Identification of a Denitrase Activity against Calmodulin in Activated Macrophages Using High-Field Liquid Chromatography−FTICR Mass Spectrometry†

Heather S. Smallwood,‡ Natacha M. Lourette,‡ Curt B. Boschek, Diana J. Bigelow, Richard D. Smith, Ljiljana Paša-Tolić, and Thomas C. Squier*

Biological Sciences Division, Pacific Northwest National Laboratory, P.O. Box 999, Richland, Washington 99352

Received May 21, 2007; Revised Manuscript Received June 20, 2007

ABSTRACT: We have identified a denitrase activity in macrophages that is upregulated following macrophage activation, which is shown by mass spectrometry to recognize nitrotyrosines in the calcium signaling protein calmodulin (CaM). The denitrase activity converts nitrotyrosines to their native tyrosine structure without the formation of any aminotyrosine. Comparable extents of methionine sulfoxide reduction are also observed that are catalyzed by endogenous methionine sulfoxide reductases. Competing with repair processes, oxidized CaM is a substrate for a peptidase activity that results in the selective cleavage of the C-terminal lysine (i.e., Lys^{148}) that is expected to diminish CaM function. Thus, competing repair and peptidase activities define the abundances and functionality of CaM in modulating cellular metabolism in response to oxidative stress, where the presence of the truncated CaM species provides a useful biomarker for the transient appearance of oxidized CaM.

Chronic inflammatory responses associated with biological aging contribute to a loss of robustness associated with aging as well as many age-related pathologies (*1*). The ability to keep the inflammatory response in check, and thus minimize associated oxidative damage, is fundamental to the retention of cell function. Endogenous antioxidant defense mechanisms (e.g., catalase and superoxide dismutase) as well as vital repair enzymes (e.g., methionine sulfoxide reductase, peroxiredoxins, and isoaspartyl methyltransfereases) function in concert to maintain protein activities critical to cell functions and healthy aging (*2*−*6*). Likewise, an endogenous denitrase enzyme activity has been proposed by Murad and co-workers that may function to repair nitrated tyrosines in proteins (*7*, *8*), and potentially to reversibly modulate protein function (*9*).

The reversible nitration of Tyr may have considerable functional significance given (i) the potential of nitration to block phosphorylation of Tyr and (ii) the involvement of tyrosine kinases in mediating cellular signaling (*10*−*15*). Thus, the accumulation of nitrotyrosines (nTyr)[1] in proteins in numerous chronic and age-related diseases may result from an aberrant repair pathway involving the proposed denitrase activity. Alternatively, the accumulation of nitrated proteins during aging may simply be the result of increases in the level of generation of reactive nitrating species or declines in overall rates of protein degradation.

A denitrase activity has been postulated in a range of different tissues on the basis of the time-dependent loss of immunoreactivity against nTyr in selected cellular proteins (*7*, *8*, *16*−*19*) (Scheme 1). In prior reports, Murad and co-workers demonstrated a denitrase activity that acted against two exogenously nitrated proteins added to activated cell lysates, i.e., albumin and histone H1.2 (*7*, *8*). In the case of histone H1.2, this protein is an endogenous substrate of the denitrase activity. In contrast, the majority of nitrated cellular proteins were insensitive to the denitrase activity, suggesting that the denitrase "activity has some substrate specificity" (*8*). Indeed, Murad and co-workers demonstrated that nitrated Cu/Zn SOD was not a substrate for the denitrase activity (*7*). Subsequent reports have demonstrated a reversible appearance of immunoreactive proteins in response to bouts of hypoxia-anoxia that were resolved using two-dimensional SDS−PAGE against nitrotyrosine antibodies in mitochondria isolated from rat liver, including manganese superoxide dismutase, enoyl-CoA hydratase, acetyl-CoA acyltransferase, electron transport flavoprotein, and malate dehydrogenase (*18*). These results suggested that the reversible nitration of proteins in response to environmental insults may have a signaling role in affecting tyrosine kinase activity (*18*). In no case were individual sites of nitration resolved, nor was the protein product that was no longer immunoreactive using antibodies against nitrotyrosine identified.

The observed denitrase activity has been suggested to involve a specific enzyme activity that acts on nitrated proteins to diminish the toxicity of reactive nitrogen species, resulting from the upregulation of inducible nitric oxide

† This work was supported by grants from the National Institutes of Health (NIA AG12993, AG17996, and RR18522), and aspects of this project were performed in the Environmental Molecular Science Laboratory, a U.S. Department of Energy (DOE) national scientific user facility at Pacific Northwest National Laboratory (PNNL), operated by Battelle Memorial Institute for the DOE under Contract DE-AC05-76RLO-1830.

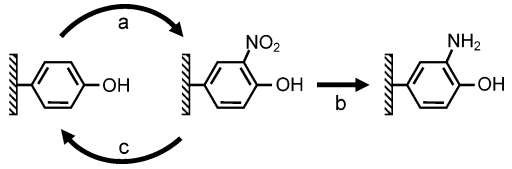
* To whom correspondence should be addressed: Pacific Northwest National Laboratory, P.O. Box 999, Mail Stop P7-53, Richland, WA 99352. E-mail: thomas.squier@pnl.gov. Telephone: (509) 376-2218. Fax: (509) 372-1632.

‡ These authors contributed equally to this work.

[1] Abbreviations: CaM, calmodulin; nTyr, nitrotyrosine; CaM_{ox}, oxidized CaM containing Met(O) or nTyr; LC, liquid chromatography; FTICR MS, Fourier-transform ion cyclotron resonance mass spectrometry; M(O) or Met(O), methionine sulfoxide; NRT, normalized retention time; UMC, unique mass classes.

10.1021/bi7009713 CCC: $37.00
Published on Web 08/21/2007

Scheme 1: Nitration of Protein-Associated Tyr (a) and Proposed Pathways Associated with the Selective Loss of Immunoreactivity against nTyr Involving either Formation of Amino-Tyr (b) or a Repair Activity Involving a Putative Denitrase Activity (c) (*7*, *8*, *23*, *40*)



synthase (iNOS), since this denitrase activity is upregulated upon exposure of animals to endotoxins, is retained by a 10 kDa membrane, is heat and protease sensitive, exhibits selectivity against specific nitrated proteins, and is preferentially observed in some tissues (e.g., spleen, lung, and activated macrophages) (*7*, *8*). However, the interpretation of a loss of nTyr immunoreactivity remains ambiguous, as antibodies against nTyr may be epitope-specific, varying with oxidant-induced changes in protein conformation and the overall efficacy of the antibody (*19*−*22*). Furthermore, the nonenzymatic modification of nTyrs to form amino-Tyr or other Tyr adducts is possible in cellular lysates (*23*). Thus, unambiguous confirmation of the involvement of a denitrating enzyme requires the identification of the reaction product(s) associated with the loss of immunoreactivity.

To this end, we have examined the nitration of macrophage proteins following activation with lipopolysaccharide (LPS) endotoxin and the possible selectivity of the denitrase activity against the calcium signaling protein calmodulin (CaM), which has previously been shown to function as a central regulator in modulating macrophage activation (*24*, *25*). High-resolution reversed-phase liquid chromatography coupled with Fourier transform ion cyclotron resonance mass spectrometry (LC−FTICR MS) facilitated the identification of various oxidized and nitrated forms of CaM isolated from activated macrophage lysates, which is distinguished from endogenously modified CaM due to the addition of a His tag at the N-terminus. We resolve that lysates obtained from activated macrophages act on nTyr in CaM to restore the native Tyr structure. Furthermore, competing with this repair process is a protease activity that selectively recognizes oxidized CaM to cleave a C-terminal Lys, which is expected to diminish CaM function.

EXPERIMENTAL PROCEDURES

Materials. The pBluescript II SK vector was purchased from Stratagene (La Jolla, CA). The following antibodies were used: a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (AbCaM, Cambridge, MA) and a polyclonal antibody against full-length CaM (sc-5537) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antibodies against nTyr were generously provided by J. Beckman of Oregon State University (Corvallis, OR) (*26*, *27*). RAW 264.7 murine macrophages were obtained from B. D. Thrall of Pacific Northwest National Laboratory.

Calmodulin Mutagenesis, Expression, and Purification. The coding region for chicken CaM [accession number MCCH (PIR database) or P02593 (SWISS-PROT database)] was excised from a pBluescript II SK vector (*28*); a histidine tag with a polyglycine linker (GHHHHHHGGGGGIL) was added to the 5′-end of the intact CaM gene (amino-terminal tag in the expressed protein), and the resulting construct was subcloned into the pET-15b vector prior to transformation into *Escherichia coli* BL21(DE3) for protein overexpression. Subsequent steps relating to CaM expression and purification are as previously described (*28*, *29*).

Oxidation of CaM. A 15−30-fold molar excess of peroxynitrite (BD Bioscience, San Diego, CA) was used to oxidize CaM, as previously described (*28*). Confirmation of nTyr was determined from the appearance of an absorbance peak at 430 nm following the addition of NaOH, where $\epsilon_{430} = 4400\ M^{-1}\ cm^{-1}$ at pH 10.0 (*30*).

Reduction of CaM. Oxidized CaM (40 μM) was incubated with a methionine sulfoxide reductase B/A fusion protein containing activities against both the *R*- and *S*-stereoisomers of Met(O) (4 μM) in 50 mM NH_4HCO_3 (pH 7.7) and 10 mM dithiothreitol (DTT) for 4 h at room temperature to enzymatically reduce Met(O) to Met (*31*). Nitrated Tyr in CaM was subsequently reduced to form an amino-Tyr upon addition of dithionite to serve as an authentic standard, made as a 1 M sodium dithionite solution in 25 mM boric acid (pH 8.9), essentially as previously described (*21*). Briefly, 2.8 μL of dithionite was added to CaM (7.7 μM) to a final volume of 1 mL, and the mixture was incubated with rotation for 5 min. CaM was subsequently extensively desalted using a spin column, and isolated CaM was buffer exchanged into 50 mM NH_4HCO_3 (pH 7.4) prior to mass spectrometric analysis. Loss of nTyr was confirmed by decreases in the absorption spectra ($\epsilon_{381} = 2200\ M^{-1}\ cm^{-1}$) (*28*), which indicated a conversion efficiency of >82%.

Cell Culture Procedures. RAW 264.7 macrophages were grown to ∼70% confluence, and cellular lysates were prepared following their activation with bacterial antigen LPS (either 10 ng/mL or 5 μg/mL) as previously described (*24*).

Lysate Incubation. His-tagged CaM (10 μg/mL) was added to cellular lysate (2−4 mg/mL) obtained from either quiescent or activated macrophages and incubated at 37 °C for 4 h. Following incubation, lysate was used for Western immunoblotting or the His-tagged CaM was affinity purified and dialyzed into 50 mM NH_4HCO_3 (pH 7.4) prior to mass spectrometry analysis.

Reversed-Phase LC Separations and Mass Spectrometric Analysis. Intact CaM oxiforms (0.5 μg) were separated using a C5 reversed-phase stationary phase, essentially as previously described (*32*). A gradient varying from mobile phase A [i.e., 0.05% trifluoroacetic acid (TFA), 0.2% acetic acid, 5% 2-propanol, 15% acetonitrile (ACN), and 79.75% water] to mobile phase B [i.e., 0.1% TFA, 9.9% water, 45% 2-propanol, and 45% ACN] at a rate of ∼0.5%/min was employed. Electrospray ionization utilized a chemically etched fused-silica needle to interface the LC system to a modified Bruker 12 T APEX-Q FTICR mass spectrometer, as previously described (*33*). One mass spectrum was recorded every 2 s, and an average of three mass spectra was used for data analysis.

Intact protein LC−FTICR mass spectra were processed using software developed in-house, available for download at http://ncrr.pnl.gov/software/, as described previously (*34*). Briefly, isotopic distributions were converted to neutral masses, which were then clustered into "unique mass classes" (UMCs) or LC−MS features, i.e., the same peptide detected repeatedly as it elutes from the LC column. The mass

measurement accuracy for on-line LC−MS operation was estimated to be <15 ppm, on the basis of the mass variation within the UMCs. Once the UMCs were defined, constituent mass spectra (members of each UMC) were summed and reprocessed to improve the signal-to-noise ratio for low-abundance species. External calibration was employed, and median masses obtained using all isotopically resolved charge states are reported. The number of distinct intact proteins was calculated by counting the UMC clusters. UMC masses were searched against the theoretical database (containing all possible combinations of oxi- and nitro-CaM forms) for tentative intact protein identifications.

CaM oxiforms common between samples prior and after macrophage lysate exposure were used for alignment of individual two-dimensional displays to provide the means of profiling intact protein abundances. To align and normalize LC−MS data sets generated for unmodified and oxidized CaM samples, we have used a stable isotope-labeled internal reference (i.e., oxidized doubly labeled [^{13}C,^{15}N]CaM).

Alternatively, following its isolation, CaM (1.0 mg/mL) was denatured (i.e., 7 M urea, 2 M thiourea, and 5 mM DTT) and incubated for 30 min at 60 °C prior to addition of trypsin (2% by weight ratio of total protein) and overnight digestion in 0.1 M NH_4HCO_3 (pH 8.4) and 1 mM $CaCl_2$ at 37 °C. LC−MS/MS analysis of the digest was performed using a reversed-phase capillary LC system described previously (*32*) and a Finnigan LTQ-FT mass spectrometer (ThermoElectron Corp., San Jose, CA). FTICR spectra (1×10^6 AGC) were collected for $400 < m/z < 2000$ at a resolution of ~10000, followed by five data-dependent LTQ-FT MS/MS acquisitions. Peptide MS/MS data were processed using Sequest (ThermoElectron, San Jose, CA), and peptide identifications were filtered using the rules suggested by Washburn et al. (*35*).

RESULTS

Loss of Immunoreactivity against nTyr in CaM following Macrophage Activation. Nitrated proteins were identified in whole cell lysates from quiescent RAW 264.7 macrophages following immunoblotting using antibodies against nTyr (Figure 1A). Consistent with the known sensitivity of CaM to nitration (*28*), there is a major nitrated protein near 16 kDa that comigrates with a CaM standard and whose immunoreactivity is preferentially lost following macrophage activation with LPS (Figure 1B). The band intensities of other nitrated proteins also decrease, albeit to a smaller extent. Likewise, there is a selective loss of immunoreactivity associated with nitrated CaM that is very sensitive to macrophage activation using minimal amounts of added LPS (Figure S1 of the Supporting Information). Under these conditions, the immunoreactivity of the majority of nitrated proteins is unaffected by macrophage activation. These results are consistent with prior observations that the immunoreactivities of the majority of nitrated proteins are not substantially affected by macrophage activation and the associated oxidative burst, despite substantial increases in the level of nitric oxide and other reactive oxygen and nitrogen species (Figure 1C) (*7*).

The LPS-induced loss of nTyr immunoreactivity in CaM was confirmed by the addition of nitrated CaM to cellular lysates prepared from either quiescent or activated macrophages (Figure 1D). In all cases, nitrated CaM (75 ng) was incubated with cellular lysates (15 μg) for 4 h at 37 °C. There is no loss of total CaM protein abundance following incubation of nitrated CaM in cellular lysates prepared from either quiescent or activated macrophages (Figure 1D). However, using lysates obtained following macrophage activation with LPS, there is a substantial loss of the immunoreactivity of nTyr in CaM. Thus, following a short period of macrophage activation (i.e., LPS exposure for 0.5 h), there is an approximately 30% decrease in immunoreactivity against nitrotyrosines, while the immunoreactivity against nitrotyrosines in CaM is largely abolished (i.e., >60% decrease in immunoreactivity) using cell lysates prepared from fully activated macrophages, i.e., resulting from exposure to LPS for 19 h. These results are consistent with the presence of a putative denitrase activity in activated macrophages. Alternatively, the formation of aminotyrosines or protein structural changes associated with other repair enzymes (involving, for example, the action of methionine sulfoxide reductases) may alter the immunoreactivity of the nitrotyrosines.

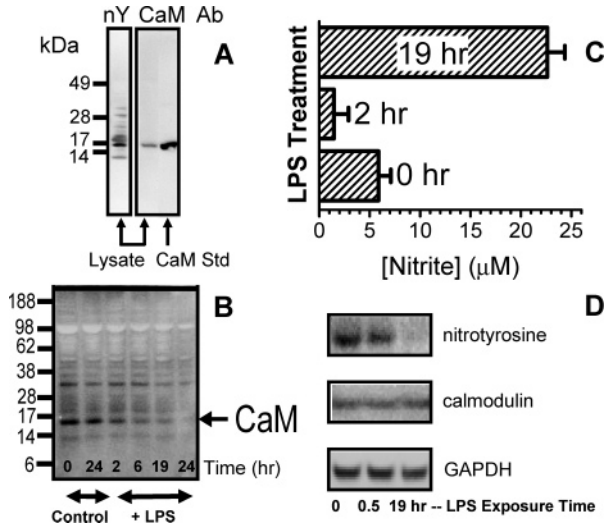


FIGURE 1: Loss of immunoreactivity against nitrated CaM upon macrophage activation. (A) Immunoblots against nTyr (left lane) and CaM (right two lanes) from lysate (15 μg) derived from RAW 264.7 macrophages or authentic CaM standard (0.2 μg). (B) LPS-dependent changes in the abundance of nitrated proteins in macrophage lysates (15 μg). (C) Nitrite production associated with macrophage activation following exposure to LPS (10 ng/mL) for the indicated times. All cells were plated for a total of 19 h before measuring nitrite levels, as previously described (*24*). (D) Immunoblots against nTyr (top panel), total CaM (middle panel), or the loading standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (bottom panel) following incubation of nitrated CaM (75 ng) in lysate (15 μg) for 4 h at 37 °C. Lysates were derived from either control (i.e., no LPS exposure) or activated macrophages prepared following exposure to LPS for either 30 min or 19 h. Endogenous CaM represents less than 20 ng of protein in 15 μg of lysate (*24*).

Repair and Cleavage of Oxidized CaM. Intact protein mass spectrometry was used to assess possible post-translational modifications or changes in the level of oxidation or nitration of His-tagged CaM following incubation with cellular lysates from fully activated macrophages (following exposure to LPS for 19 h), for which the nTyr immunoreactivities are substantially diminished (Figure 1D). A single major peak is observed for His-tagged CaM with an average mass of 18 098.02 Da that agrees with its theoretical mass (i.e.,

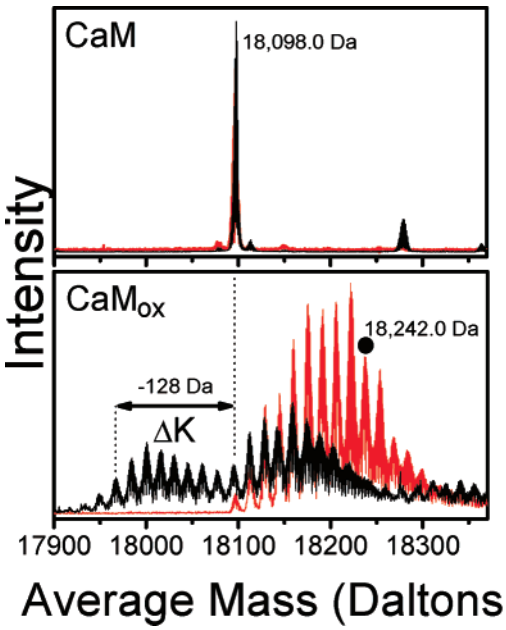


FIGURE 2: Selective repair and cleavage of oxidized CaM. Averaged intact protein mass spectra acquired during the reversed-phase LC separation of unoxidized His-tagged CaM or partially oxidized His-tagged CaM (i.e., CaM_{ox}) prior to (red spectra) and following (black spectra) incubation with lysate from activated RAW 264.7 macrophages. His-tagged CaM was incubated with a 200-fold excess (mass/mass) of lysate (approximately 4 mg/mL) for 4 h prior to affinity isolation of CaM using a Ni^{2+} affinity column and LC−MS analysis. Prior to lysis, macrophages were exposed to LPS for 19 h. Mass spectra were acquired with high resolution (i.e., ~150000) to ensure isotopic resolution for all detected protein species. The theoretical average mass of His-tagged CaM is 18 098.04 Da and increases by 16 Da upon oxidation of Met or 45 Da upon nitration of Tyr. Vertical dashed lines border CaM species separated by a loss of 128.2 Da from individual CaM oxiforms due to cleavage of Lys^{148}. The filled circle indicates the position of fully oxidized His-tagged CaM in which all nine Met residues are oxidized to their corresponding Met(O) (i.e., 18 242.0 Da).

18 098.04 Da) within 1.1 ppm (Figure 2). Following incubation of unoxidized CaM with cellular lysates, there are no significant changes in the mass of His-tagged CaM, as indicated by the retention of the major peak at 18 097.93 Da (5.5 ppm); no cleavage products or oxidative modifications are observed.

Pretreatment of CaM with a 15-fold molar excess of peroxynitrite results in multiple CaM oxiforms (CaM_{ox}) corresponding to a heterogeneous mixture of species involving the oxidation of one to nine Met residues to their corresponding methionine sulfoxides [Met(O)] and/or the nitration of either one or two of the Tyr residues to nTyr, as previously characterized (*28*). Under these conditions, there is reduced spectral intensity associated with unoxidized His-tagged CaM (i.e., 18 098 Da); CaM oxiforms are manifested as a series of peaks with masses higher than that of unoxidized His-tagged CaM (Figure 2). Significant peak intensities are associated with masses greater than 18 242 Da (i.e., CaM with all nine Met residues oxidized), which represent CaM oxiforms that also include nTyr.

Following incubation of CaM_{ox} with cellular lysates from activated macrophages, a broad shift in the distribution of CaM oxiforms toward lower masses occurs, and multiple new CaM oxiforms appear with masses of less than 18 098 Da (unoxidized CaM). The broad shifts in the spectrum toward lower masses are consistent with the ability of endogenous methionine sulfoxide reductases in lysates to reduce Met(O) in CaM (*4*, *31*, *36*). The appearance of new species with masses below that of unoxidized CaM suggests that an endogenous peptidase activity selectively cleaves the C-terminal Lys^{148} from CaM_{ox}, resulting in a mass decrease of 128 Da. Indeed, a similar cleavage of the C-terminal Lys^{148} is observed in CaM isolated from either oxidatively sensitive regions of the brain (*37*, *38*) or neurons in culture that were treated with paraquat (T. D. Williams and A. Zaidi, unpublished observations). An additional feature in the spectrum associated with the loss of water is observed for both wild-type and oxidized CaM, as previously observed (*39*).

LC−FTICR Resolution of CaM Oxiforms. Due to the high degree of similarity between various CaM oxiforms present in considerably different amounts, their separate arrival into the mass spectrometer is a prerequisite for successful MS characterization. Therefore, the combination of high-resolution protein separations and effective high-performance MS analysis of small samples proved to be critical for identification of individual CaM oxiforms corresponding to homogeneous species with variable numbers of Met(O) and nTyr residues (Figure 3).

Prior to oxidation, the majority of detected species correspond to unmodified CaM, eluting at a normalized retention time (NRT) of 0.76 with an average mass of 18 098.02 Da (Figure 3A). Following oxidation of CaM with peroxynitrate, we resolve approximately 500 different CaM oxiforms of the 2048 theoretically possible species (i.e., 2^{9+2}), differing in the position and number of Met(O) and nTyr residues, with NRTs varying between 0.4 and 0.8 and masses varying from 17 970 to 18 332 Da (Figure 3B). Two-dimensional LC−MS displays shown in Figure 3 plot detected neutral monoisotopic masses versus normalized retention times; each spot therefore corresponds to a particular CaM oxiform containing an integral number of Met(O) and nTyr residues. For example, unoxidized CaM has the same coordinates in the LC−MS display of this mixture of oxidized species (Figure 3B) as in the display obtained for unoxidized CaM (Figure 3A) [an NRT of 0.76 and an average mass of 18 098.11 Da (3.9 ppm)], while nitrated CaM containing either one or two nTyr residues [but no Met(O)] elutes with NRT values of 0.77 and 0.79 with masses of 18 142.90 Da (7.1 ppm) and 18 187.80 Da (12.6 ppm), respectively (designated as spots 1 and 2, respectively, in Figure 3B). In comparison, oxidation of Met residues to their corresponding Met(O) residues results in a reduction in retention times due to increases in polarity and corresponding increases in mass that range from 16 Da for the oxidation of a single Met to 144 Da upon oxidation of all nine Met residues in CaM. In contrast, nitration of Tyr to form nTyr results in a small increase in the retention time on the reverse-phase column, suggesting that associated protein conformational changes reduce the overall polarity of the nitrated protein. As a result of this increase in retention time upon nitration of Tyr, the relative intensities of homogeneously nitrated CaM species containing no methionine sulfoxides can be unambiguously resolved in the two-dimensional representations of the LC−FTICR data, allowing facile resolution of changes in the relative abundance of these species upon exposure of CaM_{ox} to cell lysates (Figure 3B).

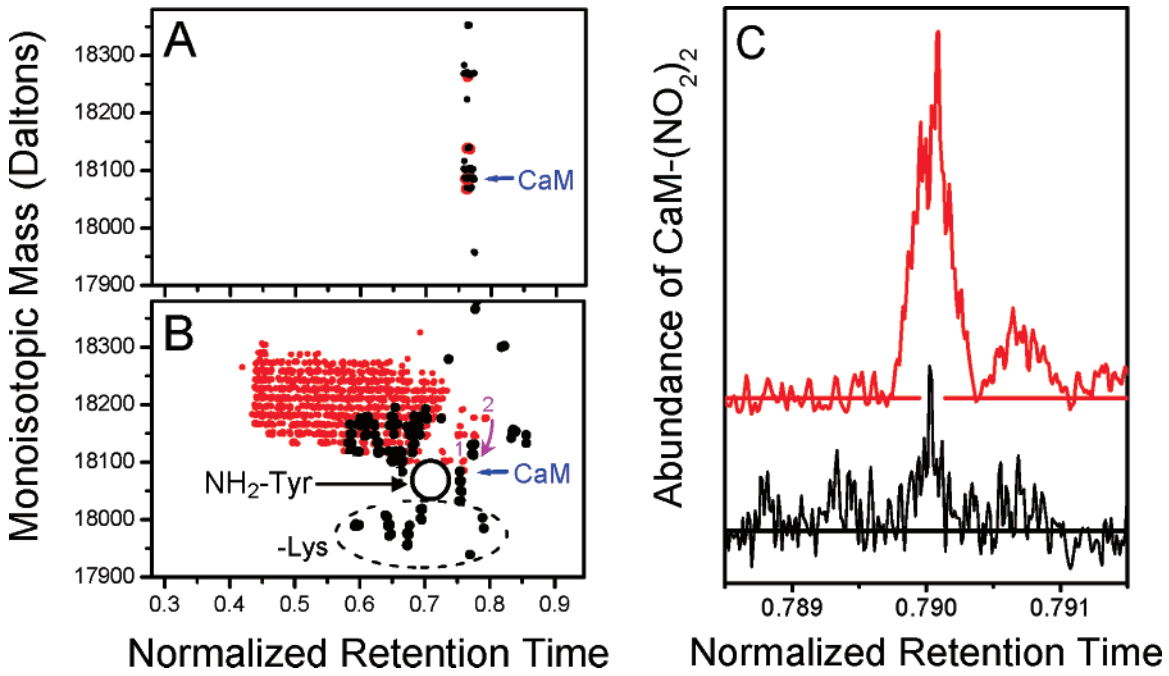


FIGURE 3: LC−FTICR identification of a denitrase activity. Two-dimensional displays depicting resolved intact protein masses of individual CaM species detected using reversed-phase separation for unoxidized CaM (A) or partially oxidized CaM (i.e., CaM_{ox}) (B), either prior to (red symbols) or following (black symbols) incubation with cell lysate from activated macrophages and subsequent purification of His-tagged CaM using Ni^{2+} affinity chromatography. The mass of unoxidized CaM is indicated with blue arrows. Positions are also indicated for nitrated CaM containing one or two nTyr residues, but no Met(O) (i.e., spots labeled 1 and 2 in panel B). Normalized spectral intensities for doubly nitrated CaM species (no oxidized Met residues) are shown in panel C and correspond to mass spectra associated with spot 2 in panel B. Retention times are normalized to allow direct comparisons between subsequent runs using oxidized and unmodified CaM as standards. A solid circle and a dashed oval in panel B represent CaM oxiforms that contain amino-Tyr (not detected) or following cleavage of Lys^{148} (see Figure 4), respectively.

Repair of Nitrotyrosines in CaM. Following incubation of His-tagged CaM_{ox} with cellular lysates, and their purification by Ni^{2+} affinity columns, similar protein yields are obtained for both unoxidized and oxidized CaM from lysates. Most importantly, there is a dramatic reduction in the complexity of the two-dimensional display obtained for CaM_{ox} following incubation with cellular lysates, consistent with the reduction of Met(O) and nTyr (Figure 3B) (see Figures S2 and S3 of the Supporting Information for assignments of individual spectra). The loss of nTyr is apparent from the large reduction in the spectral intensity associated with doubly nitrated CaM (Figure 3C) and is consistent with the substantial reduction in the overall intensity of the CaM oxiforms that include nTyr (species with masses of greater than 18 242 Da) (see Figures S2 and S3 of the Supporting Information). There are corresponding increases in the relative abundances of CaM containing either zero or one nTyr (data not shown). In no case are CaM species observed with amino-Tyr, whose coordinates in the two-dimensional display were identified using an authentic standard (data not shown; the amino-Tyr-containing CaM LC−MS coordinates are indicated as a solid circle in Figure 3B). These latter results indicate that nTyr residues in CaM are substrates for reduction following macrophage activation and rule out their conversion to amino-Tyr, formation of which is possible through both enzymatic and nonenzymatic mechanisms (*23*, *40*, *41*). Thus, these results demonstrate an inducible denitrase activity in activated macrophages that functions to restore nTyrs to their native structure.

C-Terminal Cleavage of Lys^{148} in Oxidized CaM. In addition to substantial amounts of repair, a fraction of CaM_{ox} is cleaved to generate a truncated species with a mass 128 Da below the mass of CaM_{ox} (Figure 3B). In all cases, paired species were identified linking the truncated CaM to a parent species with a mass difference of 128 Da (see Figure S3 of the Supporting Information). These results are consistent with prior observations that the C-terminal Lys^{148} is cleaved in a subpopulation of CaM isolated from oxidatively sensitive regions of the brain (*37*, *38*).

Confirmation of the identity of the cleavage product was accomplished using LC−MS/MS measurements using a more extensively oxidized CaM sample, which increases the abundance of the cleavage product. His-tagged CaM was purified from cellular lysates and digested with trypsin, permitting sequence identification of the C-terminal peptides of CaM by LC−MS/MS (Figure 4). LC−MS/MS data confidently identified eight different peptides derived from the C-terminus of CaM_{ox}. A large portion of the peptides (i.e., 25%) were cleaved at Lys^{148} (Figure 4A), indicating the presence of an endogenous peptidase activity that recognizes CaM_{ox} to selectively cleave the C-terminal Lys^{148}.

DISCUSSION

Summary of Results. Using LC−FTICR to resolve oxidized and nitrated CaM species, we report that nTyr residues in CaM are substrates for a denitrase activity that is upregulated following macrophage activation, which functions to restore the native structure of protein-bound Tyr in CaM (Figures 1−3). Furthermore, oxidized CaM is recognized for cleavage by an endogenous peptidase that selectively cleaves the C-terminal Lys^{148} (Figure 4). Additional

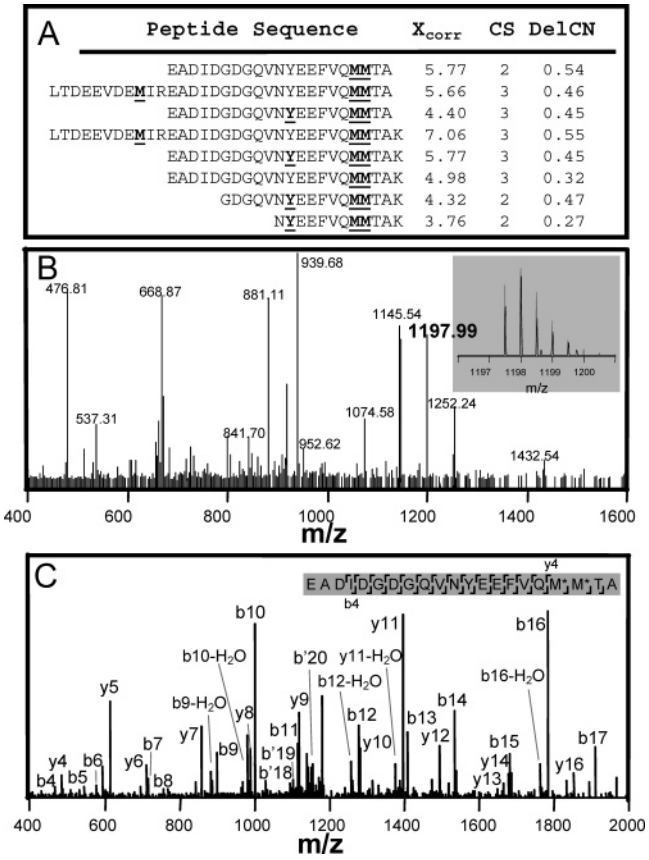


FIGURE 4: Selective cleavage of Lys^{148} from oxidized CaM. (A) C-Terminal peptides identified using LC−MS/MS and SEQUEST analysis of tryptic digests of CaM_{ox} following incubation with cell lysate and affinity purification, as described in the legend of Figure 2. Met(O) and nTyr are underlined and shown in bold. (B) Survey (i.e., parent ion) mass spectrum associated with identification of C-terminal peptide EADIDGDGQVNYEEFVQM(O)M(O)TA (*m*/*z* 1197.494, monoisotopic mass of 2392.971 Da; 1.32 ppm) is in agreement with the theoretical mass (2392.967 Da). The gray inset shows the isotopic envelope of the identified peptide. (C) Corresponding tandem mass spectrum (MS/MS) showing b- and y-ions used for peptide identification. In the schematic, oxidized Met residues are indicated with an asterisk.

reduction of Met(O) in CaM_{ox} involves the action of endogenous methionine sulfoxide reductase enzymes, which act to further reduce the extent of oxidative damage to CaM and restore the native protein fold necessary for efficient calcium signaling (*36*, *42*). Thus, the distribution of CaM species is likely to represent the product of competing pathways involving repair enzymes and proteases, which together modulate calcium signaling in macrophages through the modulation of CaM function.

CaM and Macrophage Activation. CaM represents the central regulator in mediating macrophage activation (*24*, *25*, *43*). Specifically, CaM is responsible for the formation of nitric oxide through the activation and stabilization of iNOS that is necessary for bacterial killing (*24*). Additional regulation is apparent in the CaM-dependent modulation of inflammatory cytokine release (*25*). Furthermore, both Tyr^{99} and Tyr^{138} in CaM are substrates of tyrosine kinases, which function to modulate CaM-dependent target activation (*44*−*47*). Thus, given that nitration can interfere with Tyr phosphorylation by tyrosine kinases (*2*), the presence of a cellular denitrase that is upregulated following macrophage activation is central to the maintenance of tyrosine kinase-dependent signaling pathways under conditions of oxidative stress.

Additional regulation of CaM function is possible through specific structural changes that result from the nitration of Tyr^{138} or proteolysis of Lys^{148} in CaM. Prior measurements indicate that there is a structural uncoupling between the opposing domains of CaM as a result of either the disruption of the hydrogen bond between Tyr^{138} and Glu^{82} or deletion mutations that destabilize the C-terminal helix that contains Met^{144} and Met^{145} (*48*, *49*). These results suggest that either nitration of Tyr^{138} or proteolysis of Lys^{148} is likely to disrupt the structural coupling between the opposing domains of CaM and has the potential to modify the productive association between CaM and different target proteins.

Substrate Specificities of the Denitrase Activity. The denitrase activity identified by Murad and co-workers is known to act selectively on a limited number of target proteins and, in agreement with our measurements, does not recognize the majority of nitrated proteins in activated macrophages (*7*, *8*) (Figure 1, and Figure S1 of the Supporting Information). Histone H1.2 was identified to be an endogenous substrate of the denitrase (*7*). Our observation that CaM is nitrated under cellular conditions and is a substrate for the denitrase represents the second identified physiological substrate for the denitrase. Possible reasons underlying the selectivity of the denitrase against selected target proteins, including CaM, may be related to the accessibility of protein-associated nTyr to the active site of the denitrase, which in many cases may be limited in the native structure. In the case of CaM, we know from prior experiments that there are large reductions in secondary structure following nitration with peroxynitrite that is related to the oxidation of sensitive methionines to their corresponding methionine sulfoxides (*6*, *28*). These results suggest that the denitrase activity may act selectively on nitrotyrosines within conformationally disordered protein sequences, which may explain the inability to identify a general denitrase activity that acts on the majority of nitrated proteins (*7*, *8*).

ACKNOWLEDGMENT

We thank Drs. Tatyana V. Knyushko and Joseph S. Beckman for insightful discussions and Nikola Tolić and Stephen J. Callister for assistance with data analysis.

SUPPORTING INFORMATION AVAILABLE

Additional data mentioned in the text showing immunoblots of the relationship between endogenous nitration and LPS concentrations (Figure S1) and intact protein LC−FTICR data showing resolution of CaM oxiforms with annotation prior to (Figure S2) and following (Figure S3) incubation with activated macrophage lysate. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Lane, N. (2003) A unifying view of ageing and disease: The double-agent theory, *J. Theor. Biol. 225*, 531−540.
2. Jonsson, T. J., Murray, M. S., Johnson, L. C., Poole, L. B., and Lowther, W. T. (2005) Structural basis for the retroreduction of inactivated peroxiredoxins by human sulfiredoxin, *Biochemistry 44*, 8634−8642.

3. Vigneswara, V., Lowenson, J. D., Powell, C. D., Thakur, M., Bailey, K., Clarke, S., Ray, D. E., and Carter, W. G. (2006) Proteomic identification of novel substrates of a protein isoaspartyl methyltransferase repair enzyme, *J. Biol. Chem. 281*, 32619−32629.
4. Grimaud, R., Ezraty, B., Mitchell, J. K., Lafitte, D., Briand, C., Derrick, P. J., and Barras, F. (2001) Repair of oxidized proteins. Identification of a new methionine sulfoxide reductase, *J. Biol. Chem. 276*, 48915−48920.
5. Hoshi, T., and Heinemann, S. (2001) Regulation of cell function by methionine oxidation and reduction, *J. Physiol. 531*, 1−11.
6. Bigelow, D. J., and Squier, T. C. (2005) Redox modulation of cellular signaling and metabolism through reversible oxidation of methionine sensors in calcium regulatory proteins, *Biochim. Biophys. Acta 1703*, 121−134.
7. Irie, Y., Saeki, M., Kamisaki, Y., Martin, E., and Murad, F. (2003) Histone H1.2 is a substrate for denitrase, an activity that reduces nitrotyrosine immunoreactivity in proteins, *Proc. Natl. Acad. Sci. U.S.A. 100*, 5634−5639.
8. Kamisaki, Y., Wada, K., Bian, K., Balabanli, B., Davis, K., Martin, E., Behbod, F., Lee, Y. C., and Murad, F. (1998) An activity in rat tissues that modifies nitrotyrosine-containing proteins, *Proc. Natl. Acad. Sci. U.S.A. 95*, 11584−11589.
9. Gorg, B., Qvartskhava, N., Voss, P., Grune, T., Haussinger, D., and Schliess, F. (2007) Reversible inhibition of mammalian glutamine synthetase by tyrosine nitration, *FEBS Lett. 581*, 84−90.
10. Kong, S. K., Yim, M. B., Stadtman, E. R., and Chock, P. B. (1996) Peroxynitrite disables the tyrosine phosphorylation regulatory mechanism: Lymphocyte-specific tyrosine kinase fails to phosphorylate nitrated cdc2(6−20)NH_2 peptide, *Proc. Natl. Acad. Sci. U.S.A. 93*, 3377−3382.
11. Mallozzi, C., Di Stasi, A. M., and Minetti, M. (2001) Nitrotyrosine mimics phosphotyrosine binding to the SH2 domain of the src family tyrosine kinase lyn, *FEBS Lett. 503*, 189−195.
12. Zhang, Z., Oliver, P., Lancaster, J. J., Schwarzenberger, P. O., Joshi, M. S., Cork, J., and Kolls, J. K. (2001) Reactive oxygen species mediate tumor necrosis factor α-converting, enzyme-dependent ectodomain shedding induced by phorbol myristate acetate, *FASEB J. 15*, 303−305.
13. Gow, A. J., Duran, D., Malcolm, S., and Ischiropoulos, H. (1996) Effects of peroxynitrite-induced protein modifications on tyrosine phosphorylation and degradation, *FEBS Lett. 385*, 63−66.
14. Martin, B. L., Wu, D., Jakes, S., and Graves, D. J. (1990) Chemical influences on the specificity of tyrosine phosphorylation, *J. Biol. Chem. 265*, 7108−7111.
15. Gow, A. J., Farkouh, C. R., Munson, D. A., Posencheg, M. A., and Ischiropoulos, H. (2004) Biological significance of nitric oxide-mediated protein modifications, *Am. J. Physiol. 287*, L262−L268.
16. Kuo, W. N., Kanadia, R. N., and Shanbhag, V. P. (1999) Denitration of peroxynitrite-treated proteins by "protein nitratases" from dog prostate, *Biochem. Mol. Biol. Int. 47*, 1061−1067.
17. Kuo, W. N., Kanadia, R. N., Shanbhag, V. P., and Toro, R. (1999) Denitration of peroxynitrite-treated proteins by 'protein nitratases' from rat brain and heart, *Mol. Cell. Biochem. 201*, 11−16.
18. Koeck, T., Fu, X., Hazen, S. L., Crabb, J. W., Stuehr, D. J., and Aulak, K. S. (2004) Rapid and selective oxygen-regulated protein tyrosine denitration and nitration in mitochondria, *J. Biol. Chem. 279*, 27257−27262.
19. Aulak, K. S., Koeck, T., Crabb, J. W., and Stuehr, D. J. (2004) Dynamics of protein nitration in cells and mitochondria, *Am. J. Physiol. 286*, H30−H38.
20. Aulak, K. S., Koeck, T., Crabb, J. W., and Stuehr, D. J. (2004) Proteomic method for identification of tyrosine-nitrated proteins, *Methods Mol. Biol. 279*, 151−165.
21. Viera, L., Ye, Y. Z., Estevez, A. G., and Beckman, J. S. (1999) Immunohistochemical methods to detect nitrotyrosine, *Methods Enzymol. 301*, 373−381.
22. Sacksteder, C. A., Qian, W. J., Knyushko, T. V., Wang, H., Chin, M. H., Lacan, G., Melega, W. P., Camp, D. G., II, Smith, R. D., Smith, D. J., Squier, T. C., and Bigelow, D. J. (2006) Endogenously nitrated proteins in mouse brain: Links to neurodegenerative disease, *Biochemistry 45*, 8009−8022.
23. Balabanli, B., Kamisaki, Y., Martin, E., and Murad, F. (1999) Requirements for heme and thiols for the nonenzymatic modification of nitrotyrosine, *Proc. Natl. Acad. Sci. U.S.A. 96*, 13136−13141.
24. Smallwood, H. S., Shi, L., and Squier, T. C. (2006) Increases in calmodulin abundance and stabilization of activated inducible nitric oxide synthase mediate bacterial killing in RAW 264.7 macrophages, *Biochemistry 45*, 9717−9726.
25. Weber, T. J., Smallwood, H. S., Kathmann, L. E., Markillie, L. M., Squier, T. C., and Thrall, B. D. (2006) Functional link between TNF biosynthesis and CaM-dependent activation of inducible nitric oxide synthase in RAW 264.7 macrophages, *Am. J. Physiol. 290*, C1512−C1520.
26. Kooy, N. W., Royall, J. A., Ye, Y. Z., Kelly, D. R., and Beckman, J. S. (1995) Evidence for in vivo peroxynitrite production in human acute lung injury, *Am. J. Respir. Crit. Care Med. 151*, 1250−1254.
27. Ye, Y. Z., Strong, M., Huang, Z. Q., and Beckman, J. S. (1996) Antibodies that recognize nitrotyrosine, *Methods Enzymol. 269*, 201−209.
28. Smallwood, H. S., Galeva, N. A., Bartlett, R. K., Urbauer, R. J., Williams, T. D., Urbauer, J. L., and Squier, T. C. (2003) Selective nitration of Tyr99 in calmodulin as a marker of cellular conditions of oxidative stress, *Chem. Res. Toxicol. 16*, 95−102.
29. Strasburg, G. M., Hogan, M., Birmachu, W., Thomas, D. D., and Louis, C. F. (1988) Site-specific derivatives of wheat germ calmodulin. Interactions with troponin and sarcoplasmic reticulum, *J. Biol. Chem. 263*, 542−548.
30. Richman, P. G., and Klee, C. B. (1978) Conformation-dependent nitration of the protein activator of cyclic adenosine 3′,5′-monophosphate phosphodiesterase, *Biochemistry 17*, 928−935.
31. Xiong, Y., Chen, B., Smallwood, H. S., Urbauer, R. J., Markille, L. M., Galeva, N., Williams, T. D., and Squier, T. C. (2006) High-affinity and cooperative binding of oxidized calmodulin by methionine sulfoxide reductase, *Biochemistry 45*, 14642−14654.
32. Shen, Y., Zhao, R., Belov, M. E., Conrads, T. P., Anderson, G. A., Tang, K., Pasa-Tolic, L., Veenstra, T. D., Lipton, M. S., Udseth, H. R., and Smith, R. D. (2001) Packed capillary reversed-phase liquid chromatography with high-performance electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry for proteomics, *Anal. Chem. 73*, 1766−1775.
33. Kelly, R. T., Page, J. S., Luo, Q., Moore, R. J., Orton, D. J., Tang, K., and Smith, R. D. (2006) Chemically etched open tubular and monolithic emitters for nanoelectrospray ionization mass spectrometry, *Anal. Chem. 78*, 7796−7801.
34. Sharma, S., Simpson, D. C., Tolic, N., Jaitly, N., Mayampurath, A. M., Smith, R. D., and Pasa-Tolic, L. (2007) Proteomic profiling of intact proteins using WAX-RPLC 2-D separations and FTICR mass spectrometry, *J. Proteome Res. 6*, 602−610.
35. Washburn, M. P., Wolters, D., and Yates, J. R., III (2001) Large-scale analysis of the yeast proteome by multidimensional protein identification technology, *Nat. Biotechnol. 19*, 242−247.
36. Sun, H., Gao, J., Ferrington, D. A., Biesiada, H., Williams, T. D., and Squier, T. C. (1999) Repair of oxidized calmodulin by methionine sulfoxide reductase restores ability to activate the plasma membrane Ca-ATPase, *Biochemistry 38*, 105−112.
37. Isobe, T., Ishioka, N., and Okuyama, T. (1981) Isolation and characterization of des(Ala-Lys)calmodulin in porcine brain, *Biochem. Biophys. Res. Commun. 102*, 279−286.
38. Murtaugh, T. J., Wright, L. S., and Siegel, F. L. (1986) Posttranslational modification of calmodulin in rat brain and pituitary, *J. Neurochem. 47*, 164−172.
39. Gao, J., Yin, D. H., Yao, Y., Sun, H., Qin, Z., Schoneich, C., Williams, T. D., and Squier, T. C. (1998) Loss of conformational stability in calmodulin upon methionine oxidation, *Biophys. J. 74*, 1115−1134.
40. Chen, H. J., Chen, Y. M., and Chang, C. M. (2002) Lipoyl dehydrogenase catalyzes reduction of nitrated DNA and protein adducts using dihydrolipoic acid or ubiquinol as the cofactor, *Chem.-Biol. Interact. 140*, 199−213.
41. Fang, F. C. (2004) Antimicrobial reactive oxygen and nitrogen species: Concepts and controversies, *Nat. Rev. Microbiol. 2*, 820−832.
42. Ferrington, D. A., Sun, H., Murray, K. K., Costa, J., Williams, T. D., Bigelow, D. J., and Squier, T. C. (2001) Selective degradation of oxidized calmodulin by the 20S proteasome, *J. Biol. Chem. 276*, 937−943.
43. Squier, T. C. (2006) Redox modulation of cellular metabolism through targeted degradation of signaling proteins by the proteasome, *Antioxid. Redox Signaling 8*, 217−228.
44. Benguria, A., Hernandez-Perera, O., Martinez-Pastor, M. T., Sacks, D. B., and Villalobo, A. (1994) Phosphorylation of calmodulin

by the epidermal-growth-factor-receptor tyrosine kinase, *Eur. J. Biochem. 224*, 909−916.
45. Joyal, J. L., Crimmins, D. L., Thoma, R. S., and Sacks, D. B. (1996) Identification of insulin-stimulated phosphorylation sites on calmodulin, *Biochemistry 35*, 6267−6275.
46. Sacks, D. B., Fujita-Yamaguchi, Y., Gale, R. D., and McDonald, J. M. (1989) Tyrosine-specific phosphorylation of calmodulin by the insulin receptor kinase purified from human placenta, *Biochem. J. 263*, 803−812.
47. Sacks, D. B., Mazus, B., and Joyal, J. L. (1995) The activity of calmodulin is altered by phosphorylation: Modulation of calmodulin function by the site of phosphate incorporation, *Biochem. J. 312* (Part 1), 197−204.
48. Sun, H., Yin, D., Coffeen, L. A., Shea, M. A., and Squier, T. C. (2001) Mutation of Tyr138 disrupts the structural coupling between the opposing domains in vertebrate calmodulin, *Biochemistry 40*, 9605−9617.
49. Yin, D., Sun, H., Ferrington, D. A., and Squier, T. C. (2000) Closer proximity between opposing domains of vertebrate calmodulin following deletion of Met(145)-Lys(148), *Biochemistry 39*, 10255−10268.

BI7009713