Repair of Oxidized Calmodulin by Methionine Sulfoxide Reductase Restores Ability To Activate the Plasma Membrane Ca-ATPase†

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ABSTRACT: We have investigated the ability of methionine sulfoxide reductase (MsrA) to maintain optimal calmodulin (CaM) function through the repair of oxidized methionines, which have been shown to accumulate within CaM in senescent brain [Gao, J., Yin, D. H., Yao, Y., Williams, T. D., and Squier, T. C. (1998) *Biochemistry 37*, 9536−9548]. Oxidatively modified calmodulin (CaM_{ox}) isolated from senescent brain or obtained by in vitro oxidation was incubated with MsrA. This treatment restores the functional ability of CaM_{ox} to activate the plasma membrane (PM) Ca-ATPase, confirming that (i) the decreased ability of CaM isolated from senescent animals to activate the PM Ca-ATPase results solely from methionine sulfoxide formation and (ii) MsrA can repair methionine sulfoxides within cytosolic proteins. We have used electrospray ionization mass spectrometry to investigate the extent and rates of methionine sulfoxide repair within CaM_{ox}. Upon exhaustive repair by MsrA, there remains a distribution of methionine sulfoxides within functionally reactivated CaM_{ox}, which varies from three to eight methionine sulfoxides. The rates of repair of methionine sulfoxides within individual tryptic fragments of CaM_{ox} vary by a factor of 2, where methionine sulfoxides located within hydrophobic sequences are repaired in preference to methionines that are more solvent accessible within the native structure. However, no single methionine sulfoxide is completely repaired in all CaM oxiforms. Decreases in the α-helical content and a disruption of the tertiary structure of CaM have previously been shown to result from methionine oxidation. Repair of selected methionine sulfoxides in CaM_{ox} by MsrA results in a partial refolding of the secondary structure, suggesting that MsrA repairs methionine sulfoxides within unfolded sequences until native-like structure and function are re-attained. The ability of CaM_{ox} isolated from senescent brain to fully activate the PM Ca-ATPase following repair by MsrA suggests the specific activity of MsrA is insufficient to maintain CaM function in aging brain. These results are discussed in terms of the possible regulatory role MsrA may play in the modulation of CaM function and calcium homeostasis under conditions of oxidative stress.

The site-selective oxidative modification of specific amino acids has the potential to result in the functional inactivation of a wide range of different intracellular proteins (*1*). Upon oxidative modification, proteins have two potential fates: either proteolytic breakdown into amino acids or the enzymatic repair of selected amino acids, such as cystine and methionine sulfoxide (*2*, *3*). In the case of methionine oxidation, the enzyme methionine sulfoxide reductase (MsrA) has been identified and, in conjunction with thioredoxin, has the potential to repair protein bound methionine sulfoxides on a physiologically relevant time-scale (i.e., minutes) that may promote cellular survival under conditions involving an acute oxidative stress (*4*). Furthermore, the reversible oxidation of methionine has the potential to modulate intracellular signaling under conditions involving oxidative stress in a manner analogous to other regulatory posttranslational modifications such as those involving disulfide bond formation or phosphorylation (*3*, *5*, *6*). MsrA has been found to be critical to antioxidant defense mechanisms in *Saccharomyces cerevisiae* and *Escherichia coli*, suggesting that MsrA repairs methionine sulfoxides within proteins or alternatively may prevent protein oxidation by maintaining free methionines in a reduced form. While specific examples in which MsrA has been shown to modulate the functional activity of vertebrate proteins are limited, MsrA has been shown to repair methionine sulfoxides and restore the function of in vitro oxidatively modified α-1-proteinase inhibitor (*3*, *7*−*11*).

Methionines oxidized to their corresponding methionine sulfoxides have been shown to accumulate in CaM isolated from the brains of senescent (26 months) Fischer 344 rats,

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[1] Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; CaM_{ox}, CaM containing oxidized methionines; CD, circular dichroism; DTT, dithiothreitol; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′,-tetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; MG, molten globule; MsrA, methionine sulfoxide reductase; PM, plasma membrane; ROS, reactive oxygen species.

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resulting in a reduced functional ability to activate the plasma membrane (PM) Ca-ATPase that may explain aspects associated with the loss of calcium homeostasis during aging (*12*, *13*). Since MsrA levels are particularly high in specific brain regions (*14*), a decrease in the specific activity of MsrA may result in methionine sulfoxide accumulation in CaM during a range of chronic neurodegenerative diseases (including aging) that correlate with the observed loss of calcium homeostasis and neuronal function (*15*, *16*). However, little is known regarding the role of protein structure in modulating the ability of MsrA to bind and reduce individual methionine sulfoxides in proteins. To determine the possible relevance of MsrA in maintaining functionally active calmodulin, we have investigated the in vitro ability of MsrA to repair methionine sulfoxides within CaM isolated from aged brain. In addition, we have characterized the MsrA-mediated repair of a homogeneous oxiform of CaM in which all nine methionines are oxidized to methionine sulfoxide using electrospray ionization (ESI) mass spectrometery (MS) and circular dichroism (CD) spectroscopy, permitting the measurement of the selectivity and structural consequences of methionine sulfoxide repair by MsrA in CaM_{ox}. These experiments suggest an important physiological role for MsrA in maintaining functionally active CaM.

EXPERIMENTAL PROCEDURES

Materials. Hydrogen peroxide (H_2O_2) was obtained from Fisher (Pittsburgh, PA), and the concentration was determined using the published extinction coefficient, i.e., $\epsilon_{240} = 39.4 \pm 0.2\ M^{-1}\ cm^{-1}$ (*17*). A micro BCA protein assay reagent kit was obtained from Pierce (Rockford, IL). Type XIII TPCK-treated trypsin and type I-S soybean trypsin inhibitor were obtained from Sigma (St. Louis, MO). Glutathione Sepharose 4B was obtained from Pharmacia Biotech (Sweden). All other chemicals were the purest grade commercially available. Fischer 344 rats (6 and 27 months) were obtained from the National Institute of Aging maintained rat colony (Harlan Sprague Dawley, Indianapolis), and CaM was isolated from brains essentially as previously described (*13*, *18*). A single isoform of CaM corresponding to the cDNA encoding vertebrate CaM provided by Professor Sam George (Duke University) was subcloned into the expression vector pALTER-Ex1 (Promega, Madison, WI), overexpressed in *E. coli* strain JM109 (Promega, Madison, WI), and purified essentially as previously described using phenyl Sepharose CL-4B (Pharmacia, Piscataway, NJ) and weak anion exchange HPLC (*18*, *19*). The in vitro oxidative modification of CaM involved the incubation of 60 μM CaM (1 mg/mL) in 50 mM HOMOPIPES (pH 5.0), 0.1 M KCl, and 1 mM $MgCl_2$ with 50 mM H_2O_2 for 23 h prior to removal of reactants from CaM using a Sephadex G-25 size exclusion column. Bovine liver MsrA was expressed in *E. coli* as a GST fusion protein, and purified using a glutathione Sepharose 4B affinity column as previously described (*8*). The erythrocyte ghost PM-Ca-ATPase was purified from porcine erythrocyte ghost membranes (*6*, *20*). Purified CaM, MsrA, and erythrocyte ghost membranes were stored at −70 °C.

Enzymatic Assays. To investigate the repair of methionine sulfoxides in CaM by MsrA, 30 μM CaM_{ox} was incubated for varying times at 37 °C in the presence of 1.0 μM MsrA in 20 mM Tris-HCl (pH 7.4) and 15 mM DTT (buffer A). DTT has previously been shown to function as an electron donor that can reduce MsrA (*21*). The reaction was typically stopped by applying the sample onto a Sephadex G-25 size exclusion column at varying intervals to investigate the ability of the "repaired" CaM to activate the PM-Ca-ATPase and to identify the sites of repair following tryptic digestion (see below). Alternatively, CaM was directly injected into the ESI source to measure the distribution of CaM masses (see below).

Calmodulin-dependent ATPase activity associated with the PM-Ca-ATPase was determined using the method described by Lanzetta and co-workers (*22*) for measuring phosphate release. The ghost membrane protein concentration and MsrA concentration were determined by the Biuret method (*23*), using BSA as the standard. CaM concentration was determined using the Micro-BCA assay, where a stock solution of desalted CaM was used as a protein standard ($\epsilon_{277} = 3029\ M^{-1}\ cm^{-1}$) (*18*). PM-Ca-ATPase activity (i.e., 0.16 mg mL^{-1} porcine erythrocyte ghost membranes) was measured at 37 °C in 50 mM MOPS (pH 7.0), 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5 mM ATP, and 4 μM A23187 (buffer B) in the presence and absence of 0.6 μM added CaM.

Mass Spectrometric Analysis. ESI-MS was used to identify the pattern of oxidative modification and the selectivity of repair by MsrA. To identify the distribution of oxiforms of CaM, whole protein ESI spectra were acquired on an AUTOSPEC-Q equipped with the Mark III ESI source, essentially as previously described (*24*). Whole protein CaM samples were trapped and desalted prior to ESI by washing sample onto a protein trapping column (1.5 cm × 1 mm of polymeric beads with 4000 Å pores) (Michrom BioResources, Auburn, CA) with 10 mM $(NH_4)_2CO_3$ (pH 8.0) and 100 μM EGTA at a flow rate of 250 μL min^{-1}, and retained samples were directly eluted into the ESI source with 70% (v/v) methanol in 10 mM $(NH_4)_2CO_3$ (pH 8.0) at 10 μL min^{-1} through a 130 μM i.d. stainless steel needle and nebulized with a coaxial gas flow of 10 L/h of N_2. The fractional abundance of a CaM oxiform was estimated from the observed area of the associated peak in the ESI-MS spectrum, which was corrected for fragmentation artifacts using the algorithm:

$$\text{corr area}_{\text{CaM(O)}_n} = [\text{obsd area}_{\text{CaM(O)}_n} - [0.4 \times \text{obsd area}_{\text{CaM(O)}_{n+1}}]] \times 1.4$$

which corrects (i) the loss of spectral intensity resulting from fragmentation of a particular oxiform of CaM [i.e., $CaM(O)_n$] and (ii) the increased peak intensity resulting from the collision-induced fragmentation of more highly oxidized species [$CaM(O)_{n+1}$], whose fragmentation products overlap with the species of interest [i.e., $CaM(O)_n$] (*13*).

The sites repaired on CaM by MsrA were identified by proteolytic digestion of repaired CaM and analysis by HPLC/MS. CaM (30 μM) was digested with 0.6 μM trypsin in 2% $(NH_4)_2CO_3$ at pH 8.0 for 9 h at 37 °C. Following the addition of 1.8 μM trypsin inhibitor, the resulting peptides were then separated on a chromatograph operating at 50 μL min^{-1}. Tryptic peptides were monitored with a UV detector and masses analyzed using post UV detector flow splitting of 8 μL min^{-1} to the ESI source. The chromatograph (Micro-Tech Scientific, Sunnyvale, CA) utilizes 1/16 in. OD pistons

Table 1: CaM-Dependent Activation of the PM-Ca-ATPase by MsrA[a]

sample[b]	CaM-dependent ATPase activity[c] (μmol of P_i mg^{-1} min^{-1})	
	−MsrA	+MsrA
CaM (6 months)	1.94 ± 0.09	2.04 ± 0.10
CaM (27 months)	0.97 ± 0.06[d]	1.71 ± 0.09

[a] Activity measurements were made following incubation of 30 μM CaM at 37 °C in buffer A for 1 h in the absence and presence of 1.0 μM MsrA. [b] CaM was purified from the brains of Fischer 344 rats, as described under Experimental Procedures. [c] Experimental conditions for enzyme activity measurements are as described in the legend in Figure 2. Values represent the averages of two independent measurements. [d] $P < 0.05$ in comparison to CaM-dependent ATPase activity observed using CaM isolated from young (6 month) animals.

in Model UP200M pumps, the small volume chamber in the DynaPlus mixer filled with glass beads, and is fitted with a Rheodyne 8125 injector valve (Cotati, CA). The column effluent was monitored at 214 nm with a UV detector equipped with a 3 mm path length using a 1.2 μL cell (Model UV-VIS 200, Linear Scientific, Fremont, CA). Separations involved solvent A (98% H_2O, 2% MeOH, and 0.25% formic acid) and solvent B (98% MeOH, 2% H_2O, and 0.22% formic acid), and were performed on a 1 mm i.d. × 5 cm long C18 reversed-phase column (Zorbax SBC18, 3.5 μm particles with a 300 Å pore size packed by Micro-Tech) with a 1 mm × 2 cm dry-packed guard column (Upchurch Scientific Model C.128, Oak Harbor, WA) filled with Zorbax C18 resin (SBC18, 5 μM, 300 Å, Rockland Technologies, Newport, DE). The solvent gradient was held at 5% B for 1 min, and then ramped to 20% B by 11 min, 40% by 21 min, 65% by 51 min, and finally to 95% B by 60 min. The mass spectrometer was tuned to 1800 resolving power and scanned from 2500 to 300 at 10 s/decade while collecting data in a continuum mode.

Circular Dichroism Spectroscopy. Circular dichroism (CD) spectra were measured using a Jasco J-710 spectropolarimeter (Jasco Corp., Tokyo, Japan) and a temperature-jacketed spectral cell with a path length of 1.0 cm. After 36 h dialysis against double-distilled water, spectra were recorded of desalted CaM (50 μg/mL) in 10 mM Tris-HCl (pH 7.5), 0.1 M $KClO_4$, 1 mM $Mg(ClO_4)_2$, and 0.1 mM $Ca(ClO_4)_2$ at 1 nm intervals between 200 and 240 nm at 25 °C. The apparent α-helical content was determined using the method of ridge regression (*25*), using the computer program Contin (*26, 27*).

RESULTS

Reactivation of CaM_{ox} Isolated from Senescent Brain by MsrA. CaM isolated from senescent (27 month) brain has a reduced ability to activate the PM-Ca-ATPase relative to that observed for CaM isolated from young (6 month) brains, as exhibited by the 50 ± 4% reduction in function (Table 1). This loss of function has been suggested to result from the high degree of methionine oxidation observed in CaM isolated from aged brain, since no other amino acid modifications were detected (*12, 13*). In vitro incubation of CaM isolated from senescent brain with vertebrate MsrA expressed and purified from *E. coli* restores the ability of CaM to fully activate the PM-Ca-ATPase, confirming that the oxidative modification of methionine to methionine sulfoxide is the major cause of the decreased function. These results suggest that the specific activity of MsrA in senescent brains is inadequate to maintain optimal CaM function.

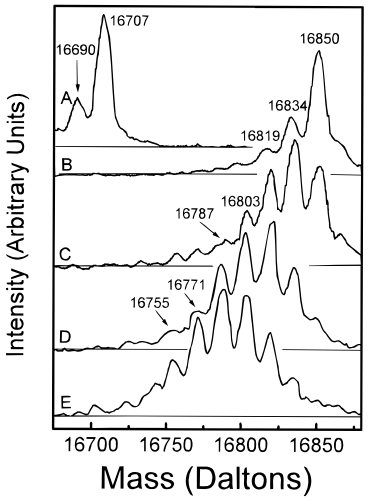


FIGURE 1: ESI-MS resolution of the distribution of CaM species. ESI-MS spectra corresponding to native CaM expressed in *E. coli* (A), CaM following the oxidative modification of all nine Met by H_2O_2 (B), or CaM_{ox} after the repair by MsrA for 8 min (C), 20 min (D), and 120 min (E). Experimental conditions: 1 μM MsrA in the presence of 30 μM CaM_{ox} in buffer A at 37 °C. At the indicated time increments, CaM (0.6 nmol) was directly injected in the ESI source to measure the distribution of CaM masses, as described under Experimental Procedures.

In Vitro Oxidative Modification of CaM. To identify the structural requirements and possible specificity of MsrA in the repair of individual methionine sulfoxides in CaM_{ox}, we have used in vitro conditions to generate a homogeneous population of oxidizatively modified CaM in which all nine methionines were oxidized to their corresponding methionine sulfoxides (see Experimental Procedures). We have used ESI-MS to measure the distribution of CaM species resulting from in vitro oxidative modification of CaM. Prior to oxidative modification, CaM exhibits two major peaks whose average molecular masses are centered at 16 690 ± 3 and 16 707 ± 3 Da. (Figure 1A). The theoretical average mass of vertebrate CaM expressed in *E. coli* is 16 705.4, indicating that the major peak corresponds to native (unoxidized) CaM. The peak at 16 690 Da has been shown to correspond to a collision-induced dissociation (CID) product generated in the mass spectrometer from the native CaM, and accounts for approximately 23% of the area (*24*). Therefore, vertebrate CaM expressed and isolated from *E. coli* consists of a single species with the expected molecular mass. The conditions used for in vitro oxidative modification of CaM result in the oxidative modification of all nine methionines of CaM (Figure 1B), as evidenced by the major peak at 16 850 ± 3 Da that corresponds to the mass of native CaM plus nine oxygens (i.e., 16 705.4 Da + 143.9 Da = 16 849.3 Da). In addition to the peak at 16 850 Da, peaks are observed at 16 819 ± 3 and 16 834 ± 3 Da that correspond to (i) the respective masses of CaM_{ox} containing seven and eight methionine sulfoxides and (ii) CID fragmentation masses of more highly oxidized CaM species that have lost either neutral H_2O or NH_3 (see above). No higher molecular mass peaks are observed, suggesting that no methionine sulfones are present in these samples. Upon correction for CID fragmentation (see Experimental Procedures), the distribution

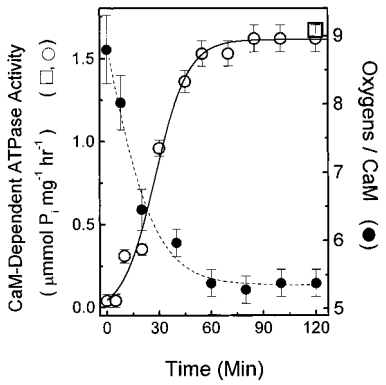


FIGURE 2: Recovery of the CaM-dependent activation of the PM-Ca-ATPase upon repair of methionine sulfoxides in CaM_{ox} by MsrA. The CaM-dependent ATPase activity of PM-Ca-ATPase was measured in buffer B in the presence of 0.6 μM native CaM (□) or CaM_{ox}, whose nine methionines were oxidized to methionine sulfoxide (○), at various time intervals following the incubation of MsrA in buffer A (see Experimental Procedures). The average number of oxygen atoms in each CaM molecule (●) is the sum of the fractional contribution of each oxiform of CaM multiplied by the number of methionine sulfoxides following correction for CID fragmentation, as described in the legend to Figure 3.

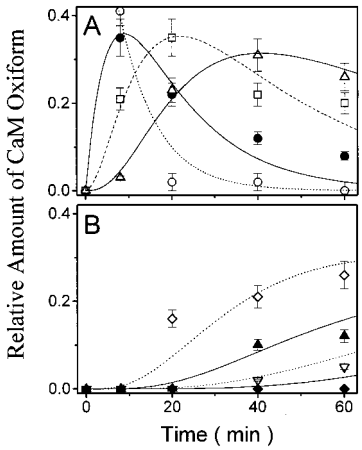


FIGURE 3: Distribution of CaM oxiforms following methionine sulfoxide repair by MsrA. Relative amounts of each oxiform of CaM containing variable amounts of methionine sulfoxide, ranging from (A) nine (○), eight (●), seven (□), six (△), or (B) five (◇), four (▲), three (▽), and two (◆) methionine sulfoxides per CaM, where the average error in the determination of the fraction of each CaM oxiform is 8% of the indicated value. Lines represent experimental fits obtained from simultaneous fitting of the complete data set to a series of eight second-order rate equations to the following model:

$$CaM(O)_n \xrightarrow{k_{10-n}} CaM(O)_{n-1}$$

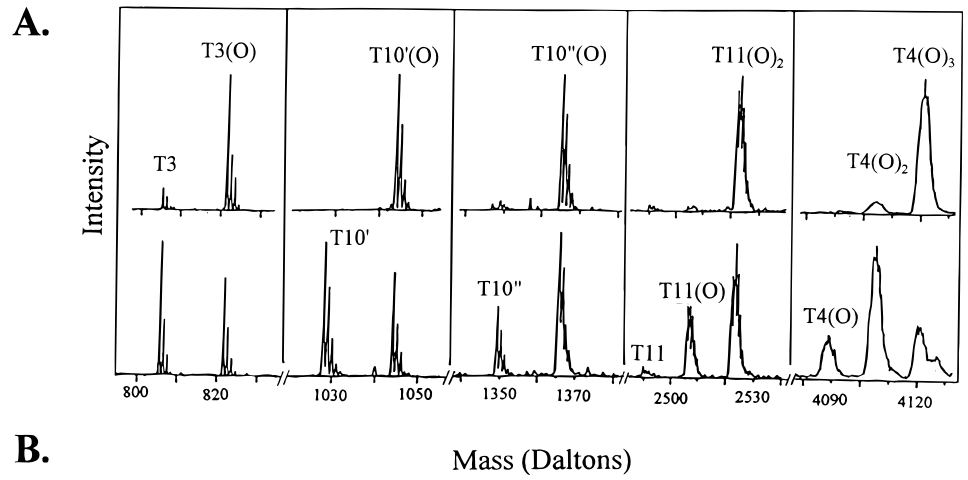
where n varies from 9 to 2. For example, $n = 9$ corresponds to the oxiform of CaM containing nine methionine sulfoxides [i.e., $CaM(O)_9$] whose corresponding rate of reduction to $CaM(O)_8$ is k_1. The calculated rate constants are $k_1 = 1900 \pm 200$ M^{-1} s^{-1}, $k_2 = 1100 \pm 100$ M^{-1} s^{-1}, $k_3 = 520 \pm 70$ M^{-1} s^{-1}, $k_4 = 300 \pm 50$ M^{-1} s^{-1}, $k_5 = 180 \pm 60$ M^{-1} s^{-1}, $k_6 = 140 \pm 100$ M^{-1} s^{-1}, $k_7 < 350$ M^{-1} s^{-1}, and $k_8 \approx 0$ M^{-1} s^{-1}. Errors represent the maximal variance, and were obtained from a global analysis of the data using the F-statistic (*49*).

of CaM oxiforms can be calculated from the respective areas apparent in the mass spectrum whose mass can be associated with individual CaM oxiforms (*24*). Thus, approximately 82% of all CaM oxiforms in this sample contain nine methionine sulfoxides, 12% of CaM oxiforms contain eight methionine sulfoxides, and approximately 6% of CaM oxiforms contain seven methionine sulfoxides. On average CaM_{ox} contains approximately 8.8 $\pm$ 0.1 methionine sulfoxides, and in the majority of CaM molecules, all 9 methionines are oxidized.

ESI-MS Resolution of the Distribution of CaM Oxiforms upon Functional Reactivation of in Vitro Oxidatively Modified CaM. Incubation of functionally inactive CaM, in which all nine methionines were oxidized to the corresponding sulfoxides (see above), with MsrA results in the full recovery of the CaM-dependent activation of the PM-Ca-ATPase (Figure 2). To identify the extent and kinetics of the repair of methionine sulfoxides in CaM_{ox} by MsrA, we have measured the distribution of CaM oxiforms resulting from incubation of in vitro oxidatively modified CaM with MsrA using ESI-MS (see above). Upon exposure of this oxidized CaM to MsrA in vitro, the gradual appearance of lower molecular mass oxiforms of CaM containing from three (16 755 $\pm$ 3 Da) to eight (16 834 $\pm$ 3 Da) oxygens is observed, which correlates with the recovery of functional activity (Figures 1 and 2). No additional methionine sulfoxide repair is observed following functional reactivation upon incubation with MsrA for longer periods of time. The inability of MsrA to repair all nine methionine sulfoxides in CaM_{ox} is not related to any reduction in the activity of MsrA, since control experiments indicate MsrA remains functionally active. Furthermore, the addition of fresh MsrA and DTT into the reaction medium after the cessation of methionine sulfoxide reduction results in no additional repair of methionine sulfoxides.

Average apparent rate constants involving the repair of methionine sulfoxides in CaM_{ox} by MsrA can be calculated from corrected areas associated with the ESI-MS spectra (Figure 3). The rate of repair of CaM_{ox} progressively declines as individual methionine sulfoxides are repaired (see legend in Figure 3 for calculated rate constants), suggesting that some residues within CaM_{ox} may be selectively repaired or that there are structural alterations (e.g., refolding) that accompany functional reactivation and prevent MsrA from repairing additional methionine sulfoxides. The repair of methionine sulfoxides is closely correlated to the recovery of the ability of CaM_{ox} to activate the PM-Ca-ATPase (Figure 2), and results in a maximal reduction of an average of 3.6 $\pm$ 0.1 methionine sulfoxides in each CaM.

HPLC-MS Resolution of Individual Tryptic Peptides. The inability of MsrA to fully repair all nine methionine sulfoxides in CaM_{ox} suggests that the ability of MsrA to recognize and repair individual methionine sulfoxides may depend on the microenvironment around individual methionine sulfoxides within the sequence of CaM_{ox}. Therefore, to identify the possible specificity of MsrA to recognize and repair individual methionine sulfoxides within CaM_{ox}, we have stopped the repair of CaM_{ox} at various times and identified the distribution of methionine sulfoxides within individual tryptic fragments using HPLC-MS. We resolve masses associated with all 12 tryptic peptides (Figure 4; Table 2), and in all cases the experimentally observed masses are within 0.03% of the theoretical mass (*19*). No modification occurs in the six tryptic fragments that contain no methionines. The cleavage pattern around Met_{76} is variable and prevents a quantitative consideration of this residue. The integrated ion currents of the other five methionine-containing peptides are shown (Figure 4; Table 2).



T1 **T2** **T3** **T4** **T5**

ADQLTEEQIAEFK ↓ EAFSLFDKDGDGTITTK ↓ ELGTV**M**$_{36}$R ↓ SLGQNPTEAELQD**M**$_{51}$INEVDADGNGTIDFPEFLT**M**$_{71}$**M**$_{72}$**AR** ↓ K ↓

T6 **T7** **T8** **T9** **T10′** **T10″** **T11**

M$_{76}$K ↓ DTDSEEEIR ↓ EAFR ↓ VFDKDGNGYISAAELR ↓ HV**M**$_{109}$TNLGEK ↓ LTDEEVDE**M**$_{124}$IR ↓ EADIDGDGQVNYEEFVQ**M**$_{144}$**M**$_{145}$TAK

FIGURE 4: ESI-MS spectra of tryptic fragments in CaM_{ox}. (A) Mass spectra of tryptic fragments isolated from CaM_{ox} before (top panels) and following (bottom panels) repair by MsrA. Masses correspond either to the native sequence or following addition of the indicated number of oxygens. Multiple oxidation states are resolved for the oxidized peptides T11 and T4, which contain two and three methionines, respectively. Repair was initiated by incubation of 30 μM CaM_{ox} with 1 μM MsrA in buffer A at 37 °C for 2.5 h prior to tryptic digestion. Each spectrum is the integration of all scans containing the tryptic peak and its oxiforms, and corresponds to the zero charge form, except T_3, which is MH^+. Spectra are normalized to the maximal peak height. (B) Positions of indicated peptides are indicated within the primary sequence of CaM relative to theoretical tryptic cleavage sites, which are designated with vertical arrows. The 12 proteolytic fragments generated following tryptic cleavage are indicated by the letter T followed by a number. Vertebrate CaM expressed in *E. coli* contains Lys_{115} instead of the trimethyl-lysine present in CaM isolated from vertebrate tissue, and thus has an additional tryptic cleavage site. Specifically, the T10 fragment observed in wild-type CaM is digested into two fragments, T10′ and T10″, in CaM expressed in *E. coli*. The tryptic fragments are numbered relative to the amino-terminal domain as previously described (*6*).

Table 2: ESI Masses of Methionine-Containing Tryptic Fragments in CaM[a]

tryptic fragment[b]	Met residue	theoretical mass[c] (daltons)	ESI mass[e] (daltons)	rate constants[f] ($M^{-1}s^{-1}$)
T3 (Glu_{31}−Arg_{37})	Met_{36}	804.4	804.6	$k_1 = 61 \pm 1$
$T3(O)_1$		820.4	820.6	
T4 (Ser_{38}−Arg_{74})	$Met_{51,71,72}$	4071.5	4069.9[d]	$k_1 = 80 \pm 20$
$T4(O)_1$		4087.5	4087.5[d]	$k_2 = 50 \pm 20$
$T4(O)_2$		4103.5	4103.8[d]	$k_3 = 15 \pm 8$
$T4(O)_3$		4119.5	4120.5[d]	
T10′ (His_{107}−Lys_{115})	Met_{109}	1027.5	1027.8	$k_1 = 53 \pm 1$
$T10'(O)_1$		1043.5	1043.7	
T10″ (Leu_{116}−Arg_{126})	Met_{124}	1348.6	1348.7	$k_1 = 30 \pm 1$
$T10''(O)_2$		1364.6	1364.7	
T11	$Met_{144,145}$	2489.1	2489.5	$k_1 = 46 \pm 2$
$T11(O)_1$		2505.1	2505.2	$k_2 = 28 \pm 13$
$T11(O)_2$		2521.1	2521.2	

[a] All peptide ions were detected with an accuracy within 0.02% of the theoretical mass of the peptide fragment, and there were no ambiguities relating to the mass assignments of the peptide ions. [b] Nomenclature regarding the relationship between peptides resulting from tryptic cleavage and the primary sequence of CaM are given in Figure 4. Five of the six methionine-containing tryptic fragments are depicted; the proteolytic cleavage around tryptic fragment T6 containing Met_{76} was dependent on the amount of repair, and was not quantitatively documented. [c] Calculated masses are monoisotopic, except for T4 which are average masses. [d] Average mass measured for T4. [e] ESI masses are measured and corrected for multiple charged forms. [f] Apparent rate constants associated with the repair of indicated tryptic peptides were calculated by simultaneously fitting the data obtained from samples incubated for various times with MsrA prior to HPLC-MS analysis to a series of rate constants corresponding to the total number of oxidized peptides, which ranges from one (i.e., T3, T10′, and T10″) to two (i.e., T11) to three (i.e., T4) rate constants.

Prior to the incubation of CaM_{ox} with MsrA, all five of the resolved methionine-containing peptides are oxidatively modified, as indicated by the large ion current associated with the molecular mass corresponding to the oxidized tryptic peptide (Figure 4, top). No higher molecular masses are observed in the mass spectra of any of these tryptic fragments, indicating that no methionine sulfones are formed under these experimental conditions and that H_2O_2 selectively oxidizes all nine methionines in CaM to their corresponding methionine sulfoxides. Upon incubation with MsrA, the fractional areas associated with the ion intensity of the fully oxidized peptides decrease in a time-dependent manner (data not shown). There are corresponding increases in the relative areas of the ion currents of the repaired peptides, indicating

that MsrA repairs individual methionine sulfoxides to their corresponding methionines (Figure 4, bottom). The time-dependent changes in the areas of these ion currents permit a determination of the relative rates of repair of individual methionines in the five resolved tryptic fragments, which are substantially different (Table 2). A consideration of the average rates of repair of T4 and T11 (which contain multiple methionine sulfoxides) requires that the multiple products be considered in the calculation of the apparent rate constants. In the case of tryptic peptides T4 and T11, which contain multiple methionines, the rate constant k_1 is the apparent initial rate of repair for all methionines in the tryptic fragment (Table 2). Therefore, a comparison of the average rates of repair of the various tryptic fragments requires that the rate of repair be normalized by the number of methionines [i.e., k_1(T4)/3 = 27 ± 7 M^{-1} s^{-1}; k_1(T11)/2 = 23 ± 1 M^{-1} s^{-1}]. Average rates of repair for methionine sulfoxides in CaM_{ox} differ by a factor of 2, where Met_{36} (k_1 = 61 M^{-1} s^{-1}) and Met_{109} (k_1 = 53 M^{-1} s^{-1}) are preferentially repaired, while Met_{124} (k_1 = 30 M^{-1} s^{-1}) is repaired more slowly. Thus, a methionine in tryptic peptide T4 (i.e., $Met_{51,71,72}$) is repaired more rapidly than T3 (Met_{36}) or T10′ (Met_{109}), which in turn are repaired more rapidly than either T11 ($Met_{144,145}$) or T10" (Met_{124}). Furthermore, following the maximal extent of repair by MsrA, no population of individual methionines within CaM_{ox} is fully repaired in all CaM oxiforms (Figure 4, bottom), providing strong evidence that conformational changes within CaM_{ox} result from the repair of individual methionine sulfoxides and limit the ability of MsrA to recognize and repair remaining methionine sulfoxides.

The extent of repair correlates with the rate of repair (see above). Thus, Met_{36} (T3) and Met_{109} (T10′) are preferentially repaired, and following exhaustive repair by MsrA only about 40% of the CaM oxiforms contain methionine sulfoxides at these positions. In contrast, Met_{124} (T10") is repaired to a much smaller extent, and following maximal repair, the majority (i.e., 70%) of CaM oxiforms contain methionine sulfoxide at this position. While the presence of multiple methionines in T4 and T11 prevents a quantitative comparison of the rates of repair of individual methionines (see below), methionine sulfoxides located in these peptides are also not fully repaired (Figure 4).

Correlation between Hydrophobicity and Rates of Repair. Since binding interactions frequently involve hydrophobic interactions (*28*), we have considered the possible relationships between the repair rates of individual tryptic fragments and the hydrophobicity of neighboring sequences around individual methionine sulfoxides in CaM_{ox}. Using the Kyte and Doolittle algorithm (*29*), we find a correlation between the calculated hydrophobicity of a seven amino acid peptide centered around individual methionine sulfoxides in CaM_{ox} and the average rates of repair of individual methionine sulfoxides (Figure 5). These results suggest that MsrA selectively binds and repairs methionine sulfoxides within hydrophobic sequences in CaM_{ox}.

Partial Refolding of CaM_{ox} upon Methionine Sulfoxide Repair. Alterations in the secondary structure of CaM resulting from methionine oxidation were assessed using CD spectroscopy to estimate changes in the apparent average α-helical content of CaM following oxidation of all nine methionines to their corresponding methionine sulfoxides (CaM_{ox}) and after partial repair of CaM_{ox} by MsrA. Prior to obtaining CD spectra, CaM was purified to homogeneity and exhaustively dialyzed against deionized water. CD spectra were measured between 202 and 240 nm at 1 nm intervals. Irrespective of calcium binding, the CD spectra of unoxidized (native) apo- and calcium-saturated CaM have substantially larger peptide ellipticities than observed for CaM_{ox} (Figure 6), indicating that methionine side chains function to stabilize the native conformation of CaM. Upon partial repair by MsrA, there is an increase in peptide ellipticity (Figure 6), indicating that repaired and functionally active CaM adopts

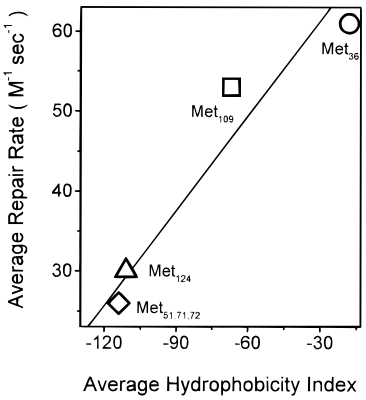


FIGURE 5: Correlation between repair rate and sequence hydrophobicity. Average rates of repair plotted against calculated average sequence hydrophobicity for tryptic fragments T3 (Met_{36}; ○), T10′ (Met_{109}; □), T4 ($Met_{51,71,72}$; ◇), and T10″ (Met_{124}; △), where larger numbers are indicative of an increased hydrophobicity (*29*). The line represents the least-squares best fit to the data. The slope is 0.37 ± 0.07, the intercept is 71 ± 6, and the linear regression coefficient is 0.96 and P = 0.04. Average hydrophobicity is the average value of three residues on either side of the indicated methionines, and in the case of T4, the calculated hydrophobicity is the average of all three positions. Calculated hydrophobicity values assume a hydrophobicity for methionine sulfoxide identical to that of Gln. Met_{144} and Met_{145} in T11 are not included in this comparison, as they are near the end of the sequence and hydrophobicity values cannot be reliably calculated.

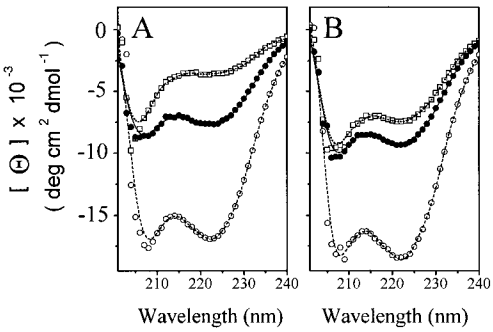


FIGURE 6: Secondary structures of CaM. CD spectra obtained for apo- (A) and calcium-saturated (B) samples of unoxidized CaM (○), CaM_{ox} (□), and repaired CaM_{ox} following incubation with MsrA (●). In all cases, spectra were acquired at 25 °C for desalted CaM (3 μM) in 50 mM Tris-HCl (pH 7.5), 0.1 M KCl, 5 mM $Mg(ClO_4)_2$, and 0.1 mM EGTA in the absence (A) and presence (B) of 0.2 mM $Ca(ClO_4)_2$. Lines represent the least-squares fit to the CD spectra obtained using the program Contin (*27*). For apo- and calcium-activated CaM (dashed lines), the calculated α-helical content was 50% and 54%, respectively. For apo- and calcium-activated CaM_{ox} (dotted lines), the calculated α-helical content was 14% and 26%, respectively. For apo- and calcium-activated CaM_{ox} repaired by MsrA (solid lines), the calculated α-helical content was 23% and 29%, respectively. Experimental errors associated with the experimental determination of the α-helical content were approximately 2% of the indicated values.

a more nativelike structure. The observation of a more nativelike protein conformation following repair of selected methionines by MsrA indicates that methionine side chains are important in stabilizing the native structure of CaM. These results are furthermore consistent with other suggestions that nonselective hydrophobic interactions between amino acid side chains play an important role in determining the global fold of a protein (*30*). Additional support that repair of selected methionine sulfoxides in CaM_{ox} by MsrA induces the formation of a nativelike structure is apparent in the calcium-dependent changes in protein structure (Figure 6). Thus, upon calcium binding to CaM_{ox}, there is a significant increase in peptide ellipticity, suggesting that following oxidative modification of all nine methionines calcium binding provides important topological constraints that function to enhance the nativelike structure of CaM. In contrast, little change in peptide ellipticity is observed following calcium activation of native (unoxidized) CaM or following partial repair of CaM_{ox} by MsrA, which are both able to fully activate the PM-Ca-ATPase (Figure 2). Furthermore, the CD spectra of the repaired CaM are similar to those of the homologous calcium binding protein parvalbumin stabilized in the nativelike molten globule (MG) state (*31, 32*). Thus, the inability of MsrA to further repair methionine sulfoxides following partial refolding suggests that they are not accessible to MsrA.

DISCUSSION

Summary of Results. Decreases in the functional ability of CaM_{ox} to activate the PM-Ca-ATPase are reversed upon incubation with MsrA, irrespective of whether CaM_{ox} is isolated from senescent brain or obtained using in vitro conditions to oxidize all nine methionines in CaM (Table 1; Figure 2). These results confirm previous suggestions that the oxidation of methionines to their corresponding methionine sulfoxides in CaM isolated from senescent brain is responsible for the decreased ability to activate the PM-Ca-ATPase (*13*). The recovery of CaM function by MsrA is an indication that the specific activity of MsrA in senescent brain may be insufficient to maintain the optimal function of CaM. Upon restoration of CaM function, a distribution of CaM oxiforms containing from three to eight methionine sulfoxides is observed, and the remaining methionine sulfoxides at any of the nine positions in CaM_{ox} become inaccessible to further repair by MsrA (Figures 1 and 4). There are corresponding increases in peptide ellipticity following functional reactivation (Figure 6), which suggest that repaired CaM_{ox} assumes a nativelike structure. These results suggest that MsrA does not recognize methionine sulfoxides within nativelike α-helical secondary structures in functionally active CaM.

Relationship between CaM Structure and MsrA Activity. The extensive oxidation of methionines in CaM isolated from senescent brain may have far-reaching effects since methionines, which comprise approximately 46% of the hydrophobic surface associated with binding target proteins, have been suggested to contribute to the productive association between CaM and a broad range of different target proteins having little sequence homology (*33*−*40*). Thus, lacking global structural changes, the oxidation of the methionine side chain from a nonpolar thioether to the corresponding polar sulfoxide will weaken binding interactions between CaM and target proteins. However, in addition, the oxidative modification of selected methionines has been shown to result in the disruption of the tertiary structure of calcium-activated CaM, which correlates with an inability to activate the PM-Ca-ATPase fully (*24, 41*). This latter result is consistent with the ability of MsrA to repair methionine sulfoxides only in CaM_{ox} oxiforms that are structurally modified and unable to activate the PM-Ca-ATPase fully (Figures 1 and 2), and suggests that MsrA can repair methionine sulfoxides only in partially unfolded protein structures. In contrast, oxidative modifications of methionines at nonessential positions in CaM that do not perturb the tertiary structure only affect the binding affinity with the PM-Ca-ATPase, and do not alter the ability of CaM to activate the PM-Ca-ATPase (*42*). The ability of partially repaired CaM_{ox} to fully activate the PM-Ca-ATPase is consistent with earlier suggestions that the partially folded MG state is in dynamic equilibrium with the native state (*43, 44*). Therefore, the subpopulation of CaM species that adopt the correct native conformation may selectively bind to the PM-Ca-ATPase. Consistent with this suggestion, previous measurements have demonstrated that large differences in the secondary and tertiary structures of mutant CaMs are largely abolished upon binding to the CaM-binding sequences of target peptides (*45, 46*).

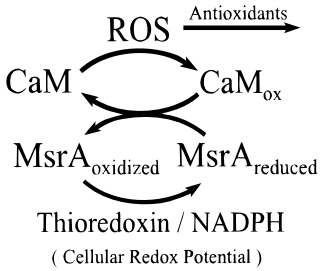


FIGURE 7: Modulation of CaM functional activity by MsrA. Multiple methionines are oxidatively modified in CaM_{ox} when reactive oxygen species (ROS) exceed antioxidant defense mechanisms, impairing the ability of CaM to activate target proteins. In the presence of NADPH, thioredoxin reductase can use thioredoxin to reduce MsrA, which can repair methionine sulfoxides in CaM and restore function.

Physiological Significance. Prolonged calcium transients and elevated basal calcium levels within excitable cells have been demonstrated to correlate with the oxidative modification of cellular proteins during biological aging (*1, 12, 47*). It has therefore been suggested that a causal linkage exists between the loss of calcium homeostasis and a range of age-related neurodegenerative diseases, including Alzheimer's disease. The reduced function of CaM_{ox} isolated from aged animals may represent a mechanism associated with the loss of calcium homeostasis under conditions of oxidative stress when reactive oxygen species (ROS) overwhelm intracellular antioxidant repair systems. Thus, depending on the oxidative load experienced by the cell, MsrA has the potential to modulate CaM function. Thus, under conditions of oxidative stress when the concentration of ROS exceeds the cellular antioxidant defense mechanisms, CaM has the potential to be oxidatively modified (Figure 7). Under these conditions, CaM_{ox} can bind, but not activate some target proteins, including the PM-Ca-ATPase (*6, 19, 24*). When normal cellular redox conditions are restored, MsrA can repair methionine sulfoxides in CaM_{ox}, and restore the CaM-

dependent activation of the PM-Ca-ATPase important to reestablishing intracellular calcium homeostasis.

In addition to the maintenance of the ability of CaM to activate the PM-Ca-ATPase and thereby maintain calcium homeostasis, MsrA function may have broader implications with respect to the maintenance of native-like protein conformations for a range of different proteins in addition to CaM. The ability of MsrA to recognize selectively and repair methionine sulfoxides within hydrophobic sequences in unfolded proteins may, for example, function to prevent protein aggregation between partially unfolded proteins. This latter function may be critical to the maintenance of protein solubility, which is necessary for recognition and degradation of oxidatively modified proteins by the multicatalytic proteosome (*48*).

Conclusions and Future Directions. The repair of methionine sulfoxides in functionally impaired CaM_{ox} isolated from senescent brain by MsrA results in the recovery of the ability of CaM to activate the PM-Ca-ATPase. These results suggest cellular changes in aged brain occur such that the specific activity of MsrA is insufficient to maintain CaM in a fully functional state. Future studies should determine whether the accumulation of functionally impaired CaM_{ox} during aging is the result of alterations in the expression levels or maximal catalytic activity of MsrA, or may instead be related to alterations in cellular redox conditions that result in a reduced specific activity of MsrA.

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