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Mutations in the SH1 helix alter the thermal properties of myosin II

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The myosin II SH1 helix is a joint that links the converter subdomain to the rest of the myosin motor domain and possibly plays a key role in the arrangement of the converter/lever arm. Several point mutations within the SH1 helix in human myosin IIs have been shown to cause diseases. To reveal whether these SH1 helix mutations affect not only motile activities but also thermal properties of myosin II, here we introduced the E683K or R686C point mutation into the SH1 helix in *Dictyostelium* myosin II. Thermal inactivation as well as thermal aggregation rates of these mutant proteins demonstrated that these mutations decreased the thermal stability of myosin II. Temperature dependence of sliding velocities of actin filaments showed that these mutations also reduced the activation energy of a rate-limiting process involved in actin movement. Given that these mutations are likely to alter coupling between the subdomains, and thus their thermal fluctuations, we propose that the SH1 helix is a key structural element that determines the flexibility and thermal properties of the myosin motor. These characteristics of the SH1 helix may contribute to the patho-genesis of the human diseases caused by mutations within this structural element.

Key words: actin filaments, thermal aggregation, myopathy

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Class II myosins are actin-based motor proteins that convert the free energy derived from ATP hydrolysis into mechanical work, driving the sliding movement of actin filaments. Myosin II molecules consist of a pair of myosin heavy chains (MHC) and two pairs of light chains. The N-terminal region of the MHC forms a globular domain with ATPase and motile activities. This motor domain consists of four major subdomains connected by structurally conserved, flexible joints [1,2]. Structural studies revealed that the converter, one of these subdomains, rotates by approximately 70° during the ATP hydrolysis cycle [3,4]. This rotation is thought to drive the long-distance swing of the light chain binding domain or "lever arm," which is an extended α-helical region, following the converter [5]. When the motor domain is firmly attached to actin filaments, this lever-arm swing is assumed to generate movement of the myosin molecule relative to the actin filaments. The SH1 helix in the reactive thiol region is a joint that links the converter subdomain to the rest of the motor domain (Fig. 1A), and possibly plays an important role in the arrangement of the converter/lever arm module [2,6].

◄ *Significance* ►

Myosin II SH1 helix is a joint that links the converter subdomain to the rest of the myosin motor domain. In this study, we introduced the E683K or R686C point mutation into the SH1 helix in *Dictyostelium* myosin II, and found that these mutations decreased both the thermal stability of myosin II and the activation energy of a rate-limiting process involved in actin movement, suggesting that the SH1 helix is a key structural element that determines the flexibility and thermal properties of the myosin motor.
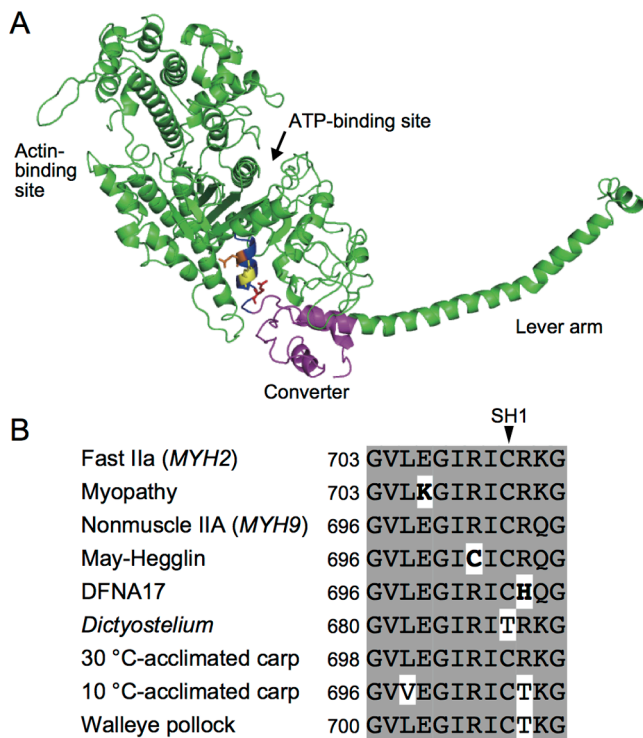
Figure 1 The SH1 helix in myosin II. (A) The crystal structure of MHC S1 of chicken skeletal muscle myosin II [1]. The SH1 helix is shown as a blue ribbon. The converter subdomain is shown as a magenta ribbon. The residues corresponding to Glu-706 in fast MHC-IIa, Arg-702 in nonmuscle MHC-IIA (*MYH9*), and Arg-705 in nonmuscle MHC-IIA are shown as orange, yellow, and red sticks, respectively. (B) Comparison of amino acid sequences of the SH1 helix region of class II MHCs from human skeletal muscle, human nonmuscle, *Dictyostelium*, 30 or 10°C-acclimated carp *Cyprinus carpio* skeletal muscle, and walleye pollock *Gadus chalcogrammus* muscle. Highly conserved residues among myosin IIs are shown in gray. The mutations (E706K linked to inclusion body myopathy, R702C linked to May-Hegglin anomaly, and R705H linked to DFNA17) are shown in bold.

Several point mutations within the SH1 helix in human myosin IIs have been shown to cause diseases (Fig. 1B). For example, E706K in fast MHC-IIa (*MYH2*) is linked to inclusion body myopathy [7]; R702C in nonmuscle MHC-IIA (*MYH9*) is linked to May-Hegglin anomaly [8,9]; and R705H in nonmuscle MHC-IIA is linked to nonsyndromic hereditary deafness DFNA17 [10]. Given the assumed role of the SH1 helix in arranging the converter/lever arm, these diseases were thought to result from altered motile activities [7,11]. Recently, however, we found that the R689H mutation in *Dictyostelium* myosin II (corresponding to R705H in human nonmuscle IIA) reduces the thermal stability and enhances thermal aggregation of the protein [12]. Wang *et al.* reported that *Drosophila* flight muscle myosin containing the E701K mutation (corresponding to E706K in human fast IIa) is more labile and has a higher tendency for aggregation [13]. Originally, Hu *et al.* pointed out that N93K, which lies in close proximity to the SH1 helix in the motor domain, induces aggregation of nonmuscle myosin IIA [11]. These

results raise the possibility that mutations in the SH1 helix affect not only enzymatic and motile activities but also thermal properties of the myosin motor.

This study aims to determine whether mutations in the SH1 helix affect the thermal properties as well as motile activities of myosin II. We introduced the E683K or R686C point mutation into the SH1 helix of myosin II in *Dictyostelium discoideum*, a tractable system for studying myosin II functions. The SH1 helix region is highly conserved among myosin IIs, including that of *Dictyostelium*; E683K and R686C in *Dictyostelium* are equivalent to E706K in fast MHC-IIa and R702C in nonmuscle MHC-IIA, respectively (Fig. 1B). We found that E683K and R686C reduced the thermal stability and activation energy of a process involved in actin movement, suggesting that the SH1 helix is a key structural element determining flexibility and thermal properties of the myosin motor. The defects in thermal stability seem to be involved in the pathogenesis of the above human diseases caused by the mutations in the SH1 helix. In addition, on the basis of the sequences of fish muscle myosin IIs, we propose that cold adaptation of fish muscle myosins is afforded by the altered thermal properties caused by other mutations in the SH1 helix.

Materials and Methods

Construction of plasmids and preparation of proteins

The E683K or R686C mutation was introduced into the *Dictyostelium discoideum mhc* gene using two-step PCR-based mutagenesis as described previously [12]. For E683K, the 5′ fragment was amplified using the 5′ primer 5′-GAGA TTCAAGATTGGTTAG-3′ and the 3′ mutagenic primer 5′-ACGAATACCTTTGAGGAC-3′. The 3′ fragment was amplified using the 3′ primer 5′-AGTGGTAGAGTCGGTGAGA-3′ and the 5′ mutagenic primer 5′-GTCCTCAAAGGTATT CGT-3′. For R686C, The 5′ fragment was amplified using the 5′ primer 5′-GAGATTCAAGATTGGTTAG-3′ and the 3′ mutagenic primer 5′-ACGAGTAATACAAATACCTTC-3′. The 3′ fragment was amplified using the 3′ primer 5′-AGT GGTAGAGTCGGTGAGA-3′ and the 5′ mutagenic primer 5′-GAAGGTATTTGTATTACTCGT-3′. Plasmids carrying either the wild-type or mutant *mhc* gene were introduced into *Dictyostelium* cells lacking the endogenous *mhc* gene [14]. For expression of the mutant S1 fragments, the corresponding regions of the plasmids carrying S1 were replaced with those of the mutants, and these plasmids were introduced into *Dictyostelium* Ax2 cells. All transformants were selected in HL5 medium containing 10 µg/mL G418.

Preparation of the proteins and phosphorylation of the regulatory light chains of myosin were performed as described previously [12]. Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard. Actin was prepared from acetone powder of rabbit skeletal muscle, and the actin concentration was determined spectrophotometrically as described previously [12].

Table 1 Enzymatic and motile activities of the wild-type and mutant myosin IIs

		Wild type	E683K	R686C
Sliding velocity at 30°C (μm/s)		0.61±0.29	0.22±0.01	0.30±0.16
Mg-ATPase kinetic parameters	Basal (s^{-1})	0.030	0.015	0.021
	V_{max} (s^{-1})	0.29	0.31	0.19
	K_{actin} (μM)	7.4	0.85	21
Activation energy (kJ/mol)		95±8	24±9	41±8

Sliding velocities and activation energies are for myosins. Basal and actin-activated Mg-ATPase activities are for S1s. Sliding velocities presented as the mean±S.D. ATPase kinetic parameters were determined by fitting the data to the Michaelis–Menten equation. Activation energies were determined as described in Figure 3.

In vitro motility assay

In vitro motility assays were performed as described previously [12] with slight modifications. Briefly, phosphorylated myosins at 0.1 mg/mL in a high salt solution (0.6 M KCl, 50 mM K-phosphate buffer [pH 6.8]) were introduced into a flow chamber made of nitrocellulose-coated coverslips. After 2 min, the flow chamber was rinsed with a solution containing 10 mM HEPES (pH 7.4), 250 mM KCl, 5 mM MgCl$_2$ and 1 mM DTT, then rinsed with 1 mg/mL BSA in the high salt solution twice. After 2 min, 10 nM F-actin labeled with rhodamine-phalloidin in a motility solution (25 mM KCl, 25 mM imidazole [pH 7.5], 4 mM MgCl$_2$, 2 mM ATP, 0.1 mM CaCl$_2$, 1 mM DTT, 0.04% [w/v] NaN$_3$, 0.2% [w/v] methylcellulose, 1% [v/v] β-mercaptoethanol, 4.5 mg/mL glucose, 0.21 mg/mL glucose oxidase, and 0.035 mg/mL catalase) was applied to the flow chamber. The temperatures of the flow chamber solution were adjusted and measured as described previously [15]. The sliding of actin filaments was analyzed as described previously [16]. To assay thermally inactivated proteins, the flow chambers into which myosins were introduced were rinsed with BSA as described above and incubated at 40°C for 1–25 min. Subsequently, the above described motility solution containing F-actin was introduced, and the number of F-actin attached to the surface was counted at 25°C.

ATPase assay

Basal and actin-activated Mg-ATPase activities of S1s were determined by measuring the amount of phosphate released at 25°C according to Kodama *et al.* [17]. Reaction mixtures contained 50 μg/ml S1 and various concentrations of F-actin (0–30 μM) in an assay buffer (25 mM HEPES (pH 7.5), 25 mM KCl, 4 mM MgCl$_2$, 5 mM ATP and 1 mM DTT). For thermal inactivation of the proteins, the reaction mixtures were incubated in a block incubator at 40°C for 5–30 min before the addition of ATP.

Thermal aggregation assay

The kinetics of thermal aggregation were studied by measuring the light scattering of S1 solutions at 500 nm as described previously [12]. Assay solutions contained 50 μg/ml S1, 25 mM HEPES (pH7.5), 25 mM KCl, 4 mM MgCl$_2$, 5 mM ATP and 1 mM DTT. Samples were maintained at 40°C in a block incubator until each measurement. Apparent first-order rate constants of aggregation were determined by fitting data to the equation for aggregation, $A=A_\infty\{1-\exp[-k(t-t_0)]\}$, where t is time, A is absorbance, A_∞ is the limiting value of A at $t=\infty$, k is the apparent first-order rate constant, and t_0 is the value of t at which $A=0$.

Results

Motile and enzymatic activities

To address whether the mutations in the SH1 helix affect thermal properties and other activities of myosin II, we expressed the E683K or R686C mutant myosin II proteins in *Dictyostelium discoideum*. In vitro actin gliding assays showed that both the E683K and R686C mutations significantly impaired motile activities of *Dictyostelium* myosin II (Table 1), as reported for the corresponding mutations in other myosin IIs [11,13,18]. Even though the sliding velocities were low, these mutants steadily translocated actin filaments, implying that these proteins were only slightly, if any, misfolded. On the other hand, these mutations affected actin-stimulated ATPase activities of *Dictyostelium* myosin subfragment 1 (S1) in a somewhat different manner (Table 1). E683K S1 showed lower K_{actin} than the wild type, while V_{max} was comparable. This result suggests a higher affinity of E683K for actin in the presence of ATP, consistent with a higher duty ratio of this mutant as described previously [19]. Given that the actin sliding velocity is dominated by the actin dissociation rate, the higher duty ratio may explain the slower motility but normal ATPase activity of E683K. In contrast, R686C S1 showed higher K_{actin} and lower V_{max} than the wild type, suggesting that this mutation lowered both the ATPase activity and affinity for actin. These results imply that the kinetic mechanisms underlying the decreased actin sliding velocities differ between E683K and R686C.

Thermal stability

To evaluate the thermal stability of wild type and mutant myosin II, we determined the thermal inactivation rates of the S1s by monitoring the remaining basal Mg-ATPase activities of proteins that were incubated at 40°C (Fig. 2A). The apparent first-order rate constants for the inactivation of wild type, E683K, and R686C S1s were estimated to be

0.017 min⁻¹, 0.14 min⁻¹, and 0.097 min⁻¹, respectively. These results indicate that both mutations significantly decreased the thermal stability of the motor domain and that E683K is still more labile than R686C. The heat labilities of these mutants are also supported by *in vitro* motility assay results indicating that the number of actin filaments attached to the mutant myosin IIs decreased faster than the wild type after thermal inactivation (Fig. 2B). In contrast, the sliding velocities were only slightly affected by thermal inactivation (data not shown), suggesting that the inactivated myosin proteins no longer associated with actin filaments and did not hamper their movements driven by the remaining active proteins. All of these results are similar to those of another SH1 helix mutation, R689H [12], suggesting that the mutations in the SH1 helix generally tend to decrease the thermal stability of myosin II.

Thermally unfolded proteins often form aggregates. This accumulation of aberrant proteins can lead to inclusion bodies in cells. With regard to myosin II, we previously observed that R689H, which decreased thermal stability, accelerated thermal aggregation of the motor domain [12]. To test whether E683K or R686C also enhances thermal aggregation of the motor domain, we studied the thermal aggregation kinetics of the wild type and S1 mutants at 40°C by monitoring light scattering at 500 nm (Fig. 2C). Apparent first-order rate constants for the aggregation of wild type, E683K, and R686C were estimated to be 0.012 min⁻¹, 0.17 min⁻¹, and 0.086 min⁻¹, respectively. These results indicate that thermal aggregation is enhanced by the R686C mutation as well as E683K, which was previously shown to accelerate thermal aggregation of *Drosophila* flight muscle myosin II [13]. Thermal inactivation and aggregation rates were similar for the wild type and the two mutants, as previously shown for R689H [12], suggesting that the irreversible aggregation step was much faster than the unfolding transition step in the thermal denaturation process of myosin.

Activation energy of actin filament sliding

In many cold-adapted enzymes, high structural flexibility leads to low activation energy and high enzymatic activity at low temperatures but also a trade-off in stability [20]. It is possible that R686C and E683K, both of which were shown to reduce thermal stability, affect the activation energy of enzymatic reactions or a process involved in actin movement. We therefore determined the activation energy of actin filament sliding movement for wild-type, R686C, and E683K myosins by examining the temperature dependence of the sliding velocity of actin filaments generated by these myosins using an in vitro motility assay (Fig. 3). We assumed that the light chain content and phosphorylation content of these myosins did not depend on temperature. Because the working stroke of myosin was shown not to be greatly affected by temperature [21], the temperature dependence of the sliding velocity would be mainly due to that of a rate-limiting process during actin movement. The apparent acti-
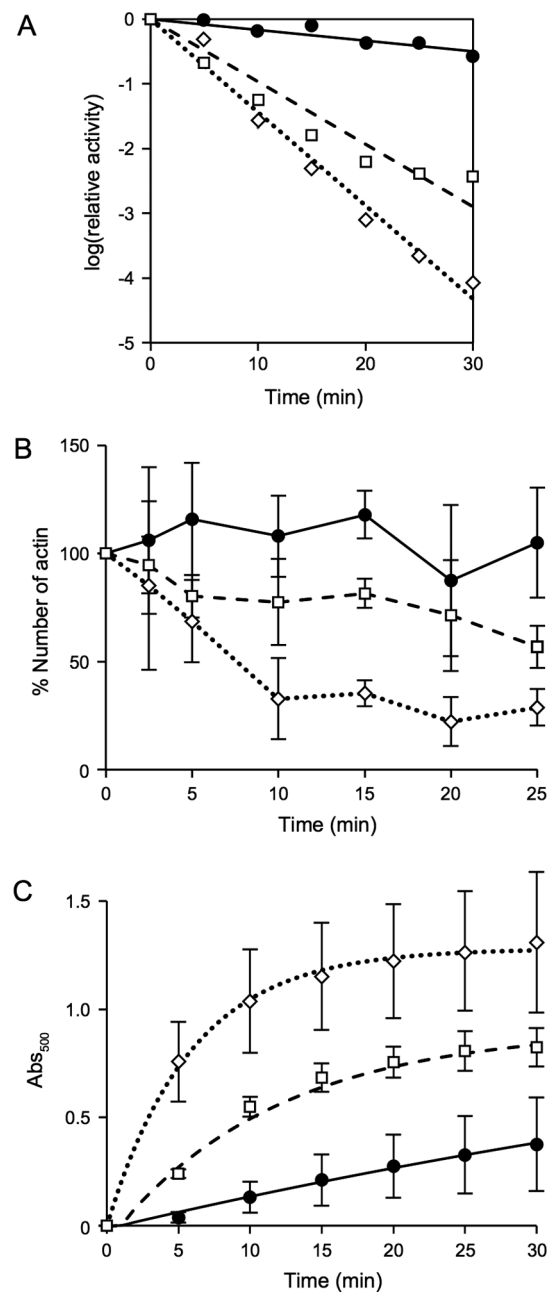

Figure 2 Thermal stability of myosins or S1s. Wild type, E683K, and R686C are represented by a closed circle, open diamond, and open square, respectively. (A) Thermal inactivation of basal Mg-ATPase activities of S1s. The proteins were incubated at 40°C for 5–30 min, and the remaining activities were measured. The semilogarithm of relative ATPase activity was plotted against the incubation time. Apparent first-order rate constants of inactivation for wild type, E683K and R686C were estimated to be 0.017 min⁻¹, 0.14 min⁻¹, and 0.097 min⁻¹, respectively. (B) Relative number of actin filaments attached to myosin-coated surface after thermal inactivation. The flow chambers into which myosins were introduced were incubated at 40°C for 1–25 min, and the number of actin filaments attached to the surface was counted. Data are presented as the mean±S.D. (C) Thermal aggregation kinetics of S1s. Data are presented as the mean±S.D. Apparent first-order rate constants of the aggregation for the wild type, E683K, and R686C were estimated to be 0.012 min⁻¹, 0.17 min⁻¹ and 0.086 min⁻¹, respectively.
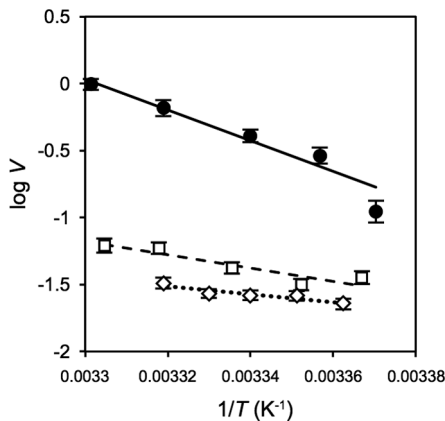
Figure 3 Temperature dependence of sliding velocities of actin filaments on myosins. The semilogarithm of the mean velocities (V) was plotted against the inverse absolute temperature (T). Wild type, E683K, and R686C are represented by a closed circle, open diamond, and open square, respectively. Error bars represent the standard errors. The solid, dotted, and dashed lines are best fits of the data for wild type, E683K, and R686C, respectively, as determined using the Arrhenius equation $V=V_0 \exp(-E_a/k_B T)$, where E_a is the activation energy and k_B is the Boltzmann constant, as described in [15].

vation energies were determined by fitting the sliding velocity data to the Arrhenius equation $V=V_0 \exp(-E_a/k_B T)$, where E_a is the activation energy and k_B is the Boltzmann constant, as described in [15], and estimated to be 95, 24, and 41 kJ/mol for the wild type, E683K, and R686C, respectively (Table 1). These results indicate that both mutations significantly lower the activation energy of a rate-limiting process during actin movement. Moreover, the activation energy of E683K was still lower than that of R686C, in agreement with the thermal stability data, implying that the activation energy is related to the thermal stability of the protein. Although E683K and R686C showed the lower activation energies, their motile activities were lower than the wild type even at low temperature. This is possibly because the values of the pre-exponential factor V_0 for these mutants are smaller than those for the wild type.

Discussion

Our results indicate that the activation energy of a rate-limiting process involved in actin movement was lowered by the mutations in the SH1 helix of E683K and R686C. As is well known, the activation energy is an energy required for crossing a energy barrier of the process by thermal activation. The SH1 helix is a joint that links the converter subdomain to the rest of the motor domain (Fig. 1A), and is thought to play an important role in the arrangement of the converter/lever arm module [2,6]. Mutations in the SH1 helix are likely to destabilize the helix, leading to looser coupling between the subdomains and higher flexibility of the motor domain, as proposed previously [11,13]. This increased flexibility would increase thermal fluctuations of the subdomains,

thereby lowering both the thermal activation energy of the swinging process of the converter/lever arm module and the thermal stability of the protein. Indeed, we previously showed that another mutation in the SH1 helix, R689H, decreased cross-bridge stiffness, thermal stability, and the activation energy of the ATP-induced conformational transition [12,22]. Meanwhile, the looser coupling between the subdomains may affect the motor conformational changes, in particular the lever arm swing, and thus slow the movement of actin filaments. Yang *et al.* suggested that in the actomyosin cycle, an energy barrier exists between the pre-power stroke and post-rigor conformational change [23]. Our results thus support the view that the main energy barrier in the actomyosin cycle is related to the swinging of the converter/lever arm module, overcome by thermal fluctuations of the subdomains. Yang *et al.* further proposed that the internally uncoupled state characterized by an unwound SH1 helix provides a pathway that lowers the energy barrier. Mutations that destabilize the SH1 helix might also facilitate the unwinding of the helix and this internally uncoupled state.

In many cold-adapted enzymes, high flexibility results in low activation energy but also low stability [20]. Our results indicate that this is also true for the SH1 helix mutations such as E683K and R686C in myosin II. Moreover, these mutations accelerate thermal aggregation of the protein owing to their heat labilities. The higher aggregation tendency explains why the diseases caused by SH1 helix mutations in human myosin IIs frequently show inclusion bodies in cells: E706K in fast MHC-IIa (E683K in *Dictyostelium*) results in cytoplasmic inclusions and accumulation of aberrant proteins in muscle [7,24]; R702C in nonmuscle MHC-IIA (R686C in *Dictyostelium*) results in leukocyte inclusions [8,25,26] or aggregation of the myosin protein itself [26,27]. In contrast to these thermosensitive mutations, G680V and G691C, which are bulky SH1 helix mutations, were previously shown to be cold sensitive in *Dictyostelium* cells [28]. Taken together, these results suggest that the SH1 helix is a key structural element that determines flexibility and thermal properties of the myosin II motor domain, consistent with the structural view that the helix acts as a clutch that controls coupling between the converter and the rest of the motor [6].

Interestingly, fast skeletal myosin isolated from cold-acclimated carp showed a lower activation energy for actin sliding movement, accompanied by lower thermal stability [16,29]. In carp fast skeletal muscle, different MHC isoforms are expressed at cold and warm environmental temperatures [29]. We therefore re-examined the sequence of the MHC isoform predominantly expressed in the 10°C-acclimated carp [30] and the published sequences of MHCs from other fishes in cold environments. We found several point mutations within the SH1 helix in myosins from the cold-acclimated carp and also from walleye pollock, which possesses one of the most heat labile myosins [31]. In particular, a mutation at

a conserved arginine was found both in 10°C-acclimated carp (R705T) and pollock (R708T) myosins (Fig. 1). Previously, we found that the arginine to histidine mutation at the same site, which in human nonmuscle myosin IIA is linked to nonsyndromic hereditary deafness DFNA17 (R705H) [10], decreased the thermal stability and activation energy of the ATP-induced conformational transition of *Dictyostelium* myosin (R689H) [12,22]. Thus, the mutation at the conserved arginine in the SH1 helix may be one of the mutations that allows for the lower activation energies of the enzymatic reactions [29,32] and a rate-limiting process involved in actin movement [16], thereby enabling adaptation of the skeletal muscles of these fish to cold environments. These observations further support our conclusion that the SH1 helix is a determinant of the flexibility and thermal properties of the myosin motor.

In conclusion, our results demonstrate that the point mutations within the SH1 helix in myosin II altered not only motile activities but also thermal properties involved in actin movement. Given that the SH1 helix is a joint that links the converter subdomain to the rest of the motor, it is relevant that these mutations alter coupling between the subdomains and thus their thermal properties. Based on these and other results, we propose that the SH1 helix is a key structural element that determines the flexibility and thermal properties of the myosin motor. These characteristics of the SH1 helix may contribute to the pathogenesis of the human diseases caused by mutations within this structural element. Furthermore, we suggest that some mutations in the SH1 helix might also contribute to the adaptation of fish muscle myosins to cold environments. Since our results in this study were obtained using *Dictyostelium* myosin II, further studies using human and fish muscle or nonmuscle myosin IIs will be necessary to confirm these speculations. Nevertheless, the proposed characteristics of the SH1 helix have potential to explain the properties of both the human diseases and the fish cold adaptation.

Acknowledgments

Conflict of Interest

None declared.

Author Contributions

S.C. and S.I. designed the work. K.S. , T.K. and S.I. performed the experiment. S.C. and S.I. wrote the manuscript.

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