Oxidation of Met^{144} and Met^{145} in Calmodulin Blocks Calmodulin Dependent Activation of the Plasma Membrane Ca-ATPase†

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Received October 7, 2002; Revised Manuscript Received January 25, 2003

ABSTRACT: Methionine oxidation in calmodulin (CaM) isolated from senescent brain results in an inability to fully activate the plasma membrane (PM) Ca-ATPase, which may contribute to observed increases in cytosolic calcium levels under conditions of oxidative stress and biological aging. To identify the functional importance of the oxidation of Met^{144} and Met^{145} near the carboxyl-terminus of CaM, we have used site-directed mutagenesis to substitute leucines for methionines at other positions in CaM, permitting the site-specific oxidation of Met^{144} and Met^{145}. Prior to their oxidation, the CaM-dependent activation of the PM-Ca-ATPase by these CaM mutants is similar to that of wild-type CaM. Likewise, oxidation of individual methionines has a minimal effect on the CaM concentration necessary for half-maximal activation of the PM-Ca-ATPase. These results are consistent with previous suggestions that no single methionine within CaM is essential for activation of the PM-Ca-ATPase. Oxidation of either Met^{144} and Met^{145} or all nine methionines in CaM results in an equivalent inhibition of the PM-Ca-ATPase, resulting in a 50−60% reduction in the level of enzyme activation. Oxidation of Met^{144} is largely responsible for the decreased extent of enzyme activation, suggesting that this site is critical in modulating the sensitivity of CaM to oxidant-induced loss-of-function. These results are discussed in terms of a possible functional role for Met^{144} and Met^{145} in CaM as redox sensors that function to modulate calcium homeostasis and energy metabolism in response to conditions of oxidative stress.

Multiple methionines in calmodulin (CaM)[1] isolated from senescent brain are oxidized to their corresponding methionine sulfoxides, resulting in an impaired ability to activate the plasma membrane (PM) Ca-ATPase. It has been proposed that these posttranslational modifications to CaM may contribute to observed alterations in intracellular calcium homeostasis that result in prolonged and blunted calcium transients (*1*−*3*). The observed increases in intracellular calcium levels have been suggested to represent an adaptive cellular response that functions to maintain cellular viability under conditions involving oxidative stress since small increases in intracellular calcium levels are known to promote cell survival (*4*). While the mechanisms that promote cell viability remain unclear, reducing the calcium gradients associated with normal signaling will minimize ATP requirements and reduce the generation of reactive oxygen species (ROS) through respiratory control mechanisms (*3*, *5*). Furthermore, methionine oxidation in CaM is reversible through the action of endogenous methionine sulfoxide reductases, suggesting that the oxidation of critical methionines in CaM may function as part of a conformational switch that regulates cellular metabolism (*6*−*8*).

To clarify the regulation of PM-Ca-ATPase function by the oxidation of CaM, it is necessary to understand whether the site-specific oxidation of specific methionines in CaM regulates enzyme function. Prior measurements have identified CaM to be highly sensitive to oxidant-induced loss of function and suggested a correlation between the oxidation of Met^{144} and Met^{145} located near the carboxyl-terminus of vertebrate CaM and inhibition of the PM-Ca-ATPase (*9*, *10*). However, these earlier measurements investigated the CaM-dependent activation of the PM-Ca-ATPase using heterogeneous samples of CaM in which the nine methionines were oxidized to differing extents. It was, therefore, not possible to identify quantitatively how the site-specific oxidation of individual methionines affects the CaM-dependent activation of the PM-Ca-ATPase.

To address how the site-specific oxidation of Met^{144} and Met^{145} affects the ability of CaM to activate the PM-Ca-ATPase, we have used site-directed mutagenesis to make conservative amino acid substitutions to replace the majority of methionines in CaM with nonoxidizable amino acids (i.e., leucines), permitting a precise identification of the functional effects resulting from the site-specific oxidation of individual methionines. Leucine was chosen because in comparison to methionine it has a similar volume, hydrophobicity, and conformational preferences that favor α-helix formation (*11*). Furthermore, substitution of the majority of leucines in each of the opposing globular domains of CaM has previously

† This work was supported by NIH (AG12993 and AG17996) and an instrumentation grant from NSF (0088931).

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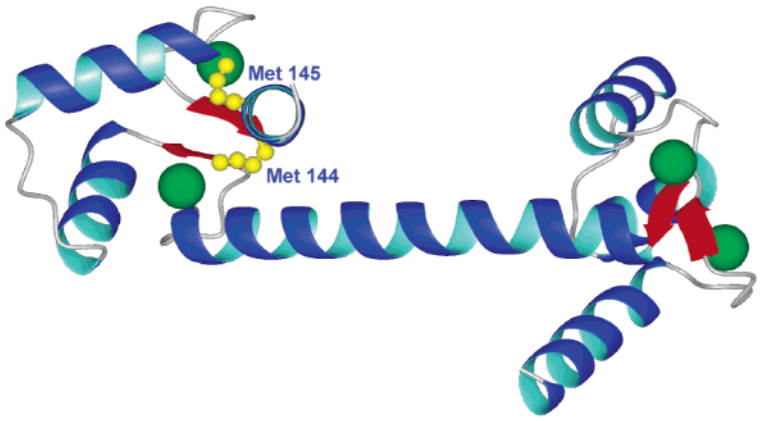
[1] Abbreviations: BSA, bovine serum albumin; CaM, calmodulin; ESI−MS; electrospray ionization mass spectrometry; HEPES, beta-(2-hydroxyethyl)piperazine-*N*-2-ethansulfonic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; PM-Ca-ATPase, plasma membrane calcium pump; ROS, reactive oxygen species.

10.1021/bi026956z CCC: $25.00
Published on Web 03/01/2003



Wild-type	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTV**M**[36]*R*SLGQNPTEAELQD**M**[51]INEVDADGNGTIDFPEFLT**M**[71]**M**[72]A*RK***M**[76]
L7	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTV**L**[36]*R*SLGQNPTEAELQD**L**[51]INEVDADGNGTIDFPEFLT**L**[71]**L**[72]A*RK***L**[76]
L8-M144	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTV**L**[36]*R*SLGQNPTEAELQD**L**[51]INEVDADGNGTIDFPEFLT**L**[71]**L**[72]A*RK***L**[76]
L8-M145	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTV**L**[36]*R*SLGQNPTEAELQD**L**[51]INEVDADGNGTIDFPEFLT**L**[71]**L**[72]A*RK***L**[76]
L9	DQLTEEQIAEF*K*EAFSLFDKDGDGTITT*K*ELGTV**L**[36]*R*SLGQNPTEAELQD**L**[51]INEVDADGNGTIDFPEFLT**L**[71]**L**[72]A*RK***L**[76]

Wild-type	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HV**M**[109]TNLGE*K*LTDEEVDE**M**[124]I*R*EADIDGDGQVNYEEFVQ**M**[144]**M**[145]TA*K*
L7	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HV**L**[109]TNLGE*K*LTDEEVDE**L**[124]I*R*EADIDGDGQVNYEEFVQ**M**[144]**M**[145]TA*K*
L8-M144	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HV**L**[109]TNLGE*K*LTDEEVDE**L**[124]I*R*EADIDGDGQVNYEEFVQ**M**[144]**L**[145]TA*K*
L8-M145	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HV**L**[109]TNLGE*K*LTDEEVDE**L**[124]I*R*EADIDGDGQVNYEEFVQ**L**[144]**M**[145]TA*K*
L9	*K*DTDSEEEI*R*EAF*R*VFDKDGNGYISAAEL*R*HV**L**[109]TNLGE*K*LTDEEVDE**L**[124]I*R*EADIDGDGQVNYEEFVQ**L**[144]**L**[145]TA*K*

FIGURE 1: CaM structure. Ribbon diagram depicting the backbone fold of calcium-saturated CaM (top) and primary sequence of wild-type and mutant CaM constructs (bottom). Secondary structural elements depicting helices (blue), sheets (red), loop structures (gray), Met^{144} and Met^{145} (yellow), and bound calcium (green spheres) are indicated in the ribbon diagram. Positions of methionines and their associated site-directed substitutions are highlighted in red in the sequence.

been shown to have minimal effects on the ability of CaM to bind and activate other CaM-dependent enzymes (*12*).

We find that all mutant CaM samples are able to activate the PM-Ca-ATPase to a degree similar to that observed for wild-type CaM. Thus, observed decreases in the ability of oxidized CaM to bind and activate the PM-Ca-ATPase can be attributed to the site-specific oxidation of either Met^{144} or Met^{145}. We report that the inability of oxidized CaM to fully activate the PM-Ca-ATPase is the result of the site-specific oxidation of Met^{144} and Met^{145}, where oxidation of the other seven methionines in CaM has a minimal effect on either the affinity or the extent of CaM-dependent activation.

MATERIALS AND METHODS

Materials. H_2O_2 was obtained from Fisher (Pittsburgh, PA). Phenyl Sepharose CL-4B was purchased from Pharmacia (Piscataway, NJ). A micro-BCA reagent assay kit was obtained from Pierce (Rockford, IL). Restriction endonucleases, primers, and dNTPs for PCR were purchased from Gibco-Invitrogen Corporation (Carlsbad, CA). The vectors pBluescript II SK and *Pfu* DNA polymerase were purchased from Stratagene (La Jolla, CA). The vector pET-15b and the *Escherichia coli* BL21(DE3) competent cells were purchased from Novagen (Madison, WI). The *E. coli* competent cell strain DH10B was purchased from Invitrogen/Life Technologies (Carlsbad, CA). The $^{15}NH_4Cl$ was purchased from Isotec, Inc. (Miamisburg, OH).

Calmodulin Mutagenesis, Expression, and Purification. The coding region for chicken CaM [accession number MCCH (PIR database) or P02593 (SWISS-PROT database)] was excised from the plasmid pCaMPL provided by Professor Samuel George (Duke University) and subcloned into the mutagenesis and expression vector pALTER-Ex1, as previously described (*13*). The cDNA encoding wild-type CaM was mutated to replace seven, eight, or all nine methionine residues with leucines (Figure 1). The mutated genes for CaM-L7, CaM-L8M144, CaM-L8M145, and CaM-L9 were produced by sequentially mutating all nine methionine residues to leucine using the PCR based mutagenesis method outlined previously (*14*, *15*). The intact mutant CaM gene for CaM-L9 was subcloned into pET-15b and transformed into the *E. coli* BL21(DE3) cell strain for protein overexpression. The bacteria were grown in minimal media, and protein production was induced with IPTG. For production of uniformly ^{15}N-labeled CaM and CaM-L9 for NMR studies, $^{15}NH_4Cl$ was used as the sole nitrogen source. Both wild-type and mutant CaM were purified by chromatography on phenyl Sepharose CL-4B, essentially as previously

described (*16*). Following column elution, CaM was dialyzed against 1 mM imidazole (pH 6.5), 0.1 M KCl, and 10 mM $CaCl_2$ (buffer A) and subsequently stored at −80 °C.

PM-Ca-ATPase Purification and Functional Assay. Erythrocyte ghost membranes containing the PM-Ca-ATPase were obtained from porcine blood and stored at −70 °C (*17*). The CaM-dependent ATPase activity of the PM-Ca-ATPase was measured as described by Lanzetta and co-workers for measuring phosphate release (*18*). The ghost membrane protein concentration was determined by the Biuret method (*19*), using BSA as a standard. The concentration of CaM standard was determined using the published extinction coefficient ($\epsilon_{277\ nm} = 3029\ M^{-1}\ cm^{-1}$) for calcium-saturated CaM (*16*, *20*) or with the micro-BCA assay using CaM as a protein standard. ATPase activity was measured in triplicate at each concentration of CaM at 37 °C in a solution containing approximately 2 nM PM-Ca-ATPase (i.e., 0.05 mg mL^{-1} porcine erythrocyte ghost membranes) in 100 mM HEPES (pH 7.5), 0.1 M KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 0.44 mM $CaCl_2$, 5 mM ATP, and 4 μM A23187 (buffer B) in a total volume of 100 μL. The reaction was initiated by the addition of ATP to the reaction mixture that was preincubated for 5 min with the indicated concentrations of calmodulin. ATPase activity was calculated from a least-squares fit to three time points at 15, 30, and 45 min for each CaM concentration. Each data point represents the average of three different measurements that were made in triplicate, and errors represent the standard deviation of each measurement. The free calcium concentration was calculated to be 100 μM, which has previously been shown to promote maximal enzymatic activation of the PM-Ca-ATPase by CaM (*2*, *9*, *21*).

Calculation of Free CaM Concentrations. The concentrations of free CaM were obtained from the following relationship:

$$[CaM]_{free} = [CaM]_{total} - \frac{(V - V_{min})}{(V_{max} - V_{min})}[PM - Ca - ATPase] \quad (1)$$

where V_{max} is the maximal CaM-dependent ATPase activity, V_{min} is the ATPase activity in the absence of added CaM, and V is the observed ATPase activity at a defined concentration of CaM. $[CaM]_{free}$ is the concentration of CaM free in solution, $[CaM]_{total}$ is the total concentration of CaM added to the solution, and [PM-Ca-ATPase] is the total binding capacity of the erythrocyte ghosts for CaM, which was measured to be 40 pmol of CaM bound per mg of porcine erythrocyte ghost (*22*). Thus, the concentration of free calmodulin was calculated by subtracting the amount of CaM bound to the PM-Ca-ATPase from the total calmodulin concentration added to the sample. Variations in the values of the calmodulin-dependent ATPase activity observed at subnanomolar concentrations of calmodulin, where the binding affinity of CaM for the PM-Ca-ATPase is not sufficient to promote enzyme activation, represent the noise in the measurement, and their average value corresponds to the ATPase activity observed in the absence of added CaM (i.e., V_{min}).

Oxidation of CaM. Quantitative oxidation of methionines in CaM to their corresponding methionine sulfoxides involved the addition of 100 mM H_2O_2 to calcium-saturated CaM (60 μM) in 1 mM imidazole (pH 6.5), 0.1 M KCl, and 10 mM $CaCl_2$ (buffer A). After incubation for 24 h, the samples were extensively dialyzed against buffer A to remove excess H_2O_2.

Mass Spectrometric Analysis. Following exposure to hydrogen peroxide, electrospray ionization mass spectrometry (ESI−MS) was used to identify the whole protein molecular weight to assess the extent of oxidative modification, adopting strategies previously described (*2*). The ESI mass spectra in positive ion mode were acquired on a Q-Tof 2 quadrupole, time-of-flight hybrid instrument (Micromass Ltd, Manchester, UK). The analysis of intact CaM was done by desalting 20 μg of protein on a trapping column (1.5 cm × 1 mm i.d.) hand-packed with Zorbax SB-C18, 5 μm (Agilent Technologies, Wilmington, DE), washing with 1% acetic acid, then eluting directly into the source with 90% MeOH, 0.5% formic acid. The instrument parameters that offered the best compromise of sensitivity and minimizing the water loss peak were 60 V on the cone and 12 V on the collision cell with no Ar.

NMR Spectroscopy. Gradient sensitivity enhanced ^1H, ^{15}N-HSQC NMR spectra were acquired as previously described (*23*) using a Varian INOVA instrument operating at 600 MHz (^1H). The spectra were recorded with 512 complex points in t_2 (^1H) and 128 complex points in t_1 (^{15}N), and the sample temperature was maintained at 25 °C. The wild-type and mutant CaM samples contained approximately 1 mM CaM, 50 mM KCl, 10 mM $CaCl_2$, 5 mM imidazole-d_4, 0.02% NaN_3, and 10% D_2O at pH 6.5. External Na^+DSS^- in D_2O was used as the ^1H chemical shift reference (0.00 ppm), and ^{15}N chemical shifts were referenced indirectly (*24*). Data processing and analysis were performed using Felix (Accelrys, San Diego, CA).

RESULTS

Expression and Purification of Calmodulin Mutants. To identify the functional consequences associated with the specific oxidation of Met^{144} and Met^{145} in CaM, we have used site-directed mutagenesis to replace the other seven methionines with the nonoxidizable amino acid leucine (i.e., L7-CaM; Figure 1). Additional CaM mutants were generated that, in addition to the seven leucine substations engineered into CaM-L7, contained site-directed substitutions of leucine for Met^{144} (CaM-L8M145), Met^{145} (CaM-L8M144), and both Met^{144} and Met^{145} (CaM-L9). All CaM species were expressed in *E. coli* and purified using the calcium-dependent changes in the hydrophobic interactions between CaM and phenyl-Sepharose CL-4B (*16*). Thus, the substitution of all nine methionines with leucines did not interfere with the normal calcium-dependent structural changes associated with calcium binding that expose hydrophobic binding clefts within each of the opposing domains.

Nonessential Role of Methionines in the Structure of CaM. Additional resolution of possible structural changes in calcium-activated CaM were assessed using NMR spectroscopy. Replacement of all nine methionine residues in CaM with leucine (CaM-L9) results in nominal chemical shift perturbations in NMR spectra as compared to wild-type CaM, suggesting that the essential structural features that define

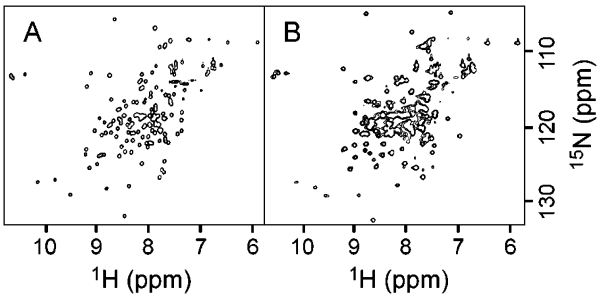


FIGURE 2: NMR spectroscopy of wild-type CaM and CaM-L9. ^{1}H, ^{15}N-HSQC spectra of ^{15}N-labeled wild-type CaM (A) and CaM-L9 (B). The slight line broadening evident in the spectrum of CaM-L9 suggested increased conformational heterogeneity or aggregation of CaM-L9 as compared to wild-type.

CaM remain intact following the replacement of methionine with leucine in CaM-L9 (Figure 2). This is consistent with previous results suggesting that the structural core in calcium-saturated CaM is relatively tolerant to substitution of residues, such as methionines, that are largely surface exposed (*25*, *26*). However, in comparison to wild-type CaM there is considerable line-broadening that is apparent in the spectra obtained for L9-CaM, which suggest an increased propensity to undergo nonspecific protein associations at the high protein concentrations used in the NMR measurements. Nevertheless, the retention of the resonance positions associated with the protein backbone of CaM-L9 in comparison to wild-type CaM strongly indicates that the three-dimensional fold of CaM is unaffected by the site-directed substitution of all nine methionines with leucines. Furthermore, under conditions associated with enzymatic activation of the PM-Ca-ATPase, there is no apparent aggregation of either wild-type CaM or CaM-L9 (data not shown).

Resolution of Oxidized CaM. Critical to an evaluation of how the oxidation of individual methionines affects the CaM-dependent activation of the PM-Ca-ATPase is an assessment of the homogeneity of oxidized CaM because unoxidized CaM samples with higher binding affinities have the potential to selectively associate with and activate the PM-Ca-ATPase. We have, therefore, used SDS−PAGE and whole-protein electrospray ionization mass spectrometry (ESI−MS) to assess the homogeneity of expressed and oxidized CaM samples. Both SDS−PAGE and ESI−MS permit the resolution of multiple oxiforms of CaM (*2*, *27*). SDS−PAGE measurements take advantage of the fact that the mobility of CaM on SDS−PAGE gels run under the conditions described by Laemmli has proven to be highly sensitive to structural alterations in the backbone fold resulting from site-directed amino acid substitutions or posttranslational modifications (*2*, *12*, *28*). For example, following the various extents of oxidation of methionines in wild-type CaM, approximately 10 distinct bands can be resolved on an SDS−PAGE gel corresponding to different oxiforms of CaM, demonstrating the sensitivity of SDS−PAGE to any heterogeneity in the extent of CaM oxidation (*29*). Likewise, in comparison to wild-type CaM, the relative mobility of CaM on SDS−PAGE is increased following the site-directed substitution of seven, eight, or nine methionines with leucines (Figure 3). Thus, an assessment of differences in the relative mobility of CaM on SDS−PAGE is a convenient method to assess the homogeneity of oxidized CaM.

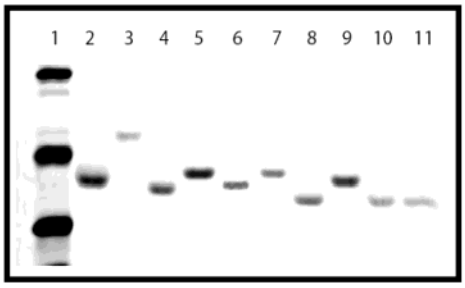


FIGURE 3: Electrophoretic mobilities of CaM. Samples correspond to wild-type CaM (lanes 2 and 3), CaM-L7 (lanes 4 and 5), CaM-L8Met144 (lanes 6 and 7), CaM-L8Met145 (lanes 8 and 9), and CaM-L9 (lanes 10 and 11) either before (lanes 2, 4, 6, 8, and 10) or after (lanes 3, 5, 7, 9, and 11) oxidation with hydrogen peroxide. Relative mobilities were assessed using SDS−PAGE [15% (w/v) acrylamide] using the conditions originally defined by Laemmli (*41*). Molecular mass standards are shown in lane 1 and include lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), and carbonic anhydrase (31.0 kDa). CaM samples were suspended in 1 mM imidazole (pH 6.5), 0.1 M KCl, and 10 mM $CaCl_2$ to ensure calcium saturation, as previously described (*29*).

Wild-type CaM migrates as a single band with an apparent molecular mass of about 19 kDa (Figure 3). Following oxidation with 100 mM H_2O_2, the mobility of wild-type CaM is reduced and migrates as a single band with an apparent molecular mass of about 24 kDa, indicating that the sample is homogeneously oxidized. The apparent 6-kDa reduction in shift of molecular mass is the result of the oxidation of all nine methionines in wild-type CaM (see below). Following the site-directed substitution of seven or eight methionines with leucines, the relative shift in CaM mobility is reduced by 1.7 and 1.1 kDa upon oxidation of Met^{144} and/or Met^{145}. All samples exhibit a single band, suggesting that the sample is homogeneously oxidized (Figure 3).

SDS−PAGE cannot fully resolve all posttranslational modifications that may result from the incubation of samples with hydrogen peroxide. Therefore, ESI−MS was used as a complementary tool to assess the homogeneity of CaM samples and to rule out additional sites of modification. A single major peak is observed in the ESI−MS spectra for each CaM mutant both before and after oxidation, which corresponds to the expected mass for each mutant protein prior to and following the quantitative oxidation of all methionines (Figure 4; Table 1). In each case, no significant spectral intensity is observed for masses higher than the mass of the species with all methionines oxidized, indicating that no other amino acids are appreciably oxidized. Moreover, despite the presence of lower mass peaks that correspond to ionization induced dehydration artifacts (*27*), the overall shape of the mass spectra for the expressed and oxidized CaM proteins are nearly identical, indicating homogeneous oxidation that reflects the homogeneity of the wild-type sample.

CaM-Dependent Activation of the PM-Ca-ATPase. The time-dependent hydrolysis of ATP by the Ca-ATPase was linear over the time scale of the measurement, permitting the calculation of the enzyme activity from a nonlinear least-squares fit to the data (Figure 5). The CaM-dependent activation of the PM-Ca-ATPase in erythrocyte ghost membranes by wild-type CaM is comparable to earlier measurements, with a maximal velocity of approximately 0.4 μmol P_i mg^{-1} hr^{-1}, and the concentration of CaM

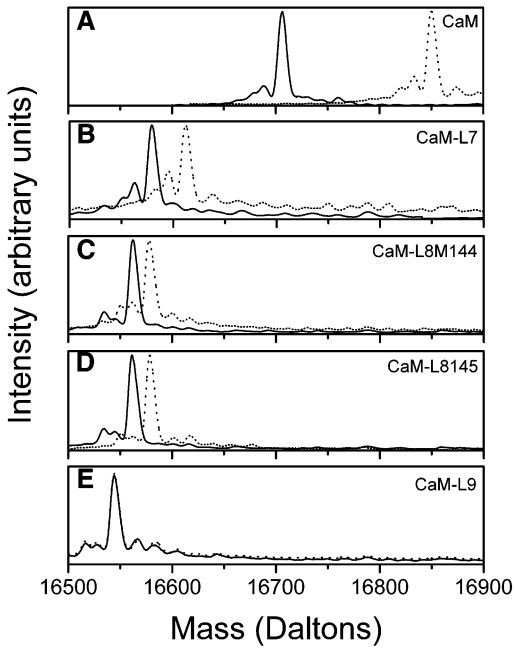


FIGURE 4: Electrospray ionization mass spectra of wild-type and mutant CaM. Spectra corresponding to wild-type CaM (A), CaM-L7 (B), CaM-L8M144 (C), CaM-L8M145 (D), and CaM-L9 (E) before (solid line) or after (dotted line) oxidation by hydrogen peroxide. Spectra were obtained following deconvolution of multiply charged ions. Experimentally, 20 μg of CaM in 0.1 mM EGTA and 10 mM $(NH_4)_2CO_3$ (pH 8.6) was trapped, desalted, and then directly infused (on-line) into a Q-Tof 2 mass spectrometer.

Table 1: Masses of Expressed and Oxidized CaM[a]

	Mass (Da)[b]			
	Expressed Protein[d]		+Oxidation[e]	
CaM sample[c]	observed	theoretical	observed	theoretical[f]
wild-type	16 707	16 706	16 850	16 851
L7	16 579	16 580	16 612	16 611
L8-M144	16 562	16 562	16 577	16 578
L8-M145	16 561	16 562	16 578	16 578
L9	16 544	16 544	16 544	16 544

[a] Whole protein masses were measured in the positive ion mode using a Q-Tof 2 quadrupole, time-of-flight hybrid instrument (Micromass Ltd, Manchester, UK). The analysis of intact CaM was done by desalting 20 μg of protein on a trapping column (1.5 cm × 1 mm i.d.) hand packed with Zorbax SB-C18, 5 μm (Agilent Technologies, Wilmington, DE), washing with 1% acetic acid, then eluting directly into the source with 90% MeOH, 0.5% formic acid. Instrument parameters were found that offered the best compromise of sensitivity and minimizing the water loss peak were 60 V on the cone and 12 V on the collision cell with no Ar. [b] Masses of deconvoluted ESI−MS spectra were determined with an accuracy of ±1 Da. [c] Wild-type CaM and CaM mutants were expressed following the site-directed substitution of seven, eight, and nine leucines for methionines, as defined in Figure 1. [d] CaM samples prior to oxidation. [e] CaM samples following exposure to the addition of 100 mM H_2O_2. [f] Calculated masses assuming the selective oxidation of methionines.

required for half-maximal activation of the PM-Ca-ATPase is approximately 7 ± 2 nM CaM (*16*) (Figure 6). Since the PM-Ca-ATPase represents approximately 0.5% of the total protein in these erythrocyte ghost membranes (*22*), the specific activity of the PM-Ca-ATPase for ATP hydrolysis is approximately 1.3 μmol P_i mg^{-1} min^{-1}. In most cases, following site-directed substitution of multiple methionines in CaM with leucines, the maximal CaM-dependent activation of the PM-Ca-ATPase is enhanced (Table 2). This effect indicates that the more hydrophobic leucine side chains enhance productive interactions with the CaM-binding sequence in the PM-Ca-ATPase that facilitate dissociation of the autoinhibitory domain and enzyme activation. This observation is consistent with earlier measurements demonstrating a nonessential role of methionines in CaM with respect to the activation of the PM-Ca-ATPase (*30*).

However, while the site-directed substitution of Met^{36}, Met^{51}, Met^{71}, Met^{72}, Met^{76}, Met^{109}, and Met^{124} with leucines in CaM-L7 has a minimal effect on the CaM concentration associated with half-maximal enzyme activation (i.e., $[CaM]_{1/2}$; Table 2), the additional replacement of Met^{144} or Met^{145} with leucines (CaM-L8M145 or CaM-L8144) results in an increase in the amount of CaM required for half-maximal enzyme activation (Table 2). These results suggest that substitution of Met^{144} and Met^{145} with leucines results in a reduced binding affinity, suggesting that these methionines play an important role in the initial association of CaM with the CaM-binding sequence of the PM-Ca-ATPase. This observation is consistent with earlier suggestions that these methionines function as hydrophobic anchors that stabilize the initial association between the carboxyl-terminal domain of CaM and the CaM-binding sequence of the PM-Ca-ATPase (*31*−*33*). However, following the substitution of methionines with leucines there is an increase in the extent of the CaM-dependent activation of the PM-Ca-ATPase. Thus, while methionines function to facilitate binding to the PM-Ca-ATPase in comparison to mutants containing leucines at these positions, they result in a diminished CaM-dependent activation.

Functional Consequences of the Site-Specific Oxidation of Met^{144} and Met^{145}. The retention of enzyme activity following the substitution of all nine methionines with leucines suggests that the site-directed oxidation of Met^{144} and/or Met^{145} in CaM-L7, CaM-L8M144, and CaM-L8M145 will permit an assessment of the functional effects of the oxidation of Met^{144} and Met^{145} on the activation of the PM-Ca-ATPase. Oxidation of all nine methionines in wild-type CaM results in a 63 ± 8% decrease in the CaM-dependent activation of the PM-Ca-ATPase (Figures 5A and 6A; Table 2). A similar decrease (52 ± 6%) is observed following the site-specific oxidation of Met^{144} and Met^{145} in CaM-L7 (Figures 5B and 6B). These results indicate that the oxidation of Met^{144} and Met^{145} are principally responsible for the oxidant-induced decrease in the maximal extent of enzyme activation and that oxidation of Met^{144} and Met^{145} results in a nonproductive association between CaM and PM-Ca-ATPase.

Additional resolution of the functional effects of oxidation of Met^{144} and Met^{145} is possible from a consideration of the site-specific oxidation of these individual methionines in CaM-L8M144 and CaM-L8M145. Oxidation of Met^{144} results in a 33 ± 11% decrease in the maximal level of enzyme activation (Figures 5C and 6C), while oxidation of Met^{145} results in a minimal perturbation with respect to the activation of the Ca-ATPase (Figures 5D and 6D). These results demonstrate that the oxidation of Met^{144} is primarily responsible for the decrease in the maximal extent of the activation of the PM-Ca-ATPase in the presence of saturating concentrations of CaM.

Oxidant-induced changes in the binding affinity of CaM for the CaM-binding sequence within the PM-Ca-ATPase

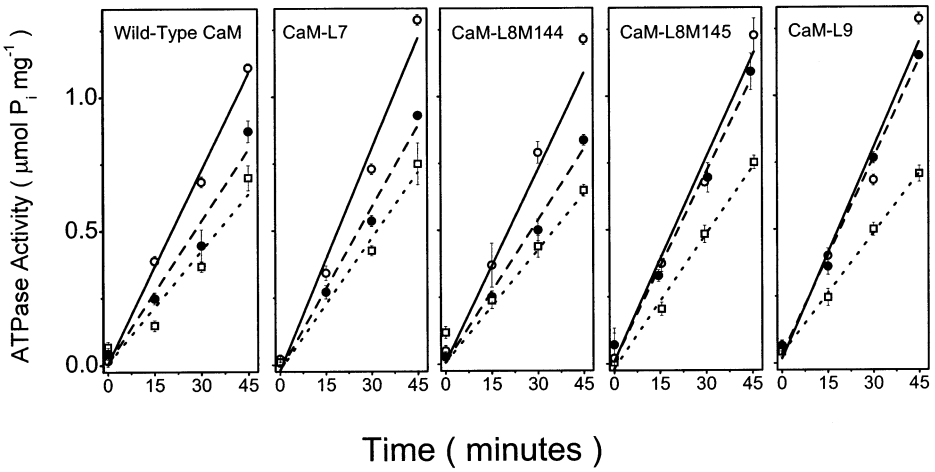


FIGURE 5: Time-dependence of plasma membrane Ca-ATPase enzyme activity. Rates of ATP hydrolysis were measured in the presence of either 10 μM wild-type CaM, CaM-L7, CaM-L8M144, CaM-L8M145, or CaM-L9 before (○) or after (●) oxidation of CaM by hydrogen peroxide in comparison to the CaM-independent activity (□). ATPase activity was measured at 37 °C in buffer B containing 0.05 mg/mL porcine erythrocyte ghosts.

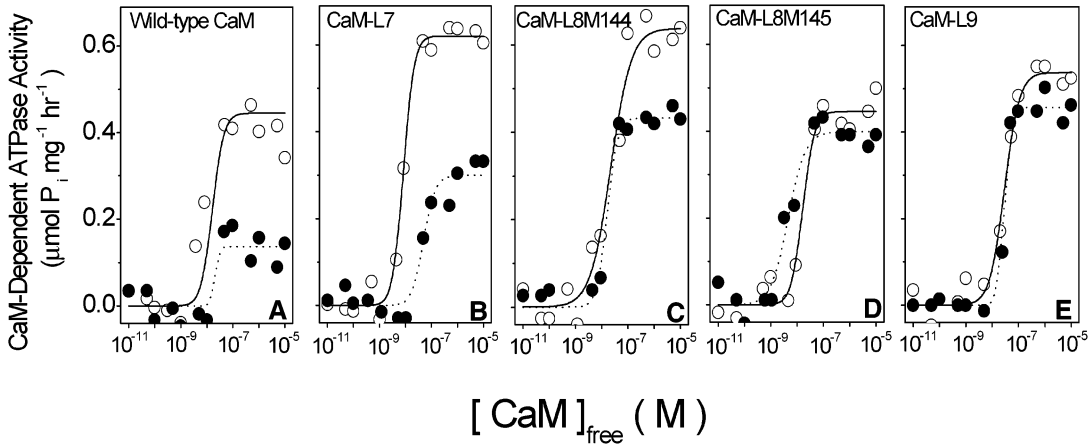


FIGURE 6: CaM-dependence of the activation of the plasma membrane Ca-ATPase. CaM-dependence of the activation of the PMCA by wild-type CaM (A), CaM-L7 (B), CaM-L8M144 (C), CaM-L8M145 (D), or CaM-L9 (E) before (○) or after (●) oxidation of CaM by hydrogen peroxide. ATPase activity was measured at 37 °C in buffer B containing 0.05 mg/mL porcine erythrocyte ghosts. Average errors in ATPase measurements were 8% of the indicated values.

can be assessed from a consideration of changes in the amount of CaM necessary for half-maximal activation of the PM-Ca-ATPase. Following oxidation of all nine methionines in wild-type CaM, the concentration of CaM necessary for half-maximal activation of the PM-Ca-ATPase increases from 7 ± 2 to 20 ± 10 nM. Similar concentrations of CaM-L8M144 (22 ± 6 nM), CaM-L8M145 (20 ± 6 nM), and CaM-L9 (26 ± 7 nM) are necessary for half-maximal activation of the PM-Ca-ATPase (Table 2). These results suggest that the polarity of the resulting methionine sulfoxides following oxidation of wild-type CaM has a similar effect on binding affinity to that associated with the substitution of the conformationally less flexible leucines for all nine methionines. However, while oxidation of Met^{144} in CaM-M144 has essentially no effect on the CaM concentration required for half-maximal activation, the oxidation of Met^{145} in CaM-L8M145 results in a substantial decrease in the CaM concentration necessary for half-maximal activation of the Ca-ATPase. In contrast, oxidation of both Met^{144} and Met^{145} in CaM-L7 results in a substantial increase in the CaM concentration necessary for half-maximal activation of the PM-Ca-ATPase (Table 2). These latter results suggest that in comparison to the methionine side chain, the more polar methionine sulfoxide at position 145 enhances the binding interaction between the CaM and the CaM-binding sequence of the PM-Ca-ATPase. In contrast, a 7-fold increase in the amount of CaM necessary for half-maximal activation of the PM-CA-ATPase is observed following oxidation of both Met^{144} and Met^{145}, suggesting that synergistic effects resulting from the oxidation of both sites results in an altered binding mechanism to the Ca-ATPase. This may be related to global structural changes that result in alterations in the relative affinities of the opposing domains of CaM. In this respect, it should be noted that prior measurements have demonstrated that the higher affinity of the carboxyl-terminal domain of CaM for the CaM-binding sequence of the PM-Ca-ATPase is critical to promoting the sequential and ordered binding of the opposing domains of CaM to the PM-Ca-ATPase that is associated with activation of the PM-Ca-ATPase (*33*). Thus, the oxidation of both Met^{144} and Met^{145} has the potential to result in global structural changes that result in a larger decrease in the apparent affinity between CaM and the PM-Ca-ATPase than would be anticipated from a consideration of either site alone.

Table 2: CaM Affinity and Activation of Plasma Membrane Ca-ATPase[a]

	Enzymatic Function[b]			
	Expressed Protein[d]		+Oxidation[e]	
CaM sample[c]	$[CaM]_{1/2\ free}$ (nM)	V_{max} (μmol P_i mg^{-1} hr^{-1})	$[CaM]_{1/2\ free}$ (nM)	V_{max} (μmol P_i mg^{-1} hr^{-1})
wild-type	7 ± 2	0.41 ± 0.03	20 ± 10	0.15 ± 0.03
L7	10 ± 2	0.62 ± 0.02	70 ± 20	0.30 ± 0.03
L8-M144	22 ± 6	0.64 ± 0.06	20 ± 7	0.43 ± 0.03
L8-M145	20 ± 6	0.44 ± 0.04	5 ± 1	0.40 ± 0.04
L9	26 ± 7	0.54 ± 0.04	30 ± 10	0.46 ± 0.02

[a] Functional parameters obtained from data in Figure 6, where $[CaM]_{1/2\ free}$ corresponds to the CaM concentration associated with half-maximal activation of the PM-Ca-ATPase, and V_{max} corresponds to the CaM-dependent activation of the PM-Ca-ATPase in the presence of saturating CaM concentrations. [b] Parameter values correspond to the CaM-dependent activation of the PM-Ca-ATPAse for wild-type and mutant CaM samples prior to (expressed protein) and following the quantitative oxidation of all methionines (+oxidation). [c] Wild-type and CaM mutants were expressed following the site-directed substitution of seven, eight, and nine leucines for methionines, as defined in Figure 1. [d] CaM samples prior to oxidation. [e] CaM samples following exposure to the addition of 100 mM H_2O_2.

DISCUSSION

Summary of Results. Oxidation of Met^{144} and Met^{145} in CaM to their corresponding methionine sulfoxides results in a 7-fold increase in the amount of CaM necessary for half-maximal activation and a 50−60% inhibition of the maximal CaM-dependent activation of the PM-Ca-ATPase (Figure 6; Table 2). Oxidation of Met^{144} is largely responsible for the decreased extent of enzyme activation, while oxidation of Met^{145} primarily alters the apparent affinity of CaM for the PM-Ca-ATPase. Similar decreases in the ability of both CaM and CaM-L7 to bind and activate the PM-Ca-ATPase are observed upon oxidation of their respective methionine residues, indicating that the oxidation of Met^{36}, Met^{51}, Met^{71}, Met^{72}, Met^{76}, Met^{109}, and Met^{124} have minimal effects on either the binding affinity or the activation of the PM-Ca-ATPase by CaM (Figure 6; Table 2).

Role of Methionines in CaM Function. Methionines contribute approximately one-half of the hydrophobic binding surface within each of the binding clefts in CaM (*34*, *35*). The flexibility of the methionine side chains has been suggested to contribute to the ability of CaM to form strong van der Waals contact interactions with a range of different target proteins with variable binding sequences (*36*, *37*). Furthermore, the polarizability of the sulfur atom in methionine is expected to reduce the activation barrier associated with calcium-dependent structural changes associated with the formation of methionine rich hydrophobic binding pockets within each of the opposing domains of CaM to solvent following calcium activation (*38*). Consistent with these expectations, the substitution of all nine methionines in CaM-L9 with leucines, which lack sulfur atoms, results in an increase in the CaM concentration necessary for one-half maximal activation of the PM-Ca-ATPase (Figure 6, Table 1). However, methionine side chains are not essential for the CaM-dependent activation of the PM-Ca-ATPase, as evidenced by the observation that their substitution with leucines in CaM-L9 results in a 30 ± 10% increase in the maximal extent of the enzymatic activation of the PM-Ca-ATPase. Thus, while methionines play an important role in defining the high-affinity association between CaM and Ca-ATPase, leucines within CaM more effectively induce protein structural changes within the Ca-ATPase required for enzyme activation. Thus, initial binding interactions associated with complex formation between CaM and Ca-ATPase are distinct from the structural changes within the CaM-binding sequence that result in the release of the autoinhibitory interaction and enzyme activation.

Relationship between Methionine Oxidation and CaM Function. During biological aging in the brain, multiple methionines in CaM are oxidized to their corresponding methionine sulfoxides, which results in a reduced ability to activate the PM-Ca-ATPase and may underlie observed age-dependent decreases in calcium regulation (*2*, *3*). Earlier measurements were unable to unambiguously identify the functional consequences associated with the site-specific oxidation of individual methionines, which is important to address the hypothesis that the sensitivity of either Met^{144} or Met^{145} in CaM to oxidation may regulate the function of CaM-dependent target proteins, including the PM-Ca-ATPase. The current results demonstrate unambiguously the correspondence between the oxidation of these methionines and the functional inhibition of the PM-Ca-ATPase and provide strong evidence that the reversible oxidation of Met^{144} (and to some extent Met^{145}) will affect the CaM-dependent activation of the PM-Ca-ATPase. Oxidation of the remaining seven methionines in wild-type CaM has a minimal effect on the ability of CaM to either bind or activate the PM-Ca-ATPase (Figure 6). This observation is consistent with earlier mutagenesis measurements that found the site-directed substitution of Val^{144} in CaM to inhibit the CaM-dependent activation of nitric oxide synthase (*39*).

Physiological Significance. CaM represents the central calcium sensor in nonmuscle cells and functions to recognize the calcium signal to coordinate changes in cellular energy metabolism involving the mobilization of energy reserves and the transcriptional regulation of genes (*40*). The ability of the site-specific oxidation of individual methionines in CaM to modulate the function of the Ca-ATPase, in conjunction with the ability of methioinine sulfoxide reductase to reduce oxidized methionines and restore CaM function, suggests a possible regulatory role for the oxidative modification of CaM in modulating calcium homeostasis and energy metabolism in response to conditions of oxidative stress. It, therefore, seems possible that sequence differences near the carboxyl terminus of CaM arose during evolution to modulate the relative sensitivity of CaM in plants and vertebrate animals to oxidation, permitting CaM to serve as a redox sensor, functioning to down-regulate energy metabolism and the associated generation of reactive oxygen species under conditions of oxidative stress.

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BI026956Z