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ScienceDirect
Biochemical and Biophysical Research Communications 357 (2007) 325–329
BBRC
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Mutation in the SH1 helix reduces the activation energy of the ATP-induced conformational transition of myosin

Sosuke Iwai *, Shigeru Chaen

Department of Integrated Sciences in Physics and Biology, College of Humanities and Sciences, Nihon University, 3-25-40 Sakurajousui, Setagaya-ku, Tokyo, 156-8550, Japan

Received 8 March 2007
Available online 2 April 2007

Abstract

The SH1 helix is a joint that links the converter subdomain to the rest of the myosin motor domain. Recently, we showed that a mutation within the SH1 helix in *Dictyostelium* myosin II (R689H) reduced the elasticity and thermal stability of the protein. To reveal the involvement of the SH1 helix in ATP-dependent conformational changes of the motor domain, we have investigated the effects of the R689H mutation on the conformational changes of the converter, using a GFP-based fluorescence resonance energy transfer method. Although the mutation does not seem to strongly affect conformations, we found that it significantly reduced the activation energy required for the ATP-induced conformational transition corresponding to the recovery stroke. Given the effects of the mutation on the mechanical properties of myosin, we propose that the SH1 helix plays an important role in the mechanochemical energy conversion underlying the conformational change of the myosin motor domain.

Keywords: Myosin; Motor domain; SH1 helix; Conformational change; Activation energy; Green fluorescent protein; Fluorescence resonance energy transfer

Myosin is a motor protein that converts chemical energy derived from the hydrolysis of ATP into the movement of actin filaments. The N-terminal globular head of the myosin molecule forms the motor domain, which is responsible for myosin's ATPase and motile activities [1]. The motor domain consists of four major subdomains connected by three flexible, highly conserved joints [2,3]. Recent structural studies revealed that the converter subdomain, which is located at the C-terminus of the motor domain, rotates through ~70° during the ATP hydrolysis cycle [4,5]. This rotation is thought to drive the long-distance swing of the lever arm domain, an extended α-helical region that follows the converter [6]. When the myosin head is firmly attached to actin, the swing will generate movement of the myosin motor relative to the actin filament (working stroke). Subsequently, myosin is detached from actin and returns the lever arm (recovery stroke) for the next working stroke.

The SH1 helix is the joint that links the converter subdomain to the rest of the motor, and is thought to play an important role in the arrangements of the converter relative to the motor. It is proposed that the SH1 helix is unwound during the ATP-hydrolysis cycle, and is thereby involved in the control of coupling between the converter and the motor [3,7]. Recently, we introduced a point mutation at a conserved arginine within the SH1 helix in *Dictyostelium* myosin II (R689H) [8]. The equivalent mutation in human non-muscle myosin IIA (R705H) is linked to non-syndromic hereditary deafness, DFNA17 [9]. We characterized the mutant protein and showed that the R689H mutation resulted in a significant impairment in motile activity, whereas actin-activated ATPase activity was only slightly affected. Single molecule mechanical

* Corresponding author. Present address: National Institute of Advanced Industrial Science and Technology, 1-1-1 Higashi, Tsukuba 305-8562, Japan. Fax: +81 298 61 3049.
E-mail address: iwai-sosuke@aist.go.jp (S. Iwai).

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doi:10.1016/j.bbrc.2007.03.155

measurements, along with motile activities produced by a mixture of wild-type and mutant myosins, suggested that the mutation reduced the elasticity of the myosin motor domain. The mutation was also shown to reduce the thermal stability of the protein and induce its thermal aggregation, which might be implicated in the disease process. These results raise the possibility that the mutation affects the flexibility of the converter and, consequently, conformational changes of the motor domain during the ATP-hydrolysis cycle.

To reveal the involvement of the SH1 helix in conformational changes of the myosin motor domain during the ATP-hydrolysis cycle, we investigated the effects of the R689H mutation on the conformational changes of the converter, using a green fluorescent protein (GFP)-based fluorescence resonance energy transfer (FRET) method developed by Suzuki et al. [10]. They observed apparent FRET signals between GFP and blue fluorescent protein (BFP) attached to the *Dictyostelium* myosin II motor domain (GFP–myosin–BFP) and concluded that myosin swings the converter at isomerization and phosphate release steps, which may correspond to the recovery and working stroke, respectively. In this study, we introduced the R689H mutation into a GFP–myosin–BFP fusion protein and assessed the steady-state conformational positions and fast kinetics of ATP-induced conformational transition using stopped-flow measurements. It seems that the mutation does not strongly affect the steady-state conformational positions of the converter domain. However, we found that the activation energy required for conformational transition was significantly reduced by the mutation, suggesting the involvement of the SH1 helix in mechanochemical energy conversion during the conformational change of the myosin motor domain.

Materials and methods

Expression and preparation of proteins. For expression of the GFP–myosin–BFP fusion protein carrying the R689H mutation, the mutation was introduced into the corresponding position of the GFP–myosin–BFP fusion gene, as described previously [8]. Plasmids carrying GFP–myosin–BFP fusion genes were introduced into *Dictyostelium* Ax2 cells. Transformants were selected in HL5 medium containing 10 μg/ml G418. GFP–myosin–BFP fusion proteins were prepared as described previously [11], with slight modifications. The proteins were extracted from the cytoskeletal fraction using a buffer containing 20 mM Hepes (pH 7.4), 250 mM NaCl, 7 mM $MgCl_2$, 5 mM ATP, and 1 mM β-mercaptoethanol. Once purified using a Ni–NTA agarose column (Qiagen), the proteins were dialyzed against a buffer containing 20 mM Hepes (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.

FRET measurements. Regarding the donor dequenching methods, the FRET efficiency, E, is given by:

$$E = 1 - F_{DA}/F_D \quad (1)$$

where F_{DA} and F_D are donor emission intensities in the presence or absence of an acceptor, respectively [12]. In this study, we did not directly determine the values of FRET efficiency, but those of the ratio of $1 - E$ in the presence of nucleotides to $1 - E$ in the absence of nucleotides. The ratio of $1 - E$ values, which we refer to as f, is defined as:

$$f = (1 - E_{nuc})/(1 - E_{no}) \quad (2)$$

where the subscripts nuc and no denote conditions in the presence or absence of nucleotides, respectively. If $f > 1$, the distance between the donor and the acceptor in the presence of nucleotides should be larger than that in the absence of nucleotides, assuming that the change in the orientation factor between the two conditions is small. Thus, the factor f can be used as a simple measure to assess the nucleotide-dependent conformational positions of the protein. If a same sample is used for the measurements under the two conditions, F_D will be invariable, regardless of the presence or absence of nucleotides. In this case, the ratio of $1 - E$ can be calculated as:

$$f = F_{DA,nuc}/F_{DA,no} \quad (3)$$

Steady-state fluorescence was measured using a Perkin-Elmer LS50B fluorophotometer as described previously [10]. Measurements were performed in a buffer comprising 20 mM Hepes (pH 7.4), 200 mM KCl, 2 mM $MgCl_2$ and 1 mM DTT, with or without 1 mM ATP, by exciting BFP at 380 nm. The emission intensities of the donor BFP were determined by integrating the emission spectrum between 410 and 460 nm, where the contribution of the acceptor GFP fluorescence is negligible. The buffer spectrum was subtracted from the others.

Stopped-flow measurements. Fast kinetic measurements were performed using an Applied Photophysics SX18 stopped-flow fluorometer as described previously [10]. Measurements were performed in a buffer comprising 20 mM Hepes (pH 7.4), 50 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT, by mixing with 1 mM ATP and exciting at 380 nm. GFP emission was detected through a 570-nm high pass filter. The time courses of the emission signals were fitted with single exponentials to determine the apparent first-order rate constants. The kinetics of GFP emission was measured 5 times or more and averaged. Temperatures were maintained in the range of 10–30 °C with circulating water. The viscosity of the solvent was adjusted by the addition of sucrose. The concentrations of sucrose were the same before and after mixing. Values for the viscosity of sucrose solutions were taken from literature [13].

Results and discussion

To examine the effects of the R689H mutation on the ATP-induced conformational changes of the myosin motor domain, we generated a GFP–myosin–BFP fusion protein carrying the R689H mutation. The steady-state fluorescence of the protein was measured and compared with that of the wild-type fusion protein. The GFP emission intensity of the mutant was high in the absence of nucleotides, and decreased upon the addition of ATP (Fig. 1A), as previously shown for the wild-type (Fig. 1B and Ref. [10]). Recently, Zeng et al. showed that these emission signals included a large non-FRET component arising from protonation of GFP [14]. This made it difficult to directly determine the FRET efficiency, E, in our experimental system. Therefore, to assess the ATP-induced conformational changes of the converter, we estimated a ratio of $1 - E$ in the presence of nucleotides to $1 - E$ in the absence of nucleotides, using BFP emission signals, as described in Materials and methods section. The ratios of $1 - E$ values were significantly larger than 1 for both the wild-type and mutant fusion proteins (Fig. 1C). The ATP-dependent changes in $1 - E$ may represent the ATP-induced swing of the converter. The ratio of the mutant protein was not significantly different from that of the wild-type protein, implying that the conformational positions of the converter during the ATP-hydrolysis cycle were not strongly affected by the R689H mutation. Given that the sliding movement