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Electrostatic interactions in the force-generating region of the human cardiac myosin modulate ADP dissociation from actomyosin

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Abstract

Human cardiac myosin has two isoforms, alpha and beta, sharing significant sequence similarity, but different in kinetics: ADP release from actomyosin is an order of magnitude faster in the alpha myosin isoform. Apparently, small differences in the sequence are responsible for distinct local inter-residue interactions within alpha and beta isoforms, leading to such a dramatic difference in the rate of ADP release. Our analysis of structural kinetics of alpha and beta isoforms using molecular dynamics simulations revealed distinct dynamics of SH1:SH2 helix within the force-generation region of myosin head. The simulations showed that the residue R694 of the helix forms two permanent salt bridges in the beta isoform, which are not present in the alpha isoform. We hypothesized that the isoform-specific electrostatic interactions play a role in the difference of kinetic properties of myosin isoforms. We prepared R694N mutant in the beta isoform background to destabilize electrostatic interactions in the force-generating region of the myosin head. Our experimental data confirm faster ADP release from R694N actomyosin mutant, but is not as dramatic as the difference of kinetics of ADP release in the alpha and beta isoforms.

Keywords

human cardiac myosin; SH2 helix; ATP; transient kinetics; mutant; force-generation region

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Introduction

Myosins are motor proteins that generate force using ATP hydrolysis and move along actin filaments. There are more than 35 classes of myosins in the myosin superfamily, each class consisting of multiple isoforms, and performing diverse cellular functions. Although the reaction pathway of actin-activated myosin ATPase is conserved for all myosin isoforms, the rate and equilibrium constants that characterize the ATPase pathway vary significantly across myosin superfamily. Therefore, determining the individual rate and equilibrium constants of ATP binding, ATP hydrolysis, and ADP release provides us with valuable information about each myosin's structural mechanism.

There are two human cardiac myosin isoforms, α and β , they share 93% sequence identity, but different kinetically. Transient kinetics assays show that ADP release from the α isoform is an order of magnitude faster than from the β isoform [1]. These differences have profound implications on the muscle physiology. The goal of our work is to determine the structural origin of such a dramatic difference of myosin isoforms kinetics. The sequence alignment of human cardiac myosin isoforms resulted in 80 different amino acid residues in the myosin head. It is not practical to mutate all 80 different amino acid residues to discover myosin regulatory sites and structural elements, responsible for the difference in kinetics. To explore potential regulatory sites within myosin head we built the homology model of the α isoform based on known crystal structure of the β isoform (4DB1.pdb). We run molecular dynamics (MD) simulations for both isoforms to detect difference in their structural dynamics and analyzed electrostatic interactions between charged residues during obtained MD trajectories for both myosin isoforms. Using this information, we pinpointed different stability of salt bridges within the force-generating region of the myosin head. Our analysis showed that the residue R694 (β isoform sequence) forms two stable salt bridges in the β isoform. These salt bridges are not present in the α isoform. We prepared R694N mutant of the human cardiac myosin (β isoform background) to disrupt such electrostatic interactions. In the transient kinetics assay we measured the kinetics of ADP release from actomyosin (step 5, Figure 1) in the wild type (WT) myosin and in the mutant. We observed a statistically significant difference in the kinetics of ADP release from actomyosin between the WT myosin and the mutant, although not as dramatic as the difference of the α and β isoforms of the WT human cardiac myosin.

Materials and Methods

Ethical approval

Actin was produced from rabbit skeletal tissue. All experimental protocols were approved by the Institutional Animal Care and Use Committee of UNC Charlotte and all experiments were performed in accordance with relevant guidelines and regulations.

Protein preparation

The β isoform construct of human cardiac myosin motor domain contains 1–843 residues and FLAG affinity tag at the C-terminus. Adenoviruses encoded with the WT and R694N myosin mutant were purchased from Vector Biolabs (Malvern, PA). Recombinant human

cardiac myosin was expressed in C2C12 (ATCC CRL-1722) cells. Actin was prepared from rabbit leg and back muscles as described elsewhere [2, 3]. Purified F-actin was labeled with pyrene iodoacetamide (Life Technologies Corporation, Grand Island, NY) [2]. Details of myosin and actin preparations can be found in the Supplementary Material.

Acquisition of fluorescent transients

In the ATP-induced actomyosin dissociation experiment, usually 0.5 μM actomyosin was rapidly mixed with ATP solution of variable concentrations. In the competitive inhibition of the ATP-induced actomyosin dissociation experiment, 0.5 μM actomyosin was rapidly mixed with the premixed ATP and ADP solution. The concentration of ATP in solution was 0.9 mM, the concentration of ADP varied from 5 μM to 200 μM . Transient fluorescence of pyrene-labeled actin was measured with Bio-Logic SFM-300 stopped flow transient fluorimeter (Bio-Logic Science Instruments SAS, Claix, France), equipped with FC-15 cuvette. All experiments were performed at $T=20^\circ\text{C}$.

Analysis of fluorescence transients

The transients obtained in each experiment were fitted by the single exponential function $S(t) = S_0 + A \cdot \exp(-k_{\text{obs}} \cdot (t - t_0))$, or the double exponential function $S(t) = S_0 + A_1 \cdot \exp(-k_{\text{obs}1} \cdot (t - t_0)) + A_2 \cdot \exp(-k_{\text{obs}2} \cdot (t - t_0))$. $S(t)$ is the observed signal at the time t , A_i is the signal amplitude, t_0 is the time before the flow stops, and $k_{\text{obs}i}$ is the observed rate constant. Transients, obtained for the same actomyosin preparation at different concentrations of the nucleotide were fitted together, assuming the known value of t_0 , measured in a separate experiment, and the constant value of S_0 , which depends on the concentration and labeling efficiency of pyrene-labeled actin in the actomyosin preparation. In the case of the two-exponential global fit, we kept amplitudes of the transients constrained, $A_1 + A_2 = \text{const}$, to account for the conservation of mass. Transients of the ATP induced actomyosin dissociation were fitted with the one-exponential function to determine the rate constant k_{obs} . The dependence of the observed rates on the ATP concentration was fitted by a hyperbola, $v = V_{\text{max}} \cdot [\text{ATP}] / (K_{\text{app}} + [\text{ATP}])$, allowing the determination of the maximum rate, V_{max} (the horizontal asymptote). In the case of the ATP induced actomyosin dissociation, the rate constant k_{+2} is V_{max} and the equilibrium constant of the collision complex formation K_1 is $1/K_{\text{app}}$. To determine the bimolecular rate ($K_1 k_{+2}$), the dependence of the observed rates on the ATP concentration was fitted by a straight line at small concentrations of ATP. Transients of the competitive inhibition experiment were fitted with the two-exponential function, where the rate constant of the fast process reflects the ATP induced actomyosin dissociation, and the slower process is the competitive inhibition of the ATP induced actomyosin dissociation by ADP, reporting the rate of ADP release from actomyosin. The ADP dependence of the reaction rate of the slower component of the observed transient was fitted to the equation $k_{\text{obs}} = V_{\text{max}} \cdot [\text{ATP}] / (K_{\text{app}} \cdot (1 + [\text{ADP}] / K_5) + [\text{ATP}])$ [4–6], using V_{max} and K_{app} determined in the experiment without ADP. The fit lets to determine K_5 , which is the equilibrium constant of ADP dissociation from actomyosin upon chasing with ATP. The rate constant k_{+5} was measured at the close to saturated ADP and ATP concentrations, the transients were fitted to the two-exponential function, with the rate constant of the fast exponent fixed at the rate of actomyosin dissociation, to account for the non-complete saturation with ADP. Rate constants were obtained in experiments with proteins from at

least three independent preparations. Obtained rate constants were averaged and mean values and standard deviations were used to fit with equations listed above. Determined constants are the mean value and the standard error. All data fits were performed with Origin 8 (OriginLab Corp, Northampton MA). Statistical significance of results was tested with ANOVA integrated in Origin 8 software. A significance level of $P < 0.05$ was used for all analyses.

Results

Isoform-specific salt bridges in myosin force-generation region

Figure 2 shows the location of salt bridges in the force-generating region, as determined in our MD computer simulations of the α and β isoforms. Salt bridges in this region exhibit different stability along the MD trajectory (Table S1), this makes electrostatic interactions within the region isoform-specific. Stable bridge R694:(E45, E98), linking the SH2 helix to the β barrel and an α helix in the N-terminal domain is β isoform-specific, this bridge is not present in the α isoform. Figure S1 shows the distance between residues R694 and E45 and E98 in both isoforms along the MD trajectory, leading to the conclusion that the bridge is isoform-specific. In our computer simulations we observed unfolding of the SH1 helix in the β isoform, the helix remains folded in the α isoform along the whole MD trajectory. SH1 helix unwinding was extensively discussed previously to explain crosslinking of C707 and C697 (rabbit skeletal muscle myosin sequence) with a pPDM linker, which is significantly shorter than the C707:C697 distance [7–12]. Unwound SH1 helix was observed in the atomic structure of scallop striated muscle myosin, complexed with ADP [13], and it was proposed that the SH1 helix melts during myosin ATPase cycle [14, 15].

ATP-induced actomyosin dissociation is slower in the myosin mutant

The rate of ATP-induced actomyosin dissociation was measured using pyrene-labeled actomyosin that was rapidly mixed with various concentrations of ATP solution. The actomyosin dissociation was monitored via an increase of pyrene fluorescence, and observed transient was fitted with a single exponent to determine the reaction rate [16]. The dependence of the reaction rates on the ATP concentration was fitted with a hyperbola (Figure 3). The maximum rate of actomyosin dissociation $V_{\max}$ of the mutant is about 70% of the $V_{\max}$ of the WT myosin (Table 1). The constant K_{app} , reflecting half saturation of actomyosin with ATP and inversely proportional to the equilibrium association constant of ATP and actomyosin, is about 60% smaller for the mutant relative to the WT myosin. The second order reaction rate constant of ATP binding to actomyosin is $2.12 \pm 0.12 \mu\text{M}^{-1}\text{s}^{-1}$, in agreement with the literature data [1]. For the mutant the rate constant is slightly smaller, $1.85 \pm 0.02 \mu\text{M}^{-1}\text{s}^{-1}$.

The equilibrium constant of ADP dissociation from actomyosin is similar for the WT and the mutant myosin

To measure the ADP dissociation equilibrium constant K_5 , we used the competitive inhibition reaction. The pyrene-labeled actomyosin was rapidly mixed with the premixed ATP and ADP solution, the concentration of ATP was constant and the concentration of ADP varied. The ADP dissociation from actomyosin was monitored via an increase in

pyrene fluorescence due to actomyosin dissociation following the dissociation of ADP and the subsequent ATP binding. The ADP dissociation is a slow process as compared to the ATP-induced actomyosin dissociation, and therefore the rate measured in the experiment is determined by the kinetics of ADP dissociation. The observed transients of pyrene fluorescence were best fitted using a double-exponential function, which is the indication of the tight actomyosin-ADP complex [16]. Fast component reflects ATP-induced actomyosin dissociation, slow component reflects the rate of ADP dissociation in the competitive inhibition reaction. Obtained reaction rates at different ADP concentrations were fitted using the model of competitive inhibition [6] (Figure 4). We found that the constant K_5 is similar for the WT and mutant myosin, $1.37 \pm 0.25 \mu\text{M}$ and $2.03 \pm 0.50 \mu\text{M}$ accordingly (Table 1). The data for the WT is in a good agreement with the previous studies [1].

ADP dissociation from actomyosin is faster in the myosin mutant

The rate of ADP release was determined by rapid mixing of actomyosin with premixed ATP and ADP solutions. The concentration of ADP was 0.1 mM, much higher than the equilibrium ADP dissociation constant K_5 , the concentration of ATP was 0.9 mM to displace ADP efficiently. The observed transients of pyrene fluorescence were fitted with the sum of two exponents [16], assuming that the fast component reflects the ATP induced actomyosin dissociation and the slow component reflects ADP release from actomyosin, followed with fast actomyosin dissociation induced by ATP binding. The obtained rate of the fast component was close to the determined value of the ATP induced actomyosin dissociation rate. The obtained reaction rates of ADP dissociation from actomyosin are about an order of magnitude slower than the rate of ATP induced actomyosin dissociation, confirming the competitive inhibition of the ATP induced actomyosin dissociation. We found that ADP dissociates faster from actomyosin with the mutant myosin than from the WT actomyosin, $k_{+5} = 57.1 \pm 4.8 \text{ s}^{-1}$ for the mutant vs $40.5 \pm 9.2 \text{ s}^{-1}$ for the WT (Table 1).

Discussion

The force-generating region is highly conserved among various myosins, and introduction of perturbations in the region affects myosin kinetics. The region connects the myosin head and the converter domain, which continues as a lever arm, connecting myosin head to the thick filament in a sarcomere. Structural elements of the region, the relay helix and the SH2-SH1 helix, position myosin head relative to the converter domain; the SH1 helix serves as a pivot, and the relay helix rotates the converter domain 60° during the power stroke [17]. Modification of the reactive cysteines SH1 and SH2 [18–20], crosslinking of the reactive cysteines [9], site-directed mutagenesis within the relay helix [21–23], relay loop [24, 25], and the SH1-SH2 helix [23, 26–30], all these perturbations change actomyosin kinetics. Restrictive perturbations, such as crosslinking of SH1 and SH2 cysteines, labeling of the reactive cysteines with bulky probes, replacement of the flexible “hinge” glycine within SH1-SH2 helix with larger alanine or valine, shift structural equilibrium of myosin towards the ADP bound conformation [9, 18, 31]. Our MD simulations show that the β isoform has the permanent salt bridge R694:(E45, E98) in the region, and we hypothesized that this permanent salt bridge plays a role of a restriction in the region, responsible for the longer lasting ADP bound state of the β isoform. The salt bridge is not present in our homology

model of the α isoform and we hypothesized that elimination of the salt bridge in the force-generating region will make it less restricted and permits faster ADP release. The residue R694 is the next residue to the reactive cysteine C695 (SH2 cysteine) and just two residues apart from G697, the proposed pivoting point of SH1 and SH2 helices. Such a location makes R694 a good candidate for the regulatory site within the SH2 helix, which introduces structural restrictions in the force generating region by forming the salt bridge R694:(E45, E98). Our transient kinetics data show that R694N mutation in the β isoform causes the increase of the rate of ADP release from actomyosin k_{+5} . Observed increase of the rate of ADP dissociation is small compared to the order of magnitude difference of the same kinetic step, observed in the WT α and β isoforms previously [1]. Our analysis of MD trajectories shows that there are several places within myosin head with electrostatic interactions, different in the α and β isoforms. Elucidation of the role of isoform-specific electrostatic interactions within myosin head is the subject of our ongoing studies; isoform-specific salt bridges in other parts of the myosin head may contribute to the observed difference of ADP retention in myosin isoforms.

Smaller rate of actomyosin dissociation upon ATP binding (k_{+2}), faster rate of ADP release (k_{+5}), and faster formation of the initial collision complex of actomyosin-ATP (K_{-1}) tell that the mutation in the force-generating region affects events at the nucleotide binding site, about 4 nm away. Our MD simulations did not reveal any correlations in the dynamics of the force-generating region and the active site, meaning the absence of a simple mechanical connector, transducing structural information from the force-generating region to the active site. Apparently, such signal transduction is governed by a series of small conformational changes along the whole myosin head, thus affecting myosin's interaction with the nucleotide and actin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. α and β isoforms of the human cardiac myosin have isoform-specific salt bridges
2. ATP-induced actomyosin dissociation is slower in the α -mimic β myosin mutant
3. ADP dissociation from actomyosin is faster in the α -mimic β myosin mutant

Figure 1.

Acto-myosin ATPase cycle reaction scheme. A = actin, M = myosin (M* and M** - myosin states with increased intrinsic fluorescence), T = ATP, D = ADP, P = phosphate. Asterisk indicates reaction rate constants describing acto-myosin kinetics. Boxed = generally accepted pathway of acto-myosin interaction.

Figure 2.

Pictorial representation of the force generation region of myosin head, α isoform (left) and β isoform (right). Double lines represent salt bridges, observed during the MD trajectory.

Figure 3.

ATP-induced actomyosin dissociation. Circles, WT, N=3, squares, R694N mutant, N=3 (the point at [ATP]=600 μM, N=1). (Right) The observed reaction rates at low [ATP] are fitted by a straight line, the second order reaction rate constant is determined from the slope of the line. $K^{-1}k_{+2} = 2.12 \pm 0.12 \mu\text{M}^{-1}\text{s}^{-1}$, WT, and $1.85 \pm 0.02 \mu\text{M}^{-1}\text{s}^{-1}$, mutant. (Left) The observed reaction rates are fitted with a hyperbola, $V_{\text{max}} = 491.5 \pm 42.8 \text{ s}^{-1}$, WT, and $338.8 \pm 27.6 \text{ s}^{-1}$, mutant.

Figure 4.

ATP induced ADP dissociation from actomyosin, [ATP] = 900 μM (circles, WT, N = 3, squares, R694N mutant, N = 3, the point at [ADP]=20 μM, N=1). Lines, fit by the model of competitive inhibition with variable parameter K_5 . $K_5 = 1.37 \pm 0.25$ μM, WT, and 2.03 ± 0.50 μM, the mutant.

Table 1

Actomyosin kinetic rate constants, mean $\pm$ SE. The data are averages of three independent protein preparations, T=20°C.

	WT	mutant
Actomyosin dissociation		
$K_{-1}k_{+2}, \mu\text{M}^{-1}\text{s}^{-1}$	2.12 $\pm$ 0.12	1.85 $\pm$ 0.02
k_{+2}, s^{-1}	491.5 $\pm$ 42.8	338.8 $\pm$ 27.6
Collision complex formation		
K_{+1}, mM^{-1}	4.5 $\pm$ 0.8	7.8 $\pm$ 1.5
ADP release from actomyosin		
$K_{-5}, \mu\text{M}$	1.37 $\pm$ 0.25	2.03 $\pm$ 0.50
k_{+5}, s^{-1}	40.5 $\pm$ 9.2	57.1 $\pm$ 4.8