

Intact Protein LC-FTICR MS Identifies Dynamics of Post Translational Modifications to Calmodulin in Macrophages

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Overview

Capillary RPLC coupled with 12 tesla FTICR MS was employed to study the effect of oxidative stress on the versatile calcium signaling protein calmodulin (CaM).

Intact protein RPLC-FTICR MS data confirmed substantial loss of nitrotyrosine in the pure protein post-incubation with the lipopolysaccharide (LPS) activated macrophage lysate, in agreement with earlier immunoblot data.

The nature of the modification clearance appears to be cleavage by cellular degradation machinery, possibly regulated by C-terminal lysine cleavage. Reversion of oxidized methionine and nitrated tyrosine to their native states was observed to a small extent, while the reduction to amino tyrosine was not observed.

Introduction

Tyrosine (Y) phosphorylation is an essential part of cellular signaling and nitrotyrosine (nY) formation can block or mimic phosphorylation. In addition to altering phosphorylation signaling, it has recently been argued that tyrosine nitration is also a dynamic process leading to cellular signaling in radical rich environments such as the mitochondria [1]. The dynamic processes of nitration and denitration, dependent upon specific cellular conditions, lead some to speculate upon the existence of a denitrase enzyme [2]. Here we show that macrophages, which produce radicals, have the capacity to nitrate and denitrinate the ubiquitous calcium sensor protein CaM.

To further clarify these results and quantify nitration, denitration and determine specificity, intact protein FTICR MS has been employed (see Figure 1 for samples analyzed). Both tyrosines in CaM are nitrated (nY), and this is typically accompanied by oxidation of some of the nine methionines to methionine sulfoxide (Mox) [3]. However, due to Mox formation in close proximity of the nY, the tryptic peptides are a complicated heterogeneous mixture of modified fragments that diminishes the overall signal resulting in a loss of resolution and challenging our ability to quantify nitration. Besides, in the context of the biological relevance, it would be preferable to determine modification patterns across the whole protein. Since multiple Tyr nitration and Met oxidations give rise to complex mixture possibly containing 2,048 (2^{11}) chemically similar forms of modified CaM, we opted to use RPLC-FTICR mass spectrometry for the analysis of *in vitro* modified CaM purified from LPS activated macrophage lysate.

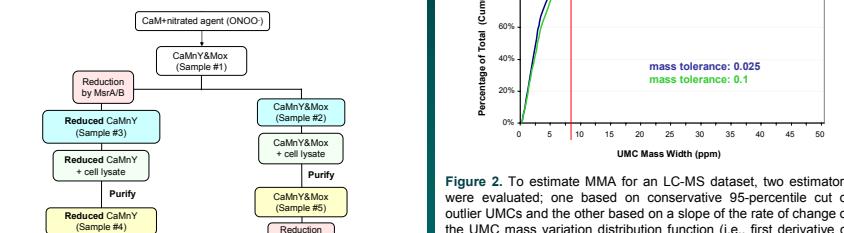


Figure 1. Experimental workflow outlining samples prepared for LC-FTICR intact protein analysis.

Methods

FTICR MS and capillary RPLC-FTICR MS

Intact protein MS analyses were performed on a modified Bruker 12T APEX-Q FTICR mass spectrometer that incorporates an ion funnel interface, various ion transfer, selection and storage multipoles.

Intact protein RPLC was performed at 8000 psi on a 80 cm \times 75 μ m column packed in-house with Phenomenex Jupiter particles (C5 stationary phase, 5 μ m particle diameter, 300 Å pore size). Mobile phase A consisted of 0.1% trifluoroacetic acid, 25% acetonitrile, 74.9% water; mobile phase B consisted of 0.1% trifluoroacetic acid, 9.9% water, 90% acetonitrile.

Sample preparation

CaM and Methionine sulfoxide reductase B/A were cloned, expressed, and purified under standard conditions, both with polyhistidine tags on the C-terminus for nickel affinity purification, and subjected to either MS analysis or modification by peroxynitrite for further experimental processing.

RAW 264.7 cells were cultured in suspension and by adherence in RPMI complete medium supplemented with 10% heat inactivated fetal bovine serum and 1% mixture of penicillin and ampicillin. The medium was collected and treated with 5 mg LPS per milliliter of medium, then redistributed equally among plates. Cells were lysed, a 1:50 ratio of *in vitro* modified CaM was added to the lysate and the mixture was incubated for 4 hours at 37°C. This was followed by purification of the CaM from the lysates as previously described above.

Data processing

Intact protein mass spectra were processed using in-house developed software to generate lists of neutral monoisotopic masses. A 2D display (mass vs. spectrum number) was generated to allow filtering of individual masses into unique mass classes (UMCs) based on several criteria such as the monoisotopic mass, abundance, isotopic fit and spectrum number (relating to LC retention time). LC-MS coordinates were used for protein and modified protein identification with mass measurement accuracy (MMA) <10 ppm. To estimate MMA for an LC-MS dataset, we analyzed the mass variation of the UMCs as described in Figure 2.

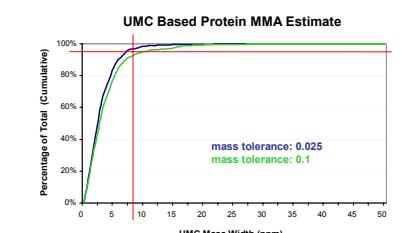


Figure 2. To estimate MMA for an LC-MS dataset, two estimators were evaluated; one based on conservative 95-percentile cut of outlier UMCs and the other based on a slope of the rate of change of the UMC mass variation distribution function (i.e., first derivative of the UMC mass variation distribution function). The latter method appears advantageous since it is invariant of the clustering parameters, but the former method yields similar results for highly optimized UMCs.

Results

Dynamics of Calmodulin Post Translational Modifications in Macrophages

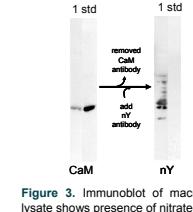


Figure 3. Immunoblot of macrophage lysate shows presence of nitrated CaM.

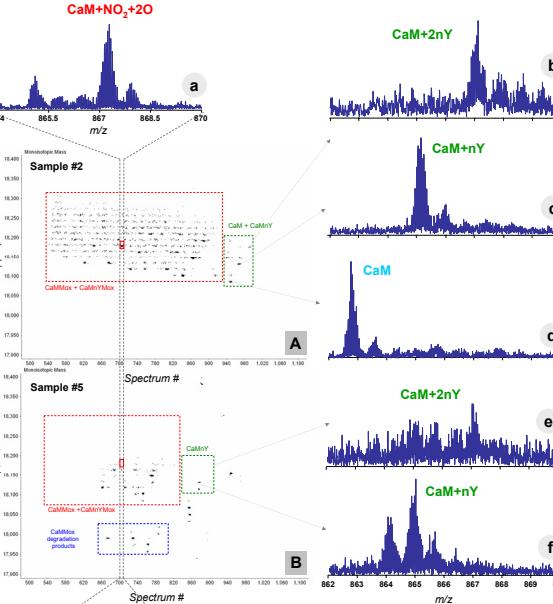


Figure 4. Immunoblot of CaMnY after incubation with macrophage lysate shows loss of nitration.

A substantial loss of nitrotyrosine post incubation with the lysate was observed; this is in agreement with earlier immunoblot data (i.e., Figure 4). The exposure to macrophage lysate also induces a substantial reduction of the oxidized CaM, accompanied by the loss of C-terminal lysine. (The loss of water and/or ammonia was observed in some instances due to activation from the increased pressure needed for efficient accumulation of low abundant species in an external hexapole.)

If nitrated proteins are denitrated by an enzyme, does reversion from nitrotyrosine to tyrosine or reduction to amino tyrosine occur?

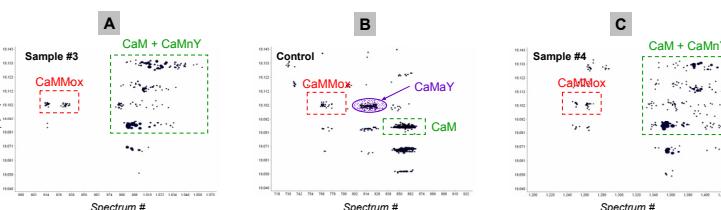


Figure 5. Portions of 2D display obtained for CaMnY&Mox samples reduced with MsrA/B prior (A) and after (B) chemical reduction (dithionite) of nitrotyrosine to amino tyrosine (aY) in comparison to 2D display obtained for CaMnY&Mox reduced with MsrA/B after exposure to LPS activated macrophage lysate (C). Note that the formation of amino tyrosine was not observed after exposure to lysate (C).

Does the loss of C-terminal lysine regulate the degradation of modified CaM?

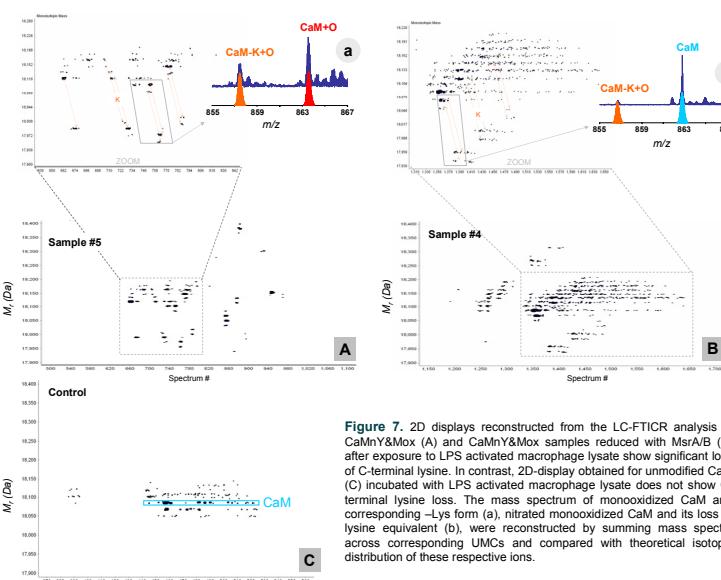


Figure 6. 2D displays reconstructed from the LC-FTICR analysis of CaMnY&Mox (A) and CaMnY&Mox samples reduced with MsrA/B (B) after exposure to LPS activated macrophage lysate show significant loss of C-terminal lysine. In contrast, 2D display obtained for unmodified CaM (C) incubated with LPS activated macrophage lysate does not show C-terminal lysine loss. The mass spectrum of monooxidized CaM and corresponding -Lys form (a), nitrated monooxidized CaM and its loss of lysine equivalent (b), were reconstructed by summing mass spectra across corresponding UMCs and compared with theoretical isotopic distribution of these respective ions.

We find a concomitant C-terminal lysine cleavage occurring selectively to modified CaM. The specificity of this cleavage may function to regulate oxidatively modified CaM. Des(Lys)calmodulin, lacking the carboxy terminal lysine residue of calmodulin has previously detected after incubation of calmodulin with cytosolic extracts of brain and anterior pituitary region [4].

Conclusions

Preliminary LC-FTICR data indicate repair of modified CaM. The biological implication for this is that when LPS causes a bacteriocidal burst of radicals within the macrophage, macrophage proteins are also oxidized and nitrated, resulting in up-regulation of repair enzymes to address the unintended intracellular damage.

The nature of the modification clearance appears to be reversion from nitrotyrosine to tyrosine (instead of reduction to amino tyrosine) accompanied with the loss of C-terminal lysine which, we hypothesize, induces cleavage of modified CaM by cellular degradation machinery.

To determine how much of various oxi- and nitro-CaM forms is lost, we initially employed "label-free" quantitation (i.e., direct comparison of ion abundances). To improve quantitation accuracy, we plan to use doubly labeled (¹³C,¹⁵N) CaM as an "internal calibrant".

We plan to employ "on-the-fly" LC-MS/MS (with CID and ECD) to thoroughly characterize various oxi- and nitro- forms of CaM. Such information will be critical for in-depth characterization of nitration clearance events (e.g., which species are lost; is the Lys cleavage dependant; does Lys cleavage mark the CaM for degradation; etc.).

Alternative separation strategies (e.g., FAIMS) coupled with FTICR will be explored.

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