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Membranous adenylyl cyclase 1 activation is regulated by oxidation of N- and C-terminal methionine residues in calmodulin

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ABSTRACT

Membranous adenylyl cyclase 1 (AC1) is associated with memory and learning. AC1 is activated by the eukaryotic Ca^{2+}-sensor calmodulin (CaM), which contains nine methionine residues (Met) important for CaM-target interactions. During ageing, Met residues are oxidized to (*S*)- and (*R*)-methionine sulfoxide (MetSO) by reactive oxygen species arising from an age-related oxidative stress. We examined how oxidation by H_2O_2 of Met in CaM regulates CaM activation of AC1. We employed a series of thirteen mutant CaM proteins never assessed before in a single study, where leucine is substituted for Met, in order to analyze the effects of oxidation of specific Met. CaM activation of AC1 is regulated by oxidation of all of the C-terminal Met in CaM, and by two N-terminal Met, M36 and M51. CaM with all Met oxidized is unable to activate AC1. Activity is fully restored by the combined catalytic activities of methionine sulfoxide reductases A and B (MsrA and B), which catalyze reduction of the (*S*)- and (*R*)-MetSO stereoisomers. A small change in secondary structure is observed in wild-type CaM upon oxidation of all nine Met, but no significant secondary structure changes occur in the mutant proteins when Met residues are oxidized by H_2O_2, suggesting that localized polarity, flexibility and structural changes promote the functional changes accompanying oxidation. The results signify that AC1 catalytic activity can be delicately adjusted by mediating CaM activation of AC1 by reversible Met oxidation in CaM. The results are important for memory, learning and possible therapeutic routes for regulating AC1.

1. Introduction

The conversion of ATP into the second messenger cAMP, which regulates numerous physiological functions including cardiac contractility [1,2], smooth muscle relaxation [3] and olfaction [4], is catalyzed by adenylyl cyclases (ACs) [5]. The family of membranous ACs consists of nine isoforms (AC1-AC9), which are classified into four subfamilies based on their regulation by stimulatory and inhibitory molecules [5]. ACs 1 and 8 are activated by the highly conserved Ca^{2+}-sensor calmodulin (CaM) [6,7]. Two CaM-binding sites on AC1 are known: a Ca^{2+}-independent binding site of 28 amino acids in the C1b domain [8] and a Ca^{2+}-dependent binding site of 14 amino acids in the C2a-region [9]. AC1 is expressed in specific brain regions (hippocampus, neocortex, entorhinal cortex and cerebellar cortex) associated with memory and learning [10]. Studies with transgenic and knockout animal models provide strong evidence that AC1 plays important roles in these processes [11,12].

Oxidative stress, resulting from increased cellular levels of reactive oxygen species (ROS), is associated with ageing and many

Abbreviations: AC, adenylyl cyclase; AD, Alzheimer's disease; CaM, calmodulin; CaM-mut, mutant calmodulin species; CaM-wt, wild-type calmodulin; CD, circular dichroism; DTT, dithiothreitol; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; H_2O_2, hydrogen peroxide; L, leucine residue; Leu, leucine; M, methionine residue; Met, methionine; MetSO, methionine sulfoxide; MsrA, methionine sulfoxide reductase A; MsrB, methionine sulfoxide reductase B; NADPH, nicotinamide adenine dinucleotide phosphate; eNOS, endothelial nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PDE, cyclic nucleotide phosphodiesterase; PMCA, plasma membrane Ca^{2+}-ATPase; ROS, reactive oxygen species; Sf9, *Spodoptera frugiperda* insect cells.

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disease states. Under conditions of oxidative stress, amino acids in proteins susceptible to oxidation, such as cysteine and methionine (Met), can be readily oxidized. Oxidation can therefore result in significant structural or functional changes to the protein [13–16]. This is particularly relevant for critical regulatory proteins, such as CaM, which interact with many different cellular target proteins [13,17]. There are nine Met in CaM, and all of these are sensitive to oxidation. These Met residues also interact directly with target proteins [18]. They are arranged into two N-terminal Met patches (M36, M51 and M71, M72, M76) and two C-terminal Met patches (M109, M124 and M144, M145) (Fig. 1). Oxidation of the hydrophobic and flexible Met results in a hydrophilic and slightly more rigid methionine sulfoxide (MetSO), as a mixture of (*S*)- and (*R*)-diastereomers [19,20].

Under conditions of oxidative stress associate with ageing, oxidized CaM is known to accumulate. For instance, in CaM from the brains of old rats, a significantly higher percentage of the Met are oxidized compared to CaM from the brains of young rats [21]. Oxidation of Met in CaM has likewise been shown to affect the ability of CaM to activate target proteins. For example, compared to wild-type CaM (CaM-wt), oxidized CaM is unable to fully activate the plasma membrane Ca^{2+}-ATPase (PMCA) [21]. In this case, it was demonstrated that even CaM with all Met residues oxidized was still able to bind to the PMCA, but not always productively [22]. In the case of CaM activation of the nitric oxide synthetases (NOS), CaM with all Met residues oxidized was unable to activate nitric oxide production by either neuronal (nNOS) or endothelial (eNOS) [23]. Interestingly, this oxidized CaM was able to partially activate other activities of nNOS and eNOS, for instance cytochrome *c* reduction [23].

In proteins with multiple Met residues, such as CaM, there is no facile means for selective oxidation of individual Met residues. Thus, determining how CaM function is regulated by oxidation of specific Met residues is challenging. One route that has proven successful is substitution of Met for leucine (Leu) by site-directed mutagenesis. Leu is comparable to Met in terms of volume, hydrophobicity and α-helical propensity [24]. Moreover, the tertiary structure of CaM is only nominally perturbed by Met to Leu substitutions, and CaM-mutants (CaM-mut) bind to, and activate, CaM targets similarly or with marginal differences compared to CaM-wt [23,25–27]. This strategy was used to demonstrate that oxidation of just M144 and M145 in CaM was responsible for the inability of CaM to fully activate the PMCA; oxidation of the remaining Met residues had no affect [26]. In contrast, oxidizing M144 and M145 in CaM had virtually no effect on the ability of CaM to fully activate eNOS and nNOS, but it did increase the concentration of CaM necessary for half-maximal activation [23]. This suggests that regulating activation of eNOS and nNOS by Met oxidation in CaM involves other Met residues in the C-terminal domain or residues in the N-terminal domain [23]. This approach was also used recently to determine that oxidation of N-terminal Met in CaM is necessary for targeting oxidized CaM for degradation by the 20S proteasome [28].

Oxidation of Met in proteins can be reversed ("repaired") in cells by the catalytic activities of the methionine sulfoxide reductases (Msr), and in the absence of irreversible consequences of the oxidation (unfolding, aggregation), the native functions of the proteins can be recovered [29–32]. In eukaryotes, there typically are two isoforms, MsrA and MsrB, that catalyze the stereospecific reduction of the (*S*)- and (*R*)-enantiomers of MetSO, respectively [33,34]. Thus, the Msr enzymes have critical regulatory functions, both rescuing proteins targeted for degradation and reversing functional consequences of oxidation. These enzymes are also very useful tools for reducing MetSO *in vitro*, for examining the reversibility of functional changes accompanying oxidation, and potentially for determining the stereospecificity of oxidation.

Given the age induced oxidative stress in the brain, the facile oxidation of Met in CaM by ROS, and the regulation of target activation by CaM *via* Met oxidation in CaM, it is prudent to examine the consequences of oxidizing specific Met residues in CaM for AC1 activation. It is conceivable that a decline in AC1 activity caused by an impaired activation of AC1 by oxidized CaM is involved in neurodegenerative diseases, such as Alzheimer's

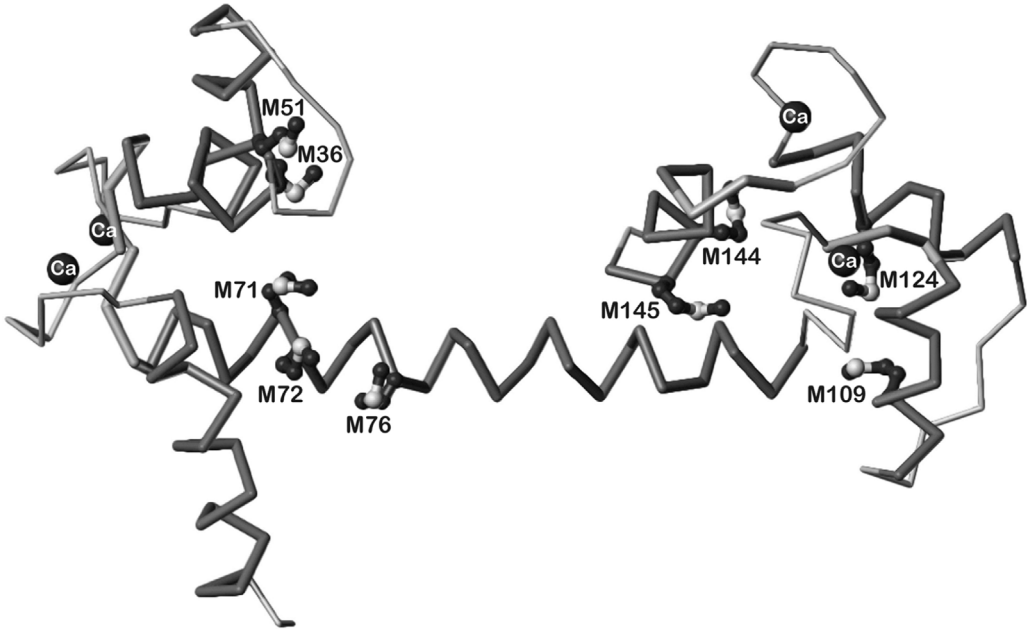

Fig. 1. Localization of the nine Met residues in Ca^{2+}-saturated CaM. The N-terminal domain is connected with the C-terminal domain *via* a flexible α-helical linker. Each domain contains two Ca^{2+}-binding motifs (EF hands). The backbone is shown as stick model (α-helices and β-sheets – thick, turns and loops – thin). The nine Met residues are represented as ball and stick models (light-coloured sulfur atoms). The Met residues are arranged into four patches: two N-terminal patches with M36, M51 and M71, M72, M76 and two C-terminal patches with M109, M124 and M144, M145. The model was generated with the modelling suite Sybyl-X (Certara, L.P., St. Louis, MO) from the high-resolution crystal structure of human CaM [76] (Brookhaven Protein Data Bank ID 1CLL).