Electron Paramagnetic Resonance Resolves Effects of Oxidative Stress on Muscle Proteins

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Abstract

We have used site-directed spin labeling and electron paramagnetic resonance (EPR) to explore the effects of oxidation on muscle function, with particular focus on the actin-myosin interaction. EPR measurements show that aging or oxidative modification cause a decrease in the fraction of myosins in the strong-binding state, which can be traced to the actin-binding cleft of the myosin catalytic domain.

Keywords

myosin; actin; muscle; Reactive Oxygen Species (ROS); methionine; aging

INTRODUCTION

The age-related deterioration of muscle function is characterized by a decrease in contractile force, progressive loss of muscle mass, and slowing of muscle movement. The defect in force generation associated with aging is due not only to muscle atrophy but also to molecular defects within the muscle contractile proteins, myosin and actin. Age-related muscle dysfunction has been attributed to oxidative damage caused by a chronic imbalance in reactive oxygen species (ROS) and the antioxidants that combat them, a condition termed oxidative stress. The production of ROS in muscle can be due to many pathophysiological and physiological conditions, including aging, inflammation, and strenuous exercise. ROS can impair force by two different mechanisms: (a) redox signaling that can decrease muscle gene expression leading to contractile protein loss and muscle atrophy and/or (b) post-translational modifications of specific amino acid side chains within contractile proteins can cause contractile dysfunction by decreasing ability to produce force (19). The sulfur-containing amino acids, cysteine (Cys) and methionine (Met), are the prime cellular targets of ROS (27). The chemical modification of the sulfur moiety can cause changes in protein folding, stability, and structure, leading to perturbations in function.

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Myosin, the molecular motor that converts chemical energy from Adenosine-5'-triophosphate (ATP) hydrolysis into mechanical force to produce muscle contraction, has been shown to be oxidatively modified at both Cys and Met residues (15, 21, 23). These modifications lead to functional decline in actomyosin interaction and structural changes necessary for force production. For myosin to generate force, the biochemical steps of ATP hydrolysis must be accompanied by structural transitions in the actin-myosin complex, beginning with a weakbinding complex characterized by dynamic disorder in both proteins (red in FIGURE 1A), and ending in a strong-binding complex that is ordered (green in FIGURE 1A) (31, 32). This force-producing weak-to-strong (W-S) transition involves the coordination of two distinct large-scale structural transitions within myosin (known as the “Minnesota Two-Step”); a disorder-to-order transition of the actin-attached catalytic domain (CD), and a straightening of the bent myosin head due to rotation of the light-chain domain (LCD; acting as a lever arm) relative to the CD (FIGURE 1B). These two structural transitions displace the associated actin filament by about 10 nm toward the center of the muscle, thus shortening the muscle. These large-scale (“global”) structural changes are accompanied by smaller scale structural changes within the myosin CD, acting as a “motor,” including the closing of the actin-binding cleft and the reciprocal opening of the nucleotide binding pocket (FIGURE 1). These changes are allostERIC and are coupled to the lever arm rotation.

Our previous work has shown that the decline in muscle function with both aging and oxidative modification can be attributed to structural changes in myosin (15, 23). Proteomics analysis revealed that functional and structural changes due to in vitro oxidation are primarily due to oxidation of multiple Met residues in myosin. Our recent work has focused on defining how oxidation of particular side chains impacts myosin structure, dynamics, and functional interactions with actin. Understanding the molecular mechanism of age-related decline in muscle strength and contractility requires the examination of the chemical modifications and subsequent functional and structural perturbations of myosin. We propose that oxidation of myosin decreases the dynamic disorder of the weakly bound actomyosin state but increases that of the strongly bound, force-generating state, thus decreasing the magnitude of the force-generating W-S transition (FIGURE 2). We hypothesize that these changes in large-scale dynamics can be attributed primarily to oxidation of specific Met residues within the myosin catalytic domain, most importantly in the actin-binding cleft.

**EPR resolves structural dynamics of actomyosin**

The structural changes that occur within the contractile proteins of muscle have been resolved primarily by the spectroscopic technique electron paramagnetic resonance (EPR), combined with site-directed spin-labeling (SDSL) (31,32, 34). In a typical experiment, site-directed mutagenesis is used to place a cysteine (Cys) amino acid at a selected site on the protein, and a Cys-reactive spin label (small molecule containing a paramagnetic nitroxide free radical) is attached covalently (FIGURE 3). Usually a monofunctional spin label is attached to a single Cys, but recently bifunctional spin labels have been introduced (attached to two Cys), resulting in rigid and stereospecific attachment, and thus reporting more accurately on the protein’s structure and dynamics (FIGURE 3A) (17, 18, 33). The spin label is usually the only paramagnetic component in the sample, so the EPR signal arises exclusively from this site. EPR and SDSL are the ideal tools to study actomyosin structural dynamics, as they resolve high-resolution structural details and dynamics of large macromolecular complexes under physiological conditions in muscle fibers and other large protein assemblies (reviewed in (31, 32, 34)). This is not feasible by other structural techniques such as x-ray crystallography (limited to static structures, not applicable to actomyosin complexes), electron microscopy (static, low resolution), or nuclear magnetic resonance (protein complexes too large).
EPR can be used to study (a) rotational motion in both the nanosecond (conventional EPR, \( V_1 \)) and microsecond (saturation transfer or STEPR, \( V_2' \)) time range, (b) orientational order or disorder of myosin relative to the actin filament, and (c) Å-resolution distance and disorder by dipolar electron-electron resonance (DEER) (FIGURE 3). Conventional EPR is sensitive to rotational motion in the picosecond to nanosecond range (FIGURE 3A, top spectrum) while STEPR can be used to detect slower motions, in the microsecond to millisecond range (FIGURE 3A, bottom spectrum). EPR is not only sensitive to spin-label mobility but also to orientation with respect to the magnetic field (FIGURE 3B). Muscle fibers can be aligned parallel or perpendicular to the magnetic field, and the resulting EPR spectra report the angle \( \theta \) between the spin label’s principle axis and the fiber axis. If a substantial difference is observed between the parallel (red) and perpendicular (blue) spectra, this indicates a highly ordered orientation (FIGURE 3B). A highly disordered orientation would have parallel and perpendicular spectra that are almost identical. EPR can also be used to measure the distance \( r \) between two spin labels (FIGURE 3C) (12). Conventional EPR can be used to measure distances from 0.7 to 2.5 nm, while the pulsed EPR technique double electron-electron resonance (DEER, FIGURE 3C), can be used to measure distances from 1.7 and 6 nm, with 0.1 nm resolution. All of these EPR techniques can be used to resolve multiple structural states of a protein present in solution at the same time.

**EPR reveals changes in weak-to-strong transitions of actomyosin with aging and oxidative modification**

The defects in force generation associated with aging are due in part to molecular defects within the contractile protein myosin (11, 15, 23). Myosin isolated from aged rats shows a decreased ability to produce force (15). To investigate age-related structural changes within myosin, EPR was used to quantify the fraction of myosin molecules in the strong-binding structural states (\( X_S \)) in permeabilized muscle fibers during contraction (2, 30) (FIGURE 4). Semimembranosus muscle fibers isolated from Fischer 344 rats showed age-related changes in \( X_S \) (15). EPR spectra in rigor (all myosin strongly bound, \( X_S = 1 \)) or relaxation (all myosin weakly bound, \( X_S = 0 \)) were not sensitive to aging; but in contraction, aging decreased \( X_S \) (FIGURE 4B, C), indicating that the force-generating W-S transition was diminished. To test the effects of ROS on muscle fibers as a model for aging, skinned muscle fibers were treated with hydrogen peroxide (23). The functional and structural changes in myosin associated with hydrogen peroxide treatment were similar to changes observed with aging (FIGURE 4C). Proteomics analysis revealed that these functional and structural changes were due primarily to oxidation of Met residues in the myosin heavy chain (23).

It has been shown that oxidative myosin modifications usually affect function in muscle fibers or solution in a nonlinear fashion, illustrating both negative (7) and positive cooperativity (22), depending on the type of modification and the aspect of muscle function being measured. We speculate that oxidatively modified myosins bind actin but fail to produce an effective powerstroke, producing drag against filament sliding; more data is needed to make a definitive conclusion about the type of cooperativity exhibited in our system.

**Focus on Methionine Oxidation**

The sulfur-containing amino acids, cysteine (Cys) and methionine (Met), are the prime cellular targets of ROS. Met oxidation by ROS to methionine sulfoxide (MetO), and reduction by Met sulfoxide reductase (Msr), has emerged as a critical cellular process with far-reaching implications in health and disease (16, 24, 28). Reports suggest that Met oxidation can perturb secondary structure and disrupt key hydrophobic interactions within...
proteins, producing both local and global changes in structural dynamics (6, 9, 35). These structural changes lead to functional perturbations such as altered enzymatic activity and protein-protein interactions. For the Ca\(^{2+}\) signaling protein calmodulin, Met oxidation is associated with decreased Ca\(^{2+}\) affinity, loss of secondary structure, significant changes to tertiary structure and increased susceptibility to degradation (3). The reversal of Met oxidation by MsrA and MsrB, which promote selective reduction of the S- and R-diastereomers of MetO respectively, partially reverses both the functional and structural defects caused by oxidation of Met residues (29).

Myosin Met are targets of modification by ROS (23). The impact of ROS on muscle function will depend on the steady-state fraction of modified proteins, which will be determined by the balance struck between oxidation by ROS and repair by antioxidant enzymes. Met oxidation in muscle is reversed by MsrA and MsrB, which are, in turn, tied to the thioredoxin and NADH redox potentials. Cardiac and skeletal muscle differ in the regulation of these antioxidant systems (5, 8). Further, protein Met reactivity toward ROS and accessibility to methionine sulfoxide reductases is sensitive to surrounding amino acids and will be “tuned” according to specific myosin (10). The current understanding of the underlying molecular mechanisms of Met oxidation is limited, and a much clearer picture linking specific oxidative modifications to discrete functional and structural impacts is needed. We aim to define how oxidation of particular side chains impacts protein structure, dynamics, and functional interactions with binding partners. Conventional methods of structural biology (microscopy, crystallography, NMR) are not sufficient, so we are carrying out the first applications of site-directed spectroscopic probes to resolve the impact of Met oxidation on protein structure and dynamics.

Changes in structural dynamics of the actin-binding cleft with oxidative modification of Met residues

As we have previously shown that both aging (15) and oxidative modification (23) perturb the weak-to-strong transition of actomyosin necessary for proper force generation, we next asked whether this perturbation could be traced to specific Met residues located in functionally important subdomains within the myosin CD.

To site-specifically study the effects of Met oxidation on myosin, a cysteine-lite construct of Dictyostelium discoideum (Dicty) myosin II was used. The Dicty myosin II motor domain (S1dC, truncated at 762) is the myosin construct that has proven most amenable to both biochemical analysis and x-ray crystallography; mutagenesis and protein overexpression are routine (26). This is not currently possible with muscle myosin. Using Dicty myosin as such a model is justified by its high level of structural and functional homology with muscle myosin (1, 4, 25). Met is an ideal side chain on which to focus for site-directed labeling, which is done by removing any reactive Cys residues (producing a Cys-lite construct of myosin that is fully functional), then introducing Cys at desired labeling sites. This allows us to focus on the functional and structural consequences of oxidation on specific Met residues in myosin.

Treatment of Dicty myosin II with hydrogen peroxide as an oxidative agent produced a myosin with significant chemical and functional perturbations (14). Mass spectrometry revealed a change in myosin’s molecular mass of ~49 kDa, probably corresponding to the addition of about three oxygen atoms. The ATPase activity of this myosin was also decreased by 50%, the same direction and magnitude as observed for myosin from aged rats and peroxide-treated rabbit fibers (15, 23). To site-specifically study the effects of Met oxidation, site-directed mutagenesis was used to protect Met residues from oxidation by mutating to leucine. Three of nine Mets in the Dicty myosin catalytic domain were found to be sensitive to oxidation: M394, M486, and M642 (14), all three of which are in functionally
important locations within the catalytic domain. M394 is located in the actomyosin binding interface, just N-terminal to the cardiomyopathy loop. M486 and M642 are both located in the force-generating region (composed of the relay helix, SH1 helix, and the converter).

To determine the structural features of myosin that are sensitive to oxidation, spin-labeling sites within the myosin actin-binding cleft (270, 401, and 537) were used to probe the oxidation induced changes in structural dynamics of myosin alone and in complex with actin (FIGURE 5A). Since aging and oxidative modifications perturb the W-S transition of actomyosin, it is likely that these modifications exert their influence via the actin-binding cleft, which undergoes a shift from an open to closed conformation with the W-S transition (FIGURE 1A).

F270C, in the corner of the actin binding cleft near the nucleotide binding site (FIGURE 5A), has been previously shown to be sensitive to actin binding (13). In the apo and strongly actin-bound biochemical states, two motional components are resolved (FIGURE 5B). In the apo state, the faster component dominates the spectrum and when myosin is strongly bound to actin, the slow component dominates. No change is observed with oxidation in the apo state of myosin. However, oxidation shifts the equilibrium toward the slower component for the actomyosin complex.

Distances across the actin-binding cleft from residues G401C in the upper 50 kDa domain to N537C on the lower 50 kDa domain were measured by DEER (FIGURE 5C). N537C and G401C are located within the actomyosin interface and are sensitive to conformational changes in the actin-binding cleft at key points in force generation (13). Notably, the actin-binding cleft populates two distinct conformations when strongly bound to actin, the shorter distance of 2 nanometers corresponding to an ‘open’ cleft and longer distance of 4 nanometers corresponding to a ‘closed’ cleft. While the effects of methionine oxidation are subtle in the apo DEER decay, they are more pronounced in the decay for the strongly actin-bound complex (FIGURE 5C). The initial decay for the oxidized actomyosin sample (red) is more rapid than for the unoxidized sample (black) (FIGURE 5C); indicating a shift toward shorter distances. Fitting the DEER decays yields distance distributions that show enhanced stability of the conformation that corresponds to the shorter ‘open’ distance. Oxidation restricted small scale conformational sampling in both the open and closed cleft conformations, and significantly stabilizes the open cleft conformation, primarily in the strongly-actin bound state. The redistribution of open and closed actin-binding cleft structural states has the potential to disrupt the actomyosin ATPase kinetic scheme as illustrated in FIGURE 1A. The reciprocal opening of the myosin nucleotide pocket that occurs with the closing of the cleft with the W-S transition will likely be affected by oxidation. The defects observed in actomyosin ATPase activity may be explained by a change in the kinetics of product release (P$i$ and/or ADP) from the nucleotide pocket.

**Proposed effect of oxidation on the dynamics of myosin**

FIGURE 6 illustrates our working hypothesis for the mechanism by which Met oxidation decreases actomyosin force. For the actin-binding cleft, myosin structural dynamics is sensitive to oxidation but mainly in the presence of actin. Oxidative modifications in the cleft lead to redistribution of open and closed cleft structural states. Both spin-label mobility inside the cleft and distances measured across the cleft in the actin-binding interface resolved two distinct components that probably correspond to two structural states. Oxidation shifts the equilibrium between states, indicating that the functional effects of oxidation are due not to the stabilization of a new structural state of myosin, but through a change in the distribution of existing structural states. M394 is located in the actin-binding interface, so it is likely that oxidation to methionine sulfoxide at this location is responsible for the changes observed in cleft structural dynamics observed in the presence of actin.
M394 is the last residue of an alpha-helix bordering the cardiomyopathy loop. Although methionine is well-suited for helical structure, methionine sulfoxide can destabilize helices. We hypothesize that oxidation of M394 destabilizes the end of the helix, effectively lengthening the cardiomyopathy loop, altering the interaction of the upper 50 kDa domain with actin, and therefore changing the distribution of cleft structural states in the presence of actin. It is likely that it is this change in actomyosin structural dynamics that underlies the functional decline in actomyosin interaction observed with peroxide treatment.

CONCLUSION

We have shown that EPR and site-directed spin labeling are powerful tools for probing structure-function relationships in muscle proteins, with particular emphasis on the effects of aging and oxidation on the actin-myosin interaction. Future studies will be carried out to test further the model illustrated in FIGURE 6, and to extend this work to other important parts of the myosin motor, such as the relay helix (20) and the converter. Understanding the functional and structural consequences of site-specific oxidation in muscle proteins is crucial in order to illuminate the mechanisms by which oxidative stress affects human health. Our studies are relevant not only to aging but any process involving oxidative stress. The pathogenesis of many cardiac and skeletal muscle diseases; including heart failure, hypertrophic cardiomyopathies, muscular dystrophies, and skeletal muscle myopathies, has been linked to oxidative stress. As myosin is the molecular motor that drives muscle contraction, we propose myosin as one of the main targets of site-specific amino acid oxidative modification induced by oxidative stress. These modifications of myosin cause contractile dysfunction leading to a decreased ability of muscle to produce force.

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REFERENCES


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FIGURE 1. Actomyosin ATPase scheme and associated structural transitions
A: Proposed coupling of biochemical states to structural states and actin movement (A = actin, M = myosin) showing changes in nucleotide bound, which determines the transition from weakly bound (red) to strongly bound (green) states of myosin when bound to actin (yellow). Black two-headed curved arrows signify dynamic orientational disorder. B: Minnesota Two-step. To produce force and actin movement, the W-S transition has two steps: The catalytic domain (CD) of myosin undergoes a disorder-to-order transition (together with actin) followed by a large-scale rotation of the light-chain domain (LCD) relative to the CD. [Adapted from (31). Copyright © 2009 Annual Reviews. Used with permission.]
FIGURE 2. Proposed effect of myosin oxidation on global dynamics of actomyosin
We propose that oxidation decreases dynamics of W states and increases dynamics of S states, decreasing the amplitude of the W-to-S transition and hence force production. The novel hypothesis is that this change in the structural dynamics of both the W and S states of actomyosin can be traced to key methionine residues (black asterisks) in functional important subdomains within the myosin catalytic domain including the actin-binding cleft and the force-generating domain.
FIGURE 3. EPR spectra of myosin
A: conventional (V1) EPR spectrum, for determination of nanosecond rotational motion, saturation transfer EPR (STEPR, V2’) spectrum, for determination of microsecond rotational motion. B: conventional (V1) EPR of myosin on oriented actin in skinned muscle fibers, with the fiber axis oriented parallel (blue) or perpendicular (red) to the applied magnetic field, indicating highly ordered orientation θ (red and blue spectra quite different). C: DEER, used to measure distance r between two attached spin labels, in the range of 2–6 nm. The oscillation period is proportional to r³. In this case r = 2.6 nm.
FIGURE 4. EPR spectra used to resolve structural states of myosin
A: Representative EPR spectra of a spin label attached to the myosin CD in skinned muscle fibers from young rats. Spectra were acquired in rigor (green), relaxation (red), and contraction (black). B: Low-field region of the same spectra (left) and spectra from aged rats (right). Spectra in contraction (black) show an age-related decrease in the S (strong-binding) peak and an increase in the W (weak-binding peak). C: The fraction of strongly bound heads $X_S$, calculated from spectra in B, show that $X_S$ decreases by nearly half due to aging. Similar effects were observed when fibers from young rabbits were oxidized with 50 mM hydrogen peroxide (23). [Adapted from (15). Copyright © 2001 The American Physiological Society. Used with permission.]

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FIGURE 5. Effect of oxidation on structural dynamics of the actin-binding cleft near the actomyosin interface
A: Labeling sites in the actin-binding cleft of myosin used to detect changes in structural dynamics (12). Labeling sites are shown as blue spheres with residue number indicated on myosin crystal structure (1FMV). Subdomains of myosin: Lower 50 kDa domain (blue); upper 50 kDa domain (green), relay helix (red); and nucleotide binding pocket (orange). B: EPR spectra of F270C spin labeled with MSL in the apo (top) and strongly actin-bound (bottom) states. C: DEER decay and distance distributions for spin labels at G401C and N537C across the actin-binding cleft in the apo (top) and strongly actin-bound (bottom) states. Red: oxidized. Black: control. [Adapted from (14). Copyright © 2011 American Chemical Society. Used with permission.]
FIGURE 6. Proposed effect of oxidation on dynamics of myosin
A: In the strongly bound state, the myosin cleft is predominantly closed. Oxidation shifts the cleft’s structural equilibrium toward the open state. B: At the atomic level, we propose that Met oxidation at 394 alters the structural dynamics of helix N (N-terminal to the oxidation site in the U50 domain) so that the cardiomyopathy loop becomes less extended, stabilizing the open cleft conformation.