

Mutation in the SH1 helix reduces the activation energy of the ATP-induced conformational transition of myosin

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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.

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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 $\sim 70^\circ$ 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

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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 MgCl₂, 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 MgCl₂, 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 MgCl₂ 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 MgCl₂, 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

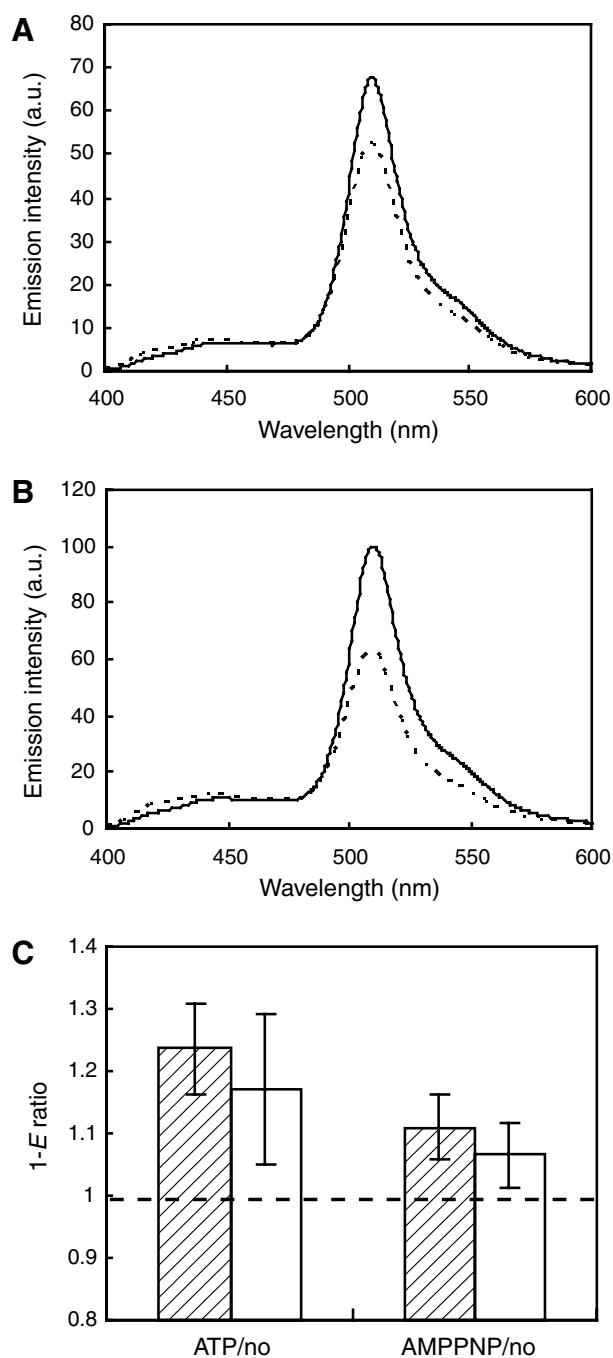


Fig. 1. Steady-state fluorescent properties of GFP-myosin-BFP fusion proteins. (A) Emission spectra of the R689H fusion protein excited at 380 nm in the absence (solid line) or presence (dashed line) of ATP. (B) Emission spectra of the wild-type fusion protein excited at 380 nm in the absence (solid line) or presence (dashed line) of ATP. (C) Ratios of $1 - E$ in the presence of nucleotides to $1 - E$ in the absence of nucleotides, for wild-type (hatched bars) and R689H (open bars) fusion proteins. The ratio of $1 - E$ values is defined as Eq. (2), and calculated using Eq. (3). Values are represented as means \pm SD for three different measurements. The position of unity is shown as a dashed line across the bar graph.

is generated as a result of the conformational changes of the converter, this finding is consistent with the previous result that the step size of the stroke is not shortened by the mutation [8].

Suzuki et al. monitored the time course of the change in the GFP emission signal upon the addition of ATP, and determined the apparent rate constant of the process [10]. From these measurements, they concluded that myosin swings the converter once during the isomerization step upon ATP binding, which may correspond to the recovery stroke. Although the signal was later shown to include a large non-FRET component, it remains a suitable empirical probe for this ATP-induced conformational transition of the motor domain [14]. As described above, the R689H mutant also showed a decrease in GFP emissions upon the addition of ATP, allowing us to determine the apparent rate constants of this process for the mutant protein. The time courses of the GFP emissions, upon the addition of 1 mM ATP, were monitored using stopped-flow measurements and fitted with single exponentials to determine the apparent first-order rate constants (Table 1). At 25 °C, the rate constant of the mutant protein was decreased to $\sim 30\%$ of that of the wild-type protein, suggesting that the ATP-induced recovery stroke step was slowed down by the R689H mutation under physiological conditions. In the previous study, we proposed that the R689H mutation might destabilize the SH1 helix and, thus, induce uncoupling of the converter from the rest of the motor [8]. This may affect the kinetics of the ATP-induced swing of the converter.

It is well established that the reaction rate, k , depends exponentially on the energy required to attain a transition state as:

$$k = A \exp(-E_a/k_B T) \quad (4)$$

where E_a is the activation energy, k_B is the Boltzmann constant, and T is the absolute temperature. To further elucidate the ATP-induced conformational transition of the myosin motor domain, we examined the temperature dependence of the process. The conformational transition decelerated with decreasing temperature (Table 1). The apparent activation energy of the process was estimated by an Arrhenius plot (Fig. 2). The values of activation energy of the wild-type and mutant myosins were estimated to be 86 and 62 kJ/mol, respectively (Table 1). This result indicated that the R689H mutation significantly lowered the activation energy of the ATP-induced conformational transition. The result that the mutation in the SH1 helix lowers the energy barrier suggests that the transition state for the conformational transition involves conformational changes within the SH1 helix, consistent with the structural findings that the SH1 helix is a joint that links the converter and the motor [3,7]. Previously, Malnasi-Csizmadia et al. showed that the conformational transition is kinetically distinct from, but coupled to, the ATP hydrolysis step [15]. Since the activation energy determined here was significantly lowered by the mutation in the SH1 helix, the energy would be predominantly composed of that for the conformational transition. The uncoupling of the converter from the motor, which may be induced by the mutation, is

Table 1
Kinetic parameters for the change in the GFP emissions of GFP–myosin–BFP fusion proteins

	Temperature (°C)	Wild-type	R689H
Apparent rate constant ^a (s ^{−1})	10	8.4	3.7
	15	18	7.2
	20	31	12
	25	52	16
	30	78	23
Activation energy ^a (kJ mol ^{−1})		86 ± 2	62 ± 4
Activation energy (37% sucrose) (kJ mol ^{−1})		69 ± 5	55 ± 1

^a In the absence of sucrose.

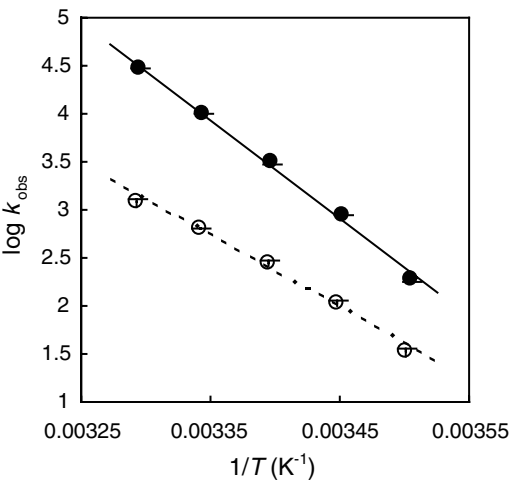


Fig. 2. Temperature dependence of the conformational transition of GFP–myosin–BFP fusion proteins. The semilogarithm of apparent rate constants (k_{obs}) of the change in GFP emissions was plotted against inverse temperature (Arrhenius plot). Wild-type and R689H proteins are represented by closed and open circles, respectively. The solid and dashed lines are best fits of the data with activation energies of 86 and 62 kJ/mol for wild-type and R689H proteins, respectively.

likely to affect the potential barrier for the swing of the converter.

We also examined the effects of a solute on the ATP-induced conformational transition, using sucrose as a model solute. The conformational transition decelerated with increasing solvent viscosity (Fig. 3A). Both the wild-type and mutant myosins showed fractional power-law dependence on viscosity, as previously shown for other protein reactions such as the dissociation of small ligands from proteins [16]. The exponents were different between wild-type and mutant myosins, which may reflect the effect of the SH1 helix mutation on the conformations of the transition state. In 1940, Kramers modeled a chemical reaction as the diffusional crossing of an energy barrier by Brownian motion [17]. Kramers showed that, in the limit of the strong friction, the reaction rate should depend on the friction within the solvent. Under the usual assumption that friction is proportional to solvent viscosity, the reaction rate finally shows a reciprocal dependence on viscosity. On the other hand, it is known that solutes such as

sucrose are preferentially excluded from protein surfaces and, consequently, may affect the free energy of the protein [18]. To reveal the mechanism underlying the effects of the solute, we examined the temperature dependence of the process in the presence of sucrose (Fig. 3B). The apparent activation energy was significantly reduced in the presence of 37% sucrose for both the wild-type and mutant myosins (Table 1), suggesting that the enthalpy of the transition state was decreased by sucrose. Nevertheless, sucrose also decreased the reaction rates, suggesting that compensation may have occurred by decreasing the entropy of the transition state. Sucrose may have decreased the entropy by enhancing the compactness of the conformations of the transition state, and thus affected the rate of the

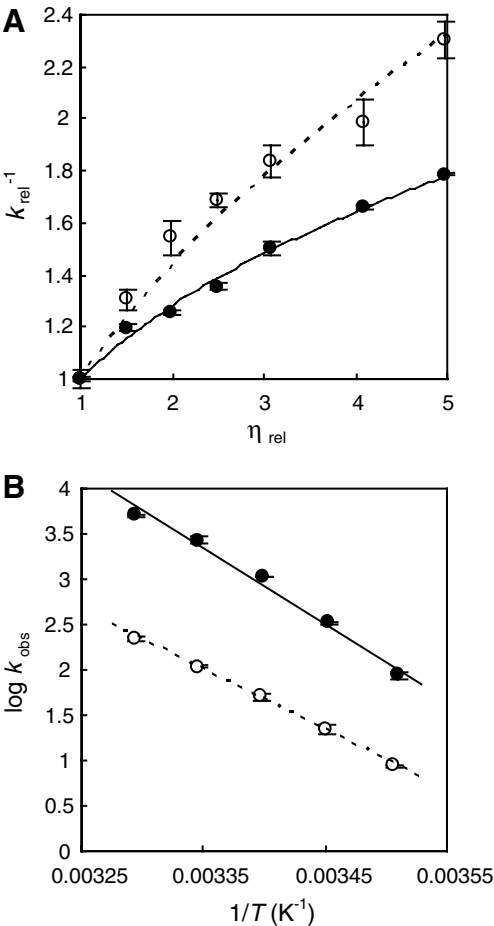


Fig. 3. The conformational transition of GFP–myosin–BFP fusion proteins in the presence of sucrose. Wild-type and R689H proteins are represented by closed and open circles, respectively. (A) Viscosity dependence of the apparent rate constant of the change in GFP emissions. Relative decreases in the rate constants ($k_{\text{rel}}^{-1} = k_{\text{obs},0}/k_{\text{obs}}$) were plotted against relative viscosity ($\eta_{\text{rel}} = \eta/\eta_0$). $k_{\text{obs},0}$ and η_0 refer to the rate constant and solvent viscosity in the absence of sucrose, respectively. The curves are best fits of the data using the equation, $k_{\text{rel}}^{-1} = \eta_{\text{rel}}^p$, with $p = 0.36$ and 0.52 for wild-type and R689H proteins, respectively. (B) Temperature dependence of the apparent rate constant of the change in GFP emissions in the presence of 37% sucrose ($\eta_{\text{rel}} = 5$). The semilogarithm of apparent rate constants (k_{obs}) was plotted against inverse temperature (Arrhenius plot). The solid and dashed lines are best fits of the data with activation energies of 69 and 55 kJ/mol for wild-type and R689H proteins, respectively.

conformational transition. The observed power-law dependence on viscosity might result from these changes. The results shown above suggest that hydration of the protein surface in the transition state is important during the ATP-induced conformational change of myosin, as previously shown by microwave dielectric spectroscopy [19].

Using a GFP-based apparent FRET method, we investigated the effects of the R689H mutation within the SH1 helix on the ATP-induced conformational change of the myosin motor domain. It seems that the mutation does not strongly affect the steady-state conformational positions of the converter domain during the ATP-hydrolysis cycle. However, we found that the mutation significantly reduced the activation energy required for the ATP-induced conformational transition that may correspond to the recovery stroke. This is accounted for by the uncoupling of the converter from the motor, which may be induced by the R689H mutation. This uncoupling may enhance thermal fluctuations in the motor domain, and consequently lower the thermal activation energy required for the conformational change. This is consistent with our previous finding that the mutation reduces the thermal stability of the protein [8]. In the previous study, we also found that the uncoupling induced by the mutation resulted in a reduction in the elasticity of the motor domain [8]. As a result of the reduced elasticity, the elastic energy that is stored in the converter or associated structures would be reduced. This might contribute, at least partly, to the reduction in the potential barrier for the ATP-induced swing of the converter, assuming that a part of the elastic energy is stored during the recovery stroke.

In conclusion, our results demonstrate that, by modulating the coupling between the converter and the motor, the SH1 helix is involved in the control of the mechanical and energetic properties of the myosin motor domain. This suggests an important role for the SH1 helix in the mechanochemical energy conversion underlying the conformational change of the motor domain, consistent with the structural significance of the helix as a joint that links the converter and the rest of the motor [3,7].

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