Journal of Gerontology: BIOLOGICAL SCIENCES
2005, Vol. 60A, No. 4, 425–431
Copyright 2005 by The Gerontological Society of America

Age-Related Decline in Actomyosin Function

Ewa Prochniewicz,[1] David D. Thomas,[1] and LaDora V. Thompson[2]

[1]Department of Biochemistry, Molecular Biology, and Biophysics, and
[2]Department of Physical Medicine and Rehabilitation, University of Minnesota, Minneapolis.

To understand the molecular basis of the functional decline in aging muscle, we examined the functional (actomyosin ATPase) and chemical (cysteine content) changes in actin and myosin purified from the muscles of young (4- to 12-month-old) and old (27- to 35-month-old) Fisher 344 rats. Using the soluble, catalytically active myosin fragment, heavy meromyosin (HMM), we determined the maximum rate (V_{max}) and actin concentration at half V_{max} (K_m) of the actomyosin ATPase, using four combinations of actin and HMM from old and young rats. V_{max} and K_m were significantly lower when both actin and HMM were obtained from old rats than when both proteins were obtained from young rats. The number of reactive cysteines in HMM significantly decreased with age, but no change was detected in the number of reactive cysteines in actin. We conclude that aging results in chemical changes in myosin (probably oxidation of cysteines) that have inhibitory effects on the actin-activated myosin ATPase.

SKELETAL muscle weakness is one of the most significant factors limiting the activities of daily living in the elderly (1). The age-related degeneration of muscle has been associated with changes in the interaction between actin and myosin, as indicated by the age-related decrease in force and speed of shortening of permeabilized muscle fibers and of myofibrils from humans as well as animals (2–6).

Among the hypotheses that have been developed to explain age-related functional changes in proteins, the one most relevant for this work involves oxidative modification of proteins by reactive oxygen species, resulting in inhibition of biological functions (7). We are particularly interested in the oxidative state of the cysteines in myosin and actin, as cysteine thiols are an important oxidation target associated with aging (8,9). Skeletal muscle myosin contains 40 cysteines (10), including two highly reactive cysteines, Cys 707 and Cys 697. Modification of these two cysteines has been shown to have dramatic effects on the functional properties of myosin, particularly in interactions with actin; spectroscopic probes attached to these sites are sensitive to ATPase activity and to actin binding (11–16). Actin contains five cysteines (17), including the highly reactive Cys 374, which is located in the region involved in the interaction with myosin. Modification of Cys 374 has been shown to affect functional interaction with myosin (18), and spectroscopic probes attached to this site are sensitive to functional interactions with myosin (19,20).

Investigations elucidating the mechanisms of the age-related degeneration of muscle have typically focused on myosin (21–24), but there is abundant evidence that modifications of actin affect actomyosin ATPase and in vitro motility (20,25–27). Therefore, to determine independently the roles of actin and myosin in age-related changes in muscle contractility, it is necessary to perform mechanical measurements on muscle from young and old animals, then purify actin and myosin from these same animals and determine separately their functional properties in the actomyosin ATPase reaction. Until the present study, this set of experiments has not been done.

In the present study, we tested the hypothesis that the age-related deterioration of muscle contractility is due to changes in the interaction between actin and myosin during the actomyosin ATPase cycle. It is difficult to study quantitatively these interactions using intact myosin, because it aggregates at low ionic strength where the interaction with actin is maximized. Therefore, quantitative measurements of the actin–myosin interaction are usually performed using soluble, catalytically active myosin fragments: two-headed heavy meromyosin (HMM) and one-headed subfragment 1 (S1). Kinetic studies have established that the mechanism of interaction between purified actin and soluble myosin heads in solution is essentially the same as that between actin and intact myosin in myofibrils (28). In the present study we purified actin and myosin from the muscles of young and old rats, prepared HMM, and determined two key parameters of the actomyosin ATPase, V_{max} (the maximum rate) and K_m (actin concentration at half V_{max}). We complemented these functional studies by measuring age-related changes in the content of reactive cysteine residues in purified actin and myosin. This approach represents a new step in exploring the mechanisms of age-related decline of actomyosin function.

Methods

Animals

Fisher 344 male and female rats aged 4–12 months (young) and 27–35 months (old) were obtained from the aging colony maintained by the Minneapolis Veterans Administration. The 50% survival rate for the Fisher 344 rat strain is about 24 months. All animals were housed in pathogen-free conditions and received food and water ad libitum. The animal care protocol was approved by the University of Minnesota Institutional Animal Care and Use

Committee. Animals were deeply anesthetized and killed, and the semimembranosus, semitendinosus, adductor magnus, and quadriceps muscles were dissected.

Single Fiber Contractile Measurements

Individual fiber segments (~2 mm long) from the permeabilized bundles were isolated and studied at 15°C as described in detail previously (2). Fiber segments were mounted in relaxing buffer (7.0 mM EGTA, 5.4 mM $MgCl_2$, 20 mM imidazole [pH 7.0], 14.5 mM creatine phosphate, 4.7 mM ATP, $CaCl_2$ to achieve pCa of 9, and enough KCl to achieve ionic strength of 180 mM). Sarcomere length was set to 2.5 μm, the diameter was measured at three places along the length of the fiber, and then maximal isometric force was determined in activating solution. Unloaded maximal shortening velocity, V_o, was determined by the slack test, in which fibers were activated by exposure to Ca^{2+} (calcium added to the relaxing buffer to bring pCa to 4.5) and then at peak isometric force rapidly shortened (slacked) by 10%–20% of fiber length such that force dropped to zero. The time between zero force and force redevelopment was measured. This procedure was performed 5 times at different slack distances. The slack distances were then regressed against the corresponding times of force redevelopment, and the slope of that line (in millimeters per second) was divided by fiber length (millimeters per fl) to obtain V_o (fiber length in millimeters per second).

Purification of Myosin and Actin

Each set of experiments consisted of determining actin-activated HMM ATPase under physiological conditions, myosin and HMM ATPase under ''high-salt'' conditions, and cysteine content in actin and HMM. For each set, two rats (one old and one young) were killed. Muscles were quickly dissected and immediately placed in ice-cold solution containing 0.3 M sucrose, 0.1 mM EDTA, and 10 mM imidazole (pH 7.0); they were subsequently homogenized on ice in rigor buffer containing 0.1 M KCl, 20 mM imidazole (pH 7.0), 0.5 mM EDTA, and the following protease inhibitors: 20 μM PMSF (phenylmethanesulfonyl fluoride), and 10 μg/ml each of aprotinin, leupeptin, antipain, pepstatin, chymostatin, TLCK (Nα-*p*-Tosyl-L-lysine-chloromethyl ketone), TPCK (N-*p*-Tosyl-L-phenylalanine chloromethyl ketone), and BAAE (*N*α-Benzoyl-L-arginine ethyl). The homogenate was centrifuged for 5 minutes at 3000 *g*, supernatant was discarded, and the pellet was extracted for 10 minutes on ice with Guba-Straub solution (0.3 M KCl, 0.1 M KH_2PO_4, and 50 mM K_2HPO_4, pH 6.4). The extract was centrifuged for 5 minutes at 3000 *g*. The supernatant was used to prepare myosin, and the pelleted muscle residue was used to prepare acetone powder, following standard methods of preparation of myosin and actin from rabbit skeletal muscle (29,30). Myosin and actin from rabbit skeletal muscle were used in control experiments.

HMM, the soluble two-headed fragment of myosin, was prepared by α-chymotryptic digestion of rat muscle myosin, following methods described for rabbit skeletal muscle myosin (31). The digest was dialyzed against 10 mM Tris (pH 7.5) and centrifuged for 45 minutes at 300,000 *g* to remove the myosin rods and undigested myosin. The resulting HMM was used without further purification. No difference in the digestion patterns of young and old myosin was observed on sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis.

Actin was extracted from rat muscle acetone powder with G-buffer (5 mM Tris (pH 7.5), 0.2 mM $CaCl_2$, 0.2 mM ATP), and was purified by the method used for *Drosophila* (32). The extract was first polymerized with 0.1 M KCl for 1 hour at room temperature, then the KCl concentration was increased to 1 M, and the extract was incubated for 30 minutes at 37°C and centrifuged 30 minutes at 300,000 *g*. The pelleted actin was suspended in G-buffer, dialyzed against G-buffer to remove KCl, and centrifuged for 10 minutes at 300,000 *g* to remove aggregates. After polymerization with 3 mM $MgCl_2$, actin was ultracentrifuged for 30 minutes at 300,000 *g*, and the final pellet was suspended in F-buffer (3 mM $MgCl_2$, 10 mM Tris, pH 7.5) containing 0.2 mM ATP. All centrifugations were performed at 4°C.

All experiments using actin, myosin, and HMM purified from one pair of rats were completed within 4 days, including the day when the animals were killed. For every given pair of young and old rats, the purification of actin and myosin as well as all experiments with purified proteins were performed in parallel to ensure that all conditions were identical, except for the rats' ages. For results presented in this paper, 8 pairs of rats were used.

Proteins purified from the young rat are designated ''young actin'' (A_y) and ''young HMM'' (M_y), and the proteins purified from the old rat are designated ''old actin'' (A_o) and ''old HMM'' (M_o). Due to the variability of available muscle tissue and the amount of purified proteins, in two of the paired experiments using old and young HMM and in one of the paired experiment using young and old actin, the proteins from the young rat were substituted with rabbit actin and myosin. Control experiments showed that the actin-activated ATPase rates for young rat proteins were the same as those for rabbit proteins.

Protein Concentration

Protein concentration was measured by ultraviolet absorption, assuming molar absorption coefficients of 0.53 mg ml^{-1} cm^{-1} for myosin at 280 nm, 0.65 mg ml^{-1} cm^{-1} for HMM at 280 nm, and 0.63 mg ml^{-1} cm^{-1} for actin at 290 nm.

ATPase Measurements

Actin-activated HMM ATPase was measured at 25°C in 3 mM $MgCl_2$, 10 mM Tris (pH 7.5), and 2.5 mM ATP, at a constant concentration of HMM (0.69 μM myosin heads) and with varying concentrations of actin (from 3.5 μM to 59 μM); concentration of the liberated phosphate was determined by the Fiske–Subbarow method (33). V_{max} and K_m of the acto-HMM ATPase were determined by fitting the data with the Michaelis-Menten equation:

$$v = V_{max}/(1 + K_m/[A]), \quad [1]$$

where v is the measured ATPase rate and [A] is actin concentration, using the software package Origin 7 (OriginLab, Northampton, MA). The high-salt ATPase activity of myosin or HMM was measured at 25°C in 0.6 M KCl,

50 mM Tris (pH 7.5), and 10 mM EDTA (K-ATPase) or 10 mM $CaCl_2$ (Ca-ATPase); protein concentrations were 0.025 mg/ml (K-ATPase of myosin and HMM), 0.3 mg/ml (Ca-ATPase of HMM), and 0.4 mg/ml (Ca-ATPase of myosin). The concentration of the liberated phosphate was determined using the malachite green method (34).

Determination of Reactive Cysteine

The reactive cysteine side chains were quantitated at 1 μM HMM and 5–8 μM actin in the presence of 0.1 M $NaHPO_4$ (pH 8), 1 mM EDTA, 1% SDS, and 10 mM Tris (pH 7.5) by the DTNB (5,5′-dithio-*bis*-(2-nitrobenzoic acid)) method (35,36). DTNB (0.5 mM) was added to the prepared protein samples, and after a 15-minute incubation at 25°C the absorbance was measured at 412 nm. The cysteine content (mol/mol protein) was calculated using the absorption coefficient of the free TNB anion as 14,150 $M^{-1}cm^{-1}$ (37). Control experiments using a standard solution of cysteine showed that buffers, including SDS, did not affect the results.

Reagents

The solutions were made in MilliQ water and degassed for ~30 minutes. All buffers, ATP, and protease inhibitors were purchased from Sigma-Aldrich (St. Louis, MO).

Data Analysis

The values of V_{max}, K_m, K-ATPase, Ca-ATPase, and cysteine content were obtained in paired experiments, where the results for each pair of young and old rats were expressed by calculating the ratio (old rat value) / (young rat value) and the difference (young rat value) – (old rat value). After the experiments with all 8 pairs of rats were completed, the ratios were averaged and presented on the figures as mean ± *SEM*. The significance of the age-related changes was determined by performing a *t* test ($\alpha = 0.05$) on the differences for all paired data. The differences were regarded as significantly different when $t > t_{crit}$.

Results

Contractility of Single Muscle Fibers

Age-related changes in muscle contractility were determined using the semimembranosus fibers dissected from the same animals which were the source of muscles for the purification of actin and myosin. Specific tension of the fibers from the old animals (82.7 ± 2.0 kN/m^2) was 77% of that of the fibers from the young animals (106.8 ± 2.8 kN/m^2), and the unloaded shortening velocity V_o of the fibers from the old animals (7.5 ± 0.5 fl/s) was 78% of that of the fibers from the young animals (9.6 ± 0.4 fl/s). These results are consistent with the age-related decline in muscle function previously observed in our laboratories (4,5). To investigate the biochemical basis of this decline, we measured the ATPase activities of the purified actin and myosin.

Effects of Aging on Actin-Activated HMM ATPase

Figure 1 is representative of a paired experimental set, in which we determined V_{max} and K_m of the actin-activated HMM ATPase. In this case, the data from young actin and

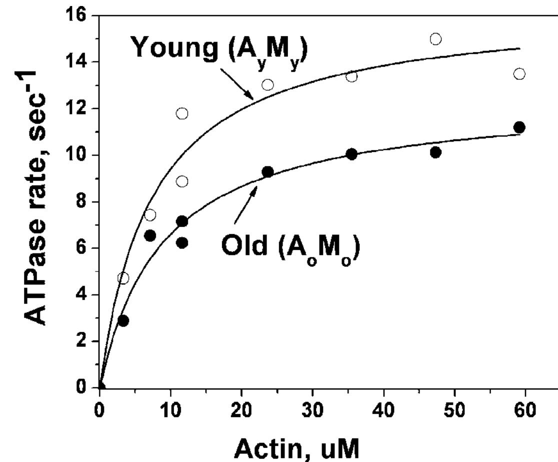

Figure 1. Representative experimental set to determine V_{max} (the maximum rate) and K_m (actin concentration at half V_{max}). The actin-activated heavy meromyosin (HMM) ATPase rate was measured at increasing concentrations of actin. The V_{max} and K_m were obtained by fitting the data to Equation 1. ○ = young actin, young HMM, V_{max} = 16.43 ± 1.00 s^{-1}, K_m = 7.44 ± 1.63 μM; ● = old actin, old HMM, V_{max} = 12.55 ± 0.62 s^{-1}, K_m = 8.98 ± 1.48 μM.

young HMM (designated A_yM_y) are compared with the data from old actin and old HMM (designated A_oM_o). In this experiment, aging results in a significant decrease in V_{max}, but no significant decrease in K_m (see Figure 1 legend). After this experiment was performed on all seven pairs of rats, the mean V_{max} for old acto-HMM (A_oM_o, 12.15 ± 0.85 s^{-1}), was 21% lower than that for young acto-HMM (A_yM_y, 15.25 ± 0.75 s^{-1}), and the *t* test on the paired data sets showed that this decrease was statistically significant ($n = 7$, $t = 11.001 > t_{crit} = 2.447$). The mean K_m for the old acto-HMM (A_oM_o, 7.51 ± 1.05 μM) was 24% lower than that for the young acto-HMM (A_yM_y, 9.90 ± 1.11 μM), and this difference was also statistically significant ($n = 7$, $t = 3.164 > t_{crit} = 2.447$).

Figure 1 shows only two of the four combinations of actin and HMM from a pair of rats. To assess which of the two proteins contributes most to the observed changes in actin-activated ATPase, we also performed experiments with two other combinations. We determined: (a) the age-related changes in HMM by comparing the activation of old and young HMM ATPase by young actin (A_yM_o vs A_yM_y) and (b) the age-related changes in actin by comparing the activation of young HMM ATPase by old and young actin (A_oM_y vs A_yM_y) (Figure 2). In the presence of young actin, V_{max} for old HMM (A_yM_o, 11.98 ± 0.87 s^{-1}) was 17% lower than that for young HMM (A_yM_y, 14.34 ± 0.86 s^{-1}). This difference was statistically significant ($n = 8$, $t = 3.981 > t_{crit} = 2.365$), suggesting that the difference detected when both actin and HMM were old (A_oM_o) is primarily due to changes in myosin. K_m for old HMM (A_yM_o, 8.58 ± 1.58 μM) was not significantly different from that for young HMM (A_yM_y, 9.60 ± 1.15 μM) ($n = 8$, $t = 0.711 < t_{crit} = 2.365$). This suggests that young actin attenuates the age-related changes in K_m for the old HMM. No significant differences were found when old and young actins were activating young HMM. The V_{max} values (15.69 ± 0.96 s^{-1} for A_oM_y and 15.68 ± 0.71 s^{-1} for A_yM_y) were not

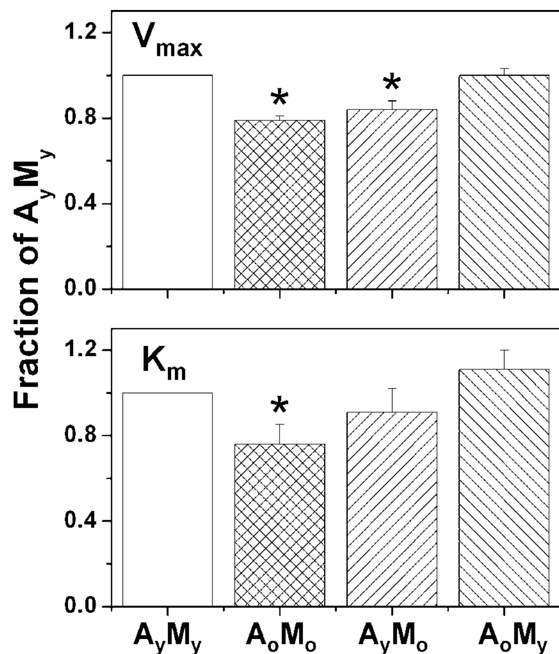

Figure 2. Age-related changes in the actomyosin function are due primarily to changes in myosin. V_{max} and K_m for actin-activated heavy meromyosin (HMM) ATPase were determined as in Figure 1. Data were normalized to the value for young actin and young HMM, AyMy. A_oM_o = old actin and old HMM; A_yM_o = young actin and old HMM, A_oM_y = old actin and young HMM. *Statistically significant.

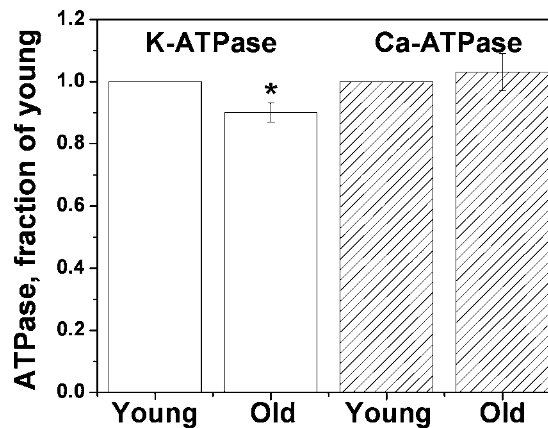

Figure 3. Age-related changes in myosin high-salt ATPase experiments. Age-related changes in the ATPase activity of heavy meromyosin (HMM). K-ATPase (open bars) was determined in the presence of 0.6 M KCl, 50 mM Tris (pH 7.5), and 10 mM EDTA; Ca-ATPase (hatched bars) was determined in the presence of 0.6 M KCl, 50 mM Tris (pH 7.5), and 10 mM $CaCl_2$. The ATPase rates for old HMM (Old) are expressed as fraction of the rates for young HMM (Young). *Statistically significant.

significantly different ($n = 6$, $t = 0.029 < t_{crit} = 2.571$). Similarly, the corresponding K_m values (10.32 ± 1.11 µM for A_oM_y and 9.54 ± 1.29 µM for A_yM_y) ($n = 6$, $t = 0.910 < t_{crit} = 2.571$) were not significantly different ($n = 6$, $t = 0.910 < t_{crit} = 2.571$). We conclude that the age-related changes in actomyosin function are due primarily to the changes in myosin.

Age-Related Changes in the ATPase of Myosin in the Absence of Actin

Because myosin is implicated in the functional effects of aging, we obtained insight into the age-related changes in myosin by measuring the high-salt K- and Ca-ATPase activity of purified myosin and HMM. It has been shown that ATPase activities measured under these conditions are quite sensitive to the modification (e.g., oxidation) of myosin's two reactive cysteines, Cys 707 and Cys 697. Increased modification of Cys 707 results in a decrease in the K-ATPase and increase in the Ca-ATPase, whereas oxidation of both Cys 707 and Cys 697 inhibits both ATPases (11,15,38) (Figure 3).

In the present study, we detected an age-related decrease of K-ATPase by about 10%, from 10.23 ± 0.76 s^{-1} to 9.10 ± 0.53 s^{-1}, which was statistically significant ($n = 8$, $t = 3.189 > t_{crit} = 2.36$). The Ca-ATPase activities of old HMM (0.83 ± 0.05 s^{-1}) and young HMM (0.82 ± 0.07 s^{-1}) were not significantly different ($n = 8$, $t = 0.201 < t_{crit} = 2.365$). Essentially the same results were observed for intact myosin, where K-ATPase of young myosin, 12.29 ± 1.26 s^{-1} was significantly ($n = 8$, $t = 3.054 > t_{crit} = 2.365$) higher than that of old myosin, 11.60 ± 1.11 s^{-1}, whereas the Ca-ATPase activities were unaffected (0.77 ± 0.03 s^{-1} for young, 0.76 ± 0.04 s^{-1} for old; $n = 8$, $t = 0.920 < t_{crit} = 2.365$). Thus, digestion of myosin to HMM does not affect age-related differences in the enzymatic properties of myosin. These results suggest age-related oxidation of cysteine residues in myosin, but the lack of activation of Ca-ATPase argues against specific modification of Cys 707.

Reactive Cysteine Content in Myosin and Actin From Young and Old Muscle

To evaluate total cysteine content in myosin's HMM and in actin, the DTNB assay was performed on proteins unfolded by treatment with 1% SDS. In control experiments using rabbit skeletal muscle HMM, this method yielded 31.4 ± 1.2 cysteines/mol of HMM, in good agreement with the 32 cysteines predicted from the amino acid sequence (10). DTNB titration of HMM from old and young rats showed an age-related decrease in free cysteine content, from 31.11 ± 0.95 moles per mol in young HMM to 28.03 ± 0.89 moles per mol in old HMM. This difference was statistically significant ($n = 6$, $t = 5.793 > t_{crit} = 2.365$) (Figure 4).

We did not detect any age-related difference in the cysteine content in actin. The cysteine content in the young actin, 4.31 ± 0.10 moles/mol, was essentially the same as that in the old actin, 4.30 ± 0.06 moles/mol (Figure 4). This finding is in agreement with the measurements using rabbit skeletal muscle actin in our previous work and work by others (36), and is consistent with 5 cysteines/mol of actin, which is predicted from the amino acid sequence (17).

Control experiments demonstrated that the purification procedure did not introduce Cys modifications in actin and myosin. We performed the initial steps of the purification process (homogenization of muscle and extraction of myosin in Guba-Straub solution) in the presence of 5 mM dithiothreitol (DTT, an antioxidant specific for Cys). Neither the actin-activated ATPase activity nor the cysteine content

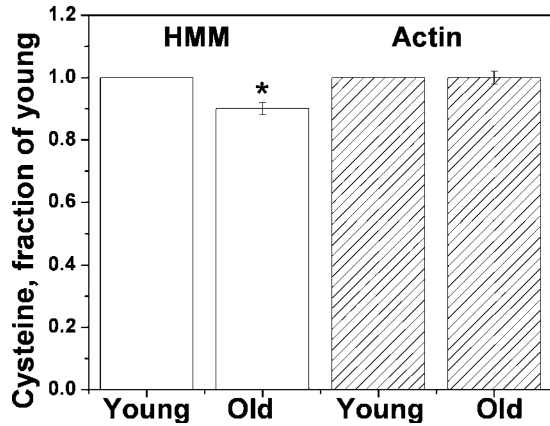

Figure 4. Age-related decline in the free cysteine content of heavy meromyosin (HMM), but not actin. Cysteine content (moles reacted per mole protein) was measured by the DTNB (5,5′-dithio-*bis*-(2-nitrobenzoic acid) method and normalized to the young value. *Statistically significant.

of purified proteins was affected by this DTT treatment. Furthermore, the cysteine content of the young as well as the old HMM purified by our standard procedure (in the absence of DTT) was not affected by a subsequent reduction at 10 mM DTT, indicating that the detected decrease in free cysteine thiols is due to irreversible modifications occurring in vivo.

Discussion

Age-Related Changes in Myosin Function and Chemical State

To perform systematic studies of age-related changes in the functional and chemical properties of actin and myosin, we have purified actin and myosin from the hamstring and quadriceps muscles of young and old animals. These muscle were chosen because the previous research in our laboratories showed that their contractility decreases with aging and that this decrease is not associated with changes in the expression of myosin heavy-chain isoforms but may be associated with chemical changes in myosin, such as cysteine oxidation (4,5,39,40). The present study has two principal findings for purified actin and myosin: (1) a significant age-related decrease in the V_{max} and K_m of actin-activated ATPase and (2) a significant age-related decrease of free cysteine content in myosin.

The age-related decrease in V_{max} (21%), detected at the level of the purified proteins, is comparable to the 22% decrease in shortening speed of muscle fibers from the same rats (see Results section) and to the 20% decrease in unloaded shortening speed of myofibrils from the semimembranosus muscle (41). The established correlation between the unloaded shortening velocity V_o of the different muscle types (e.g., fast and slow myosin isoforms) and the actomyosin ATPase (42,43) suggests that the detected decrease in V_{max} of the ATPase is due to changes in the properties of the myosin molecule. The analysis of the age-induced changes in acto-HMM, HMM alone, and actin alone supports the following conclusions: young actin activated old HMM (A_yM_o) to a lower extent than young HMM (A_yM_y), whereas there was no significant difference in the activation of young HMM by young or old actin. There is a clear difference between the observed age-induced changes in the actomyosin ATPase and the observed isoform-related changes in the actomyosin ATPase (43). The decrease in V_{max} for the different myosin isoforms is accompanied by an increase in K_m (43), whereas the age-induced decrease is observed in both V_{max} and K_m. This finding suggests that the mechanism responsible for age-induced inhibition of the ATPase is due to some unique molecular change in myosin that is distinct from changes defining the different myosin isoforms. The age-related functional changes are probably due to oxidative modifications, as will be discussed.

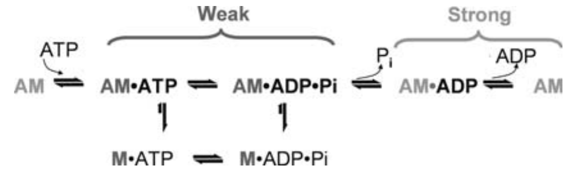

Figure 5. Age-related decrease in V_{max} suggests alterations in the structural states of myosin. Actomyosin ATPase cycle, indicating: (a) weak interactions of actin (A) with myosin (M)•nucleotide complexes, M•ATP and M•ADP•P_i, where ADP and P_i are products of ATP hydrolysis, and (b) strong interaction of actin with M•ADP and M alone.

Mechanism of Age-Related Changes in the Actomyosin ATPase

The biochemical steps of the actomyosin ATPase cycle have been associated with distinct structural states of actin and myosin. The current models postulate that muscle contraction is generated upon the transition of the actin–myosin complex from states of weak interaction (AM·ATP, AM·ADP·P) to states of strong interaction (AM·ADP, AM) (44,45) (Figure 5). In terms of this model, the age-related decrease in V_{max} suggests alterations in the structural states of myosin that affect the transition from weak to strong interactions. Possible transitions include disorder-to-order transitions in the catalytic and light-chain domains of myosin or changes in the internal structure of the catalytic domain (44,46). This possibility is supported by the observed correlation between changes in V_{max} and mutations of specific sequences within the actin-binding region of myosin (47).

The simplest interpretation of the age-related decrease in K_m is increased binding affinity of actin for myosin in the weak binding states (28,48). This interpretation of K_m is supported by studies on the effects of mutations in the actin-binding regions of myosin and myosin-binding regions of actin. These studies showed that the relative order of changes in K_m followed the relative order of changes in the weak binding affinities (25,47). We conclude that the age-related changes result in an accumulation of the weak binding complexes A·M·ATP and A·M·ADP·P, due to structural changes in myosin, which slow down the whole actomyosin ATPase cycle. This conclusion is consistent with that derived previously from our electron paramagnetic

studies on permeabilized muscle fibers, indicating an age-related decrease in the fraction of the myosin heads in the strong-binding structural states (4).

Our proposed interpretation in terms of age-related changes in the molecular interactions between actin and myosin may be oversimplified, as both K_m and V_{max} are determined by multiple transitions between intermediates of the actomyosin ATPase cycle (28,48). Thus, the understanding of the age-related changes in the molecular interaction of actin and myosin requires further biochemical as well as structural studies. The present study has taken the first step toward this goal, by demonstrating that purified actin and myosin from aged rats reveal consistent biochemical age-related changes.

Age-Related Oxidative Modifications of Myosin

We detected oxidation of 3 of 32 cysteines (10) in HMM. This finding is comparable with the previously reported oxidation of about four cysteines per myosin in the gastrocnemius muscle of rat (23). These results support the hypothesis that cysteines are important oxidative targets in the aging process (8,9). The age-induced modifications of the myosin molecule are probably facilitated by its long half-life and age-related decrease in the turnover rate (49–51).

The observed ~10% decrease in K-ATPase of HMM suggests some modification of Cys 707 and/or Cys 697. However, the lack of changes in Ca-ATPase strongly suggests that the modification is not specific for these two residues (11,12). Because oxidation of cysteines is one of the possible chemical changes in myosin that results in inhibition of the actin-activated ATPase, some of the oxidized residues could be located within or in the vicinity of the functionally relevant sequences in myosin. Possible candidates include Cys 540 and Cys 402. Cys 540 is in a region important for the strong interaction with actin. Cys 402 is of particular interest because mutation of the neighboring Arg 403 to Gln causes human heart muscle dysfunction associated with familial hypertrophic cardiomyopathy (52).

The decrease in K- and actin-activated myosin ATPases could also result from oxidative modification of sites other than cysteine. Studies on rabbit skeletal myosin (53,54) have correlated inhibition of K-ATPase with modification of Lys 84 at the interface of the catalytic and lever arm domains, and with glycation of yet unspecified lysines (55). Glycation of myosin, detected in aging rats (24), has been suggested as the mechanism for the inhibition of actin sliding on aged myosin in vitro (56,57), but localization of the glycated residues remains unknown.

Age-Related Changes in Actin

We did not detect significant age-related changes in actin's functional properties or cysteine content. However, we cannot exclude the possibility that other changes in actin occur and subsequently alter muscle function via effects on actin's interaction with proteins other than myosin. It has been shown that the oxidative modification of actin inhibits the interaction with α-actinin (58). The mutation-induced changes in α-actinin binding sites of actin were implicated in the mechanism of myocyte dysfunction and heart failure in dilated cardiomyopathy (59). Future experiments are needed to elucidate age-related structural and functional changes in actin.

Summary

The present work explores the biochemical basis of previously observed age-related degeneration of muscle contractility. Using purified actin and myosin, we have found a significant age-related inhibition of the actin-activated myosin ATPase, and a significant age-induced decrease in the free cysteine content of myosin. Future experiments will focus on a more thoroughly analyzing the age-related chemical changes in both actin and myosin, localizing sites of oxidative modifications, and determining their specific roles in the impairment of functional actin–myosin interactions.

Acknowledgments

This work was supported by grants from the National Institutes of Health to L. T. (AG17768 and AG021626) and to D. D. T. (AR32961).

We thank Dr. Dawn Lowe for stimulating discussions; Sheng Zhong for help with dissection of muscle, measurement of force and shortening velocity V_o of fibers from the semimembranosus muscle of young and old rats, and performing SDS–polyacrylamide gel electrophoresis; and Octavian Cornea for help with preparation of the manuscript. The results were presented as a poster during the Gordon Research Conference: Biology of Aging (September 2004).

Address correspondence to Ewa Prochniewicz, Department of Biochemistry, University of Minnesota, Jackson Hall 6-155, 321 Church Street, Minneapolis, MN 55455. E-mail: ewa@ddt.biochem.umn.edu

References

1. Larsson L, Ramamurthy B. Aging-related changes in skeletal muscle. Mechanisms and interventions. *Drugs Aging.* 2000;17:303–316.
2. Thompson LV, Brown M. Age-related changes in contractile properties of single skeletal fibers from the soleus muscle. *J Appl Physiol.* 1999; 86:881–886.
3. Larsson L, Li X, Frontera WR. Effects of aging on shortening velocity and myosin isoform composition in single human skeletal muscle cells. *Am J Physiol.* 1997;272(2 Pt 1):C638–C649.
4. Lowe DA, Surek JT, Thomas DD, Thompson LV. Electron paramagnetic resonance reveals age-related myosin structural changes in rat skeletal muscle fibers. *Am J Physiol Cell Physiol.* 2001;280:C540–C547.
5. Lowe DA, Thomas DD, Thompson LV. Force generation, but not myosin ATPase activity, declines with age in rat muscle fibers. *Am J Physiol Cell Physiol.* 2002;283:C187–C192.
6. D'Antona G, Pellegrino MA, Adami R, et al. The effect of ageing and immobilization on structure and function of human skeletal muscle fibres. *J Physiol.* 2003;552(Pt 2):499–511.
7. Stadtman ER, Berlett BS. Reactive oxygen-mediated protein oxidation in aging and disease. *Chem Res Toxicol.* 1997;10:485–494.
8. Thomas JA, Mallis RJ. Aging and oxidation of reactive protein sulfhydryls. *Exp Gerontol.* 2001;36:1519–1526.
9. Thomas JA, Poland B, Honzatko R. Protein sulfhydryls and their role in the antioxidant function of protein S-thiolation. *Arch Biochem Biophys.* 1995;319:1–9.
10. Maita T, Yajima E, Nagata S, Miyanishi T, Nakayama S, Matsuda G. The primary structure of skeletal muscle myosin heavy chain: IV. Sequence of the rod, and the complete 1,938-residue sequence of the heavy chain. *J Biochem (Tokyo).* 1991;110:75–87.
11. Takashi R, Duke J, Ue K, Morales MF. Defining the "fast-reacting" thiols of myosin by reaction with 1, 5 IAEDANS. *Arch Biochem Biophys.* 1976;175:279–283.

12. Reisler E. Sulfhydryl modification and labeling of myosin. *Methods Enzymol.* 1982;85 Pt B:84–93.
13. Crowder MS, Cooke R. The effect of myosin sulphydryl modification on the mechanics of fibre contraction. *J Muscle Res Cell Motil.* 1984; 5:131–146.
14. Root DD, Reisler E. Cooperativity of thiol-modified myosin filaments. ATPase and motility assays of myosin function. *Biophys J.* 1992; 63:730–740.
15. Bobkova EA, Bobkov AA, Levitsky DI, Reisler E. Effects of SH1 and SH2 modifications on myosin: similarities and differences. *Biophys J.* 1999;76:1001–1007.
16. Cakatay U, Telci A, Kayali R, Tekeli F, Akcay T, Sivas A. Relation of aging with oxidative protein damage parameters in the rat skeletal muscle. *Clin Biochem.* 2003;36:51–55.
17. Elzinga M, Collins JH. The primary structure of actin from rabbit skeletal muscle. Five cyanogen bromide peptides, including the NH2 and COOH termini. *J Biol Chem.* 1975;250:5897–5905.
18. Crosbie RH, Miller C, Cheung P, Goodnight T, Muhlrad A, Reisler E. Structural connectivity in actin: effect of C-terminal modifications on the properties of actin. *Biophys J.* 1994;67:1957–1964.
19. Prochniewicz E, Thomas DD. Perturbations of functional interactions with myosin induce long-range allosteric and cooperative structural changes in actin. *Biochemistry.* 1997;36:12845–12853.
20. Prochniewicz E, Thomas DD. Site-specific mutations in the myosin binding sites of actin affect structural transitions that control myosin binding. *Biochemistry.* 2001;40:13933–13940.
21. Ramamurthy B, Hook P, Jones AD, Larsson L. Changes in myosin structure and function in response to glycation. *FASEB J.* 2001;15: 2415–2422.
22. Hook P, Sriramoju V, Larsson L. Effects of aging on actin sliding speed on myosin from single skeletal muscle cells of mice, rats, and humans. *Am J Physiol Cell Physiol.* 2001;280:C782–C788.
23. Srivastava SK, Kanungo MS. Aging modulates some properties of skeletal myosin ATPase of rat. *Biochem Med.* 1982;28:266–272.
24. Syrovy I, Hodny Z. Non-enzymatic glycosylation of myosin: effects of diabetes and ageing. *Gen Physiol Biophys.* 1991;11:301–307.
25. Miller CJ, Reisler E. Role of charged amino acid pairs in subdomain-1 of actin in interactions with myosin. *Biochemistry.* 1995;34:2694–2700.
26. Miller CJ, Doyle TC, Bobkova E, Botstein D, Reisler E. Mutational analysis of the role of hydrophobic residues in the 338-348 helix on actin in actomyosin interactions. *Biochemistry.* 1996;35:3670–3676.
27. Prochniewicz E, Yanagida T. Inhibition of sliding movement of F-actin by crosslinking emphasizes the role of actin structure in the mechanism of motility. *J Mol Biol.* 1990;216:761–772.
28. Ma YZ, Taylor EW. Kinetic mechanism of myofibril ATPase. *Biophys J.* 1994;66:1542–1553.
29. Margossian SS, Lowey S. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol.* 1982;85 Pt B:55–71.
30. Strzelecka-Golaszewska H, Prochniewicz E, Nowak E, Zmorzynski S, Drabikowski W. Chicken-gizzard actin: polymerization and stability. *Eur J Biochem.* 1980;104:41–52.
31. Weeds AG, Pope B. Studies on the chymotryptic digestion of myosin. Effects of divalent cations on proteolytic susceptibility. *J Mol Biol.* 1977;111:129–157.
32. Razzaq A, Schmitz S, Veigel C, Molloy JE, Geeves MA, Sparrow JC. Actin residue glu(93) is identified as an amino acid affecting myosin binding. *J Biol Chem.* 1999;274:28321–28328.
33. Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem.* 1925;26:375–400.
34. Lanzetta PA, Alvarez LJ, Reinach PS, Candia OA. An improved assay for nanomole amounts of inorganic phosphate. *Anal Biochem.* 1979; 100:95–97.
35. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959; 82:70–77.
36. Knight P, Offer G. p-NN′-phenylenebismaleimide, a specific cross-linking agent for F-actin. *Biochem J.* 1978;175:1023–1032.
37. Riddles PW, Blakeley RL, Zerner B. Reassessment of Ellman's reagent. *Methods Enzymol.* 1983;91:49–60.
38. Bobkov AA, Bobkova EA, Homsher E, Reisler E. Activation of regulated actin by SH1-modified myosin subfragment 1. *Biochemistry.* 1997;36:7733–7738.
39. Larsson L, Yu F, Hook P, Ramamurthy B, Marx JO, Pircher P. Effects of aging on regulation of muscle contraction at the motor unit, muscle cell, and molecular levels. *Int J Sport Nutr Exerc Metab.* 2001;11 Suppl:S28–43.
40. Marx JO, Kraemer WJ, Nindl BC, Larsson L. Effects of aging on human skeletal muscle myosin heavy-chain mRNA content and protein isoform expression. *J Gerontol Biol Sci.* 2002;57A:B232–B238.
41. Lowe DA, Husom AD, Ferrington DA, Thompson LV. Myofibrillar myosin ATPase activity in hindlimb muscles from young and aged rats. *Mech Ageing Dev.* 2004;125:619–627.
42. Barany M. ATPase activity of myosin correlated with speed of muscle shortening. *J Gen Physiol.* 1967;50:Suppl:197–218.
43. Marston SB, Taylor EW. Comparison of the myosin and actomyosin ATPase mechanisms of the four types of vertebrate muscles. *J Mol Biol.* 1980;139:573–600.
44. Thomas DD, Prochniewicz E, Roopnarine O. Changes in actin and myosin structural dynamics due to their weak and strong interactions. *Results Probl Cell Differ.* 2002;36:7–19.
45. Prochniewicz E, Walseth TF, Thomas DD. Dynamics of actin during active interaction with myosin: the effect of weakly bound myosin heads. *Biochemistry.* 2004;43:10642–10652.
46. Thomas DD, Ramachandran S, Roopnarine O, Hayden DW, Ostap EM. The mechanism of force generation in myosin: a disorder-to-order transition, coupled to internal structural changes. *Biophys J.* 1995;68 (4 Suppl):135S–141S.
47. Murphy CT, Spudich JA. The sequence of the myosin 50-20K loop affects Myosin's affinity for actin throughout the actin-myosin ATPase cycle and its maximum ATPase activity. *Biochemistry.* 1999;38: 3785–3792.
48. Lymn RW, Taylor EW. Mechanism of adenosine triphosphate hydrolysis by actomyosin. *Biochemistry.* 1971;10:4617–4624.
49. Zak R, Martin AF, Prior G, Rabinowitz M. Comparison of turnover of several myofibrillar proteins and critical evaluation of double isotope method. *J Biol Chem.* 1977;252:3430–3435.
50. Balagopal P, Rooyackers OE, Adey DB, Ades PA, Nair KS. Effects of aging on in vivo synthesis of skeletal muscle myosin heavy-chain and sarcoplasmic protein in humans. *Am J Physiol.* 1997;273(4 Pt 1): E790–E800.
51. Husom AD, Peters EA, Kolling EA, Fugere NA, Thompson LV, Ferrington DA. Altered proteasome function and subunit composition in aged muscle. *Arch Biochem Biophys.* 2004;421:67–76.
52. Rayment I, Holden HM, Whittaker M, et al. Structure of the actin-myosin complex and its implications for muscle contraction. *Science.* 1993;261:58–65.
53. Mornet D, Pantel P, Bertrand R, Audemard E, Kassab R. Localization of the reactive trinitrophenylated lysyl residue of myosin ATPase site in the NH2-terminal (27 k domain) of S1 heavy chain. *FEBS Lett.* 1980;117:183–188.
54. Ajtai K, Peyser YM, Park S, Burghardt TP, Muhlrad A. Trinitrophenylated reactive lysine residue in myosin detects lever arm movement during the consecutive steps of ATP hydrolysis. *Biochemistry.* 1999;38:6428–6440.
55. Avigad G, Kniep A, Bailin G. Reaction of rabbit skeletal myosin with D-glucose 6-phosphate. *Biochem Mol Biol Int.* 1996;40:273–284.
56. Hook P, Li X, Sleep J, Hughes S, Larsson L. In vitro motility speed of slow myosin extracted from single soleus fibres from young and old rats. *J Physiol.* 1999;520 Pt 2:463–471.
57. Ramamurthy B, Hook P, Larsson L. An overview of carbohydrate-protein interactions with specific reference to myosin and ageing. *Acta Physiol Scand.* 1999;167:327–329.
58. DalleDonne I, Milzani A, Colombo R. H2O2-treated actin: assembly and polymer interactions with cross-linking proteins. *Biophys J.* 1995; 69:2710–2719.
59. Olson TM, Michels VV, Thibodeau SN, Tai YS, Keating MT. Actin mutations in dilated cardiomyopathy, a heritable form of heart failure. *Science.* 1998;280:750–752.

Received August 25, 2004
Accepted November 16, 2004
Decision Editor: James R. Smith, PhD