



Redox-sensitive residue in the actin-binding interface of myosin



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ABSTRACT

We have examined the chemical and functional reversibility of oxidative modification in myosin. Redox regulation has emerged as a crucial modulator of protein function, with particular relevance to aging. We previously identified a single methionine residue in *Dictyostelium discoideum* (*Dicty*) myosin II (M394, near the myosin cardiomyopathy loop in the actin-binding interface) that is functionally sensitive to oxidation. We now show that oxidation of M394 is reversible by methionine sulfoxide reductase (Msr), restoring actin-activated ATPase activity. Sequence alignment reveals that M394 of *Dicty* myosin II is a cysteine residue in all human isoforms of skeletal and cardiac myosin. Using *Dicty* myosin II as a model for site-specific redox sensitivity of this Cys residue, the M394C mutant can be glutathionylated *in vitro*, resulting in reversible inhibition of actin-activated ATPase activity, with effects similar to those of methionine oxidation at this site. This work illustrates the potential for myosin to function as a redox sensor in both non-muscle and muscle cells, modulating motility/contractility in response to oxidative stress.

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1. Introduction

To maintain functional homeostasis within cells, molecules must sensitively detect and respond to the cellular redox state. Reactive oxygen species (ROS) are a family of oxygen-derived molecules that cause covalent modification of proteins and DNA. Many physiological and pathological conditions are associated with the accumulation of ROS, which are countered by an elaborate system of antioxidant enzymes and small molecules [1]. An imbalance between the levels of ROS and antioxidants is generally termed oxidative stress. The most sensitive and selective targets of ROS and antioxidants within proteins, with particular relevance to aging, are the sulfur-containing amino acids, methionine (Met) and cysteine (Cys) [2].

The rapid rate and reversibility of sulfur oxidation give Met and Cys important roles as functional redox switches in proteins. For example, methionine sulfoxide (MetO) can be reduced to Met by

the thioredoxin-dependent enzyme methionine sulfoxide reductase (Msr). Cys oxidation can result in reversible intra- or intermolecular disulfide formation or other thiol modifications. One such modification is glutathionylation, which can covalently modify Cys by attachment of a glutathione (GSH) tripeptide. During episodes of oxidative stress, when the ratio of oxidized glutathione (glutathione disulfide, GSSG) to glutathione is high, susceptible Cys are more likely to be glutathionylated [3–5].

Sulfur-based redox switches are an integral component in cardiac and skeletal muscle function and regulation [6–10]. ROS-mediated mechanisms fundamental to muscle contractility include changes in muscle protein gene expression and post-translational modifications (PTMs) [11,12]. ROS-induced PTMs can indirectly affect muscle contractility, modifying proteins involved in calcium handling [13–15], enzymatic signaling [16,17], or proteolysis [18].

ROS-induced PTMs can also have direct effects on contractility by modifying sarcomeric proteins [18] such as myosin, the focus of the present study. Direct site-specific redox modifications to sarcomeric proteins are emerging as crucial regulators of muscle function. Met oxidation and glutathionylation in actin results in defects in polymerization [19] and activation of myosin ATPase activity [20–23]. Glutathionylation of specific Cys in troponin I and myosin binding protein C (MyBP-C) alter myofilament sensitivity to

Abbreviations: ESI-MS, electrospray ionization mass spectrometry; *Dicty*, *Dictyostelium discoideum*; S1dC, Cys-lite catalytic domain of *Dicty* myosin II; ROS, reactive oxygen species; Met, methionine; MetO, methionine sulfoxide; Msr, methionine sulfoxide reductase; Cys, cysteine; GSH, glutathione; GSSG, oxidized glutathione.

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calcium [24,25]. Site-specific glutathionylation of titin can modulate muscle elasticity in muscle [26]. Myosin has been identified as a target of protein oxidation and glutathionylation [20,22,23,27,28], but little is known about the structural and functional significance of specific redox sites within myosin.

We have shown previously that site-specific methionine oxidation in the *Dicty* myosin II catalytic domain causes a functional decline in actomyosin interaction, correlated with changes in myosin internal dynamics and structure, specifically in the actin-binding cleft [29]. Oxidation of methionine at position 394 (M394), directly N-terminal to the cardiomyopathy (CM) loop of myosin, is responsible for the decline in function [29]. Sequence alignment of the *Dicty* myosin II catalytic domain with all human myosin II isoforms reveals that this residue is a conserved cysteine residue in all isoforms of human cardiac and skeletal myosin. This conserved Cys has been identified as a site of glutathionylation in β -cardiac myosin [27].

In the present study, our goal is to examine directly the redox sensitivity of this residue. Using site-directed mutagenesis, chemical modification, and redox-specific enzymes, we have produced and reversed site-specific redox modifications, including Met oxidation and Cys glutathionylation, and determined quantitatively the associated chemical and functional changes in the myosin catalytic domain. Our results show that myosin is an oxidatively labile protein whose level of activity can be regulated by the redox status of selective sulfur-containing functional groups within its catalytic domain.

2. Methods

2.1. Protein preparations and assays

Site-directed mutagenesis of the *Dicty* myosin II gene, truncated at residue 762, containing only a single (non-reactive) cysteine at position 655 (“Cys-lite” S1dC construct [30]), was performed using the QuikChange II XL kit (Invitrogen). In addition, all constructs (except for the M394C mutant) contain a T688C mutation (needed for sufficient protein expression in *Dicty*) which was spin-labeled with IASL [4-(2-Iodoacetamido)-2,2,6,6-tetramethyl-1-piperidinyloxy] (Sigma-Aldrich, USA) to protect from oxidative modification. These proteins were expressed and purified from *Dicty* orf+ cells as previously described [31]. F-actin was prepared from rabbit skeletal muscle as previously described [32,33]. Human methionine sulfoxide reductase A protein was obtained from Abcam Biochemicals (Cambridge, England). Actin-activated ATPase activity was measured by detection of ADP using an NADH-coupled ATPase assay [34] using 0.2 μ M S1dC, 2 mM ATP, and increasing concentrations of phalloidin-stabilized F-actin in 10 mM Tris, 2 mM $MgCl_2$ (pH 7.5). The actin-dependent activity was analyzed to determine V_{max} (specific activity at saturating actin) and K_{ATPase} (actin concentration needed for $V = 0.5V_{max}$) [34].

2.2. Reversible oxidation and glutathionylation

Oxidative modification of Met in myosin was accomplished by treatment with 500 mM hydrogen peroxide (purchased as a stabilized 30% solution from Sigma-Aldrich) for 30 min on ice, followed by dialysis. Reversal of Met oxidation was achieved by incubating 20 μ M myosin for 30 min at 25 °C with 4 μ M MsrA (Abcam) and 1 mM DTT in 10 mM Tris (pH 7.5). Glutathionylation of the M394C mutant myosin was accomplished by treatment with varying concentrations of GSH in the presence of 10 mM diamide for 1 h on ice, followed by dialysis. Glutathionylation was reversed by addition of 1 mM DTT for 1 h on ice, followed by dialysis.

2.3. Mass spectrometry

Myosin samples for electrospray ionization mass spectrometry (ESI-MS) were prepared as previously described [29]. Myosin mass was determined using a QSTAR quadrupole-TOF mass spectrometer (ABI) with an electrospray ionization source. 20 μ M myosin was introduced into the solvent stream using a 10 μ L injection loop installed in the integrated loop injector with a total of five injections per sample. ESI spectra were acquired continuously over the range 500–2000 m/z , and were analyzed with BioAnalyst QS (ABI) software v 1.1.5.

3. Results

3.1. Site-directed redox modification of myosin residue 394

S1dC, a construct of the myosin II catalytic domain devoid of reactive cysteines, was used as the model system to examine the functional consequences of reversible oxidative modifications. Using *Dicty* myosin as such a model is justified by its high level of structural and functional homology with muscle myosin II [35,36]. S1dC contains nine Met residues, three of which are susceptible to oxidative modification by H_2O_2 [29]. Oxidation of Met 394, which is located at the C-terminus of an α -helix that transitions into the cardiomyopathy loop in the actin-binding interface (Fig. 1A), has been shown to be responsible for the observed functional and structural perturbations. M394 is a cysteine residue in all isoforms of human cardiac and skeletal myosin (Fig. 1).

Site-directed mutagenesis and site-specific oxidation define the key redox-sensitive residues that are chemically and functionally vulnerable to oxidants. Methionine to Leucine (M-to-L) mutations control susceptibility of Met oxidation, since Leu conservatively substitutes for Met and is not modified under the conditions used for *in vitro* oxidation. Methionine to Glutamine (M-to-Q) mutations structurally and functionally mimic Met oxidation to Met sulfoxide, since the substitution of glutamine for methionine introduces an oxygen atom at the same position in the side chain as the sulfoxide oxygen. This site-directed substitution approach of M-to-L and M-to-Q has previously been validated for the functional analysis of site-specific Met oxidation in several protein systems [37–39]. *Dicty* mutants M394L and M394Q were used here to examine the redox capabilities of this residue. M394 was also mutated to cysteine residue, as found in various human myosin II isoforms (Fig. 1B). As all other reactive Cys in S1dC have been mutated to other residues, this system is ideal for studying site-specific cysteine modification.

3.2. Reversible oxidation of Met 394

To quantify the use of methionine sulfoxide reductase (Msr) to repair individual methionine sulfoxides (MetO) in the myosin catalytic domain, we have used *in vitro* conditions to generate a homogeneous population of oxidatively modified myosin in which three of nine Mets are oxidized to their corresponding MetO, as revealed by mass spectrometry. Prior to oxidative modification, the *Dicty* myosin II catalytic domain exhibits one major peak with a molecular mass of 88,930 Da (Fig. 2A), corresponding to native S1dC expressed in *D. discoideum* spin-labeled with IASL. The conditions used for *in vitro* oxidative modification of myosin result in a 49 Da increase in mass, corresponding to the addition of three oxygen atoms (Fig. 2A) as we have previously shown [29]. Upon treatment of this oxidized myosin with MsrA, the molecular mass of S1dC decreases by 16 Da, consistent with the reversal of one MetO back to Met (Fig. 2A). Treatment of native (unoxidized) S1dC with MsrA shows no shift in molecular mass (Fig. 2A). No additional

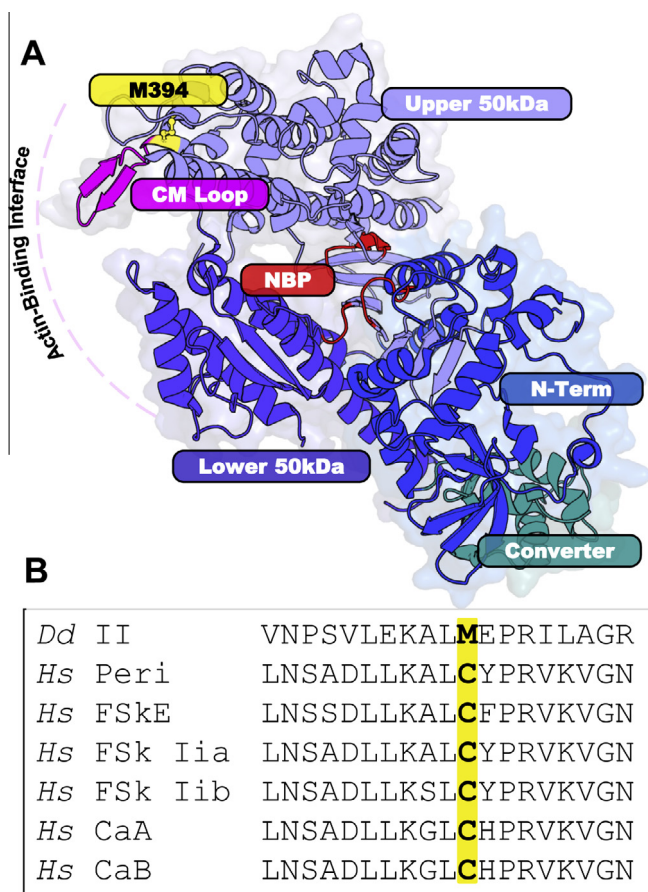


Fig. 1. (A) Structure of the myosin catalytic domain (PDB: 1FMV). Light blue: upper 50 kDa domain. Violet: lower 50 kDa domain. Dark blue: N-terminal domain. Teal: converter domain. Pink: actin-binding loops. Red: nucleotide-binding pocket. Inset: location of M394 (yellow) N-terminal to the cardiomyopathy (CM) loop (red) in the myosin actin-binding interface. (B) Sequence alignment of *Dicty* (Dd) myosin II M394 residue with *Homo sapiens* (Hs) skeletal and cardiac muscle myosin II isoforms (Peri = perinatal, FSke = fast skeletal embryonic, FSk = fast skeletal, FSk ex = fast skeletal extraocular, and Ca = cardiac). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

MetO repair is observed following incubation of myosin with MsrA for longer periods of time.

To determine whether M394 was the MetO reversed to Met by MsrA, leucine was substituted for methionine at M394 to protect from oxidation. Oxidation of the M394L mutant with H_2O_2 caused

a shift of 32 Da, corresponding to the formation of two MetOs (Fig. 2B), one less than observed in the presence of M394 (Fig. 2A). Treatment of oxidized M394L with MsrA had no effect on the mass (data not shown), showing that the other oxidized residues cannot be reduced by MsrA. These results, combined with the results of methionine sulfoxide reversal on globally oxidized *Dicty* myosin II, indicate that M394 is the only Met that, after oxidation to MetO, can be reduced back to Met by MsrA.

Oxidation of myosin with hydrogen peroxide induced a 2-fold decrease in maximum actin-activated ATPase rate (V_{max}) in the presence of saturating actin, as observed previously [29], and this inhibition was completely rescued by subsequent treatment with MsrA (Fig. 2C, M394). Treatment with DTT alone had no effect (data not shown). Mutation M394Q produced a 2-fold decrease in actin-activated ATPase activity, matching the effect of oxidation, and mutation M394L protected myosin against the effects of oxidation (Fig. 2C).

3.3. Reversible glutathionylation of M394C

ESI-MS was used to quantify the modification of Cys 394 by glutathione (Fig. 3A). Prior to modification, the M394C mutant S1dC exhibits one major peak with a molecular mass of 88,691 (Fig. 3A). Note the difference from the molecular mass in Fig. 2A and B due to the absence of spin label. Treatment with glutathione (GSH) and diamide produced a shift in molecular mass of ~305 Da, corresponding to the addition of 1 glutathione (Fig. 3A, blue to red). The reversibility of this modification was assessed by addition of DTT, which caused a shift back to 88,691 (Fig. 3A, red to magenta), indicating that the modification with glutathione is indeed reversible.

This glutathionylation treatment produced a substantial decrease in actin-activated ATPase activity that was reversed by DTT, with no effect caused by DDT or diamide alone (Fig. 3B and C). In untreated myosin, V_{max} (activity at saturating actin) and K_{ATPase} (actin concentration needed for half-maximal activation) are $7.0 \pm 1.1 s^{-1}$ and $41.0 \pm 11.8 \mu M$; glutathionylation decreased V_{max} by a factor of 5 (to $1.3 \pm 0.3 s^{-1}$), with no significant effect on K_{ATPase} ($52.7 \pm 17.5 \mu M$) (Fig. 3D). This result suggests that the effect of glutathionylation on myosin function is not simply due to decreased actin affinity, but is due to the reduced activity of actin-bound myosin.

4. Discussion

We previously identified at least three *Dicty* myosin II methionines that are susceptible to oxidation by peroxide: M394, M486 and M642, and showed that M394 is responsible for the oxida-

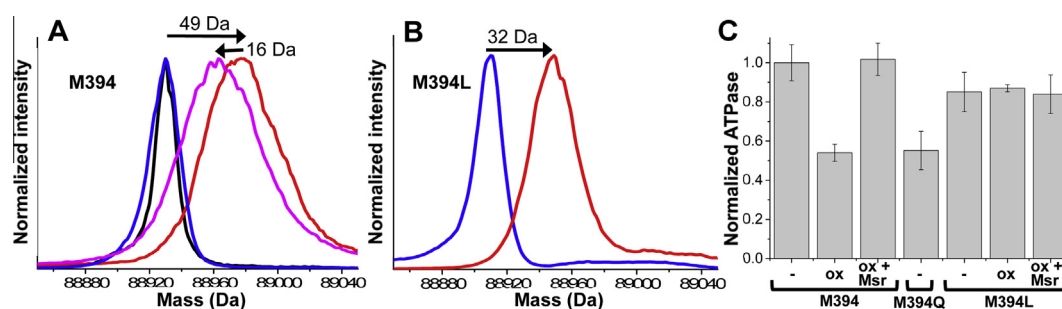


Fig. 2. Chemical and functional reversal of oxidation, using MsrA. (A) ESI-MS of wildtype residue M at position 394. Blue: no treatment. Red: after treatment with H_2O_2 . Magenta: Red sample after treatment with MsrA. Black: Blue sample after treatment with MsrA. (B) Mutant M394L, same color scheme as A. MsrA treatment had no effect on the mass of either sample in B (data not shown). (C) Recovery of S1dC actin-activated ATPase activity (V_{max}) upon repair of MetO with MsrA. Wildtype S1dC was oxidized with 500 mM H_2O_2 on ice for 30 min (ox), then treated with 0.5 μM MsrA for 30 min at 25 °C (ox + Msr). The oxido-mimetic mutant M394Q (untreated) is shown for comparison. Error bars are SEM ($n \geq 3$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

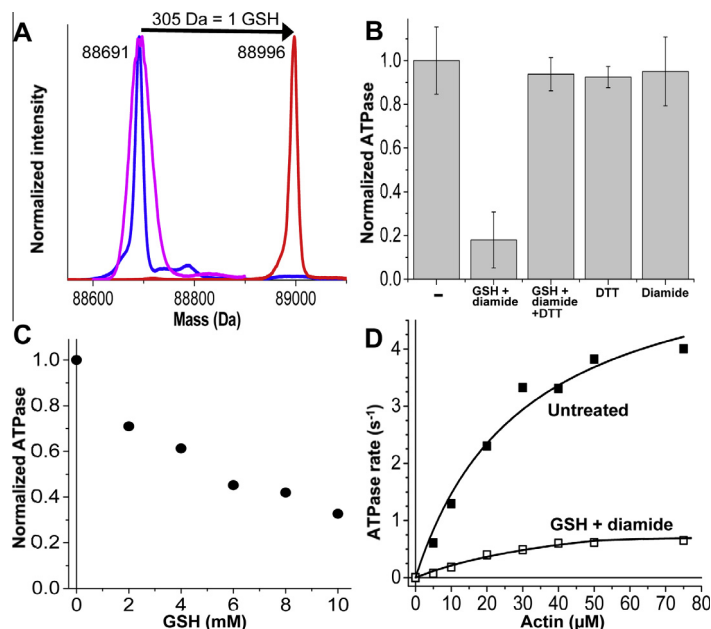


Fig. 3. (A) ESI-MS illustrating glutathionylation and reversal in *Dicty* myosin II M394C. Blue: untreated. Red: treated with 10 mM GSH plus 10 mM diamide for 1 h on ice. Magenta: treated with DTT after glutathionylation. (B) ATPase activity of M394C in the presence of 50 μ M actin. Untreated (–), after treatment with 10 mM GSH plus 10 mM diamide, 10 mM GSH plus 10 mM diamide followed by 10 mM DTT, 10 mM DTT alone, or 10 mM diamide alone. Error bars are SEM ($n \geq 3$). (C) ATPase activity of M394C S1dC, in the presence of 50 μ M actin, after treatment with increasing concentrations of GSH (with equimolar diamide). (D) Actin-dependence of ATPase activity of M394C, untreated (closed squares) and treated with 10 mM GSH plus 10 mM diamide (open squares). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tion-induced functional decline in myosin actin-activated ATPase activity and actomyosin interaction [29]. The present study shows that the oxidation of M394 is reversible by MsrA and that the reduction of this MetO completely rescues the decline in ATPase activity (Fig. 2). Mutation of this Met to Cys, mimicking the residue present in all human isoforms of skeletal and cardiac myosin, shows that this Cys can also be oxidatively modified by glutathione, decreasing actomyosin functional interaction (Fig. 3).

Methionine sulfoxide reductase most readily reverses oxidized methionines that are solvent-exposed and located in protein regions composed predominantly of random-coil [8,40,41]. It is therefore not surprising that only oxidation of M394, but not M486 and M642, is reversible. Based on the crystal structure, M394 is solvent-exposed when myosin is not bound to actin, and exists at the extreme end of an α -helix, transitioning into a random coil of the CM-loop (Fig. 1A). Although both M486 and M642 are also predicted to be solvent-exposed, they exist in highly structured regions of S1dC, in the force-generating domain. Using site-directed spin labeling (SDSL) and electron paramagnetic resonance (EPR), we previously showed that oxidation of M394 changes the structural dynamics of the actin-binding cleft, [29]. This change in structural dynamics involves a perturbation of the actin-binding cleft's open-to-closed transition, which is necessary for proper force production and movement [29,42].

Glutathionylation of Cys is a reversible oxidative modification that plays a key role in redox regulation of proteins and signal transduction. In α -cardiac myosin, three Cys within the myosin heavy chain are susceptible to glutathionylation [27], which inhibits myosin ATPase activity at high levels of GSSG but potentiates ATPase activity at low levels of GSSG [27]. Glutathionylation of these three Cys produced changes in the structure of myosin detected by changes in fluorescence and increased susceptibility to proteolytic cleavage [27]. Two of these Cys residues, Cys 400 and Cys 695, are located within the myosin catalytic domain. Cys400 is equivalent to M394 in *Dicty* myosin, located in the

actin-binding cleft adjacent to the CM-loop, while Cys695 is the reactive cysteine SH2 in the force-generating domain of myosin. Our mutagenesis strategy allows for site-specific investigation of Cys glutathionylation in the myosin catalytic domain. The M394C S1dC mutant can be modified specifically by glutathione *in vitro* (Fig. 3), resulting in a decrease in actin-activated ATPase activity (Fig. 3). In addition to this direct effect of modifying myosin function, it is likely that glutathionylation can also play a protective role [43], protecting myosin against irreversible protein thiol oxidation at Cys residues in crucial subdomains of myosin such as the actin-binding cleft.

Oxidation of methionine to methionine sulfoxide, as well as glutathionylation of cysteine, has been shown to affect protein secondary and tertiary structure. The presence of MetO can cause α -helical destabilization [44–48]. Glutathionylation has a similar effect, having been shown to decrease α -helical content of proteins [49,50]. As both Met oxidation and glutathionylation at residue 394 in *Dicty* myosin II cause a decrease in actin-activated ATPase activity of myosin (Figs. 2 and 3), we hypothesize that oxidative modification at this site, whether Met oxidation or Cys glutathionylation, destabilizes the adjacent helix, changing the structural dynamics of the entire CM-loop and hence interaction with actin. This change in structural dynamics at the actin-binding cleft probably perturbs the weak-to-strong transition of myosin, necessary for proper force production. This hypothesis will be the subject of future testing by spectroscopic methods in both muscle and nonmuscle myosin.

5. Conclusion

Redox regulation has emerged as a crucial modulator of protein function. The production of ROS in muscle can be due to many pathophysiological and physiological conditions, including aging, heart failure, inflammation, and strenuous exercise. ROS-induced

oxidative modifications occur frequently in sarcomeric proteins, including myosin. This work illustrates the potential for myosin to function as a redox sensor in both nonmuscle and muscle cells, potentially modulating motility/contractility in response to oxidative stress.

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