Calmodulin Oxidation and Methionine Substitutions
Reveal Methionine Residues Critical for Functional Interaction with Ryanodine Receptor-1*

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CaM binds to the skeletal muscle ryanodine receptor Ca2+ release channel (RyR1) with high affinity, and it may act as a Ca2+-sensing subunit of the channel. Apo-CaM increases RyR1 channel activity, but Ca2+-CaM is inhibitory. Here we examine the functional effects of CaM oxidation on RyR1 regulation by both apo-CaM and Ca2+-CaM, as assessed via determinations of [3H]ryanodine and [35S]CaM binding to skeletal muscle sarcoplasmic reticulum vesicles. Oxidation of all nine CaM Met residues abolished functional interactions of CaM with RyR1. Incomplete CaM oxidation, affecting 5–8 Met residues, increased the CaM concentration required to modulate RyR1, having a greater effect on the apo-CaM species. Mutating individual CaM Met residues to Gln demonstrated that Met-109 was required for apo-CaM activation of RyR1 but not for Ca2+-CaM inhibition of the channel. Furthermore, substitution of Gln for Met-124 decreased the apo- and Ca2+-CaM concentrations required to regulate RyR1. These results thus identify Met residues critical for the productive association of CaM with RyR1 channels and suggest that oxidation of CaM may contribute to altered regulation of sarcoplasmic reticulum Ca2+ release during oxidative stress.

Skeletal muscle contraction is initiated by Ca2+ efflux from the sarcoplasmic reticulum (SR) via the SR Ca2+ release channel/ryanodine receptor (RyR1). The homotetrameric RyR1 is the largest known ion channel having a molecular mass of more than 2,000 kDa (1). The N-terminal two-thirds of the channel forms a large cytoplasmic domain to which numerous signaling proteins are anchored, including the FK506 binding protein and calmodulin (CaM) (2, 3). In vitro, the channel is activated by Ca2+ in the μM to mM range and inactivated by μM-mM Ca2+. CaM binding to the channel enhances the sensitivity to both Ca2+ activation and inactivation.

The 148-amino acid Ca2+-binding protein, CaM, is composed of N-terminal and C-terminal globular domains connected by a flexible, central tether. CaM has an unusually high Met content, indeed 9 of the 148 amino acids are Met residues, resulting in an ~6-fold higher Met content than the average protein (4). In vertebrate CaM these Met residues are clustered primarily in the N (residues 36, 51, 71, and 72) and the C (residues 109, 124, 144, and 145) termini. A 9th Met is located in the tether at residue 76. High affinity Ca2+ binding to EF-hand motifs in each of the globular domains induces a structural rearrangement that reveals the Met-rich hydrophobic patches (5). These hydrophobic patches mediate Ca2+-CaM interaction with a large and diverse group of proteins that share little sequence homology (6). A less appreciated aspect of CaM regulation is the ability of Ca2+-free CaM (apo-CaM) to regulate certain targets (7). However, the role of Met residues in apo-CaM interaction with targets is not clear.

Although the high Met content of CaM contributes to effective target binding, the Met residues in the Ca2+-bound form of CaM are surface-exposed and susceptible to oxidation. Oxidation converts Met to Met sulfoxide, a physiologically relevant product (8). Indeed, Met sulfoxide-containing CaM has been isolated from the brains of aged animals (9).

The present study examines the functional effects of CaM oxidation on the regulation of RyR1. To identify which of the individual Met residues are important for the functional interaction of CaM with RyR1, we used site-directed mutagenesis to change each of the nine CaM Met residues to Gln, introducing an oxygen atom at the same position in the side chain as the sulfoxide. Our results define CaM Met residues that are critical for the functional interaction between CaM and RyR1 and suggest that CaM oxidation may contribute to altered regulation of SR Ca2+ release during oxidative stress.

EXPERIMENTAL PROCEDURES

Materials

Pigs were obtained from the University of Minnesota Experimental Farm. Tran35S-labeled Met and Cys were obtained from ICN Radiochemicals (Costa Mesa, CA). [3H]Ryanodine was purchased from PerkinElmer Life Sciences. Unlabeled ryanodine was obtained from Calbiochem. High performance liquid chromatography grade acetonitrile was purchased from Fisher Scientific. C4 ZipTips were from Millipore (Burlington, MA). EPMI 1640 medium was from ICN. Spectrophotometric grade trifluoroacetic acid, AMPPCP, and other reagents were from Sigma.

Received for publication, September 6, 2002, and in revised form, January 30, 2003
Published, JBC Papers in Press, February 13, 2003, DOI 10.1074/jbc.M209180200

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0.1 M NaCl, 5 mM Tris maleate buffer, pH 6.8, and centrifuged for 30 min at 2,600 × g. The supernatant was filtered through gauze and centrifuged for 30 min at 15,000 × g. Pelleted membranes were extracted in 1.1 M KCl, pH 7.0, 3 mM AMP, 100 mM [35S]methionine, and a Ca-EGTA buffer set to give the desired free Ca2+ concentration. After 90 min, SR vesicles were collected on Whatman GF/B filters and washed with 8 ml of ice-cold 100 mM KCl buffer. Estimates of maximal [35S]ryanodine binding capacity of each SR vesicle preparation were determined in medium that in addition contained 500 mM KCl, 6 mM ATP, and 100 μM Ca2+. Non-specific binding was measured in the presence of 20 μM non-radioactive ryanodine. [35S]Ryanodine binding is expressed as a percent of [3H]ryanodine binding.

[35S]Calmodulin Binding to Skeletal Muscle SR Vesicles

Ryanodine selectively binds to the open RyR and therefore provides a useful indicator of channel activity (11). RyR1 is the major CaM-binding protein in heavy SR (13, 14), thus SR vesicle [35S]CaM binding reflects primarily RyR1 CaM binding.

Oxidation of the Calmodulin

Because the thioether group of Met is not protonated at low pH, it can be oxidized selectively under acidic conditions (8). 60 μM calmodulin was incubated in 50 mM Homopipes, pH 5.0, 0.1 M KCl, 2.0 mM MgCl2, 50 mM H2O2 at room temperature for 0.5–24 h. The reaction was stopped by overnight dialysis (molecular mass cutoff = 3,500) at 4°C in distilled water (5 × 1 liter) buffered with 10 mM ammonium bicarbonate, pH 7.7.

Calmodulin Site-directed Mutagenesis, Expression, and Purification

Recombinant rat CaM was expressed in Escherichia coli using the pET-7 vector (16), purified via phenyl-Sepharose chromatography (17), and dialyzed overnight at 4°C against 2 mM HEPS, pH 7.0. CaM concentration was determined with the Micro BCA assay (Pierce) using wild-type CaM as a standard. The concentration of the CaM standard was determined using the published molar extinction coefficient, ε3.029 M C = 3,029 μM−1 cm−1 (18). Glutamine was substituted for each of the Met residues (residues 36, 51, 71, 72, 76, 109, 124, 144, and 145) using QuikChange mutagenesis kits (Stratagene, La Jolla, CA). DNA sequence analysis confirmed the correct generation of each mutant.

[35S]Methionine Incorporation

The biosynthesis of [35S]-labeled CaM was carried out essentially as described previously for [35S]FKBP12 (19). Bacterial growth was initiated in M9 medium containing ampicillin. When the A600 value of the bacteria reached 0.6 the pelleted bacteria were resuspended in RMFI 1640 medium containing ampicillin, 1/40th of the Met and Cys codons were exchanged compared with the regular RMFI 1640 medium, and iso-osmotic-thiogalactopyranoside was added to a final concentration of 1 mM. A 1.4-mCi aliquot of [35S]methionine and [35S]cysteine was then added to the medium and the cultures were incubated for 5–7 h at the same conditions.

Steady-state Fluorescence

The Ca2+-induced increase in tyrosine fluorescence intensity is thought to reflect a reduced quenching in Ca2+-bound CaM and has been used to monitor Ca2+ binding to the C terminus of CaM (20, 21). Spectra were collected at 25°C using an ISS K2 fluorometer in ratio mode. The 3 μM CaM samples were excited at 275 nm using a xenon lamp, and corrected emission spectra were acquired from 280 to 400 nm in 1-nm increments. Excitation and emission bandwidths were 8 nm. Ca2+ titrations were performed by the addition of small aliquots of concentrated CaCl2 to the sample in the apo buffer (120 mM KCl, 20 mM PIPES, 1.0 mM EGTA, pH 7.0). A matching buffer scan was subtracted from each spectrum. The fluorescence readings, at 305 nm, for each titration were normalized to the high and low end points before non-linear least squares analysis.

Circular Dichroism (CD)

CD spectra were recorded from 250 to 200 nm with a JASCO J-710 spectrophotometer coupled with a data processor. Spectra were recorded at 25°C using an ISS K2 fluorometer in ratio mode. The 3 μM CaM samples were excited at 275 nm using a xenon lamp, and corrected emission spectra were acquired from 280 to 400 nm in 1-nm increments. Excitation and emission bandwidths were 8 nm. Ca2+ titrations were performed by the addition of small aliquots of concentrated CaCl2 to the sample in the apo buffer (120 mM KCl, 20 mM PIPES, 1.0 mM EGTA, pH 7.0). A matching buffer scan was subtracted from each spectrum. The fluorescence readings, at 305 nm, for each titration were normalized to the high and low end points before non-linear least squares analysis.

MalDI-TOF Mass Spectrometry

MALDI-TOF mass spectrometry was performed at the University of Minnesota Mass Spectrometry Consortium for the Life Sciences using a Bruker Biflex III mass spectrometer (Bruker, Boston, MA) equipped with a N2 laser (337 nm, 3-ns pulse length) and a microchannel plate detector. Data were collected in the linear mode, positive polarity, with an accelerating potential of 19 kV. Each spectrum was the accumulation of ~200 laser shots. External calibration was performed using horse heart cytochrome c and horse skeletal muscle myoglobin. The matrix used for samples and standards was a saturated solution of 3,5-dimethoxy-4-hydroxybenzonic acid in 50:50 acetonitrile:water, 0.1% trifluoroacetic acid. Prior to MALDI-TOF analysis samples were desalted using Millipore’s C4 ZipTips according to the manufacturer’s protocol.

PAGE

CaM’s were analyzed under denaturing conditions using SDS-PAGE (22). Samples were incubated for 30 min in sample buffer containing either 5 mM CaCl2 or 5 mM EGTA before loading onto 15% gel. No Ca2+ or EGTA was added to the gel or running buffer.

Analysis

The CaM concentration dependence of SR vesicle [3H]ryanodine binding and the inhibition of [35S]CaM binding by unlabeled CaM were fit with the Hill equation. The Ca2+ dependence of ryanodine binding was fit with Equation 1, which assumes a high affinity Ca2+ binding site, which when bound will activate the RyR and a lower affinity Ca2+ binding site which when bound will inhibit channel opening.

\[
B = \frac{B_{\text{max}} \times ([Ca^{2+}]^{n} \times [CaM])^{n}}{EC_{50}^{n} + ([Ca^{2+}]^{n} \times [CaM])^{n} + IC_{50}^{n}}
\]

Where B is the ryanodine bound, B_{max} is the maximal ryanodine binding, EC_{50} and IC_{50} are the half-activating and half-inhibiting Ca2+ concentrations, respectively, and n, s_{a}, and s_{i} are the Hill coefficients for activation and inhibition, respectively.

Statistics

Data are presented as the means ± S.E. [3H]Ryanodine and [35S]CaM binding curves in the presence and absence of CaM and CaM mutants were studied using a one-way analysis of variance with Dunnett’s multiple comparison as a post hoc test or by Student’s paired and unpaired t tests as appropriate. The level of significance was < 0.05.

RESULTS

Effects of CaM Oxidation on Regulation of RyR1—As shown previously (14), apo-CaM activated and Ca2+-CaM inhibited RyR1. Thus, in a medium containing 100 mM Ca2+, CaM enhanced skeletal muscle SR vesicle [3H]ryanodine binding.
incubation in 50 mM H₂O₂ for 24 h abolished apo- and Ca²⁺
and is the [35S]CaM bound in the presence of unlabeled CaM, and
n
1.6 pmol/mg, and the maximal Ca²⁺
vating (EC₅₀)
B
45.7
oxiforms with 5, 6, 7, or 8 Met residues oxidized to their

FIG. 1. Effects of CaM oxidation on CaM concentration
dependence of [³H]ryanodine and [³⁵S]CaM binding to skeletal
muscle SR vesicles. Ryanodine (A and B) and CaM (C and D) binding
was carried out as described under “Experimental Procedures” in the
presence of 100 nM Ca²⁺ (A and C) or 700 µM Ca²⁺ (B and D) and either
native CaM or CaM that had been incubated in 50 mM H₂O₂ for 30 min
or 24 h. [³H]Ryanodine binding is expressed as a percent of maximal
[³H]ryanodine binding. [³⁵S]CaM binding is expressed as B/B₀, where B
is the [³⁵S]CaM bound in the presence of unlabeled CaM, and B₀ is the
[³⁵S]CaM bound in the absence of unlabeled CaM. The maximal [³H]ry-
anodine binding capacity of the SR vesicles used in this study was 7.3 ±
0.5 pmol/mg, the maximal apo-[³⁵S]CaM binding capacity was 32.4 ±
1.6 pmol/mg, and the maximal Ca²⁺-[³⁵S]CaM binding capacity was 45.7 ±
2.7 pmol/mg. Curves fit to the native CaM and CaM incubated
with 50 mM H₂O₂ for 30 min were derived from the Hill equation.

Thus, the native CaM used in these experiments consisted of a
single population of unmodified CaM. In contrast, the mass
spectrum of CaM after incubation in 50 mM H₂O₂ for 30 min
displayed four peaks corresponding to masses of 16,783,
16,798, 16,815, and 16,830 Da. Oxidation of Met to Met sulf-
oxide increased the mass by 16 Da. Consequently, this partially
oxidized CaM was composed of multiple populations of CaM
oxiforms with 5, 6, 7, or 8 Met residues oxidized to their

FIG. 2. Mass spectra of native and oxidized CaM. The mass peak
(16,711 Da) of native CaM (A) corresponds, within the experimental
error, to the theoretical CaM mass (16,706.39). The mass peaks (16,783,
16,798, 16,815, 16,830 Da) of CaM incubated in 50 mM H₂O₂ for 30 min
(B) correspond to the theoretical CaM mass with 5–8 of the Met resi-
dues oxidized to Met sulfoxide. The mass peak (16,861 Da) of CaM
incubated in 50 mM H₂O₂ for 24 h (C) corresponds to the theoretical
CaM mass with all 9 Met residues oxidized to Met sulfoxide.

Regulation of RyR1 by Met → Gln CaM Mutants—To deter-
mine the role of specific CaM Met residues in regulating RyR1,
we used site-directed mutagenesis to change each CaM Met to
Gln. This substitution introduced an oxygen atom at the same
position in the side chain as the sulfoxide. The greater polarity
of the Gln side chain relative to Met was expected to decrease
the hydrophobic interactions that normally stabilize the asso-
ciation of CaM with its target. However, substituting Gln for
Met is unlikely to significantly disturb the structure of CaM
because both amino acids have a similar propensity to form
α-helices (23).

Fig. 3 and Tables I and II summarize the effects of CaM
Met → Gln mutants on the CaM concentration dependence of
skeletal muscle SR vesicle [³H]ryanodine binding in medium
containing 100 nM Ca²⁺ or 700 µM Ca²⁺. In a medium contain-
ing 100 nM Ca²⁺, all of the N-terminal Met → Gln mutants and
M76Q, in the tether, enhanced SR vesicle [³H]ryanodine bind-
ing. However, the maximal activation by the M71Q mutant
exceeded wild-type CaM activation by ~50%. Although the
M72Q CaM mutant enhanced ryanodine binding to an extent
similar to that of wild-type CaM, the EC₅₀ was ~3-fold greater
than wild-type. In contrast, the extent and concentration de-
Fig. 3. Effects of the CaM Met → Gln mutant on the CaM concentration dependence of skeletal muscle SR vesicle [3H]ryanodine binding. Ryanodine binding was performed as described under “Experimental Procedures” in medium containing either 100 nM Ca2+ (A and B) or 700 μM Ca2+ (C and D). [3H]Ryanodine binding is expressed as a percent of maximal [3H]ryanodine binding. Solid lines, except in the case of M109Q in B, represent fits to the Hill equation.

**Table I**

CaM dependence of skeletal muscle SR vesicle [3H]ryanodine binding in media containing 100 nM Ca2+.

| EC50 (nM) | nH | % Activation |
|----------|----|--------------|
| Wild-type | 65 ± 5 | 1.4 ± 0.1 | 468 ± 57 |
| M36Q | 88 ± 22 | 1.6 ± 0.3 | 353 ± 57 |
| M51Q | 108 ± 24 | 1.7 ± 0.3 | 539 ± 204 |
| M71Q | 83 ± 8 | 1.4 ± 0.1 | 715 ± 142 |
| M72Q | 216 ± 49 | 1.2 ± 0.1 | 446 ± 37 |
| M76Q | 38 ± 1 | 1.1 ± 0.1 | 456 ± 100 |
| M109Q | 1470 ± 306 | 1.2 ± 0.1 | 543 ± 93 |
| M124Q | 43 ± 6 | 1.5 ± 0.1 | 456 ± 107 |
| M144Q | 71 ± 10 | 1.1 ± 0.1 | 540 ± 224 |

*Significantly different from wild-type; p < 0.05.

**Table II**

CaM dependence of skeletal muscle SR vesicle [3H]ryanodine binding in media containing 700 μM Ca2+.

| IC50 (nM) | nH | % Inhibition |
|----------|----|--------------|
| Wild-type | 42 ± 5 | 3.0 ± 1.0 | 49 ± 5 |
| M36Q | 31 ± 7 | 2.0 ± 0.3 | 48 ± 3 |
| M51Q | 41 ± 11 | 1.7 ± 0.6 | 34 ± 9 |
| M71Q | 60 ± 13 | 2.6 ± 1.0 | 42 ± 8 |
| M72Q | 60 ± 12 | 1.3 ± 0.3 | 40 ± 3 |
| M76Q | 47 ± 16 | 2.9 ± 1.0 | 35 ± 3 |
| M109Q | 26 ± 2 | 1.8 ± 0.5 | 47 ± 9 |
| M124Q | 204 ± 24 | 1.6 ± 0.2 | 32 ± 5 |
| M144Q | 36 ± 13 | 2.3 ± 0.2 | 41 ± 6 |
| M145Q | 68 ± 17 | 2.4 ± 0.3 | 39 ± 3 |

*Significantly different from wild-type; p < 0.05.

Fig. 4. Ca2+ dependence of skeletal muscle SR vesicle [3H]ryanodine binding in the absence of CaM and in the presence of 5 μM wild-type or Met → Gln CaM mutants. Ryanodine binding was performed as described under “Experimental Procedures” in medium containing no CaM, 5 μM wild-type CaM, 5 μM M71Q CaM, 5 μM M109Q CaM, or 5 μM M124Q CaM and either 3 mM AMPPCP and 3 mM MgCl2 (A) or 3 mM AMPPCP and no MgCl2 (B). [3H]Ryanodine binding is expressed as a percent of maximal [3H]ryanodine binding. The solid lines are the fits to Equation 1.

CaM Methionines and RyR Regulation

Dependence of Ca2+-CaM inhibition by all the N-terminal Met → Gln mutants and M76Q were similar to wild-type CaM.

With the exception of the M109Q CaM mutant, in 100 nM Ca2+, all of the C-terminal Met → Gln mutants enhanced ryanodine binding to an extent similar to that of wild-type CaM. Substitution of Gln for Met at position 109 completely abolished apo-CaM activation of RyR1. Replacing Met-124 with Gln increased the apo-CaM EC50 nearly 23-fold compared with wild-type CaM. In a medium containing 700 μM Ca2+, all of the C-terminal Met → Gln mutants, including surprisingly M109Q, inhibited ryanodine binding to an extent similar to that of wild-type CaM. Similar to the effect in 100 nM Ca2+, the M124Q mutation increased the IC50 for Ca2+-CaM inhibition of ryanodine binding. However, the 5-fold increase in the Ca2+-CaM IC50 was much smaller than the 23-fold increase in the apo-CaM EC50 caused by this mutation.

The Ca2+ dependence of wild-type or Met → Gln CaM mutants in medium containing 5 μM wild-type CaM, 5 μM M71Q CaM, 5 μM M109Q CaM, or 5 μM M124Q CaM and either 3 mM AMPPCP and 3 mM MgCl2 (A) or 3 mM AMPPCP and no MgCl2 (B). [3H]Ryanodine binding is expressed as a percent of maximal [3H]ryanodine binding. The solid lines are the fits to Equation 1.
Inhibition of skeletal muscle SR vesicle $[^{35}\text{S}]\text{CaM}$ binding by wild-type CaM and Met→Gln CaM mutants. $[^{35}\text{S}]\text{CaM}$ binding was performed as described under “Experimental Procedures” in medium containing 1 μM wild-type $[^{35}\text{S}]\text{CaM}$, either 100 nM Ca$^{2+}$ (A) or 700 μM Ca$^{2+}$ (B), and the indicated concentrations of unlabeled wild-type CaM, M71Q CaM, M72Q CaM, M76Q CaM, M109Q CaM, or M124Q CaM. Data are expressed as $B/B_0$, where $B$ is the amount of $[^{35}\text{S}]\text{CaM}$ bound in the presence of unlabeled CaM, and $B_0$ is the amount of $[^{35}\text{S}]\text{CaM}$ bound in the absence of unlabeled CaM.

**Table III.**

| % $B_{max}$ | $n_a$ | EC$_{50}$ | $n_i$ | IC$_{50}$ |
|-------------|--------|----------|------|----------|
| 3 mM AMPPCP + 3 mM MgCl$_2$ | μM | μM |
| No CaM | 31 ± 5 | 1.4 ± 0.2 | 1.86 ± 0.10 | 1.7 ± 0.6 | 886 ± 42 |
| Wild-type | 19 ± 3 | 1.0 ± 0.1 | 1.03 ± 0.17 | 1.0 ± 0.2 | 629 ± 65 |
| M71Q | 17 ± 9 | 1.0 ± 0.1 | 0.63 ± 0.51 | 0.8 ± 0.5 | 172 ± 64 |
| M109Q | 14 ± 4 | 1.0 ± 0.2 | 0.93 ± 0.21 | 1.6 ± 0.1 | 839 ± 139 |
| M124Q | 17 ± 5 | 0.8 ± 0.1 | 0.79 ± 0.38 | 1.0 ± 0.1 | 591 ± 131 |
| 3 mM AMPPCP no Mg | μM | μM |
| No CaM | 71 ± 5 | 1.0 ± 0.1 | 0.70 ± 0.28 | 1.0 ± 0.1 | 422 ± 113 |
| Wild-type | 63 ± 5 | 0.9 ± 0.1 | 0.05 ± 0.01 | 1.2 ± 0.1 | 332 ± 110 |
| M71Q | 73 ± 10 | 0.9 ± 0.1 | 0.04 ± 0.01 | 1.0 ± 0.1 | 248 ± 79 |
| M109Q | 56 ± 9 | 0.7 ± 0.2 | 1.58 ± 0.86 | 1.3 ± 0.2 | 431 ± 78 |
| M124Q | 68 ± 5 | 1.0 ± 0.1 | 0.10 ± 0.02 | 1.1 ± 0.1 | 389 ± 115 |

* Significantly different from No CaM; $p < 0.05$.

significantly lower than in the absence of CaM, but the Ca$^{2+}$ IC$_{50}$ was not significantly different. However, in the presence of M109Q CaM, the extent of channel activation was less than half that in the absence of CaM, and the shift in the EC$_{50}$ was caused by the lower extent of activation rather than an enhancement of channel opening at low Ca$^{2+}$. For example, at 0.1 μM Ca$^{2+}$, wild-type CaM and both the M71Q and M124Q CaM mutants significantly increased SR vesicle ryanodine binding compared with the absence of CaM. In contrast, ryanodine binding in the presence of the M109Q CaM was not significantly different from the binding in the absence of CaM.

To resolve better the effects of the Met→Gln substitutions on apo-CaM function, channel activation was enhanced by performing binding experiments in medium containing 3 mM AMPPCP but no MgCl$_2$ (Fig. 4B and Table III). Under these conditions, wild-type, M71Q and M124Q CaM clearly enhanced the Ca$^{2+}$ sensitivity of channel activation while the M109Q CaM did not.

**Table IV.**

| 100 nM Ca$^{2+}$ | 700 μM Ca$^{2+}$ |
|-----------------|-----------------|
| nh | IC$_{50}$ | nh | IC$_{50}$ |
| Wild-type | 1.0 ± 0.1 | 73 ± 4 | 0.9 ± 0.1 | 74 ± 5 |
| M71Q | 0.9 ± 0.1 | 65 ± 2 | 0.8 ± 0.1 | 46 ± 9 |
| M72Q | 1.0 ± 0.1 | 110 ± 4 | 0.8 ± 0.1 | 301 ± 124 |
| M76Q | 1.0 ± 0.1 | 37 ± 2 | 0.8 ± 0.1 | 61 ± 2 |
| M109Q | 1.0 ± 0.1 | 2304 ± 532* | 0.7 ± 0.1 | 145 ± 46 |
| M124Q | 0.7 ± 0.1 | 641 ± 168* | 0.9 ± 0.2 | 321 ± 129* |

* Significantly different from wild-type CaM; $p < 0.05$. 

Inhibition of $[^{35}\text{S}]\text{CaM}$ Binding to RyR1 by Met→Gln CaM Mutants—To determine whether the Met→Gln mutations affected CaM regulation of RyR1 via changes in the affinity of CaM binding to the RyR or via alterations in CaM regulatory efficacy, we compared inhibition of SR vesicle $[^{35}\text{S}]\text{CaM}$ binding by wild-type and Met→Gln CaM mutants (Fig. 5 and Table IV). Compared with wild-type CaM, greater concentrations of the M124Q CaM mutant were required to inhibit SR vesicle $[^{35}\text{S}]\text{CaM}$ binding in medium containing either 100 nM or 700 μM Ca$^{2+}$. Thus, the effects of the M124Q mutation on CaM
affinity for RyR1 were similar to the effects of the mutation on SR vesicle [35S]ryanodine binding. At high Ca\textsuperscript{2+} concentrations, the M109Q CaM mutant inhibited SR vesicle [35S]CaM binding in a manner similar to wild-type CaM. However, in contrast to the inability of the M109Q apo-CaM to enhance SR vesicle ryanodine binding, this mutant inhibited [35S]CaM binding to SR vesicles. This suggests that at low Ca\textsuperscript{2+}, the M109Q apo-CaM mutant does bind to the RyR1, but that binding does not affect channel opening.

SDS-PAGE, CD, and Intrinsic Tyrosine Fluorescence—When CaM is denatured for SDS-PAGE in the presence of Ca\textsuperscript{2+}, the mobility of the protein on SDS-PAGE is increased relative to that seen after denaturation in the presence of EGTA (26). Although the mechanisms underlying the mobility shift are not understood, it is thought to reflect some difference in the ability of SDS to bind and/or denature apo- and Ca\textsuperscript{2+}-CaM (27). Thus, mutation-induced structural changes in the Met → Gln mutant CaMs might be reflected in altered mobility on SDS-PAGE. As can be seen from Fig. 6, none of the Met → Gln mutations altered the mobility of CaM in the presence of EGTA. Furthermore, none of the mutations prevented the mobility shift upon the addition of Ca\textsuperscript{2+}.

To assess further the potential structural alterations induced by the M109Q and M124Q CaM mutations, the CD spectra arising from these two mutants were compared with the spectra of wild-type CaM. The far UV spectra of wild-type CaM and the M109Q and M124Q mutant CaMs were not significantly different, either in the presence of 500 μM EGTA (Fig. 7A) or in the presence of 500 μM Ca\textsuperscript{2+} (Fig. 7B). Thus the Met → Gln mutations did not cause major changes in the secondary structure of CaM.

To determine whether the M109Q or M124Q mutation altered the CaM Ca\textsuperscript{2+} affinity we determined the Ca\textsuperscript{2+} dependence of the change in intrinsic tyrosine fluorescence (20, 21). The apparent Ca\textsuperscript{2+} affinities of wild-type CaM, M109Q CaM, and M124Q CaM did not significantly differ (K_{Ca}: wild-type CaM, 2.5 ± 0.3 μM; M109Q CaM, 1.5 ± 0.4 μM; M124Q CaM, 2.0 ± 0.3 μM).

**DISCUSSION**

The unusually high CaM Met content (9 of 148 residues) is thought to allow CaM to associate with and regulate a large number of structurally diverse proteins. Here we defined the role of specific CaM Met residues in the regulation of RyR1. Oxidation of all 9 CaM Met residues to their corresponding sulfoxide abolished both apo- and Ca\textsuperscript{2+}-CaM binding and modulation of RyR1. In comparison, incomplete oxidation, i.e. oxidation of 5–8 CaM Met residues, did not alter the extent of either apo-CaM activation or Ca\textsuperscript{2+}-CaM inhibition, but rather increased the CaM concentration required for these effects. Thus it appears that the presence of 4 unoxidized Met residues is sufficient for the full extent of CaM regulation of RyR1 but not to provide CaM with the normal high affinity for RyR1. It is not clear, however, whether the ability of the incompletely oxidized CaM to regulate RyR1 fully was the result of the preservation of specific, vital Met residues or a critical Met surface area.

Apo-CaM function appeared to be more sensitive to Met modification than Ca\textsuperscript{2+}-CaM function. Incomplete oxidation caused a larger increase in apo-CaM EC_{50} than in Ca\textsuperscript{2+}-CaM IC_{50}. The differential effect of oxidation on the function of apo- and Ca\textsuperscript{2+}-CaM was also reflected in the differing effects of some of the Met → Gln mutants. Thus, the M124Q mutation increased the apo-CaM EC_{50} by more than 20-fold but increased the Ca\textsuperscript{2+}-CaM IC_{50} by only 5-fold. Even more dramatic were the effects of the M109Q mutant on apo-CaM versus Ca\textsuperscript{2+}-CaM function. This single Met → Gln substitution completely abolished activation of RyR1 but did not alter Ca\textsuperscript{2+}-CaM inhibition of the channel.

Met-109 and Met-124 were necessary for the high affinity interaction of CaM with RyR1. Yuan et al. (28) found that in solution, the N-terminal Met residues in apo-CaM are largely buried, whereas the C-terminal Met residues are more exposed. They suggest that these C-terminal Met residues may play a role in the binding of apo-CaM to targets. Thus, in apo-CaM, Met-109 and Met-124 may be available to interact with RyR1. Consequently, a mutation in either of these residues significantly affects the functional interaction of apo-CaM with RyR1. Upon Ca\textsuperscript{2+} binding to CaM, there is an increased Met exposure
altered the interaction of Ca\(^{2+}\)-addition, M124Q was the only substitution that significantly

tations clearly demonstrate the importance of the C-terminal

membrane Ca\(^{2+}\) of CaM-dependent protein kinases (29, 31) and the plasma

channel.

The initial nonspecific association of CaM with targets is

thought to be followed by more precise interactions between

the Met residues required for normal enzyme regulation, sub-

stitution of Gln for Met-124 decreased the affinity of CaM for

the Met-109 and Met-124 CaM mutants were indistinguishable from wild-type CaM in the absence of Ca\(^{2+}\)

and, also in agreement with Chin and Means (29), in the presence of Ca\(^{2+}\). Thus we were unable to detect any substantial

structural modification in these mutants.

Alternatively, the M109Q mutation could increase the Ca\(^{2+}\)

affinity of the CaM such that a substantial fraction of the mutant CaM would exist as inhibitory Ca\(^{2+}\)-CaM in medium containing 100 \(\mu\)M Ca\(^{2+}\). However, the Ca\(^{2+}\) dependence of the change in intrinsic fluorescence did not differ between wild-
type CaM and either M109Q or M124Q CaM.

Finally, a Met residue might be required in position 109 to

make specific interactions with RyR1, albeit with a low affinity, but did not activate the

channel. Therefore, it is likely apo-CaM activation of RyR1 requires a specific interaction between Met-109 and the channel.

Met \(\rightarrow\) Gln substitutions have been used previously to define

the Met residues required for Ca\(^{2+}\)-CaM activation of a number of CaM-dependent protein kinases (29, 31) and the plasma membrane Ca\(^{2+}\)-pump (32). Although there was variability in the Met residues required for normal enzyme regulation, sub-

stitution of Gln for Met-124 decreased the affinity of CaM for all of these targets. The M124Q mutation also decreased the maximal CaM-dependent kinase activation but not CaM activation

of the Ca\(^{2+}\) pump. Whereas apo- and Ca\(^{2+}\)-bound M124Q CaM fully regulated RyR1, the substitution decreased the affinity of CaM for the channel. Thus, Met-124 appears to

be an important determinant of the CaM affinity for all of these targets; however, its importance in determining CaM regulatory
efficacy is target-dependent.

CaM is functionally bifurcated (29, 33), thus the N and C
termini of CaM may serve different roles in both apo- and

Ca\(^{2+}\)-CaM regulation of RyR1. The effects of Met \(\rightarrow\) Gln

mutations clearly demonstrate the importance of the C-terminal

lobe Met-109 and Met-124 in apo-CaM activation of RyR1. In

addition, M124Q was the only substitution that significantly altered the interaction of Ca\(^{2+}\)-CaM with RyR1. Rodney et al. (34) proposed a model of RyR1 regulation by CaM in which Ca\(^{2+}\) binding to the CaM C-terminal pair of EF-hands mediates the conversion of CaM from an activator of RyR1 to an inhibitor. The requirement of Met-109 for apo-CaM activation of RyR1 but not for Ca\(^{2+}\)-CaM inhibition of the channel sug-

gests that a critical component of the Ca\(^{2+}\)-induced structural change converting CaM from an activator to an inhibitor en-
tails altering the interaction between CaM Met-109 and RyR1.

In summary, oxidation of all 9 CaM Met residues abolished the functional interaction between CaM and RyR1. Incomplete oxidation decreased CaM affinity for RyR1 but not the extent of channel regulation. Site-specific substitution of Met with Gln at residue 109 abolished apo-CaM activation of RyR1 without altering Ca\(^{2+}\)-CaM inhibition of the channel. Substitution of Met-124 with Gln decreased the affinity of both apo- and Ca\(^{2+}\)-

CaM for RyR1. Thus these results identify Met residues critical for the productive interaction of CaM for RyR1 and suggest that oxidation of CaM may contribute to RyR1 dysfunction during oxidative stress.

Acknowledgments—We thank Drs. Deb Ferrington for helpful discus-
sions and LeeAnn Higgins for assistance with the mass spectrometry.

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