Temperature dependence of speed of actin filaments propelled by slow and fast skeletal myosin isoforms

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Rossi, R., M. Maffei, R. Bottinelli, and M. Canepari. Temperature dependence of speed of actin filaments propelled by slow and fast skeletal myosin isoforms. J Appl Physiol 99: 2239–2245, 2005. First published August 11, 2005; doi:10.1152/japplphysiol.00543.2005.—It was shown that the temperature sensitivity of shortening velocity of skeletal muscles is higher at temperatures below physiological (10–25°C) than at temperatures closer to physiological (25–35°C) and is higher in slow than fast muscles. However, because intact muscles invariably express several myosin isoforms, they are not the ideal model to compare the temperature sensitivity of slow and fast myosin isoforms. Moreover, temperature sensitivity of intact muscles and single muscle fibers cannot be unequivocally attributed to a modulation of myosin function itself, as in such specimen myosin works in the structure of the sarcomere together with other myofibrillar proteins. We have used an in vitro motility assay approach in which the impact of temperature on velocity can be studied at a molecular level, as in such assays acto-myosin interaction occurs in the absence of sarcomere structure and of the other myofibrillar proteins. Moreover, the temperature modulation of velocity could be studied in pure myosin isoforms (rat type 1, 2A, and 2B and rabbit type 1 and 2X) that could be extracted from single fibers and in a wide range of temperatures (10–35°C) because isolated myosin is stable up to physiological temperature. The data show that, at the molecular level, the temperature sensitivity is higher at lower (10–25°C) than at higher (25–35°C) temperatures, consistent with experiments on isolated muscles. However, slow myosin isoforms did not show a higher temperature sensitivity than fast isoforms, contrary to what was observed in intact slow and fast muscles.

Oxygen inside the specimen (15), and this phenomenon might be more evident for the fast, more energy-demanding muscles. Moreover, intact muscles, being invariably composed of different fiber types, cannot provide a precise assessment of temperature sensitivity of a single fiber type. The analysis of isolated single muscle fibers would overcome the latter problem because single muscle fiber often contains only one isoform and their myosin heavy chain (MHC) isoform content can be precisely assessed (36). However, chemically skinned fibers have been studied mostly at low (12–15°C) temperature. At physiological temperature, they are mechanically unstable owing to increasing disorder in the striation pattern. Moreover, at physiological temperature, skinned fibers might suffer from insufficient energy supply because mitochondria are lacking, and energy supply depends on diffusion of a regenerating system (phosphocreatine and creatine kinase) inside the fiber and on its rate of ATP resynthesis. A significant improvement in the use of single muscle fibers at physiological temperature has been achieved using a “temperature-jump” protocol, in which the fiber was activated at low temperature and then transferred to high temperature (30). However, such an approach might not be free from problems because it cannot fully address the energetic issue and the fiber can go through some warming and striation disorder during transit from low to high temperature. Furthermore, because in both intact muscles and single fibers myosin works together with other sarcomere proteins in the complex structure of the sarcomere, temperature sensitivity might not solely depend on the properties of the myosin molecule.

The precise understanding of the temperature sensitivity of slow and fast myosin isoforms is relevant in defining their biological role. A very recent work suggested that ATPase activity, a well-known determinant of velocity (4), of slow and fast myofilbrils becomes very similar at physiological temperature (10). The latter work underlines the importance of investigating the properties of pure myosin isoforms at temperatures up to physiological.

To overcome the above problems and define the temperature sensitivity of fast and slow myosin isoforms at a molecular level we used an in vitro motility assay (IVMA) approach (1) in which the speed of sliding of actin filaments propelled by myosin molecules immobilized on a coverslip is analyzed. In IVMA, actin sliding velocity depends solely on the properties of myosin because the sarcomeric organization is lost and the other myofibrillar proteins are not present. Therefore, IVMA...
enabled study of the effects of temperature on the function of myosin molecules independently from the possible modulatory role of other sarcomeric proteins. Moreover, the analysis could be performed on pure myosin isoforms that were extracted from single muscle fibers of known myosin isoform content and loaded in IVMA (11–13, 22, 31, 39). Finally, temperature sensitivity could be studied in a wide range of temperatures (10–35°C) because myosin molecules isolated from the sarcotendinous structure are stable at physiological temperature (1). In IVMA, in fact, myosin function cannot be affected by disorder of striation pattern and by lack of energy supply because myosin being distributed at low concentrations on a surface has very easy access to the abundant ATP in the bathing solution.

The present data show that at the molecular level the temperature sensitivity of pure myosin isoforms is higher at the lowest than at the highest temperatures consistent with what is shown in isolated muscles and single muscle fibers (30, 33, 34). However, the velocity of pure slow and fast myosin isoforms showed the same temperature sensitivity at a molecular level, contrary to that observed for slow and fast muscles and muscle fibers (10, 33, 42). The latter finding suggests that at physiological temperature myosin isoforms maintain their large differences in shortening velocity, conferring to muscles the remarkable capacity to finely adjust their performance by recruiting fibers containing different myosin isoforms.

**MATERIALS AND METHODS**

**Animals and Muscle Sampling**

Two New Zealand adult male rabbits (3.0–3.3 kg) and six Wistar adult male rats (0.34–0.37 kg) were used in this study. Rabbits were killed by stunning; rats were anesthetized with ether and then decapitated. Psosas and soleus muscles were dissected from the animals, placed in cold skinning solution (6), and divided into several fiber bundles that were stored at −20°C in skinning solution with 50% glycerol for up to 3 wk. On each experimental day, fiber bundles were removed from the freezer and used for myosin extraction.

The experimental protocol for the study on animals was approved by the local Animal Ethics Committee; all the experimental procedures conformed with the U.K. Animal (Scientific Procedures) Act 1986.

**Experimental Approach**

Pure rabbit myosin isoforms were extracted from bulk soleus (almost 100% type 1 myosin) and psosas (almost 100% type 2X myosin) muscles (23, 40).

To obtain pure rat myosin isoforms, an approach based on extraction of myosin from pure muscle fibers was used (12, 13). Because single fibers often contain only one type of MHC isoform, they are a convenient source of pure myosin isoforms. However, because of the short length of the fibers, one fiber segment does not provide sufficient myosin to be used more times in IVMA experiments. Therefore, 1) single fibers were dissected, 2) SDS-PAGE was performed (6) to identify MHC isoform content, 3) ~30–40 fibers shown to contain the same MHC isoform were pooled, and 4) myosin was extracted from pooled fibers. To maintain myosin function in IVMA, all the above procedures had to be performed within 48 h after the removal of a fiber bundle from the freezer.

**Experimental Procedures**

*Fiber typing and MHC identification.* Single fibers (at least 12 mm long) were manually dissected from a single bundle, chemically skinned for 1 h in skinning solution containing 1% Triton, and cut into two segments. The shorter segment (~2 mm long) was characterized for MHC isoform composition by 8% SDS-PAGE, as described by Pellegrino et al. (31). The same electrophoresis protocol was used to determine the MHC isoform composition of whole muscle samples.

*Myosin extraction from single fibers.* The longest segments of dissected fibers (at least 10 mm long) containing the same MHC composition were pooled together, and myosin was extracted according to Canepari et al. (13). About 60–80 µg of pure myosin could be obtained from a pool of 30–40 fibers. This amount of myosin was enough to let us prepare more samples to be loaded in IVMA and analyzed in a temperature range from 10 to 35°C. This protocol was repeated two times for each myosin isoform; thus ~240 fibers were analyzed.

*Myosin extraction from bulk muscles.* Pure rabbit myosin isoforms were extracted from bulk muscles according a procedure previously described in detail (11).

Rat myosin was also prepared from bulk soleus (80% type 1 and 20% type 2A myosin) and bulk psosas (80% type 2B and 20% type 2X myosin) muscles. Rat myosin extracted from bulk muscles was used only to prepare heavy meromyosin fraction.

*Heavy meromyosin preparation.* Heavy meromyosin (HMM) was obtained by a proteolytic digestion with α-chymotrypsin of myosin according to a modification of the method of Margossian and Lowey (27) previously described in detail (11).

*Actin preparation.* G actin was extracted as described by Pardee and Spudich (29) from acetone powder prepared from the residues of rabbit muscles after myosin extraction. After polymerization, F actin was labeled by incubation for several hours with rhodamine-phalloidin (Molecular Probes R415) as described by Kron et al. (26).

*IVMA.* Myosin (or HMM) was diluted to 0.1 mg/ml in a high (or low) ionic strength buffer and infused in a flow cell treated with nitrocellulose and prepared according to Anson et al. (3). The IVMA analysis was performed according to Canepari et al. (11, 13) in the temperature range 10–35°C. A water-glycol ethylene solution from a (Thermo Haake DC10) thermostat was circulated through a coil pipe created in the microscope stage and in a jacket of Perspex surrounding the microscope objective to have the desired temperature in the flow cell. The flow-cell temperature was continuously monitored in all experiments by a thermometer probe (Delta Ohm HD8601P Thermometer) placed outside but very closely in contact with the flow-cell.

The composition of the experimental buffer was MOPS 25 mM (pH = 7.4 at 25°C), KCl 25 mM, MgCl₂ 4 mM, EGTA 1 mM, DTT 1 mM, glucose oxidase 200 µg/ml, catalase 36 µg/ml, glucose 5 mg/ml, and ATP 2 mM. Average velocities of actin filaments were determined with single preparations of rat myosin sample, the velocities of at least 50 filaments were measured and their distribution was characterized according to parametric statistics. At each temperature at least five samples of each isoform were analyzed.

*Effect of MgADP on filament velocity.* The increase in MgADP concentration in the IVMA buffer is known to decrease actin sliding velocity (Vf) (20). As MgADP production from ATP hydrolysis increases with increasing temperature, IVMA experiments were performed with both slow and fast rat myosin at 35°C in presence or in absence of 5 mM creatine phosphate (CP) and 100 units/ml creatine phosphokinase (CPK) in the experimental buffer. The CP/CPK system is often used in muscle fiber works (18, 42) because it prevents the accumulation of ADP.

**Data Analysis**

Data were expressed as means ± SE. Statistical significance of the differences was assessed by two-way ANOVA for repeated measures
Where $R$ is the gas constant, and Bottinelli et al. (7):

No significant differences were found among different isoforms.

Less than 5% ($P < 0.05$) was considered to be significant.

At all temperatures $V_f$ was significantly lower in slow type 1 than in fast type 2A myosin and in fast type 2A than in fast type 2B myosin ($P < 0.05$). Figure 1A shows that $V_f$ of all isoforms increased with increasing temperature and that the relative differences in $V_f$ among the three isoforms were maintained at all temperatures.

In Fig. 1B, the relation between $V_f$ and temperature is described by an Arrhenius plot. For each isoform the data points were best fitted by two separate regression lines, one in the range 10–25°C and one in the range 25–35°C. The slopes of the two regressions were significantly different ($P < 0.05$) for all isoforms. From the slopes of the regressions, $E_a$ could be determined, and from $E_a$, $Q_{10}$ values were calculated (Table 1). The transition in the Arrhenius plot indicates a change in $E_a$ occurring at 25°C. For all isoforms, both $E_a$ and $Q_{10}$ were 1.5-fold higher in the 10–25°C than in the 25–35°C range (Table 1).

In both temperature ranges, the slopes of the Arrhenius plot of the three myosin isoforms were compared. No significant difference was found, indicating that the three myosin isoforms had a similar temperature dependence (Fig. 1B, Table 1).

**RESULTS**

**Temperature Dependence of $V_f$ on Pure Rat Myosin Isoforms**

We have determined the speed of $V_f$ in an IVMA on pure type 1, 2A, and 2B myosin isoforms in a wide temperature range (10–35°C). Myosin 2X was not studied because it was very difficult to obtain a sufficiently high number of pure type 2X fibers to extract myosin from. Moreover, we did not analyze slower isoforms at 10°C because at this temperature the speed was too slow to be correctly measured by our analysis system.

The same myosin samples were subjected to different temperatures in the same experiment. $V_f$ was found to depend solely on the temperature tested regardless of the fact that such temperature was obtained by cooling from a higher temperature or warming from a lower temperature. Such finding suggests that the effect of temperature on $V_f$ was fully reversible.

At all temperatures $V_f$ was significantly lower in slow type 1 than in fast type 2A myosin and in fast type 2A than in fast type 2B myosin ($P < 0.05$). From the slopes of the regressions, $E_a$ and $Q_{10}$ of all myosin isoforms analyzed, or warming from a lower temperature. Such finding suggests that the effect of temperature on $V_f$ was fully reversible.

From the slopes of the linear regression lines, the kinetic energy ($E_a$) and the temperature coefficient ($Q_{10}$) were calculated as described by Bottinelli et al. (7):

$$E_a = -\text{slope} \times 2.303 \times R$$

where $R$ is the gas constant, and

$$\log Q_{10} = \frac{E_a}{R} \times \left[\frac{1}{T_1} - 1/(T_1 + 10)\right]$$

where $T$ is absolute temperature.

**Temperature Dependence of $V_f$ on Pure Rabbit Myosin Isoforms**

The temperature dependence of $V_f$ on pure type 1 and 2X rabbit myosin isoforms was determined in the temperature range 15–25 and 25–35°C.

**Table 1. $E_a$ and $Q_{10}$ of all myosin isoforms analyzed, in the temperature range 15–25 and 25–35°C**

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<th>15–25°C</th>
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<td>$E_a$</td>
<td>$Q_{10}$</td>
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<tr>
<td>Rat type 1 myosin</td>
<td>114±11</td>
<td>4.92</td>
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<tr>
<td>Rat type 2A myosin</td>
<td>114±12</td>
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<tr>
<td>Rat type 2B myosin</td>
<td>112±5</td>
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<tr>
<td>Rabbit type 1 myosin</td>
<td>121±4</td>
<td>5.46</td>
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<tr>
<td>Rabbit type 2X myosin</td>
<td>110±5</td>
<td>4.68</td>
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<tr>
<td>Rabbit fast (80% type 2B) HMM</td>
<td>105±7</td>
<td>4.35</td>
</tr>
<tr>
<td>Rabbit fast (80% type 2B) HMM</td>
<td>110±3</td>
<td>4.66</td>
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<tr>
<td>Rabbit type 2X HMM</td>
<td>112±6</td>
<td>4.83</td>
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*Values are means ± SD. $E_a$, activation energy. $Q_{10}$, temperature coefficient.

**Table 1. $E_a$ and $Q_{10}$ of all myosin isoforms analyzed, in the temperature range 15–25 and 25–35°C**

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*Values are means ± SD. $E_a$, activation energy. $Q_{10}$, temperature coefficient.

*Significantly different from the value measured in the range 15–25°C ($P < 0.05$). No significant differences were found among different myosin isoforms.

No significant differences were found between myosin and its heavy meromyosin (HMM) fraction.

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range 10–35°C. Vf of both rabbit myosin isoforms increased with increasing temperature (Fig. 2A). At all temperatures, Vf of myosin 2X was faster than Vf of myosin 1 (P < 0.001). The ratio between Vf of fast and slow isoforms slightly decreased increasing temperature, being 7.7 at 15°C and 6.1 at 35°C.

The Arrhenius plots are shown in Fig. 2B. For both myosin isoforms data points were best fitted by separate regression lines in the 15–25°C and 25–35°C temperature ranges (P < 0.05), with a transition in the Arrhenius plot at 25°C. For both isoforms, both Ea and Q10 were ~1.8-fold higher in the 10–25°C than in the 25–35°C range (Table 1).

Although the ratio between Vf of fast and slow isoforms decreased with increasing temperature, the comparison of the slopes of the regression lines showed that the temperature dependence of the two rabbit myosin isoforms was not significantly different.

Temperature Dependence of Vf on Rat and Rabbit HMM Fragments

The HMM fragment of myosin is known to propel actin filaments at higher (almost twofold) velocities than myosin in the IVMA (16, 41). To test whether the temperature sensitivity of HMM and native myosin is different, we analyzed the temperature dependence of Vf on HMM prepared from a slow and a fast rat and rabbit muscles. The Arrhenius plots and Ea and Q10 values were found to be similar to those of myosin molecules (Fig. 3 and Table 1).

Effect of MgADP on Filament Velocity at 35°C

To assess whether the drop of temperature sensitivity of the speed of actin filaments at the highest temperature was due to an excess of ADP in the experimental buffer, experiments were performed with rat slow and fast myosin at 35°C in the absence...
Figure 4 shows a deviation from linearity at 25°C (Figs. 1 and 2) which indicates a higher variation in temperature sensitivity of Vf with the temperature range observed in intact muscles, and that slow and fast myosin isoforms have the same temperature sensitivity, contrary to that observed comparing slow and fast muscles in vitro.

No significant differences were observed between the velocity of sliding in the presence or in the absence of CP + CPK (Fig. 4) in both slow and fast myosins.

**DISCUSSION**

In this work, we studied the effects of temperature on the velocity of slow and fast skeletal myosin isoforms at the molecular level in a wide temperature range up to physiological. The findings show that in all isoforms the temperature sensitivity is higher at low temperatures than at higher temperatures, as observed in intact muscles, and that slow and fast isoforms have the same temperature sensitivity, contrary to that observed comparing slow and fast muscles in vitro.

Speed of actin filaments propelled by myosin isoforms, from both rat and rabbit, increased with increasing temperature. The variation in temperature sensitivity of Vf with the temperature range is suggested by the analysis of the Arrhenius plots. The latter plots show a deviation from linearity at 25°C (Figs. 1B and 2B) with a Q10 higher at lower (15–25°C) than at higher temperature (25–35°C) in all rat and rabbit myosin isoforms. The lower sensitivity of myosin at the higher temperatures was not related to denaturation of the molecule or to accumulation of ADP, known to inhibit velocity, in the experimental buffer or to a change of the buffer pH at different temperatures. In fact, the observation that motility speeds at any temperature were independent from whether such temperature was tested before or after higher temperatures indicates that irreversible denaturation of the myosin at the highest temperature did not occur. Moreover, no significant differences were observed between the velocity of sliding in the absence or in the presence of an ATP regenerating system (CP + CPK) at 35°C (Fig. 4), excluding that the drop of temperature sensitivity of Vf at the highest temperature could be due to an excess of ADP in the experimental buffer. Interestingly, in single muscle fibers, in the absence of CP and CPK, velocity of shortening can actually drop after the long activations used for force-velocity determinations (9). At last, the small differences in pH of the bathing solution between 10°C (7.57) and 35°C (7.28) were very unlikely to affect the data as very little changes in Vf have been observed in a much larger pH range (7.0–8.0) (20).

A deviation of the Arrhenius plot at ~25°C was previously observed in studies of shortening velocity (33) and in IVMA studies (20, 22). The deviation of linearity suggests a change in the catalytic activity or in the step limiting the rate of the cross-bridge cycle, which defines shortening velocity at the molecular level, at temperatures ~25°C. The values of Ea and Q10 here reported are fully comparable with those determined in previous studies using IVMA (19–22, 25) but higher than those found in studies on isolated muscles and single fibers. It is unlikely that the greater sensitivity to temperature in IVMA may be associated to the lack of regulatory proteins as Homsher et al. (19) and Anson and Canepari (2) found that the temperature dependence of the velocity of regulated actin filaments in an IVMA is higher, not lower, than that of unregulated filaments at temperature higher than 12.5°C. In the motility assay the myosin molecules work isolated from the sarcomeric structure, randomly distributed on a slide and in a very low ionic strength buffer. Thus it is possible that these experimental conditions could make them more responsive to the increase of temperature.

The present work indicates that the temperature sensitivity of velocity of slow and fast myosin isoforms is very similar at the molecular level. The latter finding strongly suggests that at physiological temperature myosin isoforms should maintain their large differences in shortening velocity and, therefore, that they can actually play a major role in the large functional heterogeneity and plasticity of skeletal muscles. The latter conclusion is not in agreement with recent findings in which ATPase activity, which is well known to be related to velocity (4), of slow and fast skeletal myofibrils has been shown to become very similar at physiological temperatures (10).

The observation of the same temperature sensitivity of the velocity of slow and fast myosin isoforms was somewhat unexpected. On the basis of the classical work by Ranatunga (33–35), it is generally believed that temperature sensitivity of mechanical and biochemical properties of slow muscles is greater than that of fast muscles. It has been also shown that some kinetics steps might be differently affected by temperature in slow and in fast skinned fibers (42), although Stienen et al. (37) reported a similar Q10 of the ATPase activity of fast and slow human fibers contracting isometrically.

The discrepancy between a similar temperature sensitivity of Vf of slow and fast isoforms in IVMA and a higher sensitivity of shortening velocity of slow muscles and fibers might have multiple causes. At high temperature the energy supply of whole muscles and single fibers might be difficult, and fast muscles might be more exposed to metabolic problems owing to their higher energy requirements (8). In IVMA no problems of energy supply are likely because myosin molecules, being distributed on a surface at low concentrations, can easily use the abundant ATP present in the medium. The IVMA results cannot be affected by the disorganization of the striation pattern, that can play a significant role in skinned muscle fibers, as in such assays sarcomere structure is absent. Moreover, it can be argued that, on the contrary of IVMA analysis, the analysis of whole muscles and single muscle fibers assesses velocity of myosin within a complex ensemble of proteins that can have a modulatory role on shortening velocity and its temperature sensitivity. Slow and fast fibers express not only different myosin isoforms but also different isoforms of other myofibrillar proteins among which are regulatory proteins (36).
Harrison and Bers (17) supposed that the diversity between troponin C-fast and troponin C-slow might explain the different sensitivity of fast and slow fibers to temperature changes but the role of different isoforms of regulatory proteins in this respect is still unknown. Moreover, Mutungi and Ranatunga (28) found that proteins determining passive tension were characteristically different in fast and slow fibers and being sensitive to temperature might differentially affect velocity of slow and fast isoforms with changing temperatures.

The results obtained with HMM confirm and strengthen those obtained with myosin: 1) the Arrhenius plot shows a deviation from linearity at 25°C, and 2) the temperature sensitivity of slow and fast isoforms is similar. Moreover, the data obtained show that myosin molecule and its enzymatic fraction HMM have the same temperature dependence even if they have a different velocity in IVMA (Ref. 41; our results). Recently Guo and Guilford (16) found that the high velocity of actin filaments on HMM results from the absence of the drag generated by the myosin tail and not from the proteolytic nicking of the myosin motor domain. Consistent with their findings, our results show that the temperature dependence of Vf on myosin and HMM is similar (Fig. 3, Table 1), suggesting that the temperature sensitivity is a property of the motor domain itself.

In conclusion, the present data, obtained on rat and rabbit myosin and HMM, show 1) a temperature sensitivity of pure myosin isoforms higher at the lowest than at the highest temperature, 2) a similar temperature sensitivity of slow and fast isoforms in a wide range of temperature, and 3) large differences in velocity among myosin isoforms also at physiological temperature.

GRANTS
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