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

The 148-amino acid Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 the apo-CaM and Ca\(^{2+}\)-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 Ca\(^{2+}\) release during oxidative stress.

Skeletal muscle contraction is initiated by Ca\(^{2+}\) efflux from the sarcoplasmic reticulum (SR) via the SR Ca\(^{2+}\) 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 Ca\(^{2+}\) in the nM to \(\mu\)M range and inactivated by \(\mu\)-mM Ca\(^{2+}\). CaM binding to the channel enhances the sensitivity to both Ca\(^{2+}\) activation and inactivation.

Calmodulin (CaM) binds to the skeletal muscle ryanodine receptor Ca\(^{2+}\) release channel (RyR1) with high affinity, and it may act as a Ca\(^{2+}\)-sensing subunit of the channel. Apo-CaM increases RyR1 channel activity, but Ca\(^{2+}\)-CaM is inhibitory. Here we examine the functional effects of CaM oxidation on RyR1 regulation by both apo-CaM and Ca\(^{2+}\)-CaM, as assessed via determinations of \(^{3}H\)ryanodine and \(^{35}S\)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 Glu demonstrated that Met-109 was required for apo-CaM activation of RyR1 but not for Ca\(^{2+}\)-CaM inhibition of the channel. Furthermore, substitution of Gln for Met-124 increased the apo- and Ca\(^{2+}\)-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 Ca\(^{2+}\) release during oxidative stress.

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

Edward M. Balog§, Laura E. Norton‡, Rachel A. Bloomquist‡, Razvan L. Cornea‡, D. J. Black‡, Charles F. Louis, David D. Thomas‡, and Bradley R. Fruen‡

From the From §Department of Biochemistry, Molecular Biology, and Biophysics, University of Minnesota, Minneapolis, Minnesota 55455, the ‡Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, Ohio 43210, and the ¶Department of Biology, Georgia State University, Atlanta, Georgia 30303

* This work was supported by National Institutes of Health Grant GM 31382 and by the American Heart Association, Northland Affiliate. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: 6-155 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455. Tel.: 612-625-3292; Fax: 612-625-3263; E-mail: balog0046@ce.umn.edu.

§ The abbreviations used are: SR, sarcoplasmic reticulum; AMPPCP, adenosine 5′-(3′,5′)-monophosphate; apo-CaM, Ca\(^{2+}\)-free CaM; CaM, calmodulin; HOMOPIPES, homopiperazine-N,N,N′,N′-bis-(ethan-sulfonic acid); MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PIPES, 1,4-piperazinediethanesulfonic acid; RyR, ryanodine receptor.

**[^3H]Ryanodine Binding to Skeletal Muscle Heavy Sarcoplasmic Reticulum**

Isolation of SR Vesicles—Skeletal muscle SR vesicles were prepared from porcine longissimus dorsi muscle (10). Muscle was homogenized in

This paper is available online at http://www.jbc.org

1 The abbreviations used are: SR, sarcoplasmic reticulum; AMPPCP, adenosine 5′-(3′,5′)-monophosphate; apo-CaM, Ca\(^{2+}\)-free CaM; CaM, calmodulin; HOMOPIPES, homopiperazine-N,N,N′,N′-bis-(ethan-sulfonic acid); MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; PIPES, 1,4-piperazinediethanesulfonic acid; RyR, ryanodine receptor.

3 The 148-amino acid Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 Ca\(^{2+}\)-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 the apo-CaM and Ca\(^{2+}\)-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 Ca\(^{2+}\) release during oxidative stress.

**[3H]Ryanodine Binding to Skeletal Muscle Heavy Sarcoplasmic Reticulum**

Isolation of SR Vesicles—Skeletal muscle SR vesicles were prepared from porcine longissimus dorsi muscle (10). Muscle was homogenized in
0.1 to 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 resuspended in 1.1 M KCl, 5 mM Tris, pH 6.8, centrifuged at 130,000 × g for 45 min, and then resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Tris, pH 6.8, buffer. SR was then centrifuged through a discontinuous sucrose gradient (22, 36, and 45% sucrose) at 130,000 × g for 5 h, and the heavy SR fraction was collected from the 36 and 45% sucrose interface. Heavy SR vesicles were resuspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Tris, pH 6.8, flash frozen in liquid nitrogen, and stored at −70 °C. All buffers contained a protease inhibitor mixture (100 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, 1 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride).

**[35S]Calmodulin Binding—Ryndione selectively binds to the open RyR and therefore provides a useful indicator of channel activity (11). SR vesicles (0.2 mg/ml) were incubated at 37 °C in medium containing 120 mM potassium propionate, 10 mM PIPES, pH 7.0, 3 mM AMPPCP, 120 mM potassium chloride, and purified as described previously for [35S]FKBP12 (19). Briefly, bacterial growth was observed when the A600 value of the bacteria reached 0.6, the pelleted bacteria were resuspended in 150 mM KCl, 20 mM Pipes, pH 7.0, 5 mM CaCl2, and 50 mM Na2 EGTA or CaCl2 using quartz cuvettes with a path length of 1.0 mm. Spectra were recorded with a scan speed of 20 nm/min, signal-averaged six times, and an equally signal-averaged solvent base line was subtracted.

**Oxidation of Calmodulin**

Because the thiouger 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 mM KCl, 2 mM MgCl2, 50 mM H2O2 at room temperature for 0.5–24 h. The reaction was stopped by overnight incubation (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 HEPES, 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, ε277–280 nm = 3,029 M−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). Briefly, 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 concentrations compared with the regular RPMI 1640 medium, and isopropyl-β-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 bacteria cultured for 5–7 h at the same conditions.

**Matrix-assisted Laser Desorption/ionization-Time-of-Flight (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-hydroxycinnamic acid in 50:50, acetonitrile:nanopure water, 0.1% trifluoroacetic acid. Prior to MALDI-TOF analysis samples were desalted using Millipore's C4 ZipTips according to the manufacturer's protocol.

**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 excised 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 nonlinear 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 digitally and fed through the data processor for signal averaging and base line subtraction. Spectra were recorded at 25 °C with a CaM concentration of 150 μM in a solution of 2 mM HEPES, pH 7.0, and either 500 μM Na2 EGTA or CaCl2 using quartz cuvettes with a path length of 1.0 mm. Spectra were recorded with a scan speed of 20 nm/min, signal-averaged six times, and an equally signal-averaged solvent base line was subtracted.

**PAGE**

CaMs 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 [35S]Ryndione binding and the inhibition of [35S]CaM binding by unlabeled CaM were fit with the Hill equation. The Ca2+-dependence of ryndione 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,

\[
B = B_{\text{max}} \times ((\text{Ca}^{2+})^n (\text{Ca}^{2+})^p + \text{EC}_{50}^n) \times (1 - (\text{Ca}^{2+})^n (\text{Ca}^{2+})^p + \text{IC}_{50}^p) \quad \text{(Eq. 1)}
\]

where B is the ryndione bound, B_{\text{max}} is the maximal ryndione binding, EC_{50}^n and IC_{50}^p are the half-activating and half-inhibiting Ca2+ concentrations, respectively, and n and p are the Hill coefficients for activation and inhibition, respectively.

**Statistics**

Data are presented as the means ± S.E. [35S]Ryndione 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 nM Ca2+, CaM enhanced skeletal muscle SR vesicle [35S]Ryndione binding.
is the [35S]CaM bound in the presence of unlabeled CaM, and of oxidative modification of CaM incubated with 50 mM H2O2 TOF mass spectrometry was performed to determine the extent alters the productive association of CaM with the RyR1. [3H]ryanodine binding. [35S]CaM binding is expressed as 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 and [35S]CaM binding to skeletal

oxiforms with 5, 6, 7, or 8 Met residues oxidized to their oxidized CaM was composed of multiple populations of CaM oxide increased the mass by 16 Da. Consequently, this partially displayed four peaks corresponding to masses of 16,783, 16,798, 16,815, and 16,830 Da. Oxidation of Met to Met sulf-

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]ryanodine 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.

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 residues 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.

Mass Spectrometry of Oxidatively Modified CaM—MALDI-TOF mass spectrometry was performed to determine the extent of oxidative modification of CaM incubated with 50 mM H2O2 for either 30 min or 24 h. As shown in Fig. 2, the mass spectrum of native CaM displayed a single peak corresponding to a mass of 16,711 Da, which corresponds, within the measurement error, to the theoretical mass of unmodified CaM (16706.39 Da). 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 sulfoxide increased the mass by 16 Da. Consequently, this partially oxidized CaM was composed of multiple populations of CaM oxidiforms with 5, 6, 7, or 8 Met residues oxidized to their corresponding sulfoxide. CaM incubated in 50 mM H2O2 for 24 h displayed a single peak corresponding to a mass of 16,861 Da. Therefore, incubation of CaM in 50 mM H2O2 for 24 h produced a single population of CaM with all 9 Met residues oxidized to their corresponding Met sulfoxides.

Regulation of RyR1 by Met → Gln CaM Mutants—To determine 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 association 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 containing 100 nM Ca2+, all of the N-terminal Met → Gln mutants and M76Q, in the tether, enhanced SR vesicle [3H]ryanodine binding. However, the maximal activation by the M71Q mutant exceeded wild-type CaM activation by ~50%. Although the M72Q CaM mutant enhanced ryano dine binding to an extent similar to that of wild-type CaM, the EC50 of wild-type CaM incubated in 50 mM H2O2 for 24 h displayed a single peak corresponding to a mass of 16,861 Da. Therefore, incubation of CaM in 50 mM H2O2 for 24 h produced a single population of CaM with all 9 Met residues oxidized to their corresponding Met sulfoxides.

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 residues 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.
Wild-type CaM. In a medium containing 700 nM CaCl2 (A and B) or 700 μM CaCl2 (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.

Effects of the CaM Met→Gln mutants 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 CaCl2 (A and B) or 5 μM wild-type CaM, or 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.

Table I

<table>
<thead>
<tr>
<th>CaM dependence of skeletal muscle SR vesicle [3H]ryanodine binding in media containing 100 nM CaCl2</th>
<th>% Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 (nM)</td>
<td>nH</td>
</tr>
<tr>
<td>Wild-type</td>
<td>65 ± 5</td>
</tr>
<tr>
<td>M36Q</td>
<td>88 ± 22</td>
</tr>
<tr>
<td>M51Q</td>
<td>108 ± 24</td>
</tr>
<tr>
<td>M71Q</td>
<td>83 ± 8</td>
</tr>
<tr>
<td>M72Q</td>
<td>216 ± 49</td>
</tr>
<tr>
<td>M76Q</td>
<td>38 ± 1</td>
</tr>
<tr>
<td>M109Q</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>M124Q</td>
<td>1470 ± 306</td>
</tr>
<tr>
<td>M144Q</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>M145Q</td>
<td>71 ± 10</td>
</tr>
</tbody>
</table>

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

Table II

<table>
<thead>
<tr>
<th>CaM dependence of skeletal muscle SR vesicle [3H]ryanodine binding in media containing 700 μM CaCl2</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (nM)</td>
<td>nH</td>
</tr>
<tr>
<td>Wild-type</td>
<td>42 ± 5</td>
</tr>
<tr>
<td>M36Q</td>
<td>31 ± 7</td>
</tr>
<tr>
<td>M51Q</td>
<td>41 ± 11</td>
</tr>
<tr>
<td>M71Q</td>
<td>60 ± 13</td>
</tr>
<tr>
<td>M72Q</td>
<td>60 ± 12</td>
</tr>
<tr>
<td>M76Q</td>
<td>47 ± 16</td>
</tr>
<tr>
<td>M109Q</td>
<td>26 ± 2</td>
</tr>
<tr>
<td>M124Q</td>
<td>204 ± 24</td>
</tr>
<tr>
<td>M144Q</td>
<td>36 ± 13</td>
</tr>
<tr>
<td>M145Q</td>
<td>68 ± 17</td>
</tr>
</tbody>
</table>

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

Effects of the CaM Met→Gln mutants 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 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.

that of wild-type CaM. Similar to the effect in 100 nM CaCl2, the M124Q mutation increased the IC50 for CaCl2 -CaM inhibition of ryanodine binding. However, the 5-fold increase in the CaCl2 -CaM IC50 was much smaller than the 23-fold increase in the apo-CaM EC50 caused by this mutation.

The Ca2+ dependence of SR vesicle [3H]ryanodine binding was examined in the absence and presence of wild-type CaM and selected Met→Gln CaM mutants in medium containing 3 mM AMPPCP and 3 mM MgCl2 (Fig. 4A and Table III). Similar to previous reports (24, 25), wild-type CaM significantly decreased both the CaCl2 -EC50 and IC50. Similarly, the M71Q and M124Q CaM also lowered the Ca2+ -EC50 and IC50. In contrast, in the presence of the M109Q CaM mutant, the Ca2+ -EC50 was
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 \(\mu\)M 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 \(\rightarrow\) 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.

**Inhibition of \[^{35}\text{S}\text{CaM}\] binding to skeletal muscle SR vesicles by wild-type and met \(\rightarrow\) Gln CaM mutants—**To determine whether the Met \(\rightarrow\) 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 \(\rightarrow\) 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 \(\mu\)M 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 [³H]ryanodine binding.

At high Ca²⁺ concentrations, the M109Q CaM mutant inhibited SR vesicle [³H]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 [³S]CaM binding to SR vesicles. This suggests that at low Ca²⁺, 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 Ca²⁺, 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 Ca²⁺-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 Ca²⁺.

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 Ca²⁺ (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 Ca²⁺ affinity we determined the Ca²⁺ dependence of the change in intrinsic tyrosine fluorescence (20, 21). The apparent Ca²⁺ affinities of wild-type CaM, M109Q CaM, and M124Q CaM did not significantly differ (KCa: 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 Ca²⁺-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 Ca²⁺-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 Ca²⁺-CaM function. Incomplete oxidation caused a larger increase in apo-CaM EC₅₀ than in Ca²⁺-CaM EC₅₀. The differential effect of oxidation on the function of apo- and Ca²⁺-CaM was also reflected in the differing effects of some of the Met → Gln mutants. Thus, the M124Q mutation increased the apo-CaM EC₅₀ by more than 20-fold but increased the Ca²⁺-CaM IC₅₀ by only 5-fold. Even more dramatic were the effects of the M109Q mutant on apo-CaM *versus* Ca²⁺-CaM function. This single Met → Gln substitution completely abolished activation of RyR1 but did not alter Ca²⁺-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 Ca²⁺ binding to CaM, there is an increased Met exposure...
the Met residues required for Ca\(^{2+}\)–CaM than to apo-CaM function. The M109Q mutation could potentially abolish apo-CaM activation of RyR1 via three mechanisms. The mutation could disrupt the structure of CaM to such an extent that the mutant apo-CaM could not functionally interact with the channel. In agreement with Chin and Means (29), the Ca\(^{2+}\)–induced mobility shifts on an SDS gel by M109Q CaM and wild-type CaM were similar. In addition, the mutant could be purified via phenyl-Sepharose chromatography. Thus M109Q CaM preserved sufficient hydrophobic surface to be retained by the phenyl-Sepharose column and underwent a Ca\(^{2+}\)–induced structural rearrangement similar to wild-type CaM. Finally, the CD spectra of the M109Q and M124Q 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 could exist as inhibitory Ca\(^{2+}\), CaM in medium containing 100 nM 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. All of the Met \(\rightarrow\) Gln mutants interacted, although with varying affinity, with RyR1. The initial nonspecific association of CaM with targets is thought to be followed by more precise interactions between specific residues (30). In low Ca\(^{2+}\), M109Q CaM associated 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, substitution of Gln for Met-124 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 suggests that a critical component of the Ca\(^{2+}\), induced structural change converting CaM from an activator to an inhibitor entails 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 discussions and LeeAnn Higgins for assistance with the mass spectrometry.

REFERENCES
Calmodulin Oxidation and Methionine to Glutamine Substitutions Reveal Methionine Residues Critical for Functional Interaction with Ryanodine Receptor-1
Edward M. Balog, Laura E. Norton, Rachel A. Bloomquist, Razvan L. Cornea, D. J. Black, Charles F. Louis, David D. Thomas and Bradley R. Fruen


Access the most updated version of this article at doi: 10.1074/jbc.M209180200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 11 of which can be accessed free at http://www.jbc.org/content/278/18/15615.full.html#ref-list-1