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Calorimetry and mass spectrometry study of oxidized calmodulin interaction with target and differential repair by methionine sulfoxide reductases

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Abstract

Calmodulin is known to be a target for oxidation, which leads to conversion of methionine residues to methionine sulfoxides. Previously, we reported that both methionine sulfoxide reductases MsrA and MsrB were able to reduce methionine sulfoxide residues in oxidized calmodulin. In the present study, we have made use of the interaction between calmodulin and RS20, a peptide model for calmodulin targets, to probe the structural consequences of oxidation and mode of repair both by MsrA and MsrB. Isothermal titration calorimetry and differential scanning calorimetry showed that oxidized calmodulin interacts with RS20 via its C-terminal domain only, resulting in a non-productive complex. As shown by spectrofluorometry, oxidized calmodulin treated with MsrA exhibited native binding affinity for RS20. In contrast, MsrB-treatment of oxidized calmodulin resulted in 10-fold reduced affinity. Mass spectrometry revealed that the sulfoxide derivative of methionine residue 124 was differentially repaired by MsrA and MsrB. This provided a basis for rationalizing the difference in binding affinities of oxidized calmodulin reported above, since Met124 residue had been shown to be critical for interaction with some targets. This study provides the first evidence that in an oxidized polypeptide chain MetSO residues might be differentially repaired by the two Msr enzymes.

Keywords: Calmodulin; RS20 target peptide; Oxidation; Reparation; Methionine sulfoxide reductases

1. Introduction

Reactive oxygen species (ROS) are paradoxical molecules since they are essential for the regulation of cellular functions but also toxic by-products of aerobic metabolism causing damage to proteins, lipids, and DNA (for a review see [1]). Cells are protected from these damages by several anti-oxidant systems. Imbalance between the production and degradation of ROS yields the so-called oxidative stress which is involved in many pathologies such as neurodegenerative diseases, cancer or atherosclerosis [2]. ROS are able to diffuse across membranes and modify amino acids of a protein. Cysteins, tyrosines, and methionines can be modified depending on pH and ROS concentration [3]. Methionine is the most sensitive residue to oxidation, which reversibly converts it to methionine sulfoxide (MetSO) [4]. The product of methionine oxidation is a diastereoisomeric mixture of methionine-*S*-sulfoxide (MetS^SO) and methionine-*R*-sulfoxide (MetS^RO).

Abbreviations: CaM, calmodulin; CaMox, oxidized calmodulin; apo-CaM, calcium-free calmodulin; SynCaM, hybrid calmodulin; MLCK, myosin light chain kinase; MetSO, methionine sulfoxide; MetS^SO, methionine-*S*-sulfoxide; MetS^RO, methionine-*R*-sulfoxide; Msr, methionine sulfoxide reductase; ITC, isothermal titration calorimetry; DSC, differential scanning calorimetry; ESI, electrospray ionization; FTICR, Fourier transform ion cyclotron resonance.

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Repair of protein-bound MetSO is catalyzed by methionine sulfoxide reductases (Msr). These enzymes, present in all living organisms [5], belong to two classes, referred to as MsrA and MsrB [6–8]. Recent studies established that MsrA and MsrB are specific for $MetS^SO$ and $MetS^RO$, respectively [7–9]. This diastereo-specificity accounts for the fact that both MsrA and MsrB are required for reduction of all MetSO present in oxidized calmodulin [6].

Calmodulin (CaM), one of the most important signal transducers in cells [10,11], is very sensitive to oxidation and oxidized CaM (CaMox) is found in the brain of elderly animals [12]. CaM is unusually rich in methionines which are essential for CaM folding and interaction with the targets (Fig. 1) [13]. As an example, nearly all Met side chains participate in the binding to the myosin light chain kinase (MLCK) [14]. A number of studies have been undertaken to demonstrate the consequences of oxidative stress to CaM structure and function [12,15–17]. Oxidation of CaM leads to a drastic decrease in the stability of both domains, alters the cooperativity between the calcium binding sites [15,18] and diminishes the CaM ability to activate target enzymes, for example, the plasma membrane Ca^{2+}-ATPase [17,19,20].

We have shown previously that MsrA and MsrB act on oxidized CaM, each by repairing 4–6 of the eight MetSO residues initially present, and, as demonstrated by Fourier transform ion cyclotron resonance (FTICR) mass spectrometry, CaMox is fully repaired by the combined action of MsrA and MsrB [6]. As observed by Squier et al. [21], MsrA restores the ability of oxidized CaM to activate the plasma membrane Ca^{2+}-ATPase.

In this paper, we examine (i) the influence of oxidation on the interaction of CaM domains with a 20-residue-long peptide RS20 corresponding to the CaM-binding domain of MLCK, and (ii) the separate and simultaneous MsrA and MsrB repairing actions on the recovery of oxidized CaM ability to activate the target. We clearly demonstrate that only C-terminal domain of CaMox interacts with the target peptide, which explains why oxidized CaM lose the ability for target activation. Combined action of MsrA and MsrB completely restores CaM functionality. Separate action of MsrA or MsrB produces functionally different calmodulins, which was found to correlate with differential reduction of the C-terminal Met124.

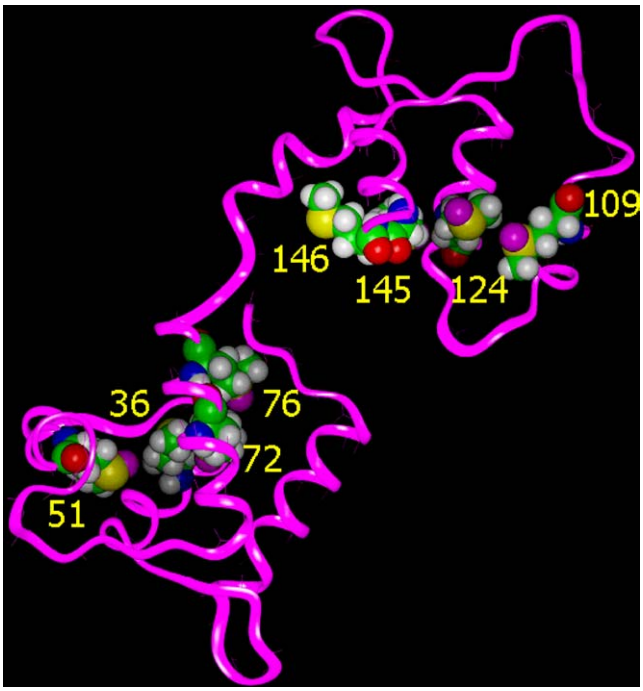

Fig. 1. Distribution of methionines in the SynCaM model. The model was created with the help of the PDB file 1CFD, amino acids from mammalian calmodulin were replaced by those of SynCaM.

2. Materials and methods

2.1. Proteins

Hybrid of mammalian and plant CaM (SynCaM) was expressed and purified as described previously [22]. It contains eight methionines thoroughly distributed through the molecule (Fig. 1). Protein concentration was measured with an extinction coefficient of 1560 M^{-1} cm^{-1} at 280 nm [23]. CaM was decalcified using TCA treatment prior to oxidation [24]. Batches of 10 mg of calcium-free CaM (apoCaM) were oxidized for 4.5 h in the presence of 50 mM H_2O_2. To remove H_2O_2, gel filtration through G-25 Sephadex equilibrated with 50 mM Hepes, pH 7.5 was performed. The 20-residue peptide RS20 corresponding to the CaM-binding domain of the myosin light chain kinase was synthesized and purified as previously described [25]. Peptide concentration was measured using an extinction coefficient of 5600 M^{-1} cm^{-1} at 280 nm. MsrA and MsrB were expressed and purified as previously described [6]. CaM reparation was performed according to [6]. Oxidation and reparation were performed on apoCaM.

2.2. Tryptic digestion of CaM

Oxidized calmodulins repaired by one or another Msr were digested using sequencing grade porcine trypsin (12 ng/μl, Promega). The peptides were extracted and dried in a vacuum centrifuge and redissolved in 50 μl of 50/50 v/v acetonitrile/water, 1% formic acid before analysis. Monoisotopic masses were obtained by FTICR mass spectrometry.

2.3. Electrospray FTICR mass spectrometry

Measurements were made using an FTICR mass spectrometer (Bruker Daltonics) equipped with a passively shielded 9.4 superconducting magnet (Magnex Scientific Ltd.), a cylindrical infinity ICR cell with 0.06-m diameter and an external ESI source (Analytica of Brandford). The ESI source was equipped with a pyrex capillary which was coated on both ends with platinum paint. Carbon dioxide was used as the drying gas in the electrospray source, and its temperature was carefully controlled. The background pressure in the ICR ana-

lyzer was typically below 2×10^{-13} bar. Flow rate was 10 μl/min [26].

2.4. Isothermal titration calorimetry (ITC)

Thermodynamic parameters of peptide binding to oxidized and repaired CaM were measured using a MicroCal MCS instrument. Experiments were carried out at 25 °C in 50 mM Hepes, 2 mM $CaCl_2$, pH 7.5. CaM concentration in the cell ranged from 0.02 to 0.06 mM and peptide concentration in the syringe ranged from 0.2 to 0.8 mM. Heats of dilution were measured by injecting the ligand into the buffer solution and injecting the buffer into the protein solution. These heats were subtracted from the experimental curves prior to data analysis. Titration curves were fitted using MicroCal Origin software, assuming one set of sites. Affinity constants (K_a), enthalpy changes (ΔH) and stoichiometry (N) were obtained. Consequently, the Gibbs energy (ΔG) and the entropy variations (ΔS) were calculated according to the standard equations.

2.5. Differential scanning calorimetry (DSC)

Heat denaturation measurements were carried out on a MicroCal VP-DSC instrument in 0.51-ml cells at a heating rate of 1 K/min. Experiments were performed in 10 mM Hepes or cacodylate buffer, pH 7.5 with 1 mM $CaCl_2$ or 10 mM EGTA. Protein concentrations varied from 1.0 to 2.5 mg/ml. Curves were corrected for the instrumental baseline obtained by heating the solvent used for protein solution. The reversibility of denaturation was checked routinely by sample reheating after cooling in the calorimetric cell. The calorimetric denaturation enthalpy (ΔH_{cal}) and the partial molar heat capacity of the protein (C_p) were determined as described elsewhere [27], assuming that the molecular mass of CaM (CaMox) is 16628 (16756) Da and the partial specific volume is 0.72 cm^3/g. To analyze functions of excess heat capacity, MicroCal Origin software was used. The accuracy of the calorimetric denaturation enthalpy was 8%. The errors in the temperatures of individual transitions obtained by deconvolution of complex endotherms did not exceed 0.4 °C.

2.6. Fluorescence

Titrations of RS20 peptide with CaM, CaMox, and repaired CaM were performed using 300 nM RS20 in 50 mM Hepes, 1 mM $CaCl_2$, pH 7.5 on a Kontron SFM 25 spectrofluorimeter with slit widths of 5/5 nm, using 0.2 cm (excitation direction) and 1 cm (emission direction) cells (Hellma) thermostated at 25 °C, with excitation at 280 nm and emission scanned from 300 to 500 nm. RS20 is known to undergo a blue shift and an increase in fluorescence signal in presence of CaM [23]. This property was used to measure RS20 binding to different forms of CaM. Spectra were not further corrected since the concentrations used gave no appreciable inner filter effect ($A < 0.05$ at any wavelength used). Then the fluorescence intensities at 330 nm were fitted to the saturation curve equation by means of non-linear least-squares regression analysis as described in [28].

3. Results

3.1. Characterization of CaMox

Standard conditions of oxidation led to a partial oxidation of CaM. As detected by electrospray FTICR mass spectrometry, several species were found in the mixture with 5–8 methionines oxidized to their corresponding sulfoxide, in agreement with the previously published data [6]. Peptide mass fingerprinting showed that the C-terminus half of CaM was fully oxidized while the N-terminus contained most of the reduced methionines. This result is in agreement with Yuan et al. [14] who found that the Met methyl groups in apo-CaM are fully buried in the N-lobe and partially exposed in the C-lobe. The binding of Ca^{2+} to CaMox was drastically modified; saturation of CaMox occurred at higher Ca^{2+} level (ITC data not shown). Therefore, 1–2 mM $CaCl_2$ was used to ensure the complete saturation of CaMox.

3.2. RS20 peptide binding to native and oxidized Ca^{2+}–CaM

A typical set of ITC data for the RS20 interaction with the Ca^{2+}–CaM is shown in Fig. 2A. The higher panel presents the raw calorimetric data for the ligand-into-protein titration and the lower panel presents the binding isotherm. The binding affinity between native Ca^{2+}–CaM and RS20 was too close to the experimental ITC limits and could be underestimated [29]. The binding affinity was thus obtained by fluorescence and the binding enthalpy was obtained by ITC which allowed calculation of the thermodynamic parameters (Table 1). Thermodynamic data show that there is a one-to-one complex between RS20 and Ca^{2+}–CaM.

The binding isotherm and the fluorometric titrations of RS20 to Ca^{2+}–CaMox were monophasic (Fig. 2B). Therefore, taking into account the heterogeneity of oxidized population, we provide a mean value, which is in fact an apparent binding constant (Table 1). The stoichiometry was always slightly under one, clearly showing that a small part of the mixture is not able to bind the peptide. The binding affinity of Ca^{2+}–CaMox for RS20 was 17 times weaker than the value obtained for the native CaM (Table 1). The binding enthalpy of the peptide interaction with Ca^{2+}–CaMox was less than that for the native CaM by a factor of 1.7.

From the ITC data we can assume that, opposite to native CaM, CaMox interacts with the target by only one lobe. To test this supposition and to define the domain interacting with the target in CaMox, we used DSC to follow the individual domains of a protein. Temperature dependence of the partial molar heat capacity of Ca^{2+}–CaMox exhibited two peaks cor-

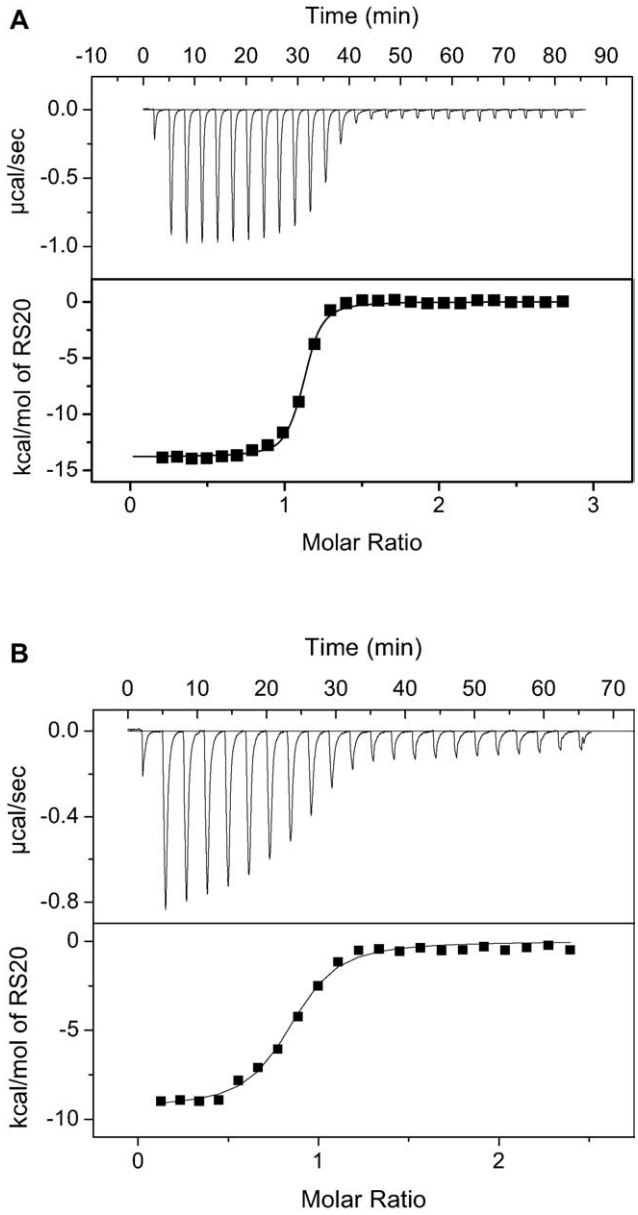

Fig. 2. ITC data curves and binding isotherms of RS20 interaction with Ca^{2+}–CaM (A) and Ca^{2+}–CaMox (B).

responding to the denaturation of the C- (first peak) and N-terminal (second peak) domains of CaM [30,31] (Fig. 3). The reversibility was almost complete for both peaks. Upon complex formation with RS20 the temperature of the first calorimetric peak increased by 10.1 °C while the temperature of the second peak was not changed (Table 2). In addition, the enthalpy of the first peak increased by a factor of two (Fig. 3). These data are a proof of the RS20 peptide binding to the C-terminal lobe of CaMox.

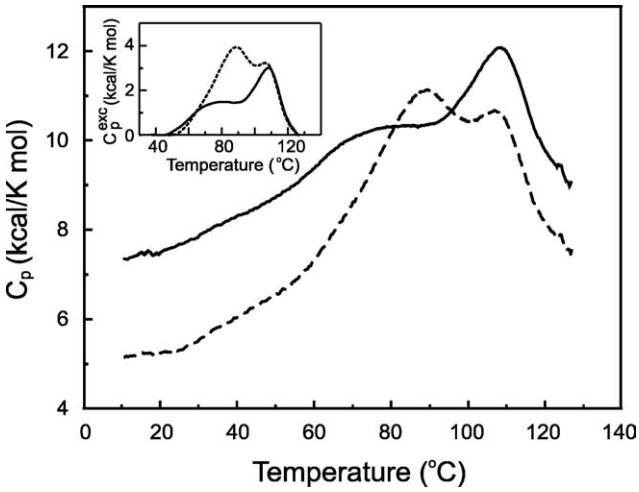

Fig. 3. Temperature dependence of the partial molar heat capacity of Ca^{2+}–CaMox (solid line) and its complex with RS20 peptide (dotted line). The inset shows the temperature dependences of the excess heat capacity of CaMox (solid line) and CaMox–RS20 (dotted line).

3.3. Reparation by MsrA

Oxidation was performed on the apostate of CaM, the basic physiological relevant state of CaM, for which it has been reported that Met124 and Met144 are partially exposed while Met109 and Met145 are fully buried [14]. The apoform was then subjected to reparation. Contrary to binding of Ca^{2+} (data not shown), the thermodynamic parameters of RS20 binding to Ca^{2+}–CaMox repaired by MsrA are equal to those obtained for peptide binding on native CaM (Fig. 4, Table 1), although methionine sulfoxides are still present in the repaired protein, as demonstrated by Grimaud et al. [6] and by mass spectrometry in the present work (data not shown). DSC experiments carried out on apoCaMox treated with MsrA showed a complete recovery of the C-terminal domain thermostability while this parameter was not modified for the N-terminal domain (Table 2).

3.4. Reparation by MsrB

MsrB is also able to repair some of the CaM methionine sulfoxides [6]. The mass spectrum of oxidized CaM obtained

Table 1
Thermodynamic parameters of RS20 peptide binding to Ca^{2+}-calmodulin, to oxidized calmodulin, and to oxidized calmodulin after repair by MsrA or MsrB or by both enzymes simultaneously at pH 7.5

Sample	K_a (m^{-1})	ΔG (kJ/mol)	ΔH (kJ/mol)	$T\Delta S$ (kJ/mol)	N
CaM [a]	$3.8 \pm (0.7) \times 10^7$	–43.3 ± 0.9	–55.3 ± 2.9	–11.8 ± 3.0	1.1 ± 0.2
CaMox [b]	$2.2 \pm (0.4) \times 10^6$	–36.3 ± 1.2	–32.4 ± 4	3.9 ± 4.1	0.7 ± 0.1
CaMox + MsrA [a]	$3.1 \pm (0.9) \times 10^7$	–42.8 ± 1.8	–56.3 ± 3.2	–13.5 ± 3.2	0.7 ± 0.2
CaMox + MsrB [b]	$4.1 \pm (0.9) \times 10^6$	–37.8 ± 1.4	–54.7 ± 2.7	–17.7 ± 3.0	0.8 ± 0.1
CaMox + MsrAB [a]	$2.1 \pm (0.3) \times 10^7$	–41.9 ± 2.0	–58.1 ± 3.7	–16.2 ± 4.2	0.9 ± 0.1

[a] K_a values were determined only by fluorescence.
[b] K_a values were determined by fluorescence and ITC.

Table 2
Thermal denaturation parameters of oxidized calmodulin, its complex with RS20 peptide, and oxidized apocalmodulin after repair by MsrA or by MsrB or by both enzymes simultaneously at pH 7.5

Sample	ΔH_{cal} [a] (kJ/mol)	T_d^1 (°C)	T_d^2 (°C)
Ca^{2+}–CaMox[b]	450	78.2	108.0
Ca^{2+}–CaMox–RS20[b]	660	88.3	108.5
ApoCaM[c]	276	48.4	64.0
ApoCaMox[c]	130	45.8	62.0
ApoCaMox + MsrA[c]	150	48.7	62.3
ApoCaMox + MsrB[c]	170	47.9	63.0
ApoCaMox + MsrAB[c]	274	49.5	64.0

Transitions were determined by deconvolution analysis of the heat absorption curves to non-two-state transitions [27].
[a] Total calorimetric enthalpy of denaturation.
[b] 10 mM Hepes, 1 mM $CaCl_2$.
[c] 10 mM cacodylate, 150 mM KCl, 10 mM EGTA.

after reparation with this enzyme is similar to the one obtained after reparation with MsrA. Therefore, it was tempting to conclude that both Msr repaired CaM in the same manner [6]. The Ca^{2+} binding thermogram after CaMox reparation with MsrB is exactly the same as the one obtained after reparation with MsrA (data not shown). The thermodynamic parameters of RS20 binding to CaM showed that the binding enthalpies were equal for the oxidized protein repaired by MsrA or MsrB, while the binding constant is about 10 times lower for CaM repaired by MsrB (Fig. 4, Table 1). DSC of apoCaMox repaired by MsrB showed that the thermostability of the C-terminal domain was practically restored which is associated with a slight increase in thermostability of the N-terminal domain (Table 2).

3.5. Differential repair of CaMox methionine sulfoxides

The difference in the binding affinity for RS20 between oxidized CaMs repaired by MsrA or MsrB may rely on a different repair of some methionine sulfoxides. To give credit to this hypothesis, we analyzed by electrospray FTICR mass spectrometry the tryptic digests of CaMox repaired by one or the other Msr. Table 3 contains the peptides resulting from the digestion of CaMox. When a tryptic peptide contains a

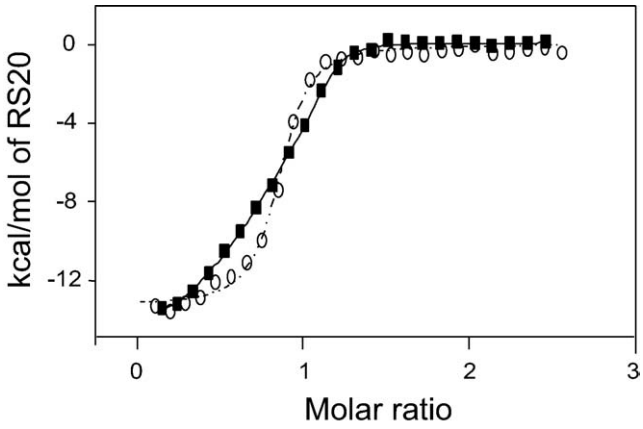

Fig. 4. ITC binding isotherms of RS20 interaction with Ca^{2+}–CaM repaired by MsrA (chain line) or MsrB (solid line).

Table 3
Peptides obtained by tryptic digestion of oxidized calmodulin

Residues	Measured monoisotopic mass (Da)	Sequence
1–13	1506.7	ADQLTDEQIAEFK
14–21	955.4	EAFSLFDK
22–30	906.4	DGDGTITTK
31–37	804.4, 820.4 [a]	ELGTVMR
38–74	4063.8, 4079.8 [a], 4095.8 [b]	SLGQNPTEAELQDMINEVD ADGNGTIDFPEFLNLMAR
75	146.2	K
76–77	277.1, 293.1 [a]	MK
78–86	1064.4	DTDSEEELK
87–90	521.2	EAFR
91–94	507.3	VFDK
95–106	1248.6	DGNGFISAAELR
107–115	1027.5, 1043.5 [a]	HVMTNLGEK
116–126	1348.6, 1364.6 [a]	LTDEEVDEMIR
127–148	2473.0, 2489.0 [a], 2505.0 [b]	EADVDGDGQVNYEEFVQ VMMAK

[a] One methionine oxidized.
[b] Two methionines oxidized.

methionine two peaks are detected: the first one corresponding to the mass of the reduced methionine and the second one to the mass of the oxidized (+16 Da). If two methionines are present, up to three peaks can be detected (+16 and +32 Da). For each peptide containing methionine the ratio between its reduced and oxidized form was determined. Only Met124 shows a significant difference in the ratio reduced/oxidized. While on average MsrA repairs 50% of Met124SO species, MsrB repairs only 25% (Fig. 5). For all other methionines each enzyme repairs on average 50% of the oxidized form.

3.6. Combined effect of MsrA and MsrB

As previously shown by mass spectrometry, no methionine sulfoxides were detected after CaMox reparation by the

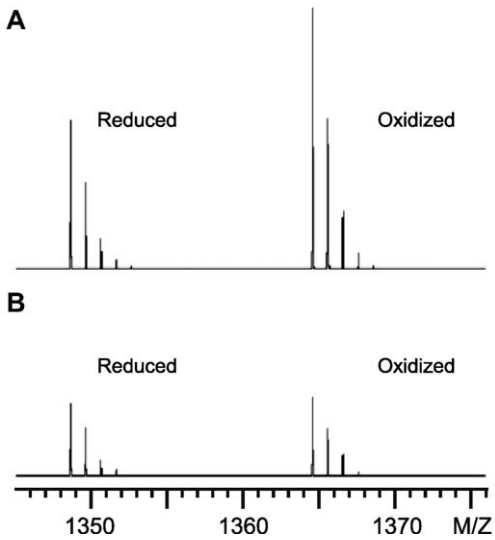

Fig. 5. ESI-FTICR mass spectra of tryptic peptide 116–126 of CaMox repaired by MsrB (A) and MsrA (B).

combined action of MsrA and MsrB [6]. Thermodynamic parameters of Ca^{2+} binding to CaMox (data not shown) and RS20 binding to Ca^{2+}–CaMox repaired by both Msr are similar to those of native CaM (Table 1). As shown by DSC, the synergetic action of both enzymes on oxidized apoCaM leads to a total recovery of the thermostability of both domains and total enthalpy of denaturation (Table 2). Thus, the thermodynamic parameters confirm the restoring of CaM functionality under the joint action of MsrA and MsrB. This result was not affected by the order of Msr action on the protein and whether Msr were applied on CaMox sequentially or simultaneously, as shown by mass spectrometry and ITC.

4. Discussion

Oxidative stress is linked nowadays to reduced survival rate and various pathological situations, and, accordingly, several protection and repair systems help cells to cope with the presence of ROS [1,2,32]. These systems include methionine sulfoxide reductases catalyzing reduction of MetSO back to methionine residues in polypeptides that have suffered oxidative damages [5–8]. An illustration is provided by CaM, oxidized forms of which accumulate in senescent brains [12]. Our previous mass spectrometry analyses of CaMox showed that Met residues were the only residues altered by oxidation and they were converted to MetSO [6]. Several of the Met residues are functionally important, and their oxidation decreases dramatically CaM affinity for target enzymes [16,17,19–21], a basis to rationalize enzymes' dysfunction during oxidative stress. Therefore, we characterized the structural effects associated with the incomplete target activation by the oxidized CaM, and, subsequently, the repair action of MsrA and MsrB enzymes on CaMox-target interaction by using microcalorimetry and peptide mass fingerprinting. Surprisingly, each Msr exhibited different abilities to act upon CaMox, and, in particular, exhibit different abilities to repair Met124, a residue of critical importance for CaM-target interaction [33].

Ability of CaMox to activate the targets has been analyzed by studying the interaction between CaMox and RS20, the CaM-binding sequence of the smooth muscle myosin light chain kinase. Oxidation altered drastically the mode of interaction of CaM with the RS20 peptide. In particular, the oxidized form of CaM was found to interact with RS20 via its C-terminal domain only, as demonstrated by the following. First, enthalpy values obtained by ITC in the RS20/CaMox complex were less than those obtained with the RS20/CaM association by a factor of 1.7, suggesting that RS20 interacted with only one of the two domains. Second, the C-terminal domain of CaMox was found to be stabilized by RS20, confirming the previous assumption. In stark contrast, binding of an MLCK peptide or MLCK protein leads to a compact structure of Ca^{2+}–CaM [13,34], in other words both C- and N-terminal lobes of CaM interact with RS20 [13]. Hence, a consequence of these results is that, in the cell, CaMox will interact in a non-productive manner with its targets by its C-terminal domain only, an interaction that is not sufficient for target activation. Interestingly, the mode of interaction between CaMox and RS20 exhibits similar features to those reported in describing apoCaM/RS20 interaction which also involved only the C-terminal lobe [35]. Hence, our results provide a structural interpretation of the well established fact that oxidation of calmodulin prevents the transduction of calcium signal into physiological function.

The recent discovery of MsrB prompted us to investigate its activity on CaMox and to compare it with MsrA activity. Efficiency of MsrB in repairing CaMox was indicated by DSC denaturation study since MsrB stabilized the C-terminal lobe though less efficiently than MsrA (Table 2). Moreover, ITC data showed that upon repair by MsrB, both lobes of CaM interacted with the target, suggesting that MsrB repair might be sufficient for CaM to activate target proteins. Neither of the two enzymes allowed full thermostability of the N-terminal domain. However, an unexpected finding appeared when we compared binding affinities of CaMox repaired with MsrA with those of CaMox repaired with MsrB. Indeed, CaMox repaired by MsrA recovered binding affinity similar to that of native CaM, an observation consistent with results previously reported by Squiers and collaborators [21]. In contrast, MsrB repair did not allow CaMox to recover affinity for RS20. The basis for this difference is discussed below.

Previously, we showed that MsrA and MsrB enzymes each reduced a subset of MetSO residues present in CaMox while concomitant action of both led to reduction of almost all MetSO initially present [6]. The difference in binding affinity of CaMox repaired by MsrA compared to that of CaMox repaired by MsrB indicated that, in this case at least, MsrA and MsrB enzymes acted in different ways. A possibility was that one, or more, of the oxidized Met residues was determinant for binding affinity and was acted upon differently by MsrA and MsrB. In other words, this raised the question of how efficiently MetSO residues had been reduced by MsrA as compared to MsrB. Mass spectrometry analysis of tryptic digests showed that all, but one, MetSO residues were reduced with similar efficiency by either one of the two enzymes. This was the expected result since oxidation of Met residues is thought to be diastereospecific and to yield both substrates of MsrA and MsrB, i.e. $MetS^{S}O$ and $MetS^{R}O$, respectively, in equal amounts. Surprisingly, however, reduction of Met124SO residue varied depending upon which Msr was used for repair. Indeed, analysis of the tryptic peptides of CaMox repaired by MsrA or MsrB demonstrated significant difference in the reduced/oxidized ratio for Met124 (Fig. 5). While MsrA repairs on average 50% of Met124SO, MsrB repairs only 25%. This fact can be explained by the close vicinity of some other methionine ($MetSO^{X}$) preventing by structural constraints the reparation of Met124SO by MsrB. Indeed, Met109, Met145, and Met146 are within 5 Å to Met124. A suitable hypothesis is that $Met124S^{R}O$ cannot be repaired by MsrB if Met^{X} is still in oxidized state. Met124 and Met^{X} can be oxidized to $MetS^{S}O$ or $MetS^{R}O$ with equal probability, so

there are four combinations: Met124S^{R}O–Met^{X}S^{R}O (a), Met124S^{S}O–Met^{X}S^{S}O (b), Met124S^{R}O–Met^{X}S^{S}O (c) and Met124S^{S}O–Met^{X}S^{R}O (d), each with 25% probability. Met124SO in (a) and (c) species should normally be repaired by MsrB but Met^{X} in (c) is an *S*-sulfoxide and can only be repaired by MsrA, therefore MsrB can only repair Msr124SO in (a) species, i.e., 25% of Met124SO. If we repair CaMox by the combined action of both reductases, MsrA repairs Met^{X}S^{S}O of species (c) unlocking the reparation of Met124S^{R}O by MsrB and leading to full reparation of the molecule.

Could the differential repair efficiency be somehow responsible for the difference in binding affinity of CaMox to RS20? In fact, this possibility appears to be very likely when considering that in a series of Met to Gln mutants, only the M124Q mutant of CaM had a seriously reduced activation potential for CaM-dependent protein kinase I [36]. The M124L mutation in CaM impaired the activation of the MLCK enzyme, while mutation of Met72 or Met145 to a Leu residue had little effect [37,38]. Hence, we now have an explanation for the fact that MsrA, and not MsrB, was able to rescue CaMox for binding to RS20 with native efficiency.

Previously, we reported that CaMox was fully repaired by the concomitant action of MsrA and MsrB, pointing both to a racemic oxidation of Met residues, and, subsequently, to a complementary action of both enzymes [6]. Here, additional results have been provided that confirmed complementary action of both enzymes. For instance, while neither MsrA, nor MsrB could completely revive the initial thermostability of apoCaMox domains and the total enthalpy of denaturation, the combined action of both enzymes led to a total recovery of thermodynamic parameters of apoCaMox denaturation. The same was valid for the Ca^{2+} binding. Therefore, CaMox that had been reduced by MsrA remained a substrate for MsrB and vice versa. Moreover, increase in the total calorimetric enthalpy of denaturation under the simultaneous action of both MsrA and MsrB exceeded the sum of their separate action more than two times. Hence, instead of a simple complementary and additive effect of MsrA and MsrB, this last result opens the possibility that both reductases act in synergy. This hypothesis is of particular interest when considering that in several organisms, MsrA and MsrB evolved such as to form two domains within a single polypeptide [39].

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