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Stereospecific Oxidation of Calmodulin by Methionine Sulfoxide Reductase A

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Abstract

Methionine sulfoxide reductase A has long been known to reduce S-methionine sulfoxide, both as a free amino acid and within proteins. Recently the enzyme was shown to be bidirectional, capable of oxidizing free methionine and methionine in proteins to S-methionine sulfoxide. A feasible mechanism for controlling the directionality has been proposed, raising the possibility that reversible oxidation and reduction of methionine residues within proteins is a redox-based mechanism for cellular regulation. We undertook studies aimed at identifying proteins that are subject to site-specific, stereospecific oxidation and reduction of methionine residues. We found that calmodulin, which has 9 methionine residues, is such a substrate for methionine sulfoxide reductase A. When calmodulin is in its calcium bound form, Met77 is oxidized to S-methionine sulfoxide by methionine sulfoxide reductase A. When methionine sulfoxide reductase A operates in the reducing direction, the oxidized calmodulin is fully reduced back to its native form. We conclude that reversible covalent modification of Met77 may regulate the interaction of calmodulin with one or more of its many targets.

Keywords

Calmodulin; methionine oxidation; methionine reduction; methionine sulfoxide reductase; signaling

Methionine and cysteine are the two sulfur-containing amino acids that are present in peptides and proteins. Well-known roles of cysteine include antioxidant defense, catalysis, protein structure, and redox sensing and regulation. Other than its common role in protein initiation, the functions of methionine have been poorly defined. Biochemistry texts often treat it as a generic hydrophobic amino acid, but recent investigations from several laboratories are changing this view. Cysteine provides antioxidant defense through reversible oxidation and reduction of the tripeptide glutathione. We proposed some 15 years ago that methionine also serves as an antioxidant, but it does so through reversible oxidation

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and reduction of methionine residues in proteins [1]. While cysteine is well-recognized for the ease of its oxidation, it is often not appreciated that methionine is quite readily oxidized to methionine sulfoxide (metO) [2, 3]. However, oxidation by hydrogen peroxide, hypochlorous acid, and other reactive species creates a chiral center at the sulfur so that the metO produced is a mixture of the S and R epimers [4]. Reduction of the sulfoxide back to methionine is catalyzed by methionine sulfoxide reductases (Msr), enzymes found in almost all organisms from microbes to humans. Two distinct classes of reductases are required, one to reduce the S-epimer (MsrA) and one to reduce the R-epimer (MsrB). MsrA was described many years before MsrB. As a consequence most studies published so far have focused on MsrA. Review articles for both classes are available [5–7], as are those which focus on methionine oxidation in proteins and its effects [3, 8].

Reduction of methionine residues in proteins allows them to react again with reactive species, creating a system with catalytic efficiency in scavenging potentially damaging species. Thioredoxin and thioredoxin reductase act sequentially to regenerate active Msr, with the net result being the catalytic scavenging of reactive oxygen species at the expense of NADPH. A scheme of the system has been published [9]. There is substantial experimental evidence to support the importance of this system, both *in vivo* and *in vitro*. Knocking out MsrA caused increased susceptibility to oxidative stress in mice [10], yeast [11], and bacteria [12–14]. Conversely, overexpressing MsrA conferred increased resistance to *Drosophila* [15], *Saccharomyces* [16], *Arabidopsis* [17], PC-12 cells [18], and human T cells [16]. Overexpression in *Drosophila* doubled the lifespan of the flies [15]. Studies on the evolution of mitochondria and their use of an alternate genetic code also support the proposition that methionine in proteins acts as an antioxidant [19, 20].

Thus, cysteine and methionine residues both have important antioxidant functions. Other recent studies also demonstrate that methionine, like cysteine, has a substantive role in stabilizing protein structure. Methionine does so through hydrophobic interaction with nearby aromatic residues [21]. As mentioned above, the importance of cysteine residues in redox sensing and regulation is now well established [22, 23]. Since methionine oxidation is a reversible covalent modification, investigators had suggested that it may also have a role in cellular signaling and regulation [24–26]. Hydrogen peroxide, typically produced in the cell by NADPH oxidases, usually serves as the oxidizing agent in cysteine-mediated redox regulation through oxidation of an active site, low pK_a cysteine to sulfenic acid [23]. We recently demonstrated that MsrA itself catalyzes the stereospecific oxidation of protein methionine residues to S-metO [27], and that micromolar hydrogen peroxide is sufficient to oxidize the low-pK_a active site cysteine of MsrA to sulfenic acid [28]. MsrA is thus a bifunctional enzyme, and a structural basis for regulation of MsrA to prevent a futile cycle has been proposed [27]. When operating in the oxidizing direction, MsrA is a methionine peroxidase[1] catalyzing the formation of methionine sulfoxide:

$$H_2O_2 + \text{methionine} \rightarrow \text{methionine sulfoxide}$$

[1]We had previously referred to MsrA as a methionine oxidase, but as pointed out by a knowledgeable reviewer, that term is incorrect because MsrA does not use oxygen as a substrate.

In addition to hydrogen peroxide, free or protein-bound metO can also serve as the oxidizing agent, although the concentration required to drive the reaction is much higher than for hydrogen peroxide. Enzymes whose catalytic cycle includes formation of a cysteine sulfenic acid are at risk of inactivation by hyperoxidation of the sulfenic acid (−SOH) to the sulfinic acid ($-SO_2H$) [29]. Hydrogen peroxide and organic hydroperoxides mediate the hyperoxidation, but metO cannot. Thus, it is experimentally simpler to use metO as the oxidant when studying MsrA in the oxidizing direction. While MsrA can mediate the oxidation of methionine residues in proteins, MsrB does not catalyze the reaction [27]. Having established that MsrA is a bifunctional enzyme, the next logical step in elucidating its role in cellular regulation would be the identification of proteins that undergo site-specific, stereospecific oxidation of their methionine residues. We describe here studies aimed at finding those proteins, and we demonstrate that calmodulin is one such target of MsrA mediated oxidation.

EXPERIMENTAL PROCEDURES

Bovine lens crystallin was purchased from Sigma (C4163). Gifts of purified proteins from colleagues in the National Heart, Lung, and Blood Institute were: human recombinant apolipoprotein A from Alan Remaley; rabbit actin from Ikuko Fujiwara; recombinant human α-synuclein from Nelson Cole; and human recombinant thioredoxin-1 from Duck-Yeon Lee. Glutamine synthetase was produced in our laboratory following a published procedure [30]. Recombinant human calmodulin, human α_1-antitrypsin, human peroxiredoxin 6, and mouse 14-3-3 zeta/delta were produced in BL21 (DE3) *E. coli* and purified as follows. Cells were disrupted by sonication in buffer A (20 mM Tris, 1 mM EDTA, pH 7.5) containing 1 mM phenylmethylsulfonyl fluoride. After centrifugation at 27,000*g* for 30 min, the supernatant was made 1% in streptomycin sulfate to remove nucleic acids followed by protein precipitation by 80% ammonium sulfate. For calmodulin, 14-3-3 zeta/delta, and peroxiredoxin 6, the protein precipitates were redissolved in and dialyzed against buffer A. The solution was loaded onto an anion exchange column (TSKgel DEAE-5PW, 21.5 mm×15 cm) equilibrated with buffer A and run at 5 ml/min. The column was eluted with a linear gradient of NaCl in buffer A: 0 to 500 mM over 30 min and then 500 to 1000 mM over the next 15 min. Fractions were analyzed by SDS-PAGE and visualized with Coomassie Brilliant Blue R-250. Fractions containing protein were pooled, and concentrated to 4 ml with a 10 kD cutoff Amicon Ultra-15 centrifugal filter units. Solid ammonium sulfate then was added with stirring to give a final concentration of 1 M. The solution was applied to a phenyl column run at 1.0 ml/min (TSKgel Phenyl-5PW, 7.5 mm×7.5 cm) equilibrated with buffer B (20 mM HEPES, 1 mM EDTA, 1 M ammonium sulfate, pH 7.5) and eluted with a linear gradient of decreasing ammonium sulfate: 1.0 to 0.5 M over 30 min and then 0.5 M to 0 M over the next 15 min. Fractions with the purified protein were again visualized after SDS-PAGE gel electrophoresis. Calmodulin was not bound to the column while eluted fractions with 14-3-3 zeta/delta or peroxiredoxin 6 were pooled. The purified proteins were then dialyzed against buffer A. α_1-antitrypsin was purified by gel filtration (Sephacryl S-200 packed manually in a Bio-Rad EconoColumn, 2.5×120 cm), followed by anion exchange chromatography as above. Protein concentrations were determined

spectrophotometrically at 280 nm from their molar extinction coefficients. Preparations were stored at −80° C until use.

Mouse liver cytosolic and mitochondrial fractions were prepared as published [31]. The activity of calmodulin targets was assayed following published procedures for phosphodiesterase [32], calcineurin [33], and myosin light chain kinase [34]. Recombinant MsrAs, both myristoylated and non-myristoylated, were produced in *E. coli* and purified and assayed as described [31] except that the carboxyl-His tagged C72A active site mutant was purified on a nickel-NTA column (Qiagen). Residues of each protein are numbered from the first initiating Met residue, regardless of whether it is present in the mature protein. The numbering of calmodulin residues can especially be a source of confusion since previous literature often did not count the initiating methionine. Thus, Met77 to which we often refer in this paper was identified as Met76 by some workers.

Affinity Chromatography

MsrA columns were prepared by covalently linking MsrA to Affi-Gel 10 (BioRad) through primary amino groups. Five hundred to 600 μg recombinant protein (myristoylated or nonmyristoylated and wild-type or the C72A active site mutant) was dialyzed against 50 mM HEPES pH 7.6, 1 mM EDTA, mixed with 500 μl resin and incubated at 4 °C for 4 hr. The resin was poured into an Econo column (Bio-Rad) and washed with 10 bed volumes of buffer A (10 mM Tris, pH 7.5) followed by an additional 10 bed volumes of buffer A with 1 M KCl. The column was then washed again with 10 bed volumes of buffer A. Mouse liver extracts (300 mg) were prepared by homogenization in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM PMSF, and 1% (v/v) protease inhibitor cocktail (Sigma P8340). The extracts were passed over the affinity column three times which were then washed with 20 bed volumes of buffer A. Completeness of washing was confirming by monitoring absorbance at 280 nm. Bound proteins were eluted stepwise with 20 bed volumes of buffer A containing different salt concentrations (0.1, 0.25, 0.5, 1 M KCl). Each eluent was concentrated to 150 μl and analyzed by SDS-PAGE electrophoresis, imaged by either Coomassie Blue or silver staining.

Immunoprecipitation

Homogenates were prepared in 50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM PMSF, and 1% (v/v) protease inhibitor cocktail. Extracts were prepared from mouse liver, kidney, brain, and heart from wild-type mice, from transgenic mice overexpressing myristoylatable MsrA, and from knockout mice lacking MsrA [35, 36]. HEK293 cells were transiently transfected using the calcium phosphate method with PCR3.1 vectors expressing one of these MsrA: wild-type, G22A (nonmyristoylatable), C72A (active site mutant) or G22A/C72A [31]. Cells were harvested 36 h after transfection and disrupted by sonication. Tissue or HEK293 extracts were centrifuged for 30 min at 15,700*g* at 4° C. Protein concentrations were determined by Bradford's method [37]. HEK293 supernatant containing 1 mg protein or tissue supernatant containing 2–5 mg protein was mixed with 3 μl monoclonal anti-MsrA (Abfrontier, South Korea) and brought to 1 ml with buffer. Binding was allowed to proceed for 2 h at 4 °C in 1 ml total volume. Thirty μl protein G Sepharose (Sigma) was added and incubation continued for an additional 2 h. The Sepharose was pelleted by centrifugation for

30 s at 800g and washed 5 times with rocking for 5 min using 1 ml 50 mM Tris-HCl pH 7.5, 1 mM EDTA. Bound proteins were eluted with 30 μl 2x SDS-PAGE sample buffer (100 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 10% β-mercaptoethanol).

Oxidation and reduction of proteins

The purified protein being tested as an MsrA substrate (20 μM) was incubated for 1 h at 37 °C with 5 μM MsrA, 20 mM Tris, pH 7.5, 1 mM diethylene triamine pentaacetic acid (DTPA), 10 mM metO in a total volume of 40 μl [27]. The reaction was stopped by addition of 2 μl 10% trifluoroacetic acid. Proteins were then analyzed by reverse phase HPLC-mass spectrometry using the gradient and instrumentation as described [27]. In the case of calmodulin, 0.5 mM $CaCl_2$ was present when the calcium-bound form was tested, and the incubation time was varied to generate a time course. The reversibility of MsrA-mediated oxidations was tested by addition of 10 mM DTT and further incubation for 30 min. Oxidation and reduction were analyzed by reverse phase HPLC-mass spectrometry as above.

Larger scale production of Met77 oxidized calmodulin was accomplished by incubating calmodulin (20 μM) with 5 μM myristoylated MsrA in 20 mM Tris-HCl, pH 7.5, 1 mM $CaCl_2$ at 37° C for 7 hr. The total volume was 1.5 ml. Calmodulin with all 9 met oxidized to metO was prepared by incubating calmodulin (20 μM) with 20 mM H_2O_2 in 20 mM Tris-HCl pH 7.5, 1 mM DTPA in the absence of calcium at 37° C for 4 h, again in a total volume of 1.5 ml. The oxidized calmodulins were purified using DEAE chromatography on a Toso TSKGel DEAE-5PW (7.5 cm long, 7.5 mm diameter, 10 μm beads). The column was equilibrated in 20 mM Tris, pH 7.5 and run at 1.0 ml/min. One mM $CaCl_2$ was included when the MsrA oxidized calmodulin was purified but not when the H_2O_2 oxidized calmodulin was purified. Calmodulin was loaded in the same buffer. Keeping the buffer concentration constant, proteins were eluted by a linear NaCl gradient: 0–500 mM from 0–30 min and 500–1000 mM from 30 to 45 min. Fractions were analyzed by HPLC-mass spectrometry and those containing oxidized calmodulin were pooled and then dialyzed against 20 mM Tris, pH 7.5, 1 mM $CaCl_2$. The concentration of calmodulin was determined spectrophotometrically at 280 nm using a molar absorbtivity of 2,560 M^{-1} cm^{-1}.

RESULTS

Affinity Chromatography and Immunoprecipitation

As an unbiased approach to identification of binding partners or substrates of MsrA, we made columns with attached MsrA [38]. Cytosolic MsrA is myristoylated, a post-translational modification which enhances the interaction of MsrA with certain substrates [38], the columns were made with either myristoylated or non-myristoylated MsrA, and extracts from mouse liver were passed over the columns. Eluates were analyzed by SDS-PAGE and compared to control columns with either no protein attached or with bovine serum albumin. The only interacting protein we found was cellular MsrA itself (not shown), presumably because of the weak propensity of MsrA to interact with itself. We considered the possibility that substrates containing metO interacted with MsrA but were released

following reduction of the metO to met. We therefore made affinity columns in which the active site Cys72 was mutated to Ala72, and the protein was either myristoylated or not. They were loaded with either the unfractionated liver extract, the cytosolic fraction, or the mitochondrial fraction, but again no interacting proteins were found. We then examined whether immunoprecipitation of MsrA would coprecipitate binding partners. We tested extracts of liver, kidney, brain, and heart from wild-type mice, from transgenic mice overexpressing myristoylatable MsrA, and from knockout mice lacking MsrA [35, 36]. We also tested extracts from control HEK293 cells and cells transfected to express high levels of wild-type, myristoylatable, non-myristoylatable, or Cys72Ala inactive MsrA. No interacting proteins were detected (not shown). We recognize that some may consider the results presented in this paragraph to be “negative”. We believe the experiments were executed with attention to experimental and technical details, and therefore the results support the conclusion that the interaction of MsrA with other proteins is transient in nature.

Interaction with Selected Proteins Detected by Their Oxidation

Given the inability of unbiased methods to reveal interacting proteins, we chose to test specific, recombinant proteins for interaction with MsrA by assaying for their oxidation by MsrA. We selected proteins whose methionine residues might be substrates for the myristoylated MsrA peroxidase and whose oxidation might serve a regulatory role. Two of the 11 tested proteins were substrates, α_1-antitrypsin and calmodulin (Table 1). The surface exposed Met358 of α_1-antitrypsin was readily oxidized (Fig. 1, Table 2), a modification known to abolish its anti-protease activity [39]. Incubation of the oxidized α_1-antitrypsin with MsrA operating in the reductase direction completely restored the native form of α_1-antitrypsin, confirming that oxidation generated only the S-epimer of MetO358 (Fig. 1). Because α_1-antitrypsin is a circulating antiprotease and MsrA is not normally found in the circulation, we thought it unlikely that oxidative modification of $\alpha_1$antitrypsin by MsrA occurs *in vivo*. In contrast, the important regulatory protein calmodulin is an intracellular protein, and as we document below, the calcium bound form of calmodulin was stereospecifically oxidized at Met77.

Site Specific and Stereospecific Oxidation of Calmodulin

Calmodulin was oxidized by MsrA both in the presence and absence of calcium (Fig. 2). When incubated with MsrA in the presence of calcium, the mass of calmodulin increased by 16.0 Da (Fig. 2A), consistent with oxidation of a single met residue to metO. When the oxidized calmodulin was incubated with MsrA under reducing conditions, it was fully recovered as native calmodulin, establishing that the oxidation generated only the S-metO form. In the absence of calcium, methionine residues were promiscuously oxidized giving rise to forms which differed in mass by 16 Da steps (Fig. 2B). Sequencing demonstrated that all 9 methionines could be oxidized (not shown), as is the case when hydrogen peroxide alone is used to oxidize the protein [40].

Site-specificity is a hallmark of post-translational covalent modifications that function as regulators, and we therefore focused our attention on the characterization of calmodulin oxidized by MsrA in the presence of calcium. Control and calmodulin oxidized in the presence of calcium were digested with trypsin and the peptides mapped by HPLC-mass

spectrometry. Peptides containing 8 of the 9 met in calmodulin were separated and identified, but none were modified in the oxidized calmodulin. The tryptic peptide expected to include Met77 is only a dipeptide (Met-Lys) and was not recovered in either the control nor oxidized protein digests, presumably because it did not bind to the reverse phase column. The proteins were then digested with chymotrypsin and mapped. The protease generated 2 peptides containing Met77, one with residues 73–90 and one with 74–90. The corresponding peptides from the oxidized calmodulin eluted at a slightly earlier time, as expected for the conversion of met to metO, and in both cases their masses were increased by 16 Da: The calculated mass of $P_{73–90}$ is 2,185.01 and the observed mass from oxidized calmodulin was 2,201.00. The calculated mass of $P_{74–90}$ is 2,053.97 and the observed mass from oxidized calmodulin was 2,069.96. Sequencing of both oxidized peptides confirmed that Met77 was oxidized to metO. The sequencing of $P_{74–90}$ is shown in Table 3. The recombinant protein used in these studies lacks the amino-terminal acetylation and trimethylation of Lys116 found in mammalian calmodulin. We therefore purified calmodulin from mouse brain and tested its susceptibility to oxidation by MsrA. It was also stereospecifically oxidized at Met77 (not shown).

MetO containing proteins often exhibit earlier elution times on reverse phase chromatography and slower mobility on SDS-PAGE. A marked difference in mobility during SDS-PAGE was observed for calmodulin in which all 9 met were oxidized by hydrogen peroxide, and there was no difference in mobility in the presence or absence of calcium (Fig. 3A). When only Met77 was oxidized by MsrA, a smaller but reproducibly observed change in mobility was also observed, and the increased mobility caused by binding of calcium in the native calmodulin was also observed in the MsrA-oxidized protein. A clear separation of control and MsrA-oxidized calmodulin can be effected by reverse phase chromatography (Fig. 3B). The difference in elution time from the reverse phase column may prove to be useful as an analytical tool for detecting oxidatively modified calmodulin.

Effect of Myristoylation of MsrA

Mammalian cytosolic MsrA is myristoylated on its amino-terminal glycine [31], and the myristoyl group is bound on the surface of the protein to form a novel “myristoyl nest” which appears to promote protein-protein interactions with the MsrA [38]. Consistent with that function, the rate of oxidation of calmodulin by non-myristoylated MsrA was extremely slow (Fig. 4). We determined kinetic parameters for the oxidation reaction of myr-MsrA with holo-calmodulin with 10 mM metO as the oxidizing substrate. The V_{max} was 12.7 ± 0.3 nmol·min^{-1}mg^{-1}, and the K_m was 197 ± 2 μM. (The cytosolic concentration of calmodulin in smooth muscle is 35–50 μM [41].)

Calcium Binding Is Required for Site Specificity

The site-specific oxidation of Met77 in calmodulin described above was for holo-calmodulin, that is, the calcium bound form. Removal of calcium to give apo-calmodulin causes large changes in its tertiary structure that include changes in exposure of the met residues in both the amino and carboxyl calcium-binding domains [42]. We therefore investigated the oxidation of apo-calmodulin by myristoylated MsrA. The specificity

observed with the calcium-bound holo-calmodulin was lost with the apo form, and most molecules contained multiple metO residues (Fig. 2B). All 9 met residues were oxidizable, although Met110 and Met145 were oxidized most rapidly (not shown). The oxidation remained stereospecific in generating S-metO, as evidenced by the ability of MsrA to completely reduce all the metO in oxidized apo-calmodulin.

Regulation by Reversible Oxidation of Calmodulin

Reversible oxidation and reduction of Met77 is a potential mechanism for regulating the interaction of calmodulin with one or more of its target proteins. We have no *a priori* basis for deducing which of the hundreds of known calmodulin targets [43, 44] would be regulated by such a redox mechanism. However, we still elected to assay the activity of three calmodulin-activated enzymes, the phosphatase calcineurin, the kinase CaMKII, and the hydrolase phosphodiesterase 1. We employed purified preparations of these enzymes, and determined the concentration dependence of their activation (Fig. 5). There was no difference in activation between reduced and oxidized calmodulin.

DISCUSSION

MsrA has long been known to catalyze the reduction of free S-metO and that of S-metO residues in peptides and proteins. The active site cysteine, Cys72, is sited in the amino half of the protein, and it is oxidized to the cysteine sulfenic acid as it reduces the metO. Two cysteine residues in the flexible carboxyl terminus reduce the sulfenic acid back to cysteine and themselves form a disulfide bond, which in turn will be reduced by thioredoxin [7]. We demonstrated that if the carboxyl terminal cysteines are not available or reducing power is not available, then MsrA functions in the opposite direction, oxidizing free met or met residues in peptides and proteins to S-metO [27]. MsrA is therefore a bifunctional enzyme capable of interconverting met and S-metO within proteins. Oxidation and reduction of met residues could therefore constitute a regulatory mechanism analogous to phosphorylation and dephosphorylation. We noted that a futile cycle could be prevented by the reversible binding of a putative regulatory protein to the carboxyl terminus of MsrA. When the regulatory protein is bound, the reducing cysteines would not have access to the active site and MsrA functions as a peroxidase. When dissociated, MsrA would function as a reductase [27].

We show in this report that the key regulator of calcium action, calmodulin, is a substrate for site-specific, stereospecific met oxidation. Calmodulin is a highly conserved 148-residue protein found in almost all eukaryotic cells. It binds calcium whose intracellular concentration has increased following an extracellular stimulus. The calcium-bound form transduces the extracellular signal by forming high affinity complexes with its target proteins[45], currently numbering over 350 [44]. Squier and colleagues have studied in great detail the effects of chemical oxidation of met residues in calmodulin [40, 46–48]. All methionines in apo-calmodulin can be oxidized by MsrA, as they can be by the chemical oxidants. While it is possible that MsrA mediated oxidation of the apo form could be a regulatory event, we consider it more likely that the site-specific oxidation of Met77 in holo-calmodulin would participate in regulation. Comparison of the proteins which interact with

native, holo-calmodulin but not with the MetO77 form may identify calmodulin targets that are regulated by reversible oxidation of Met77. Our studies to date utilizing co-immunoprecipitation or affinity chromatography have not yet identified such candidates. Finding them, from among the many targets, may require development of a more specialized selection method.

In the crystal structure, calmodulin is a dumbbell shaped molecule in which the amino and carboxyl lobes form the calcium-binding sites and are connected by a long α-helix referred to as the “central helix” or “central linker” [49, 50]. Calmodulin has 9 met residues, and those located in the calcium-binding lobes are thought to be important in the high affinity binding of calmodulin to its targets [42]. Met77, the residue subject to modification by MsrA, is located in the carboxyl region of the central linker. While the central linker is a rigid helix in the crystal structure, NMR studies demonstrate that it is rather unstructured and flexible in solution [51]. However, Qin and Squier reported that binding of calcium stabilizes the helical structure of the sequence between Met77 and Ser82 [52], presumably contributing to the site-specific oxidation of Met77 by MsrA.

Although there are many detailed structural studies of calmodulin itself, there are relatively few of calmodulin with its target, and virtually all of these use only a peptide from the target that constitutes the critical binding site for interaction with calmodulin. As a consequence, we were not able to use the available structures to identify calmodulin targets whose binding might be modulated by oxidation of Met77. However, Valley and colleagues recently pointed out that the sulfur of methionine commonly interacts with the rings of aromatic amino acids, and that this interaction is important for stabilizing protein structure and function [21]. They found that the Met-aromatic interaction occurred at a greater distance (~5–6 Å) than that of a salt bridge (<4 Å), but the energy associated with the two interactions was comparable. The crystal structure of calmodulin places Phe12 at a distance of 5.6 Å from the sulfur of Met77, suggesting that oxidation of Met77 may disrupt binding of targets which utilize the amino terminal lobe of calmodulin. Identification of these targets is the logical next step in assessing the physiological significance of reversible modification of Met77 by MsrA.

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ABBREVIATIONS

DTPA	diethylene triamine pentaacetic acid
metO	methionine sulfoxide

MLCK	myosin light chain kinase
MsrA	methionine sulfoxide reductase A
PDE1	phosphodiesterase 1

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Highlights

- Methionine oxidation and reduction is a potential regulatory mechanism.
- Methionine sulfoxide reductase A mediates both oxidation and reduction.
- Regulatory targets of methionine sulfoxide reductase were unknown.
- We show that calmodulin is a target, specifically at Met77.

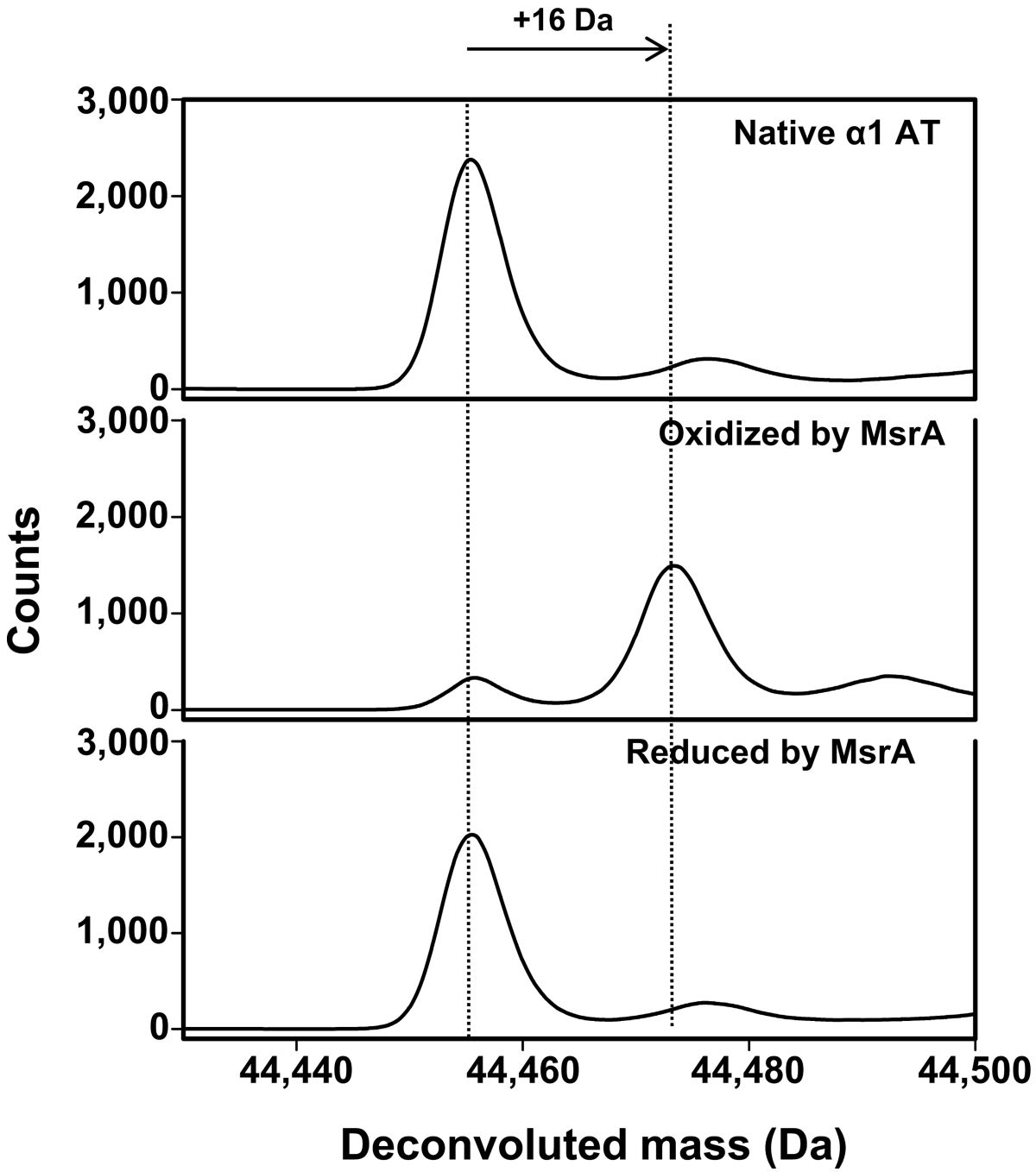

Figure 1.

Oxidation and reduction of α_1-antitrypsin by MsrA. The deconvoluted mass spectrum of control α_1-antitrypsin is shown in the upper panel. The middle panel shows oxidation by MsrA with a mass increase of 16 Da. The lower panel demonstrates the reversibility of oxidation by MsrA, establishing that the oxidation generated only the S-metO form.

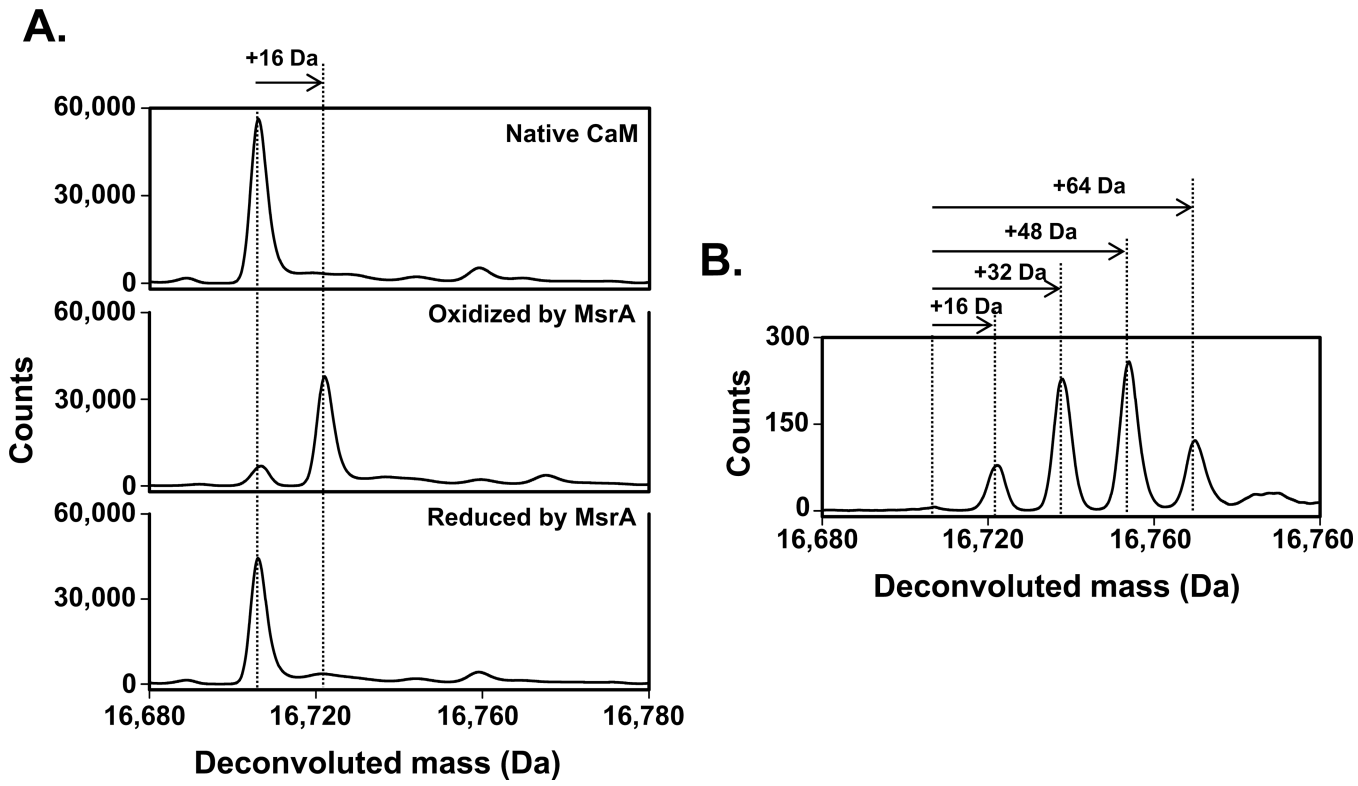

Figure 2.

Oxidation and reduction of calmodulin by MsrA. Results for the calcium-bound form of calmodulin are in the A panels. The deconvoluted mass spectrum of control calmodulin is shown in the upper panel. The middle panel shows oxidation by MsrA with a mass increase of 16 Da. The lower panel demonstrates the reversibility of oxidation by MsrA, establishing that the oxidation generated only the S-metO form. Results for the apo form of calmodulin oxidized by MsrA are in panel B and demonstrate multiple oxidations, all of which were completely reversed when incubated with MsrA under reducing conditions (not shown).

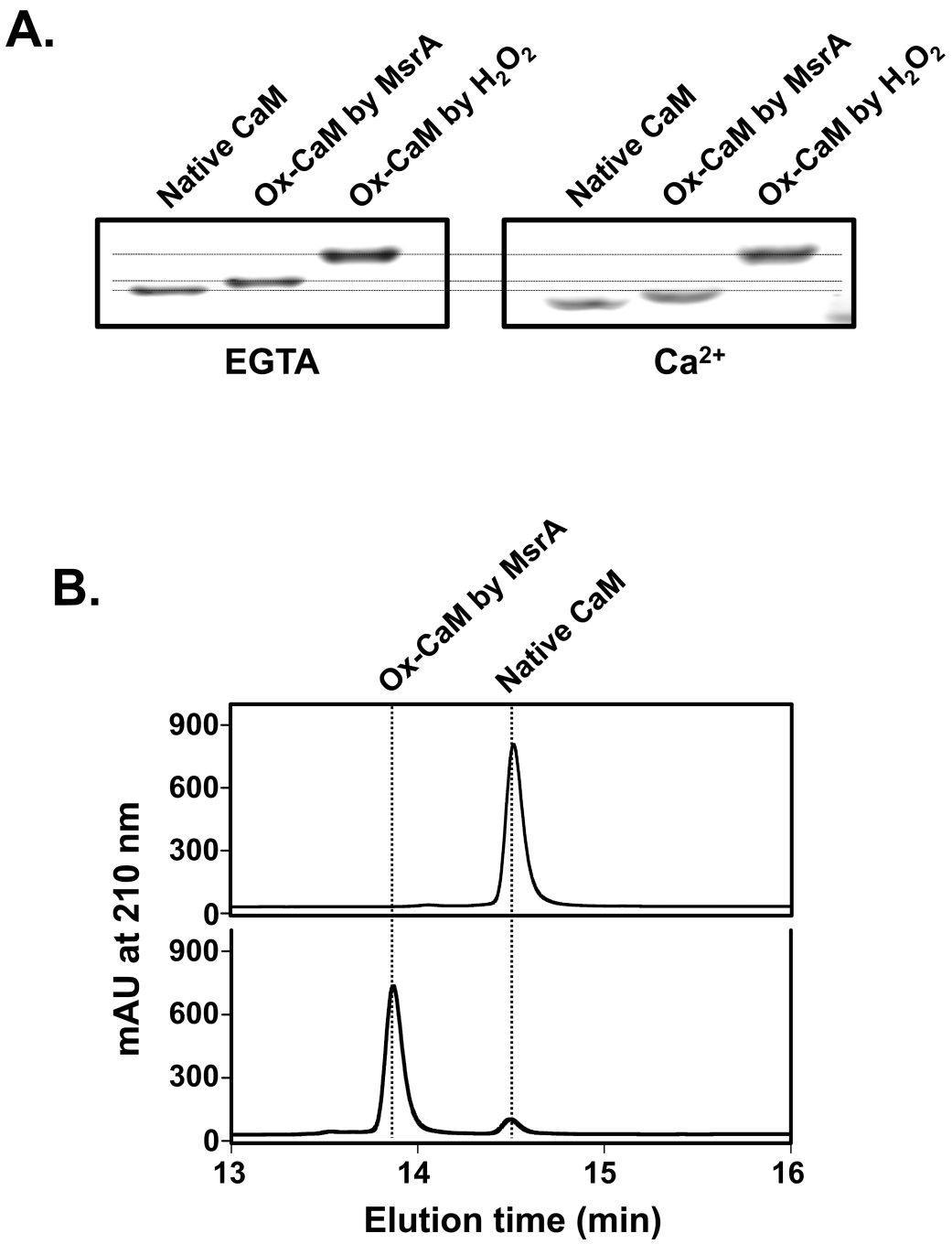

Figure 3.
Mobility of MsrA-oxidized calmodulin during SDS gel electrophoresis (A) and reverse phase chromatography (B).

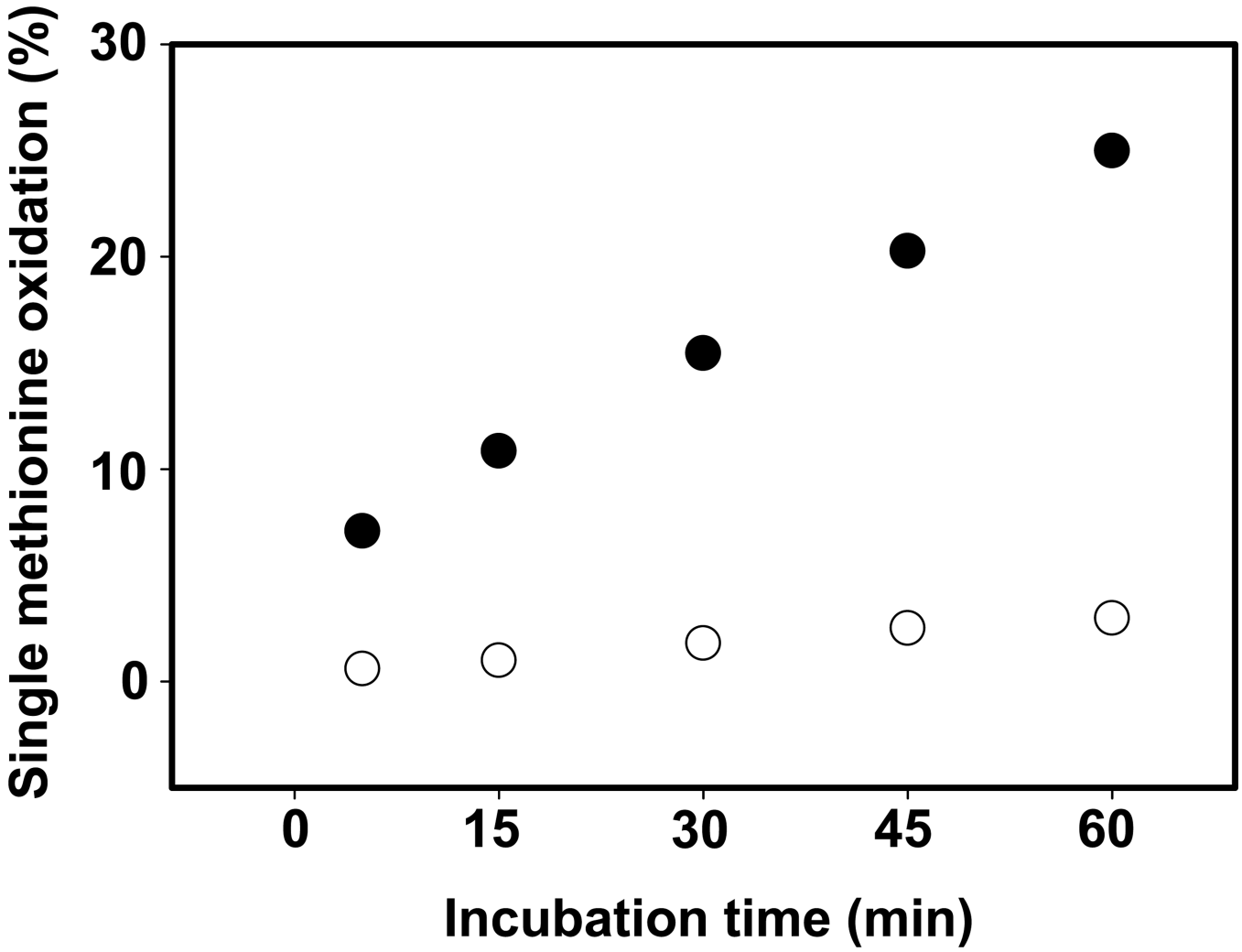

Figure 4.

Effect of myristoylation of MsrA on the rate of oxidation of holo-calmodulin. ●, myristoylated MsrA; ○, non-myristoylated MsrA.

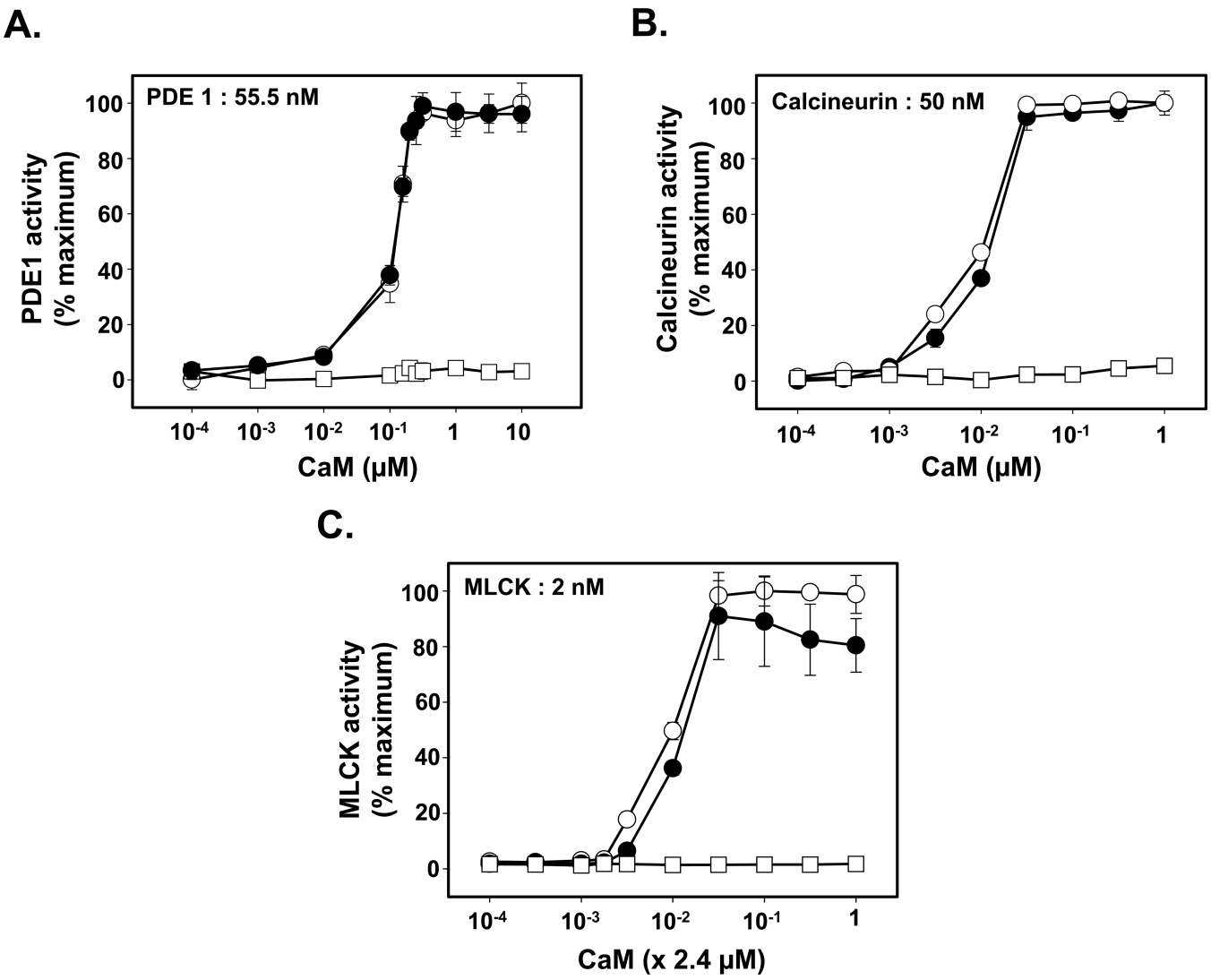

Figure 5.

Activity of calmodulin on (A) Phosphodiesterase 1, (B) calcineurin, and (C) Ca^{2+}/calmodulin-dependent kinase II. Each assay was replicated in separate experiments on different days. ○, control calmodulin; ●, Msr-A oxidized calmodulin; □, H_2O_2-oxidized calmodulin.

Table 1

Susceptibility of Proteins to Stereospecific Oxidation and Reduction by Myristoylated MsrA

Protein	Oxidation and Reduction	Residue Modified
Calmodulin	Yes	Met77
α_1-Antitrypsin	Yes	Met358
14-3-3 zeta/delta	No	-
Actin	No	-
α-Crystallin A	No	-
α-Crystallin B	No	-
α-Synuclein[a]	Yes	Met1, Met5
Apolipoprotein A	No	-
Glutamine synthetase	No	-
Peroxiredoxin 6	No	-
Thioredoxin	No	-

[a] Reported in [27]

Table 2

Sequencing of the MsrA-Oxidized α_1-Antitrypsin Peptide by Tandem Mass Spectrometry[a]

	b-ions			y-ions		
Residue	**Calculated**		**Observed**	**Calculated**		**Observed**
Gly343	b1	58.029	58.068		-	
Thr344	b2	159.077	159.078	y21	2218.11	ND
Glu345	b3	288.120	288.122	y20	2117.07	ND
Ala346	b4	359.157	359.157	y19	1988.02	1988.04
Ala347	b5	430.194	430.195	y18	1916.99	1916.98
Gly348	b6	487.215	487.216	y17	1845.95	1845.92
Ala350	b7	558.252	558.261	y16	1788.93	1788.94
Met351	b8	689.293	689.295	y15	1717.89	1717.88
Phe352	b9	836.361	836.357	y14	1586.85	1586.85
Leu353	b10	949.445	ND	y13	1439.78	1439.77
Glu354	b11	1078.49	1078.48	y12	1326.70	1326.70
Ala355	b12	1149.52	1149.52	y11	1197.65	1197.65
Ile356	b13	1262.61	1262.70	y10	1126.62	1126.62
Pro357	b14	1359.66	1359.70	y9	1013.53	1013.53
MetO358	**b15**	**1506.70**	**1506.70**	**y8**	**916.481**	**916.476**
Ser359	b16	1593.73	1593.73	y7	769.4454	769.442
Ile360	b17	1706.81	1706.82	y6	682.413	682.414
Pro361	b18	1803.87	ND	y5	569.330	569.329
Pro362	b19	1900.92	1900.90	y4	472.277	472.278
Glu363	b20	2029.96	2029.95	y3	375.224	375.223
Val364	b21	2129.03	ND	y2	246.181	246.183
Lys365	b22	-		y1	147.113	147.114

[a]The expected mass of each b and y ion was calculated by GPMAW and compared to the observed mass, demonstrating that Met358 was oxidized to MetO. The error in mass measurement is ≤ 10 ppm. ND, not detected.

Table 3

Sequencing of the MsrA-Oxidized Calmodulin Peptide by Tandem Mass Spectrometry[a]

	b-ions			y-ions		
Residue	**Calculated**		**Observed**	**Calculated**		**Observed**
Ala74	b1	72.045	ND		-	-
Arg75	b2	228.146	228.145	y16	1999.94	ND
Lys76	b3	356.241	356.2396	Y15	1843.84	1843.86
MetO77	**b4**	**503.276**	**503.279**	**Y14**	**1715.74**	**1715.74**
Lys78	b5	631.371	631.346	Y13	1568.71	1568.69
Asp79	b6	746.398	746.392	Y12	1440.61	1440.62
Thr80	b7	847.446	847.443	Y11	1325.58	1325.59
Asp81	b8	962.473	962.466	Y10	1224.54	1224.54
Ser82	b9	1049.50	1049.51	y9	1109.51	1109.51
Glu83	b10	1178.55	1178.54	y8	1022.48	1022.46
Glu84	b11	1307.59	1307.60	y7	893.436	893.436
Glu85	b12	1436.63	1436.65	y6	764.394	764.396
Ile86	b13	1549.72	1549.74	y5	635.351	635.348
Arg87	b14	1705.82	1705.96	y4	522.267	522.261
Glu88	b15	1834.86	ND	y3	366.166	366.162
Ala89	b16	1905.90	ND	y2	237.123	237.130
Phe90	b17	-	-	y1	166.086	166.087

[a]The expected mass of each b and y ion was calculated by GPMAW and compared to the observed mass, demonstrating that Met77 was oxidized to metO. The error in mass measurement is ≤ 10 ppm. ND, not detected.