Methionine Oxidation in the Calmodulin-Binding Domain of Calcineurin Disrupts Calmodulin Binding and Calcineurin Activation†

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ABSTRACT: Calcineurin is a Ca^{2+}/calmodulin-activated Ser/Thr phosphatase important in cellular actions resulting in memory formation, cardiac hypertrophy, and T-cell activation. This enzyme is subject to oxidative inactivation by superoxide at low micromolar concentrations and by H_2O_2 at low millimolar concentrations. On the basis of the hypothesis that oxidation of Met residues in calmodulin-binding domains inhibits binding to calmodulin, purified calcineurin was used to study the susceptibility of Met residues to oxidation by H_2O_2. The rate for oxidation of Met_{406} in the calmodulin-binding domain was determined to be 4.4×10^{-3} M^{-1} s^{-1}, indicating a high susceptibility to oxidation. Functional repercussions of Met_{406} oxidation were evaluated using native enzyme and a calcineurin mutant in which Met_{406} was exchanged for Leu. Measurement of fluorescent calmodulin binding demonstrated that oxidation of Met_{406} results in a 3.3-fold decrease in the affinity of calmodulin for calcineurin. Calcineurin activation exhibited a loss in cooperativity with respect to calmodulin following Met_{406} oxidation as shown by a reduction in the Hill slope from 1.88 to 0.86. Maximum phosphatase activity was unaffected by Met oxidation. Changes in the calcineurin−calmodulin interaction were accompanied by a 40% loss in the ability of calmodulin to stimulate binding of immunophilin/immunosuppressant to calcineurin. All effects on calmodulin binding to the native enzyme by the treatment with H_2O_2 could be reversed by treating the enzyme with methionine sulfoxide reductase. These results indicate that the calmodulin-binding domain of calcineurin is susceptible to oxidation at Met_{406} and that oxidation disrupts calmodulin binding and enzyme activation. Oxidation-dependent decreases in the affinity of calmodulin for calcineurin can potentially modulate calmodulin-dependent signaling and calmodulin distribution.

Although all amino acids can be oxidized, Cys and Met are among the most susceptible to oxidative modification. Furthermore, these two amino acids are the only residues that are targets for reduction by cellular antioxidant systems. The ability of amino acids to be modified and then to have the modification reversed introduces the possibility of a regulated cycle of posttranslational modifications contributing to cellular regulation. This is certainly a primary regulatory mechanism for signaling proteins that modulate, and are modulated by, phosphorylation and dephosphorylation. To establish cyclic oxidative modification as a regulatory mechanism, it is necessary to document the mechanisms that reverse the oxidation and to identify targets that are modified. Chemical and enzymatic mechanisms to reverse oxidation of Cys and Met residues are well-established. In fact, cyclic oxidative modification and reductive regeneration of Met and Cys residues are considered to be a part of the cellular antioxidant defense system used to remove reactive oxygen and nitrogen species (*1*). In addition, evidence that cellular ROS production can modulate enzymatic activity when redox-sensitive residues reside in the active site of an enzyme has also been presented. For example, oxidation of the active site Cys residue in protein tyrosine phosphatases disrupts enzymatic activity (*2*). Analogously, in proteins like GroEL and calmodulin that require hydrophobic interaction to work, Met residues in the interaction domains have been implicated in conferring redox sensitivity (*3, 4*). In this work, we examine the effect of Met oxidation in the calmodulin-binding domain of calcineurin. Establishing the susceptibility of calmodulin-binding domains to cyclic oxidative modification and reductive regeneration introduces an additional mechanism for regulation of signaling pathways.

Calmodulin is a regulator of cellular signaling that binds to and activates more than 40 known target proteins (*5*). Because calmodulin contains no Cys residues, oxidation of Met residues is hypothesized to play a role in the compromised calcium metabolism that is observed in aged animals. Met residues within calmodulin that are critical for protein–protein interaction are potential targets for oxidation (*6*). For example, the conversion of Met_{144} and Met_{145} in calmodulin to MetSO disrupts calmodulin-dependent activation of the sarcoplasmic/endoplasmic Ca^{2+}-ATPase (*7*).

Calcineurin (protein phosphatase 2B, PP2B) is a serine/threonine phosphatase and a highly abundant calmodulin target in selected cells, including some neurons. Calcineurin activation contributes to hypertrophy of cardiac myocytes (*8*), to memory formation in neurons (*9*), and to cellular

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activation in T-lymphocytes (*10*). Like calmodulin, calcineurin is susceptible to oxidation-dependent inactivation. Calcineurin is inhibited by superoxide, which acts at micromolar concentrations to oxidize a catalytic Fe^{2+} to Fe^{3+} (*11–13*). Purified calcineurin is also inactivated at low millimolar H_2O_2 concentrations (*12–15*). Two different mechanisms have been proposed for the oxidative inactivation of calcineurin: oxidation of Cys residues (*15*) and oxidation of the catalytic iron/zinc center (*16*). Regardless of the predominant mechanism, the concentrations of H_2O_2 required to inhibit calcineurin in vitro are approximately equal to the concentration required to oxidize key Met residues in calmodulin (*17*). Due to the similar sensitivity of calcineurin and calmodulin to in vitro oxidation by H_2O_2, Met oxidation is potentially a relevant mechanism that contributes to perturbations of calmodulin- and calcineurin-dependent cellular actions.

Calcineurin activation by calmodulin occurs via a disinhibition mechanism. Binding of calmodulin to the calmodulin-binding domain in the regulatory region of calcineurin causes a conformational change in the protein, resulting in displacement of the autoinhibitory domain from the catalytic site. The result of calmodulin binding is a 100-fold stimulation of calcineurin phosphatase activity (*18*). The structurally undefined (*10*) regulatory region of calcineurin-A, which includes the calmodulin-binding domain, contains seven Met residues. One of these Met residues, Met_{406} (*19*), contributes to the stabilization of the complex with calmodulin (*20*).

The degree to which Met residues are accessible to solvent correlates with their sensitivity to oxidation (*4, 21*). On the basis of its role as a participant in calmodulin binding, Met_{406} of calcineurin is predicted to be highly solvent accessible (*10*). In addition, the calmodulin-binding domain of calcineurin is known to be sensitive to proteolysis (*22*). Taken together, these observations suggest that Met_{406} of calcineurin will exhibit a high sensitivity to oxidation. Furthermore, because calmodulin binding is necessary for calcineurin activity, inhibition of calcineurin−calmodulin binding as a result of Met_{406} oxidation is expected to inhibit calcineurin activation.

In many cell types, the concentration of calmodulin is lower than the total concentrations of its targets, making calmodulin a limiting factor in their activation (*23*). In cells, or subcellular compartments, that have limiting calmodulin, changes in the affinity of calmodulin for highly abundant targets can alter the amount of calmodulin available for other targets. Therefore, in cells in which calcineurin is highly abundant, oxidation of Met_{406} in the calmodulin-binding domain of calcineurin might increase the concentration of calmodulin available for activation of other calmodulin-dependent enzymes. On the basis of the hypothesis that oxidation of Met residues in calmodulin-binding domains inhibits binding to calmodulin, the effect of oxidation of Met_{406} in calcineurin was examined. First, a peptide mapping procedure was used to show that Met residues in the regulatory region of calcineurin, including Met_{406}, are susceptible to oxidation. A mutant of calcineurin in which Met_{406} was changed to Leu was used to confirm that when Met_{406} oxidation is blocked, H_2O_2-dependent changes in the calmodulin activation profile of calcineurin, and decreases in calmodulin affinity, are also blocked. Finally, reversal of Met oxidation using methionine sulfoxide reductase was found to restore the calmodulin binding properties of native calcineurin. Taken together, these results indicate that Met oxidation in the calmodulin-binding domain of calcineurin is a reversible posttranslational mechanism for regulating enzymatic activity.

MATERIALS AND METHODS

Chemicals. A peptide corresponding to the calmodulin-binding domain of calcineurin, spanning amino acid residues 391−414 (calcineurin peptide, ARKEVIRNKIRAIGKMARVFSVLR) was purchased from ResGen (Invitrogen Corp., Carlsbad, CA). HPLC solvents were purchased from Burdick and Jackson (Honeywell Burdick and Jackson, Morristown, NJ), LysC was purchased from Roche Diagnostics (Basel, Switzerland).

Protein Expression in Escherichia coli. Wild-type calcineurin was expressed and isolated as described previously (*24*) using 10 units/mL SOD and 0.2 μg/mL catalase in the isolation buffer. The plasmids pETCN-α (*25*), encoding both human calcineurin-Aα and calcineurin-B, and pBB279 (*26*), encoding myristoyl transferase, were used to express calcineurin-A with an N-terminal polyhistidine tag and myristoylated calcineurin-B. The calcineurin was purified using calmodulin sepharose affinity chromatography. Calmodulin was expressed in *E. coli* and purified by sequential phenyl sepharose and G-75 sephadex chromatography steps.

Calcineurin Mutant. The site-directed mutant was constructed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) using the pETCN-α plasmid as a template and the following primers: forward (5′ to 3′), CAA GAT CCG AGC AAT AGG CAA ACT GGC CAG AGT GTT CTC AGT GCT C; reverse (5′ to 3′), GAG CAC TGA GAA CAC TCT GGC CAG TTT GCC TAT TGC TCG GAT CTT G.

Oxidation. Purified calcineurin (2.5 μM) was oxidized in 10 mM HEPES (pH 7.6), 100 mM KCl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$ with 12 mM H_2O_2 at room temperature in the dark for 4 h unless otherwise stated. The concentration of H_2O_2 was estimated using an ϵ_{240} of 39.4 cm^{-1} M^{-1} (*27*). If the calcineurin was to be used for binding or activity assays, the reaction was stopped by at least 25-fold dilution, and addition of catalase to a final concentration of 5 μg/mL. For the peptide mapping assay, oxidation was stopped by addition of catalase to a final concentration of 5 μg/mL, with a 5 min incubation at room temperature, before reduction and alkylation. The calcineurin peptide was oxidized at a concentration of 0.5 mg/mL in 10 mM HEPES (pH 7.6), 100 mM KCl, and 1 mM $MgCl_2$.

Methionine Sulfoxide Reductase. Drosophila methionine sulfoxide reductase B (MSR-B)[1] with an N-terminal His tag was expressed in *E. coli* using the pET28_MSRB expression construct (*28*) and purified using a His Bind column (Merck KGaA, Darmstadt, Germany). MSR-B was included at the concentrations indicated, and 5 mM DTT was included as a reducing agent in lieu of endogenous thioredoxin (*29*). Calcineurin to be used in phosphatase assays was incubated at 0.1 μM with 1 μM MSR-B (10× with respect to

[1] Abbreviations: DTT, dithiothreitol; pNPP, *p*-nitrophenol phosphate; Fl-CaM, fluorescent calmodulin; Fl-FKBP-12, fluorescent FK506-binding protein; AUC_{os}, area under the curve of the oxidized form; MSR-B, methionine sulfoxide reductase B; SASA, solvent accessible surface area.

calcineurin). Calcineurin and Fl-CaM used in the fluorescence polarization assay were incubated at 0−20 nM calcineurin with MSR-B added to 0.1 μM (5× or greater). Calcineurin to be used in the peptide mapping assay was incubated at 2.5 μM calcineurin with 0.25, 2.5, or 5 μM MSR-B (0.1, 1, or 2×).

Phosphatase Assay. Calcineurin-catalyzed hydrolysis of *p*-nitrophenyl phosphate (pNPP) was assessed using a colorimetric assay. Calcineurin (25 nM) was incubated with 2.5 mM pNPP and calmodulin, in reaction buffer containing 40 mM Tris (pH 8.0), 100 mM KCl, 0.1 mM DTT, 5 mM ascorbate, 100 units/mL superoxide dismutase, 20 μg/mL catalase, 6 mM $MgCl_2$, 1 mM EGTA, and sufficient $CaCl_2$ to yield 3 μM Ca^{2+}_{free}. The reaction mixture was incubated at 37 °C for 2 h, and *p*-nitrophenyl hydroxide formation was assessed as the absorbance at 405 nm.

Fluorescence Anisotropy. Changes in the rotational freedom of fluorescent probes were measured using a Polarion microplate fluorometer (Tecan U.S. Inc., Research Triangle Park, NC) or a PanVera Beacon 2000 variable-temperature fluorescence polarization system (PanVera Corp., Madison, WI). The assay conditions included 40 mM Tris (pH 7.5), 100 mM KCl, 6 mM $MgCl_2$, 0.1 mg/mL bovine serum albumin, 1% NP-40, and 3 μM added $CaCl_2$. Each sample was measured three times. FKBP-12 was labeled with fluorescein 5-maleimide (Pierce Biotechnology Inc., Rockford, IL) by incubation with a 25-fold molar excess of fluorescein 5-maleimide for 2 h at room temperature, followed by quenching with excess ethanolamine, and purification by gel filtration. The concentration of labeled FKBP-12 (Fl-FKBP-12) was estimated using an ϵ_{497} of 78000 cm^{-1} M^{-1}. Calmodulin-I6 which has been engineered to contain a Cys three residues from the C-terminus (*30*) was labeled with Alexafluor-C_5-maleimide (Molecular Probes, Inc., Eugene, OR) as follows. Calmodulin-I6 was incubated with a 25-fold molar excess of Alexafluor-C_5-maleimide for 2 h at room temperature, and unligated Alexafluor-C_5-maleimide was quenched with excess ethanolamine and separated from labeled calmodulin-I6 by gel filtration. The concentration of labeled calmodulin-I6 (Fl-CaM) was estimated using an ϵ_{497} of 78000 cm^{-1} M^{-1}.

The lowest concentration of Fl-CaM giving reliable fluorescence polarization measurements was 0.2 nM. Because the K_d for binding of calmodulin to calcineurin may be as low as 28 pM (*31*), fluorescence polarization cannot be used to measure the dissociation constant, and results are reported as EC_{50} values. The fluorescence intensity of Fl-CaM increased by up to 25% following calcineurin binding. The following equation was used to correct anisotropy values for intensity: $F_B = (A - A_F)/[(A_B - A)(Q_B/Q_F) + A - A_F]$ (*32*), where F_B represents the fraction of Fl-CaM bound to calcineurin, A, A_F, and A_B represent the measured anisotropy, the anisotropy of free Fl-CaM, and the anisotropy of fully bound Fl-CaM, respectively, and Q_B and Q_F represent the intensity of fluorescence from bound and free Fl-CaM, respectively. The fluorescence intensity of Fl-FKBP-12 was unchanged under all conditions that were tested.

Circular Dichroism Spectroscopy. Absorption of circularly polarized light was measured using an Applied Photophysics Chirascan high-performance circular dichroism spectrometer (Applied Photophysics, Surrey, U.K.). Measurements were taken in 2.5 mM HEPES (pH 7.6) at 25 °C. The calcineurin peptide was quantified using a molecular mass of 2812.5 Da, and calmodulin was quantified using an ϵ_{276} of 3300 cm^{-1} M^{-1}.

Peptide Mapping. Calcineurin was reduced by incubation with 4 mM dithiothreitol (DTT) for 30 min at 60 °C and alkylated by incubation with 10 mM iodoacetamide at room temperature for 1 h. Excess iodoacetamide was inactivated by addition of 4 mM DTT and incubation at room temperature for 30 min. Digestion was carried out by incubation with LysC (Roche Diagnostics) at a LysC:calcineurin concentration ratio of 1:10 (w/w) overnight at 37 °C. Peptides released from 1.5 μg of calcineurin were trapped and desalted in line with a peptide captrap (Michrom Bioresources Inc., Auburn, CA) using a Paradigm MS4 HPLC system (Michrom) and loaded onto a 0.2 mm × 5 mm C_{18}AQ column (3 μm, 200 Å, Michrom) at a flow rate of 5 μL/min. Elution directly into a ThermoFinnigan LTQ-XL mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with an Advance source (Michrom) was carried out with a gradient from 5% solution B (5% H_2O, 0.1% formic acid, and 95% acetonitrile) and 95% solution A (2% acetonitrile, 0.1% formic acid, and 98% H_2O) to 45% solution B over 30 min. For quantitation of the calcineurin peptide, 500 pg was loaded onto the trap and analyzed by the same method as the calcineurin digest. The LTQ was tuned to maximize the signal of angiotensin and set to alternate between MS and MS^2 scans. The extracted ion chromatogram of the parent masses for peptides of interest was analyzed using QualBrowser (Thermo Fisher Scientific). Peak identities were confirmed by MS^2 spectra, and the presence of oxidized Met was confirmed by the neutral loss of the 64 amu sulfonium ion. Peaks were selected by hand and background-subtracted, and the proportion of the peptide in native form was calculated as $[AUC_{nat}/(AUC_{ox} + AUC_{nat})]$. The oxidations of Met_{483} and Met_{490} in the Gly_{475}−Lys_{501} fragment could not be quantified individually because the two singly oxidized forms coeluted. The rate of oxidation of these residues is, therefore, described as the average of the two, calculated as $[(AUC_{nat} + 0.5 \times AUC_{1MetSO})/(AUC_{nat} + AUC_{1MetSO} + AUC_{2MetSO})]$, where 1MetSO refers to the two singly oxidized forms of the peptide that coelute and 2MetSO refers to the peptide with two methionine sulfoxides. The Val_{361}−Lys_{393} fragment, containing Cys_{372} and Met_{364}, was not quantified because of interference of Cys oxidation with sulfonic acid in that peptide. In all other peptides, the Cys residues were found only in the alkylated form, indicating that they were not irreversibly oxidized by the exposure to H_2O_2. Peaks were considered free from interference caused by coeluting peptides if their area was greater than 70% of the area under the curve of the base peak chromatogram over the same time.

Because of the abundance of Lys and Arg residues in the calmodulin-binding domain, trypsin was not useful for analysis of calcineurin Met_{406}. *Staphylococcus aureus* V8 protease theoretically allows analysis of nine of the 14 calcineurin-A Met residues, including Met_{406}, but was found to result in poor sequence coverage of the catalytic domain. Therefore, LysC was chosen for this analysis to balance analysis of the calmodulin-binding domain and overall sequence coverage.

Solvent Accessible Surface Area Calculation. The accessible areas of the Met sulfur atoms were calculated using

Surface Racer 3.0 (*33*) using a 1.4 Å sphere to probe PDB entry 1AUI.

Crystal Structure Visualization. The image of calmodulin binding to the calmodulin-binding domain of calcineurin was generated from PDB entry 2F2O using RasMol version 2.6 (*34*).

Data Analysis. Binding and phosphatase activity were analyzed using Prism (GraphPad, San Diego, CA) with the sigmoidal dose–response equation allowing for variable slope: $Y = Y_{min} + (Y_{max} - Y_{min})/[1 + 10^{(\log EC_{50} - X)} \times \text{Hill slope}]$, where X is the logarithm of the concentration of the independent variable and Y is the response which starts at Y_{min} and goes to Y_{max} with a sigmoid shape. EC_{50}, Hill coefficient, and curve maximum and minimum values were considered to be significantly different when their 95% confidence ranges did not overlap.

Pseudo-first-order reaction rate constants for Met oxidation were calculated on the basis of the equation $d(A)/dt = -k'(A)$, where A is the proportion of a fragment in the native form and $-k'$ was estimated as the slope of a plot of $\ln(A)$ versus time by linear regression. Second-order rate constants were determined from the slope of a plot of pseudo-first-order rate constants versus H_2O_2 concentration following oxidation with 1, 12, or 24 mM H_2O_2.

RESULTS

Met_{406} in Calcineurin-A Is Sensitive to Oxidation. The calmodulin-binding domain of calcineurin belongs to the 1-14 motif class of calmodulin-binding domains. The numbers in this nomenclature refer to the placement of bulky hydrophobic residues (Ile_{396} and Val_{409}) that interact with the two lobes of calmodulin. Met_{406} in the calmodulin-binding domain is one turn of the α-helix from Val_{409} and interacts with the hydrophobic pocket of the N-terminal lobe of calmodulin when bound (Figure 1) (*20*). The residue is also close to the hydrophobic residues Met_{72}, Ala_{15}, and Leu_{39} (3.5, 3.9, and 4.7 Å, respectively) in calmodulin, suggesting that its conversion to the more hydrophilic methionine sulfoxide would disrupt calmodulin binding and the activation of calcineurin.

To determine if Met_{406} oxidation contributes to the oxidant-dependent calcineurin inactivation, we examined the sensitivity of this and other Met residues in calcineurin to oxidation by H_2O_2. The abundances of oxidized and native LysC fragments from calcineurin were estimated using a peptide mapping procedure in which peak areas of individual peptides in extracted ion chromatograms were determined.

Of the 19 Met residues in the A and B subunits of calcineurin, the degree of oxidation for five of them could be quantified unambiguously and two of them could be quantified with some interference from coeluting peptides. Representative extracted ion chromatograms showing the LysC fragment Met_{406}–Lys_{424} in the +3 charge state in both the native (m/z 732.7 ± 0.5) and oxidized (m/z 738.1 ± 0.5) forms are displayed in Figure 2. In the absence of any treatment, 92% of Met_{406} was in the native form while 8% was detected as MetSO (Figure 2A). Following treatment with 12 mM H_2O_2 for 4 h and exposure to DTT alone, 75% of Met_{406} was detected as MetSO (Figure 2B); 49% of the oxidation was reversed by incubation with DTT and MSR-B (Figure 2C). The proportion of peptide in the native form was determined for all quantifiable Met-containing peptides as a function of the time of exposure to 12 mM H_2O_2 and is shown in Figure 3.

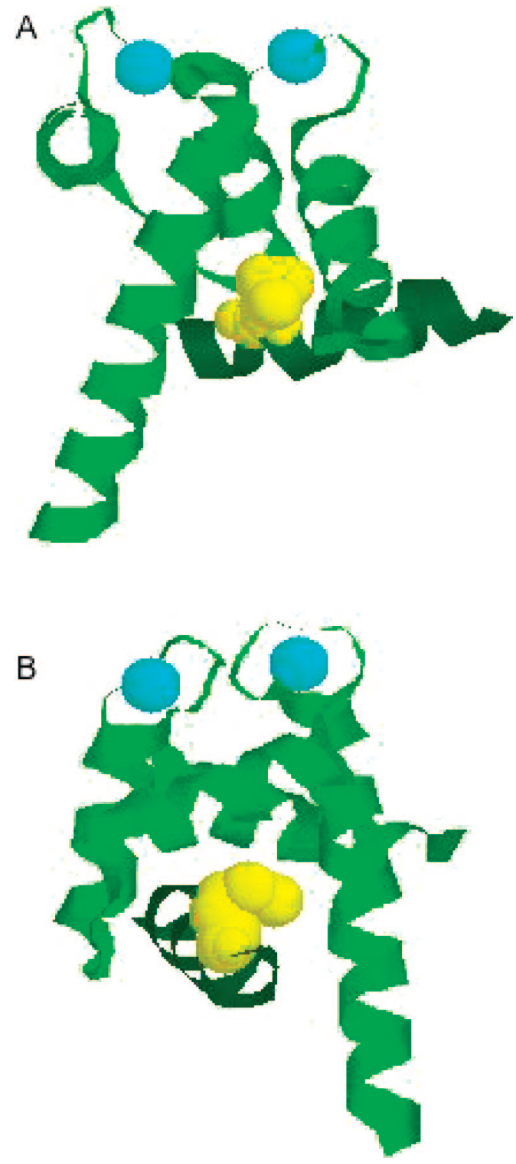

FIGURE 1: Molecular representation of the interacting region of a fusion protein consisting of calmodulin and the calcineurin calmodulin-binding domain (*20*) (PDB entry 2F2O). Amino acids 4–85 of calmodulin are colored light green, and the calcineurin calmodulin-binding peptide (amino acids 161–175) is colored dark green. Space filling representations for Met_{171}, corresponding to Met_{406} in calcineurin, and calcium ions are colored yellow and cyan, respectively.

Data for the ratio of native to oxidized peptide with respect to time of H_2O_2 exposure were used to calculate pseudo-first-order reaction rate constants for oxidation of the individual Met residues. The experiment was repeated with several different concentrations of H_2O_2, and the slope of a plot of pseudo-first-order rate constants versus H_2O_2 concentration was taken as the bimolecular rate constant for Met oxidation (Tables 1 and 2). The same procedure was carried out with the synthetic calcineurin peptide representing the calmodulin-binding domain to determine the oxidation rate of a Met residue with solvent access unrestricted by protein tertiary structure.

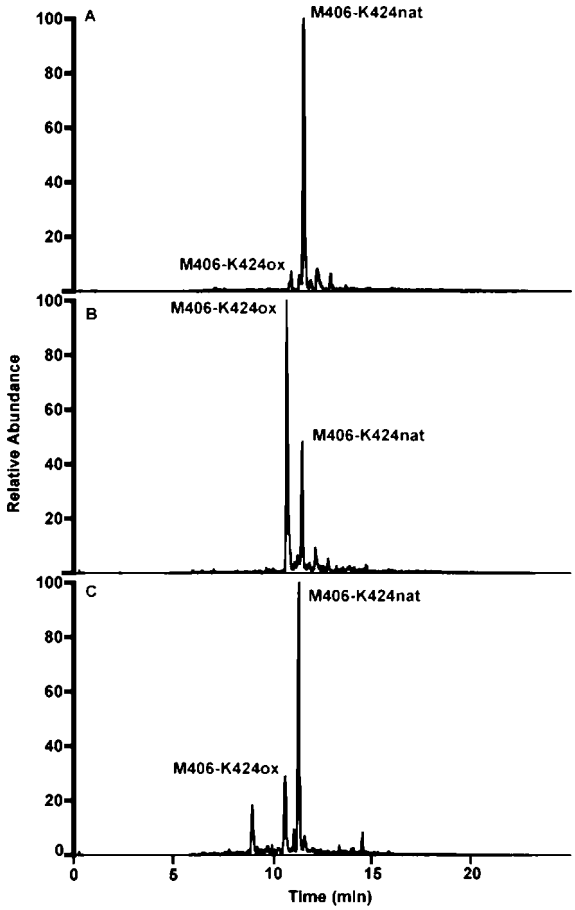

FIGURE 2: Representative extracted ion chromatograms showing the intensity of the *m*/*z* ranges of 732.7 ± 0.5 and 738.2 ± 0.5 used to quantify the calcineurin fragment Met_{406}−Lys_{424} in the native and oxidized states. Spectra for peptides from calcineurin that was untreated (A), oxidized with 12 mM H_2O_2 for 4 h (B), or oxidized and then reduced with 2× MSR-B (C) are shown.

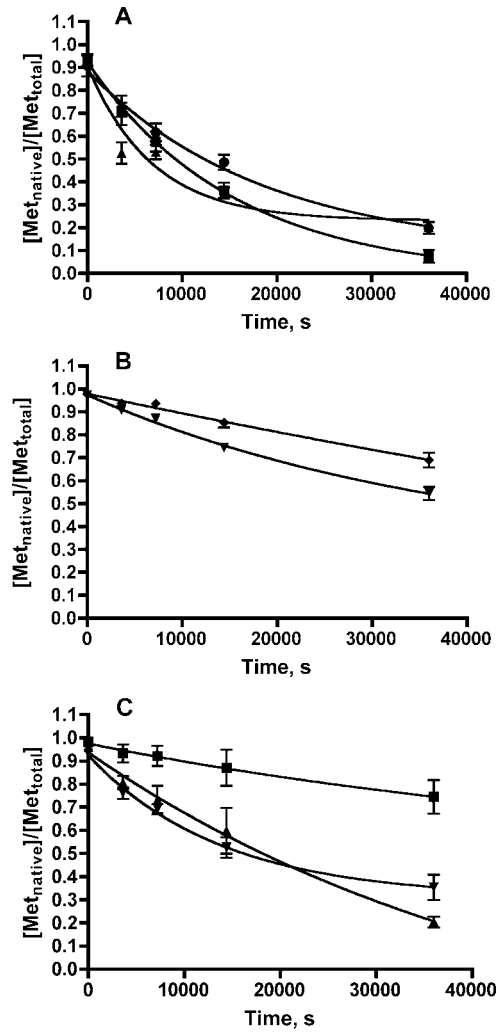

FIGURE 3: Calcineurin peptides remaining unoxidized following treatment with 12 mM H_2O_2 for the indicated times are shown as a fraction of the total peptide. Data for fragments containing Met_{406} (▲), Met_{431} (■), and Met_{483}/Met_{490} (●) in the regulatory region of calcineurin-A are shown in panel A, fragments containing Met_{99} (▼) and Met_{227} (◆) in the catalytic domain in panel B, and fragments containing Met_{11} (▲), Met_{44} (▼), and Met_{101} (■) in calcineurin-B in panel C. All data are the mean of two experiments, each analyzed in duplicate, ± the standard error of the mean.

Met residues in the regulatory region of calcineurin-A, which encompasses amino acids 345−521, are more sensitive to oxidation than Met residues in the catalytic domain of the enzyme. The rates for oxidation of Met_{406} and Met_{431} in the regulatory region of calcineurin-A approach the rate of Met oxidation in the synthetic calcineurin peptide, indicating a high degree of solvent exposure for these residues. The average oxidation rates for Met_{483} and Met_{490} are lower than those measured for other residues in the regulatory region, possibly due to the interaction of one or both with the substrate binding cleft of the catalytic domain of calcineurin resulting in low solvent accessibility (*10*). Oxidation rates for Met_{99} and Met_{227} in the catalytic region of calcineurin-A (from amino acid 1 to 345) are lower than the rates for Met in the regulatory region, making Met_{99} and Met_{227} less likely targets for oxidative modulation of calcineurin.

We determined the oxidation rate for three Met residues in calcineurin-B: residues 11, 44, and 101. All of these residues have oxidation rates that are less than 60% of the rate for the calcineurin peptide (2.8×10^{-3}, 2.8×10^{-3}, and 0.55×10^{-3} M^{-1} s^{-1}, respectively), indicating that they all are less susceptible to oxidation than Met residues in the regulatory region of calcineurin-A. Although they all have similar solvent exposures (1.9, 1.01, and 2.49 Å^2, respectively), the oxidation rate for Met_{101} of calcineurin-B at 0.55×10^{-3} M^{-1} s^{-1} is low in comparison with the rates for Met_{11} and Met_{44}, which are both 2.8×10^{-3} M^{-1} s^{-1}. This apparent discrepancy may be a reflection of molecular dynamics or structural information that is not captured in the surface area calculation based on the crystal structure of calcineurin.

Reduction of Exposed MetSO Residues. We used the peptide mapping procedure to measure the effectiveness of MSR-B in reversing the oxidation of Met in calcineurin. H_2O_2 oxidation results in an equal mixture of the *R* and *S* stereoisomers of MetSO. MSR-B can only reduce Met(R)SO and was expected to reduce up to 50% of total MetSO. Figure 4 shows the fraction of Met-containing, LysC-generated peptides that are in the unoxidized state after treatment with H_2O_2 and reversal of the oxidation by MSR-B. Peptides released from untreated calcineurin, calcineurin oxidized by treatment with 12 mM H_2O_2 for 4 h, calcineurin incubated with 5 mM DTT for 2 h after oxidation, and calcineurin incubated with DTT and 0.1×, 1×, or 2× MSR-B after oxidation are shown. Met residues 99 and 227 of calcineurin-A as well as Met_{101} of calcineurin-B all have low susceptibility to oxidation and were not affected by any of

Table 1: Oxidation Rates of Methionine Residues in Calcineurin-A[a]

Met residue	LysC fragment	SASA (Å^2)	k_{ox} ($\times 10^{-3}$ M^{-1} s^{-1})[c]	% of peptide k_{ox}
CN peptide	−	43[d]	4.9 ± 0.29	100
51	$A_{48}-K_{52}$	11.3	−	−
99	$N_{77}-K_{100}$	0.658	2.2 ± 0.23[e]	45
179, 191	$Y_{170}-K_{214}$	0, 0	−	−
227	$E_{220}-K_{243}$	0	0.86 ± 0.058	18
290	$T_{244}-K_{293}$	2.17	−	−
329, 347	$Y_{324}-K_{360}$	0, 7.06	−	−
364	$V_{361}-K_{393}$	0	−	−
406	$M_{406}-K_{424}$	n/a[b]	4.4 ± 0.38	91
431	$G_{425}-K_{441}$	n/a[b]	4.6 ± 0.60[e]	95
483/490	$G_{475}-K_{501}$	0, n/a[b]	2.6 ± 0.19[f]	54

[a] Residues reported here are based on UniProt entry Q08209. [b] There was no structural information regarding that residue in PDB entry 1AUI. [c] Rate constants are reported with the standard error of the linear regression of pseudo-first-order rate constants vs hydrogen peroxide concentration. [d] The SASA of the sulfur atom in the Met in the CN peptide is approximated by the reported value for the SASA in Gly-Met-Gly as determined using a 1.4 Å sphere (*48*). [e] Peptides that coeluted with other peptides, which may affect the accuracy of the results. [f] Met_{483} and Met_{490} exist in the same LysC fragment; the k_{ox} reported is the average of the two.

Table 2: Oxidation Rates of Met Residues in Calcineurin-B[a]

Met residue	LysC fragment	SASA (Å^2)	k_{ox} ($\times 10^{-3}$ M^{-1} s^{-1})[b]	% of peptide k_{ox}
11	G_2-K_{21}	1.90	2.8 ± 0.35	57
44	$K_{29}-K_{73}$[c]	1.01	2.8 ± 0.20	59
101	$L_{92}-K_{103}$	2.49	0.55 ± 0.091	11
118, 119	$M_{118}-K_{125}$	2.72, 0	−	−
166	$M_{166}-V_{170}$	0	−	−

[a] Residues reported here are based on UniProt entry P63098. [b] Rate constants are reported with the standard error of the linear regression of pseudo-first-order rate constants vs hydrogen peroxide concentration. [c] Fragment $Lys_{29}-Lys_{73}$ results from cleavage between a pair of lysine residues (Lys_{28} and Lys_{29}). No other missed cleavages were detected in methionine-containing peptides.

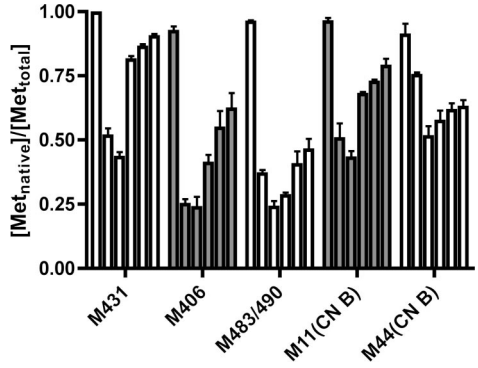

FIGURE 4: Oxidation and reversal of the oxidation of Met in calcineurin. The fractions of nonoxidized Met in LysC fragments containing Met_{431}, Met_{406}, and Met_{483}/Met_{490} from calcineurin-A and Met_{11} and Met_{44} from calcineurin-B are shown. Each series of five bars represents the following treatments from left to right: untreated, oxidized with 12 mM H_2O_2 for 4 h, oxidized and then reduced with 5 mM DTT only, and oxidized and then reduced with DTT and 0.1×, 1×, or 2× MSR-B. All data are the mean of two experiments, each analyzed in duplicate, ± the standard error of the mean.

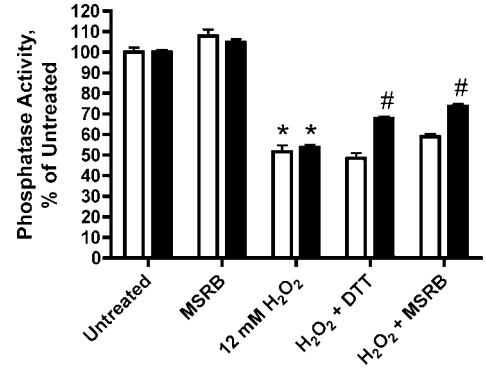

FIGURE 5: Phosphatase activity of wild-type (white bars) and $M_{406}L$ (black bars) calcineurin with saturating Ca^{2+} and calmodulin levels as a percentage of activity in an untreated sample. For all values, $n = 3$, ± the standard error of the mean. Asterisks indicate a significant difference from the untreated value, and number signs indicate a significant difference from samples treated only with 12 mM H_2O_2 as assessed by 95% confidence intervals that do not overlap.

the treatments (data not shown). Met_{431} and Met_{406}, the residues with the highest susceptibility to oxidation in calcineurin-A, also exhibited the greatest reversal of oxidation by MSR-B with 80 and 49% of MetSO being reduced, respectively, while 57% of oxidized Met_{11} in calcineurin-B was reduced by the MSR-B treatment.

Calcineurin Activity Is Insensitive to Met Oxidation. Calcineurin is sensitive to oxidative inactivation by H_2O_2 (*12, 15, 16*). To examine the possible involvement of Met_{406} oxidation in modulating calcineurin activation by calmodulin, calcineurin mutants in which Met_{406} was changed to Leu ($M_{406}L$) or Ala ($M_{406}A$) were created. These conservative mutations eliminate the possibility of oxidation in the calmodulin-binding domain. The calcineurin $M_{406}L$ enzyme has 65.5% of the wild-type enzyme activity, whereas calcineurin $M_{406}A$ has only 36.0% of the activity measured with the wild-type enzyme. Therefore, all further experiments examining effects of Met_{406} oxidation were conducted with the $M_{406}L$ mutant as a control. Calcineurin $M_{406}L$ had EC_{50} values for calmodulin binding and for activation by calmodulin that were not significantly different from the wild-type values (Figures 6 and 7). A 4 h incubation of the wild-type enzyme or of calcineurin $M_{406}L$ with 12 mM H_2O_2 decreased the phosphatase activity of wild-type or $M_{406}L$ calcineurin to 51.6 or 53.7% of that of the untreated enzyme, respectively (Figure 5). Incubation with 1 mM H_2O_2 caused a decrease in phosphatase activity of wild-type or $M_{406}L$ calcineurin

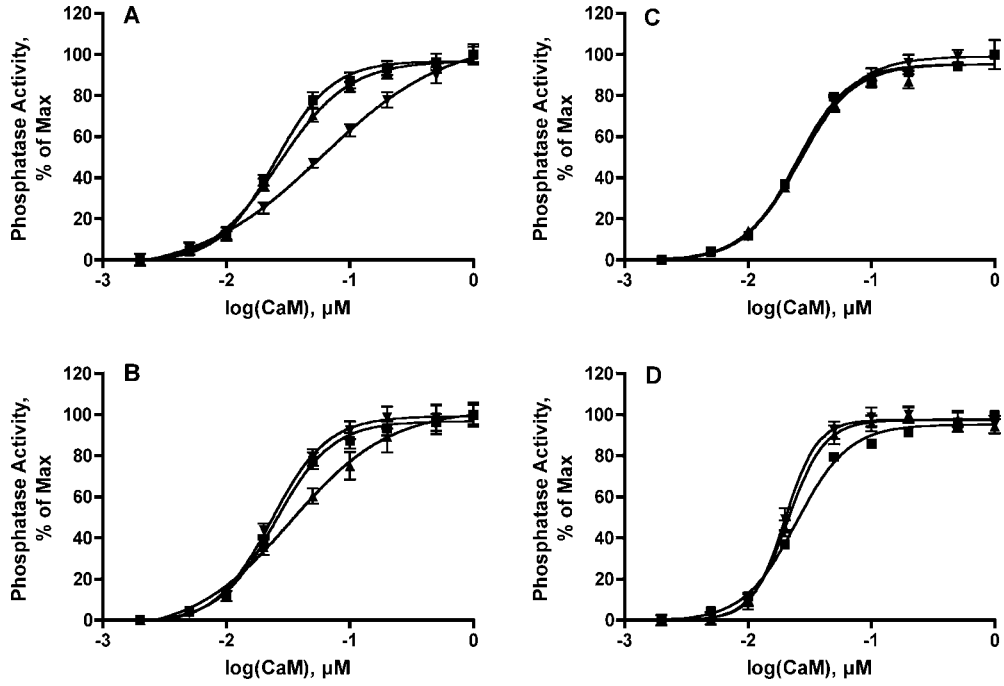

FIGURE 6: Activation profiles for wild-type (A and B) and $M_{406}L$ (C and D) calcineurin as a function of calmodulin concentration. Data are presented as the percentage of the maximum phosphatase activity for each treatment. Panels A and C show the activity of calcineurin that was untreated (■), oxidized with 1 mM H_2O_2 (▲), or oxidized with 12 mM H_2O_2 (▼). Panels B and D show the activity of calcineurin that was reduced with DTT alone (■), oxidized with 12 mM H_2O_2 and reduced with DTT (▲), or oxidized and reduced with DTT and MSR-B (▼). For all data, $n = 3$, ± the standard error of the mean.

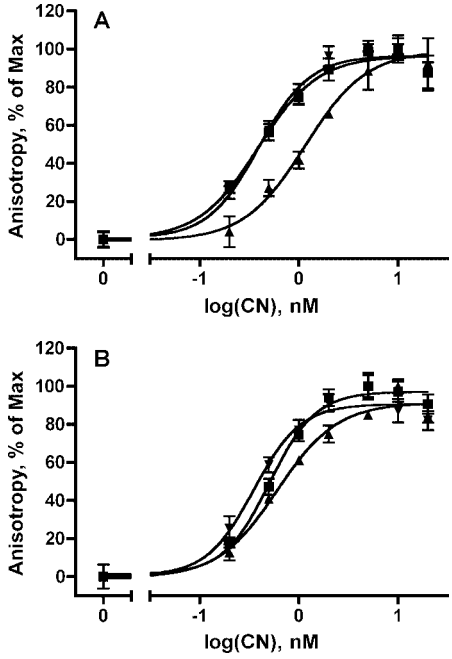

FIGURE 7: Fl-CaM binding to wild-type (A) or $M_{406}L$ (B) calcineurin as determined by changes in fluorescence anisotropy displayed as the percentage of the maximum change in fluorescence anisotropy. The indicated concentrations of enzyme that was untreated (■), oxidized (▲), or oxidized and reduced with DTT and MSR-B (▼) and then incubated with 0.2 nM Fl-CaM prior to measurement of the fluorescence polarization. For all values, $n = 3$, ± the standard error of the mean.

to 72.1 or 71.5% of the activity of the untreated enzyme, respectively (data not shown).

To determine if reversal of the Met oxidation could restore enzyme activity that was lost due to the incubation with H_2O_2, samples that had been treated with 12 mM H_2O_2 for 4 h were incubated with DTT or DTT with MSR-B to catalyze the reduction of MetSO to Met. Treatment with DTT or DTT with MSR-B produced a modest increase in the activity of the H_2O_2-treated $M_{406}L$ calcineurin to 67.7 or 73.8% of the untreated value, respectively, but had no effect on H_2O_2-treated wild-type calcineurin phosphatase activity (Figure 5).

The identical decrease in the maximal activity of the wild-type and the $M_{406}L$ calcineurin enzymes after exposure to H_2O_2 indicates that the inhibition of maximal phosphatase activity caused by the incubation with H_2O_2 is not mediated by Met oxidation in the calmodulin-binding domain. Indeed, the minimal reversal produced by the incubation with MSR-B shows that any contribution of Met oxidation to the loss of calcineurin phosphatase activity caused by H_2O_2 must be modest. Since Met_{406} is oxidized under these conditions (Figure 2A,B), it is likely that any effect its oxidation has on maximum phosphatase activity is masked by concurrent oxidation of residues in the catalytic domain. The reason for the increased response of $M_{406}L$ calcineurin to reduction with DTT or DTT with MSR-B in comparison to the wild-type enzyme is unclear.

Met Oxidation in Calcineurin Modulates Calmodulin-Dependent Activation. Binding of Ca^{2+} and calmodulin to target peptides and proteins is a highly cooperative process resulting in a sharp concentration dependence for the activation of calcineurin by Ca^{2+} and calmodulin (*18*). Positive cooperativity is shown in Figure 6A, where stimulation from 10 to 90% of the maximal calcineurin phosphatase activity occurs over a change in calmodulin concentration of less than 1 log unit, resulting in a calculated Hill slope of 1.88. Oxidation of calcineurin with 12 mM H_2O_2 produced a decrease in the Hill slope to 0.86, indicating a loss of cooperativity in binding of calmodulin to calcineurin. Oxidation with 1 mM hydrogen peroxide had no effect on the

calmodulin activation profile (Figure 6A). The positive cooperativity in calmodulin binding was restored following incubation of the oxidized enzyme with DTT and MSR-B. The calculated Hill slope for this enzyme is 1.92 (Figure 6B), indicating that Met oxidation is responsible for the loss of cooperativity in CaM-dependent calcineurin activation. In contrast, calmodulin stimulation of $M_{406}L$ calcineurin was not affected by treatment with H_2O_2 or reduction of the oxidized enzyme with MSR-B (Figure 6C,D), indicating that Met_{406} is solely responsible for the loss of cooperativity in CaM binding when calcineurin is oxidized.

Met_{406} Oxidation Decreases the Affinity of Calcineurin for Calmodulin. The residues that contribute to calcineurin−calmodulin binding are known on the basis of the crystal structure of a fusion between calmodulin and its binding domain in calcineurin (*20*) as well as by analogy to the interaction between smooth muscle myosin light chain kinase and calmodulin (*35*). Met_{406} is in the calmodulin-binding domain of calcineurin and is directly involved in binding through hydrophobic interaction with calmodulin. A fluorescence anisotropy binding assay was used to determine the affinity of calmodulin for native calcineurin and for calcineurin after oxidation of Met_{406}. Incubation of wild-type calcineurin with 12 mM H_2O_2 is expected to convert 65% of Met_{406} to MetSO (Figure 3A). This treatment caused an increase in the EC_{50} for Fl-CaM binding from 0.36 to 1.1 nM (Figure 7A). Treatment with 1 mM hydrogen peroxide had no effect on calmodulin binding (data not shown). The affinity of calcineurin for Fl-CaM was fully restored to an EC_{50} of 0.35 nM following incubation of the oxidized enzyme with MSR-B and DTT. The affinity of $M_{406}L$ calcineurin for Fl-CaM was not affected by treatment with H_2O_2 as the EC_{50} values were 0.47 and 0.55 nM for the untreated and H_2O_2-treated enzyme, respectively (Figure 7B). While neither oxidized nor reduced $M_{406}L$ calcineurin had calmodulin affinities that were different from those of the untreated enzyme, treatment of oxidized $M_{406}L$ calcineurin with MSR-B did decrease the EC_{50} for Fl-CaM binding from 0.55 to 0.27 nM (Figure 7B), indicating that Met at sites other than at Met_{406} may contribute to the calcineurin−calmodulin interaction.

Calcineurin Oxidation Inhibits FK506−Fl-FKBP-12 Binding in the Presence of Calmodulin. Calmodulin increases the affinity of immunosuppressant−immunophilin complexes for calcineurin (*36*). Affinities of the FK506−Fl-FKBP-12 complex for native and for oxidized calcineurin were measured using a fluorescence anisotropy assay (Figure 8). Fl-FKBP-12 does not bind to calcineurin in the absence of FK506 (data not shown). In the absence of calmodulin, calcineurin oxidation had no effect on the affinity of the FK506−Fl-FKBP-12 complex for calcineurin. Addition of calmodulin in 10% excess of calcineurin produced the expected increase in affinity of the enzyme for the FK506−Fl-FKBP-12 complex as the K_d decreased 5.7-fold from 0.57 to 0.099 μM. Calmodulin was less effective in stimulating binding of the FK506−Fl-FKBP-12 complex to oxidized calcineurin as the K_d decreased only 3.8-fold from 0.73 to 0.19 μM. These data are consistent with an oxidation-dependent decrease in calmodulin affinity for calcineurin and a subsequent decrease in the affinity of calcineurin for the FK506−Fl-FKBP-12 complex.

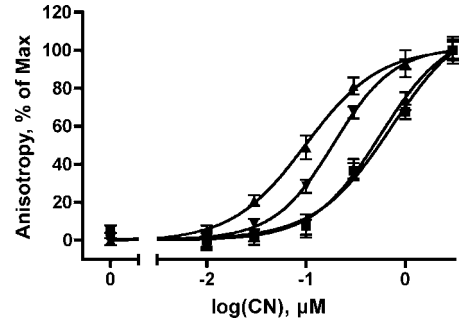

FIGURE 8: Changes in the fluorescence anisotropy induced by binding of the FK506−Fl-FKBP-12 complex to untreated (◆) or oxidized calcineurin (■), in the absence of calmodulin, were measured and are shown as a function of calcineurin concentration. Calmodulin was then added to the same samples, and binding to untreated (▲) and oxidized (▼) calcineurin was assessed. For all values, $n = 3$, ± the standard error of the mean.

Circular Dichroism of Native and Oxidized Calmodulin-Binding Peptides. Met oxidation in peptides can promote conformational "switching" from a preference for an α-helical to a β-sheet structure (*37*). Calmodulin-binding domains generally assume an α-helical structure when they are interacting with calmodulin (*20, 35*), and chemical modifications which disrupt α-helical structures are expected to inhibit calmodulin−protein interactions. To determine if Met oxidation-induced changes in the calmodulin-binding domain secondary structure could disrupt calmodulin binding, we measured the α-helicity of the calmodulin-binding domain peptide from calcineurin (ARKEVIRNKIRAIGKMARVFSVLR). Solutions of 25 μM native calcineurin peptide or calcineurin peptide oxidized by exposure to 12 mM H_2O_2 for 4 h each had the circular dichroic characteristics of a random coil. An α-helical structure, as measured by minima in the circular dichroic spectra at 208 and 222 nm, could be induced in both the native and oxidized peptides by adding trifluoroethanol to a final concentration of up to 40%. The TFE concentration dependence for the induction of the α-helix structure was not changed by Met oxidation (data not shown). Both the native and oxidized peptides induced an increase in the α-helicity of a solution in which they were equimolar with calmodulin, and in those solutions, addition of Ca^{2+} caused a further increase in α-helicity. However, the degree of α-helicity induced was not affected by the oxidation state of the peptide in solution with calmodulin. Therefore, the secondary structure of the CaM-binding domain peptide appears to be insensitive to the oxidation state of Met within the resolution of this technique.

DISCUSSION

The data presented here indicate that Met_{406} in the calmodulin-binding domain of calcineurin is sensitive to oxidation and that oxidation of Met_{406} decreases the affinity of the enzyme for calmodulin and is thus able to modulate the activation of calcineurin by Ca^{2+} and calmodulin. Following a treatment with H_2O_2 that increased the percentage of Met_{406} in the sulfoxide form from 8 to 75% (Figure 2A,B), the positive cooperativity for activation by Ca^{2+} and calmodulin was abolished, and affinity for calmodulin was decreased 3-fold. Disruption of the calmodulin−calcineurin interaction was further demonstrated by a weakened ability of calmodulin to stimulate binding of the FK506−Fl-FKBP-

12 complex to calcineurin. The inhibition of calcineurin phosphatase activity following exposure to H_2O_2 is not mediated by Met oxidation. This is shown by the following observations: the decrease in phosphatase activity is independent of the introduction of nonoxidizable mutation at Met_{406}; the enzyme inhibition cannot be reversed by MSR-B; enzyme inhibition occurs at 1 mM H_2O_2, a concentration which was shown to have a minimal effect on the calcineurin Met oxidation state.

Calmodulin binding to calcineurin and the displacement of the autoinhibitory domain can still occur if Met_{406} is oxidized. This is shown by the data presented in Figure 5 which shows that under conditions that produce 65% oxidation of Met_{406}, the degree to which the enzyme is inhibited is the same for the native enzyme and the $M_{406}L$ control. It is further supported by the finding that the calmodulin-induced increase in affinity of the FK506−Fl-FKBP-12 complex for calcineurin was decreased by only 44% for oxidized calcineurin. In both cases, if calmodulin binding were abolished by Met_{406} oxidation, the calmodulin-dependent effect would be expected to be decreased by 65% in comparison to the $M_{406}L$ control. The ability of calmodulin to bind to oxidized calmodulin-binding domains from calcineurin is confirmed by the circular dichroism data. Changes in circular dichroism upon addition of an equimolar concentration of calcineurin peptide to a solution of calmodulin indicate that there is an increase in the α-helicity of the calmodulin solution regardless of the oxidation state of the peptide. These data indicate that either the secondary structure of the peptide is not changed by the Met oxidation or binding to calmodulin stabilizes the α-helical conformation in the peptide to a much greater extent than Met oxidation inhibits binding. Although calmodulin can bind to oxidized calcineurin, the characteristics of calmodulin activation are altered as shown in Figure 6. The positive cooperativity that is characteristic of calmodulin activation of its targets is abolished by oxidation of Met_{406}. This eliminates the "all or nothing" response of calcineurin to a small changes in Ca^{2+} concentration that is essential for calcineurin function at the cellular concentrations of Ca^{2+} and calmodulin (*18*).

H_2O_2 has been used as a model oxidant to study several proteins in which Met oxidation is of known physiological (*4, 21*) or pharmacological (*38, 39*) relevance. In the absence of metal-catalyzed oxidation, the most susceptible Met residues are those that have sulfur atom solvent exposure and oxidation rates approaching those of free Met. We have used the Met residue in the calcineurin peptide as a reference point for the Met oxidation rate because it is expected to have unrestricted solvent exposure. The oxidation rate of calcineurin Met_{406} in the calmodulin-binding domain was among the most susceptible of the residues quantified, with a bimolecular rate constant of 4.4×10^{-3} M^{-1} s^{-1}, 91% of the rate for the calcineurin peptide (Table 1). The rate of oxidation by H_2O_2, relative to free Met, is similar to the rates of the most susceptible residues in calmodulin (*17*) and α1-antitrypsin (*21*), both physiologically relevant targets for Met oxidation, validating Met_{406} in the calmodulin-binding domain of calcineurin as a potential target for oxidation in vivo.

Calcineurin-B residues Met_{11}, Met_{44}, and Met_{101} have similar solvent accessible surface areas (1.90, 1.01, and 2.49 $Å^2$, respectively), but different rates of oxidation [2.8, 2.8, and 0.55×10^{-3} M^{-1} s^{-1}, respectively (Table 2)]. This discrepancy is likely a reflection of the limitations of surface area calculations from a static crystal structure that does not take into account molecular dynamics (*4*). It is possible that the N-terminal half of calcineurin-B, which has a lower calcium affinity and a more dynamic role in calcineurin regulation than the C-terminal half (*24*), may be flexible in solution and have a greater exposure to the solvent than suggested by the crystal structure.

As shown in Figure 4, peptides containing Met_{483}/Met_{490} of calcineurin-A and Met_{44} of calcineurin-B demonstrated minimal reduction of MetSO when treated with MSR despite their moderate sensitivity to oxidation. Methionine sulfoxide reductase A activity is very sensitive to the solvent exposure of its substrate (*40*), and it is possible that, although these residues have sufficient solvent exposure for reaction with H_2O_2, they may not be sufficiently exposed for the *Drosophila* MSR-B enzyme used in these studies to fully reduce Met(R)SO in these positions. Met_{406} and Met_{431}, because of their apparent high solvent exposure (Table 1), are expected to have free access to MSR-B. The addition of DTT in the absence of MSR consistently decreases the ratio of native to oxidized Met residues in the peptides that were examined (third bar of each group, Figure 4). This may be due to the formation of the pro-oxidants $O_2^{\bullet -}$ and Fe^{2+} by reduction of O_2 and Fe^{3+}, respectively (*41*). DTT-induced oxidation of Met residues in these peptides has the greatest effect on Met_{483}/Met_{490} of calcineurin-A and Met_{44} of calcineurin-B and may contribute to the relatively modest effect of MSR-B-induced reduction of Met(R)SO in these residues.

Treatment with MSR-B completely reversed the effects of oxidation on the calmodulin affinity and activation profile of calcineurin. The complete reversal was unexpected, as oxidation with H_2O_2 produces equal concentrations of Met(R)SO and Met(S)SO, only one of which, Met(R)SO, is a substrate for MSR-B. In addition, we have shown only 49% reduction of oxidized Met_{406} following incubation with 2× MSR-B. There are two possible explanations for the complete reversal observed in the functional assays. First, it is possible that there is a stereochemical specificity to the inhibition and that only Met(R)SO inhibits binding of calmodulin to calcineurin. If this were the case, then reduction of the *R* isomer by MSR-B would be sufficient to restore calmodulin affinity and the Met(S)SO would have no effect on calmodulin binding or activation. A second possibility is that MSR-B has a low level of activity toward Met(S)SO that is not apparent under the experimental conditions used to generate the data shown in Figure 4. This activity may be undetected in our examination of Met_{406} reduction (Figure 4) because of the lower concentrations of MSR-B used in those experiments. The ratio of 2× MSR-B to substrate was used in these experiments, whereas levels of 5× and 10× MSR-B to substrate were used in the calmodulin binding and phosphatase activity assays. MSR-B activity toward Met(S)SO may also be masked in Figure 4 by Met oxidation, induced by DTT, which counteracts MetSO reduction.

Although total cellular calmodulin concentrations range from 2 to 25 μM (*42*), at high intracellular Ca^{2+} concentrations the amount of free calmodulin available in cells is less than 200 nM (*23*), and calmodulin targets compete for a limiting pool of calmodulin. Due to its high affinity for

calmodulin and slow rate of dissociation (28 pM and 0.0012 s^{-1}, respectively) (*31*), calcineurin is a preferred calmodulin binding partner, and in cells and subcellular regions where it is abundant, as in brain where it makes up 1% of total protein (*43*), calcineurin can monopolize cellular calmodulin. This creates a dual role for calmodulin binding to calcineurin: stimulation of calcineurin phosphatase activity and regulation of other calmodulin-dependent processes through sequestration of available calmodulin. High-affinity calmodulin-binding proteins can modulate the activity of unrelated calmodulin-dependent processes by acting as dominant affecters in cells where they are present at high concentrations (*44*). Several posttranslational modifications have been proposed to modulate calmodulin affinity, including phosphorylation (*44, 45*), disulfide bond formation (*46*), and S-nitrosylation (*47*). Here, we have demonstrated that Met oxidation in the calmodulin-binding domain of calcineurin can reversibly modulate calmodulin affinity and that calcineurin meets the theoretical requirements (*44*) to act as a dominant affecter in calmodulin signaling.

Modulation of calmodulin binding by Met oxidation may apply to other calmodulin-binding proteins, as 60 of 143 calmodulin-binding domains (42%) of the human calmodulin-binding proteins listed in the Calmodulin Target Database (*5*) contain one or more Met residues in their calmodulin-binding domains. Calmodulin-binding domains may be a significant target for Met oxidation, and we are currently developing methodology to measure methionine oxidation in more complex samples so that we can determine the degree of calmodulin-binding domain oxidation in calcineurin as well as in other calmodulin targets in cell culture models or in animal tissue.

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