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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.

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 μM 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 μM myosin for 30 min at 25 °C with 4 μM 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 μM myosin was introduced into the solvent stream using a 10 μL 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 alpha-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 to 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