides with changes in monomer actin orientation [43]. Taking into consideration these data, we suggest that the ATPase cycle is accompanied by multistep change in the slope and mobility of actin.

This work was supported by the Russian Foundation for Basic Research (grant No. 01-04-49310), Polish Foundation of Scientific Studies (grant 0757/P04/99/17), and the Institute of Experimental Biology of the Polish Academy of Sciences.

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