Thermal Stability of Contractile Proteins in Bat Wing Muscles Explains Differences in Temperature Dependence of Whole-Muscle Shortening Velocity

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ABSTRACT

Muscle contractile properties are dependent on temperature: cooler temperatures generally slow contractile rates. Contraction and relaxation are driven by underlying biochemical systems, which are inherently sensitive to temperature. *Carollia perspicillata*, a small Neotropical bat, experiences large temperature differentials among body regions, resulting in a steep gradient in temperature along the wing. Although the bats maintain high core body temperatures during flight, the wing muscles may operate at more than 10°C below body temperature. Partially compensating for these colder operating temperatures, distal wing muscles have lower temperature sensitivities in their contractile properties, including shortening velocity, relative to the proximal pectoralis. Shortening velocity is correlated with the activity of myosin ATPase, an enzyme that drives the cross-bridge cycle. We hypothesized that the thermal properties of myofibrillar ATPase from the pectoralis and forearm muscles of the bat wing would correlate with the temperature sensitivity of those muscles. Using myofibrillar ATPases from the proximal and distal muscles, we measured enzyme activity across a range of temperatures and enzyme thermal stability after heat incubation across a range of time points. We found that forearm muscle myofibrillar ATPase was significantly less thermally stable than pectoralis myofibrillar ATPase but that there was no significant difference in the acute temperature dependence of enzyme activity between the two muscles.

Keywords: myofibrillar protein, thermal stability, enzyme activity, bat muscle, bat flight.

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Introduction

Muscle is composed of both passive mechanical and biochemical components, and properties of both components impact whole-muscle performance. Passive mechanical properties (e.g., elasticity) are generally temperature insensitive, while biochemical processes are under the kinetic constraints common to physiological or chemical systems (Hochachka and Somero 2002; Olberding and Deban 2021). Biochemical systems are thus inherently temperature dependent because reaction rates are temperature dependent. The evolution of high and relatively constant body temperatures is hypothesized to have enabled the optimization of component biochemical systems at temperatures at which reaction rates are generally high (Hochachka and Somero 2002; Angilletta 2009). However, considerable variation in body temperature, including differences in temperature among body regions, is present in many endotherms. This regional heterothermy may result in significant temperature differentials between the limbs, which are located peripherally, and the core. Limb muscles are important for locomotion, but depending on their size and location, they may be susceptible to heat loss and may operate at temperatures lower than core body temperature (Doubt 1991; Adán et al. 1995; Rummel et al. 2019).

In muscle, faster reaction kinetics at warmer temperatures result in faster force development, activation, and relaxation and in greater shortening velocity. The hydrolysis of ATP is tightly coupled to muscle contraction: myosin cyclically binds ATP and actin during muscle contraction to power the cross-bridge cycle and muscle movement. The rate of ATP hydrolysis by myosin is correlated with shortening velocity (Bárány 1967; Spudich 1994), and the thermal properties of myosin ATPases are targets of selection (Johnston and Walesby 1977; Altringham and Johnston 1986; Goldspink 1995).

Bat wings are modified forelimb appendages, unique among mammalian appendages, that enable powered flight. Greatly elongated digits and a wing membrane composed of very thin skin (Madej et al. 2013) make the wings particularly vulnerable to heat loss during flight, which can result in differences in temperature between distal wing muscles and the core. Temperature differentials of up to 12°C when flying at an air temperature of 22°C have been measured in *Carollia perspicillata*, a small Neotropical fruit bat (Rummel et al. 2019). Such low temperatures may compromise performance in the distal muscles. However, the contractile rate properties of distal bat wing muscles are less temperature sensitive than those of proximal wing muscles and of other

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mammalian muscles, allowing for partial compensation for the low operating temperature (Rummel et al. 2018, 2021). Here, we explore the potential mechanisms of low temperature sensitivity in the shortening velocity of distal wing muscles in *C. perspicillata* by comparing the temperature sensitivity and thermal stability of myofibrillar ATPase from the proximal pectoralis to those of myofibrillar ATPase from the forearm muscles, which are located more distally. We hypothesized that thermal properties of myofibrillar ATPase would correlate with the thermal sensitivity of shortening velocity and specifically that activity would be less temperature sensitive in the distal muscles, mirroring the trend in whole-muscle shortening velocity. We also hypothesized that the myofibrillar ATPase from the distal muscles would be less stable at warm temperatures.

Methods

Tissue

Muscles were collected from captive-bred *Carollia perspicillata*. Bats in this colony were housed in the Center for Animal Resources and Education at Brown University under a 12D:12L cycle and provided with food and water ad lib. All experiments were conducted in accordance with a protocol approved by the Brown University Institutional Animal Care and Use Committee and with USDA regulations. The study sample was comprised of muscles from male and female adult bats. Body masses ranged from 14.4 to 20.4 g with a mean of 17.4 ± 0.7 g ($n = 8$). The extensor carpi radialis longus muscle (ECRL), a wrist extensor located in the forearm, was removed from one side of the animal and used for an unrelated experiment. Pieces of the pectoralis, the entirety of the ECRL and the extensor carpi radialis brevis muscle (ECRB; located lateral to the ECRL) from one side, and the remainder of the forearm muscles from both sides were removed, and each muscle or group of muscles was weighed to the nearest 0.1 mg. Each muscle or group of muscles was frozen on dry ice and stored in tightly sealed tubes at −80°C until experiments were to be conducted.

For these experiments, at least 60 mg of tissue was needed to measure activity and stability at a range of temperatures and incubation times, respectively. The average mass of the *C. perspicillata* ECRL is approximately 43 mg (Rummel et al. 2018), and only one ECRL was available for these experiments, so we grouped the ECRL and ECRB for the activity assays. Tissue samples containing the rest of the forearm musculature were used for the thermal stability assays. Hereafter, we refer to both sets of muscles as forearm muscles.

Myofibrillar ATPase Preparation and Assay

Myofibrillar ATPase activity was assayed using the method of Marsh and Wickler (1982). Muscles were homogenized in a glass-glass tissue homogenizer at 0°C in approximately 15 volumes of 100 mM phosphate, 20 mM Tris, and 5 mM EDTA at pH 6.8. The homogenate was vortexed and then centrifuged at 1,000 *g* for 10 min. The pellet was resuspended in 100 mM KCl, 20 mM Tris, 0.5% Triton X-100, and 5 mM EDTA at pH 6.8 and centrifuged at 1,000 *g* for 10 min. This step was performed twice. The resulting pellet was resuspended in 100 mM KCl and 20 mM Tris at pH 6.8. This step was performed thrice, and the final suspension was put on ice. The assays were carried out at 27°C, 32°C, 37°C, 42°C, and 47°C in stirred microcentrifuge tubes; for some preparations, we also measured activity at 22°C. Assay temperature was maintained by submerging the reaction tube in a small chamber of recirculating water from a water bath, and it was monitored with a Keithley 871 digital thermometer inserted into the chamber adjacent to the submerged reaction tube. The final volume of the assay tubes was 0.8 mL and contained 100 mM KCl, 20 mM imidazole, 2 mM $MgCl_2$, 0.25 mM $CaCl_2$, 2 mM MgATP, and approximately 0.2 mg of myofibrillar protein. The ATP solution was buffered with imidazole and adjusted to a pH of 7.2 at 22°C. With increases in experimental temperature, the assay solution would be expected to decrease in pH to approximately 7 at 37°C, in accordance with measured muscle intracellular pH values (Rahn et al. 1975; Aickin and Thomas 1977). The assays were started by the addition of ATP and stopped by the addition of 30% trichloroacetic acid (TCA) after 90 s at 22°C, 60 s at 27°C and 32°C, and 30 s at the remaining temperatures. Zero time controls were run by adding TCA first. The reaction tubes were allowed to equilibrate to experimental temperature for 30 s before the addition of ATP or TCA for both assays and controls. Assays were run in triplicate, and controls were run in duplicate. Protein was measured by the biuret method, with bovine serum albumin as a standard. Once the ATP reactions had proceeded and been quenched by TCA, assay and control tubes were centrifuged at 3,220 *g* and then placed on ice. Free phosphate was measured in the supernatant by the colorimetric detection of molybdenum blue by the method of LeBel et al. (1978).

Thermal Stability Assay

The myofibril suspension was prepared as above. Aliquots of the myofibril suspension were pipetted into reaction tubes and preincubated at 44.5°C for 10, 20, 40, and 60 min or placed directly on ice for a baseline activity measurement (0 min). After preincubation at 44.5°C, tubes were placed on ice until assayed for residual activity (Johnston et al. 1973). For each time point, assays were run in triplicate, and controls were run in duplicate. All assays and controls were conducted at 37°C, as outlined above. Phosphate was then measured as above.

Statistics

Log-transformed activity data, including the interaction of muscle and temperature or time where appropriate, were compared between muscles and across temperatures or time points using linear regression in R (R Core Team 2021). Body mass was also included as a predictor variable because myosin ATPase and shortening velocity are known to vary with body mass interspecifically (Bárány 1967; Syrovy and Gutmann 1975; Seow and Ford 1991; Pellegrino et al. 2004). A significant interaction of muscle and temperature or time was taken to indicate a significant difference in temperature

dependence of the muscles' myofibrillar ATPase activity. Activation energy (E_a) was determined from the slope of the Arrhenius plot of the activity data using linear regression. For the thermal stability regression, the activity at time zero was included as a predictor variable to correct for the effect of starting activity on activity over time. The thermal inactivation constant (K_D) was calculated from the first-order reaction analysis

$$a = \frac{\partial[E]}{\partial[E]_0} = e^{-K_D t}, \quad (1)$$

where a is the normalized activity, $\partial[E]$ and $\partial[E]_0$ are the specific activities of the initial enzyme state and the inactivation state, respectively, and t is time (Sadana 1988). Log transforming equation (1) yields a linear equation in which the slope is equal to K_D.

Results

Activity

Myofibrillar ATPase activity was strongly dependent on temperature for both the forearm muscles and the pectoralis muscle. The interaction of muscle and temperature was not significant, indicating no difference in thermal sensitivity (fig. 1). When the interaction was dropped from the model, both muscle and temperature were significant predictors of activity, though body mass was not significant (linear regression, $F_{3,76} = 206.7$, $P < 0.001$, $R^2 = 0.886$; table 1). At every temperature, the ATPase activity of the pectoralis muscle was higher than that of the forearm muscles (intercept estimates $= -3.17$ and -3.46, respectively; $P < 0.001$). The E_a calculated from the slope of the Arrhenius plot was 57.0 ± 2.2 kJ/mol over the temperature range of 22°C–47°C and did not differ significantly between muscles (linear regression, $F_{3,76} = 218.9, P < 0.001, R^2 = 0.892$).

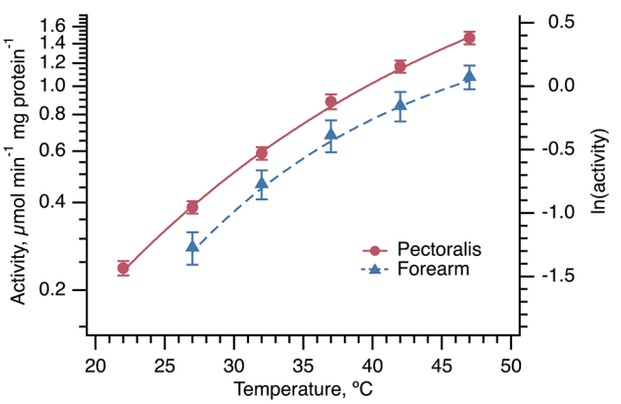

Figure 1. Specific activity of myofibrillar ATPases from the pectoralis muscle and the forearm muscles that is measured in micromoles of ATP hydrolyzed per minute per milligram of protein. Points are mean ± SEM at each temperature. At each temperature, $n = 8$ for the pectoralis muscle, and $n = 6$ for the forearm muscles. Data were not collected at 22°C for the forearm muscles.

Table 1: Results of the multiple linear regression of temperature, muscle, and body mass on ln(activity)

	Estimate	SE	t	P
Intercept	−3.464878	.245691	−14.103	<2E−16
Temperature	.072066	.002928	24.612	<2E−16
Pectoralis muscle	.298209	.049627	6.009	6.02E−08
Body mass	.014610	.012257	1.192	.237

Note. Model statistics: $F_{3,76} = 206.7$, $P < 0.001$, $R^2 = 0.886$.

Thermal Stability

The myofibrillar ATPase from the forearm muscles was significantly less thermally stable when preincubated at 44.5°C and then measured at 37°C, as indicated by a significant muscle × time interaction (slope estimates $= -0.017$ and -0.011, respectively; linear regression, $F_{5,43} = 361.6$, $P < 0.001$, $R^2 = 0.97$ [fig. 2; table 2]). Body mass was not a significant predictor of residual activity. K_D was 1.82 × 10^{-4} s^{-1} for the pectoralis and 2.86 × 10^{-4} s^{-1} for the forearm muscles.

Discussion

As hypothesized, we found significant differences in the thermal stability of pectoralis and forearm muscle myofibrillar ATPases but not in the temperature dependence of their activities, contrary to our prediction. Enzyme activity temperature dependence is influenced by both the protein structural stability and the catalytic performance of enzymes, here measured as enzyme stability and specific activity, respectively. In our measures of specific activity, the myofibrillar preparations spent only a brief period of time at the assay temperature (30 s at the warmer temperatures), and thus thermal stability effects would have been minimal. Fish that are adapted to different water temperatures have been shown variously to display substantial differences in myofibrillar ATPase thermal stability and activity (Johnston et al. 1975; Johnston and Walesby 1977; Watabe et al. 1992). Myofibrillar ATPases from cold-acclimated carp are much less thermally stable than those from warm-acclimated carp, but the differences in the temperature dependence of activity are less pronounced between the cold- and warm-acclimated fish (Watabe 2002). Thus, the pattern we observed, in which the activation energies of myofibrillar ATPases from the bat muscles were the same but the thermal stabilities were different, was consistent with data from other vertebrates. The activation energies of the bat muscles fell within the large range of other values reported in the literature (Johnston et al. 1973; Stein et al. 1982; Johnston and Sidell 1984).

The lower thermal stability of myofibrillar ATPase from the distal forearm muscles compared to myofibrillar ATPase from the pectoralis muscle, located in the body core, is consistent with the hypothesis that the lower temperature sensitivity in the shortening velocity of the ECRL in *Carollia perspicillata* arises by changes to the thermal properties of the underlying biochemistry. Because differences in muscle temperature result in changes to contractile rates, maintaining minimum contractile speeds for locomotion at operating temperatures may be the selective pressure driving

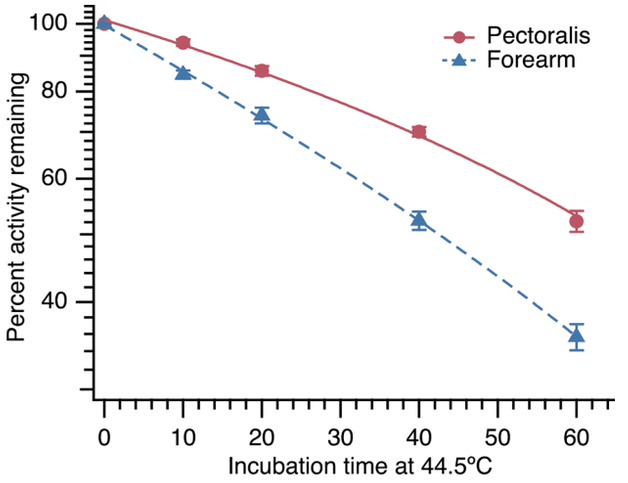

Figure 2. Residual activity of myofibrillar ATPase from the pectoralis muscle and the forearm muscles after preincubation at 44.5°C. Activity was measured at 37°C, and for each time point activities from a given preparation were normalized to the activity at time zero. Points are the mean of the normalized data at each time point.

the evolution of differences in thermal properties of the pectoralis myofibrillar ATPases versus forearm muscle myofibrillar ATPases. Although the function of the forearm muscles in bats during flight has been relatively poorly studied, because the ECRL is active cyclically during the wingbeat cycle and its temperature sensitivity is lower than in other mammalian muscles, its function is time dependent (Rummel et al. 2018, 2021). Because the kinetics of the cross bridges influence both shortening velocity and the rate of force development, the thermal dependence of this variable is equally important in muscles that shorten and do work and in muscles that must produce force cyclically within a defined period. Temperature differentials between the core and distal wing muscles are as high as 12°C in *C. perspicillata* flying at ~22°C, but they may be even higher under certain environmental conditions or in other species (Rummel et al. 2019, 2022). The decreased thermal sensitivity of forearm muscle contractile components likely compensates somewhat for these low operating temperatures, which slow contractile rates. Molecular adaptation to temperature has been poorly documented in endotherms, such as mammals, and even in hibernating mammals in which body temperature varies over time (van Breukelen and Martin 2002). To our knowledge, this is the first study of the properties of myofibrillar ATPase from muscles along the length of a single locomotor appendage.

The lower K_D in the forearm muscle myofibrillar ATPase likely reflects a difference in the global stability of the enzyme. Lower thermal stability reflects increased molecular flexibility and is an adaptation to accommodate the decrease in kinetic energy available to the system at lower temperatures (Hochachka and Somero 2002). Our thermal stability assays were conducted at 44.5°C, which was a convenient temperature for assessing differences in global enzyme stability within a reasonable time frame. Although this temperature is above the normal resting body temperature of bats, it is noteworthy that it is only a couple of degrees above the rectal temperatures of bats flying at air temperatures of approximately 30°C, which can be greater than 42°C (Carpenter 1986; Thomas et al. 1991), and the active pectoralis muscles during flight may be warmer than rectal temperature (Rummel et al. 2019). The myofibrillar preparation we used was a complex of many proteins, and thus further research would be needed to determine which components are less thermally stable in the forearm muscles.

The greater activity of the pectoralis myofibrillar ATPase compared to the forearm muscle myofibrillar ATPase is somewhat surprising. Intrinsic contractile velocities measured in lengths per second are faster in the ECRL than in the pectoralis (Rummel et al. 2018, 2021). Because myosin ATPase activities are, in general, related to shortening velocity (Bárány 1967), we hypothesized that the forearm muscle myofibrillar ATPases would have higher activities than pectoralis myofibrillar ATPases. Importantly, however, the relationship between myosin ATPase activity in vitro and shortening velocity is correlative and not causative (Rall 2014). For example, Candau et al. (2003) compared the ATPase rates and thermal stabilities of rabbit soleus and psoas myofibrils, finding that the ATPase rates were similar at physiological temperatures for the two muscles, despite differences in whole-muscle mechanical properties. Several additional factors make connecting enzyme activities and mechanical properties difficult in our data. Constraints on tissue availability and the quantity of myofibrillar suspension needed for the ranges of temperatures and incubation times studied required that we mix tissues from different muscles of the forearm. Though fiber velocities in the ECRL are faster than in the pectoralis (Rummel

Table 2: Results of the multiple linear regression of incubation time, starting activity, muscle, and body mass on ln(activity)

	Estimate	SE	*t*	*P*
Intercept	−1.1314382	.1763093	−6.417	9.03E−08
Activity at time 0	1.3803112	.0954933	14.455	<2E−16
Body mass	−.0114288	.0124057	−.921	.362
Time	−.0171908	.0005925	−29.015	<2E−16
Pectoralis muscle	.0062997	.0306323	.206	.838
Time : pectoralis muscle	.0062985	.0008326	7.565	1.98E−09

Note. Model statistics: $F_{5,43} = 361.6$, $P < 0.001$, $R^2 = 0.97$.

et al. 2021), if fiber velocities in the adjacent ECRB are slower, measured myofibrillar ATPase activity of the combined tissues will be an average of the two fiber velocities. We also assumed that the temperature sensitivity of contractile properties/myofibrillar ATPases would be similar throughout the forearm muscles based on their similar anatomical location. Moreover, we report specific enzyme activities, which are influenced by enzyme purity. Possibly, the ECRL/forearm muscle myofibrillar preparations contained more nonmyofibrillar protein because these muscles are more tendinous than the pectoralis. Tendon proteins, such as collagen, in the sample would increase the apparent protein content of the preparation.

Our measures of specific enzyme activity (rate of catalysis) and thermal stability represent two potential avenues for thermal adaptation of enzymes. Thermal adaptation in enzymes is complex, and different proteins, even from the same organism, may undergo different functional adaptations to maintain performance at relevant temperatures (Fields and Somero 1998; Lockwood and Somero 2012; Fields et al. 2015). For example, the Michaelis-Menten constant (K_M), which is a measure of substrate affinity, is another functionally important parameter, and it has been shown to differ among orthologous proteins adapted to different temperatures (Lockwood and Somero 2012; Fields et al. 2015). Though not addressed in this study, there may be differences in the temperature dependence of substrate-binding competency that may be functionally important and disconnected from our measure of catalytic activity. Other proteins that are important in muscle contraction, such as the sarcoplasmic reticulum Ca^{2+}-ATPase, may be differently adapted to the thermal environment of the bat wing, in terms of global protein stability or the kinetics of substrate binding or turnover.

Conclusions

Temperature effects, which may impair the function of distal bat wing muscles during flight, are likely buffered by changes to the thermal properties of myofibrillar enzymes governing muscle contraction. The decreased thermal stability in myofibrillar ATPases from distal muscles relative to that in myofibrillar ATPases from proximal muscles parallels both decreased operating temperatures and decreased temperature sensitivity of shortening velocity in the distal wing muscles (Rummel et al. 2018, 2019). The differences in temperature between muscles of the bat wing due to regional heterothermy are extreme enough to result in selection pressure on the biochemical systems that underlie muscle contractile physiology. Although physiological adaptation to temperature is associated more frequently with ectotherms, it may be a common phenomenon among endotherms that experience variation in body temperature among body regions.

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