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Protein oxidation and age-dependent alterations in calcium homeostasis

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1. ABSTRACT

Alterations in the capacity to maintain normal calcium homeostasis have been suggested to underlie the reduced cellular function characteristic of the aging process, and to predispose the senescent organism to a host of diverse pathologies including cancer, heart disease, and a range of muscle and neurodegenerative diseases. Therefore, critical to the eventual treatment of many age-related diseases has been the identification of both post-translational modifications and the underlying structural changes that result in an age-related decline in the function of critical calcium regulatory proteins. In brain, multiple methionines within the calcium signaling protein calmodulin (CaM) are oxidized to their corresponding methionine sulfoxides during aging, resulting in an inability to activate a range of target proteins, including the plasma membrane (PM) Ca-ATPase involved in the maintenance of the low intracellular calcium levels necessary for intracellular signaling. Likewise, changes in the transport activity of the PM-Ca-ATPase occur during aging. In muscle, the function of the SERCA2a isoform of the Ca-ATPase within the sarcoplasmic reticulum (SR) declines during aging as a result of the nitration of selected tyrosines. The age-related loss-of-function of these critical calcium regulatory proteins are consistent with observed increases in intracellular calcium levels within senescent cells. A possible regulatory role for these post-translational modifications is discussed, since they have the potential to be reversed following the restoration of normal cellular redox conditions by intracellular repair enzymes that are specific for these post-translational modifications. It is suggested that the reversible oxidation of critical calcium regulatory proteins within excitable cells by reactive oxygen species functions to enhance cellular survival under conditions of oxidative stress by reducing the energy expenditure within excitable cells. Thus, a diminished ability to efficiently generate cellular ATP may ultimately

underlie the loss of calcium homeostasis and cellular function during aging.

2. INTRODUCTION

Biological aging represents a fundamental process that results in a progressive decline in cellular function over time, and represents the major risk factor with respect to the development of cancer, neurodegenerative, and cardiovascular diseases. Therefore, an understanding of the basic process of aging is probably fundamental to the development of new therapies for a wide range of human diseases. A certain dichotomy currently exists with respect to current views of biological aging, and it remains uncertain whether aging is a developmental process that is ultimately controlled by a genetic program involving a limited number of genes, or rather represents an accumulation of stochastic events that damage the soma and result in the physiological decline of the organism. Furthermore, fundamental differences may exist from species to species (1), suggesting the importance of investigating the mechanisms of aging in both genetically tractable model organisms and using mammalian systems that are more easily interpreted with respect to the study of human aging.

Dramatic increases in both mean and maximal lifespan have been reported following genetic selection using *C. elegans* and *Drosophila*, which suggest that reduced rates of reproduction results in significant increases in lifespan within the same genetic background (2-5). These results are often interpreted to suggest that there is a tradeoff between reproduction and the maintenance of somatic cells associated with long life-span. While the signals associated with this linkage have not been fully elucidated, two-fold increases in lifespan have been reported using *C. elegans* in which insulin-like signaling pathways are down-regulated that correlates with decreased rates of metabolism (6, 7). Likewise, up-regulation of some antioxidant enzymes such as superoxide dismutase (SOD) and catalase can result in significant increases in lifespan, suggesting that there is a fundamental linkage between metabolism and aging (8, 9). In these latter cases, strong correlations have been observed between the accumulation of oxidized proteins and lifespan (10), consistent with a causative role for reactive oxygen species in the aging process.

A similar age-related accumulation of oxidatively modified proteins has been observed in mammals (11-14). Furthermore, caloric restriction results in an enhanced lifespan that correlates with the differential expression of critical proteins involved in energy metabolism, stress responses, and calcium regulation. These results suggest a possible linkage between the energetic costs associated with the maintenance of intracellular calcium gradients necessary for cellular signaling, oxidative damage, and the loss of physiological responses associated with the aging process. Consistent with this hypothesis, oxidative stress, the associated production of reactive oxygen species (ROS), and the loss of calcium regulation has been implicated as primary factors associated with a range of neurodegenerative and debilitating diseases associated with aging (e.g., senile dementia of the Alzheimer type, familial amyotrophic lateral sclerosis, cancer, Parkinson's disease, diabetes mellitus, and ischemia) (15-21). Furthermore, the administration of free-radical scavengers to aged gerbils both reverses the level of protein oxidation in aged brain and restores the animals performance on standard behavior tests, suggesting an important age-related decline in mental performance associated with a reversible process involving the oxidation of protein components in the brain (22). Likewise, cardiovascular damage associated with reperfusion injury is dramatically reduced upon prior administration of antioxidants (23).

A major unresolved issue is the mechanisms that result in the observed correlation between oxidative damage and the loss of calcium homeostasis. In this respect, the calcium regulatory protein CaM, Ca-ATPase pumps in both sarcoplasmic reticulum and the plasma membrane, and calcium channels have been suggested to represent major targets that are preferentially damaged by ROS (20, 24-27). In the case the CaM and the SR Ca-ATPase, the structural and functional consequences associated with the oxidative modification of unique sites have been identified (28-31). These latter results strongly suggest that the loss of calcium homeostasis observed during biological aging is the result of oxidative modifications to these critical calcium regulatory proteins.

3. CALCIUM SIGNALING MECHANISMS WITHIN EXCITABLE CELLS

Calcium has evolved as the major signaling molecule in all eukaryotes, and functions to modulate intracellular metabolism rapidly and to coordinate a range of diverse cellular processes involved in neurotransmission, neuronal plasticity, muscle contraction, and a host of reactions involved in the energy and biosynthetic metabolism of the cell (32). Critical to these calcium signaling mechanisms is the maintenance of the large (i.e., 10,000 fold) concentration gradients across the plasma membrane and endo/sarcoplasmic reticulum by Ca-ATPases and the Na/Ca exchanger, which permit the rapid and localized increases in cytosolic calcium upon modulation of calcium channels (Figure 1). The maintenance of these steep calcium gradients represents a major energy expenditure within excitable cells, and through respiratory control mechanisms is tightly coupled to rates of oxidative phosphorylation and the generation of ROS. Therefore, observed decreases in rates of calcium release and re-sequestration have the potential to both reduce energy consumption and the concomitant production of ROS, which may represent adaptive responses under conditions of oxidative stress that enhances the probability of cellular survival (33). However, a quantitative understanding of the possible role of oxidative stress to the loss of calcium homeostasis requires that specific post-translational modification within key calcium regulatory proteins be identified. Summarized below is the current information concerning the structure and function of critical calcium regulatory proteins, and when possible what is known regarding age-related alterations in function

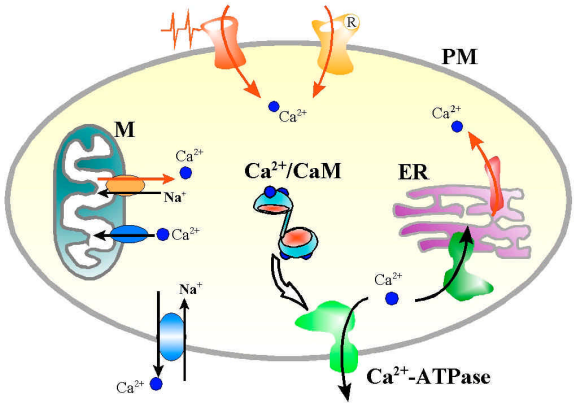

Figure 1: Calcium Transport Systems in Eukaryotic Cells**.** Ca^{2+}-activated CaM functions to enhance the activities of the Ca-ATPase (green) in the plasma membrane (PM) and endoplasmic reticulum (ER) through either direct binding to the auto-inhibitory domain of the PM-Ca-ATPase or through the phosphorylation of phospholamban (not shown), the protein which regulates the SR Ca-ATPase. Similarly, CaM associates with voltage and ligand gated calcium release channels and functions to inhibit calcium release at high intracellular calcium concentrations. Additional regulation of calcium involves sodium/calcium antiporters and calcium symporters in the mitochondria (M) and PM. Additional details of the respective transporter systems are discussed in the text.

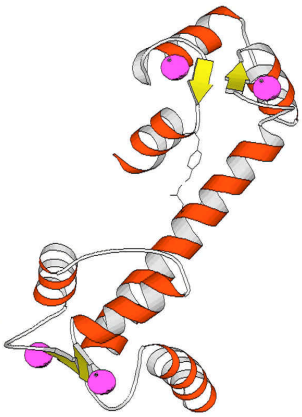

Figure 2: Ribbon Diagram Depicting Backbone Fold of Calmodulin. Hydrogen bond is illustrated between Tyr^{138} and Glu^{82} of calcium-saturated vertebrate CaM, which is suggested to stabilize the central helix (36, 124). Secondary structural elements representing the ∀-helical backbone (red) and antiparallel ∃-sheet structure (yellow) are indicated, and side chains associated with Tyr^{138} and Glu^{82} are illustrated using a stick representation. Calcium ligands are drawn using their van der Waals radii (purple).

and the sensitivity of these proteins to oxidative stress.

3.1. Calmodulin (CaM)

CaM is an intracellular calcium sensor involved in the rapid coordinate regulation of the activity of approximately 30 enzymes, channels, and receptors associated with intracellular signaling and metabolism (34). The crystal structure of the calcium-liganded form of CaM contains two structurally homologous globular domains connected by a seven-turn ∀-helix referred to as the central helix (Figure 2) (35, 36). Five high-resolution structures are currently available of CaM in association with target peptides derived from the auto-inhibitory domains of smooth and skeletal muscle myosin light chain kinase, CaM-dependent protein kinase II∀, CaM-dependent kinase kinase, and the PM-Ca-ATPase (37-41). While fluorescence and small angle X-ray and neutron diffraction measurements of CaM bound to target peptides indicate that many general features associated with the binding of the opposing globular domains in CaM to recognition sites on target proteins are conserved (42, 43), the details of the binding interactions are highly variable and suggest that differences between the CaM binding mechanisms of target enzymes will be important with respect to the coordinate regulation of target proteins. Thus, a comparison of the crystal structures demonstrates that critical groups on the target peptides involved in the binding interaction with CaM have a variable spacing within the primary structure of the peptide, and in the case of CaM-dependent kinase kinase the binding orientation of CaM to the peptide is opposite to that previously observed.

Measurements relating to the hydrodynamic properties of CaM indicate that the two opposing globular domains in calcium-activated CaM are structurally coupled (44-47). This suggests that the structural properties of the central helix may be critical in defining the optimal spatial relationship between the opposing globular domains in CaM for the rapid and efficient activation of target proteins. Consistent with this suggestion, structurally distinct conformations of the central helix are present in both apo- and calcium-activated CaM at physiological ionic strengths (46, 47). Large changes in the spatial arrangement of the opposing globular domains of CaM occurs following oxidative modification of methionines in CaM isolated from senescent rats, and result in the nonproductive interaction between oxidized CaM (CaM_{ox}) and the PM-Ca-ATPase (26, 28, 29). Thus, methionine oxidation alters the spatial arrangement of the opposing globular domains of CaM as a result of modifications to the structure of the central helix. Oxidation has been suggested to result in alterations in specific binding interactions between important ionizable groups (e.g., Tyr^{138} and Glu^{82}) following oxidation of Met^{144} or Met^{145} near the carboxyl-terminus that modulates the stability of the central helix, resulting in alterations in the binding mechanisms between the opposing globular domains of CaM and target proteins that are normally responsible for enzyme activation (28, 29). Alternatively, methionine oxidation may alter specific side-chain interactions between CaM_{ox} and the CaM-binding sequence that result in a decreased binding energy that fails to promote the normal conformational changes within the target protein associated with enzyme activation.

3.2. Ca-ATPases

3.2.1 Plasma Membrane Ca-ATPases

Multiple isoforms of the plasma membrane Ca-ATPase have been isolated from rat brain (48), which all contain an arginine-rich sequence near the carboxyl-terminus associated with the calmodulin binding domain.

The additional CaM binding domain increases the molecular mass of the PM-Ca-ATPase (130 kdaltons) relative to the better studied sarcoplasmic reticulum Ca-ATPase (110 kdaltons), whose structure has been solved at 8-Δ (49). Previous spectroscopic measurements between site-directed fluorophores on the SR Ca-ATPase have provided information relating to the folding pattern of the ATPase (50-52). It is therefore clear that the nucleotide binding cleft and the associated phosphorylation site at Asp_{351} are about 60 Δ and 40 Δ, respectively, from the calcium binding sites that reside at the level of the bilayer, and that the mechanisms involving energy transduction must involve long-range structural coupling between these sites (53). These functionally significant regions that include the phosphorylation site, the nucleotide binding cleft, and the 2nd, 4th, 5th, 6th, and 10th major hydrophobic sequences (predicted to be transmembrane segments) are homologous between the SR Ca-ATPase and the plasma membrane Ca-ATPase. CaM binding to the PM-Ca-ATPase results in the dissociation of the auto-inhibitory domain, which undergoes essentially unconstrained rotational diffusion (54). This result is consistent with earlier ideas that suggest the CaM-binding domain of the PM-Ca-ATPase functions as an auto-inhibitory domain that prevents substrate binding or utilization (55). The PM-Ca-ATPase in brain synaptic membranes isolated from either Fischer 344 rats or a Fischer 344/BN F1 hybrid is highly sensitive to oxidation and has an impaired transport function when isolated from aged animals (20, 56). The loss of transport function is unrelated to any changes in membrane lipid physical properties (57).

3.2.2.Ca-ATPases in the Sarco/Endo-plasmic Reticulum

The SR/ER Ca-ATPase is expressed in mammalian tissues as three major isoforms: the fast-twitch skeletal isoform (SERCA1), the cardiac/slow-twitch isoform (SERCA2a) and its alternatively spliced gene product, the smooth/nonmuscle isoform (SERCA2b), and an additional nonmuscle isoform, (SERCA3). SERCA1 is selectively and most abundantly expressed in fast-twitch skeletal muscle, and has been the most extensively characterized (58, 59). The SERCA1 isoform of the Ca-ATPase is a single polypeptide chain of 110 kDa (1001 amino acids), and is of the E-P class of enzymes, forming a covalent aspartyl-phosphoryl enzyme intermediate originating from the (-phosphoryl group of ATP during the transport cycle (52). SERCA2a consists of 997 amino acids, is 84% identical in sequence to SERCA1, is the major isoform of the Ca-ATPase expressed in brain, heart, and smooth or slow-twitch muscle (59-61), and also transports calcium via an aspartyl-phosphoryl enzyme intermediate. The selective co-expression of the membrane protein, phospholamban (PLB), with SERCA2a allows regulation of calcium transport in response to ∃-adrenergic stimulation. In reconstituted membranes and various co-expression cell systems SERCA1 can also be regulated by PLB (62). Phosphorylation of PLB at Ser^{16} or Thr^{17} by cAMP-dependent protein kinase or calmodulin-dependent protein kinase, respectively, induces up-regulation of Ca-ATPase transport rates, manifested by a decrease in the calcium concentrations required for Ca-ATPase activity, but not by changes in calcium affinity (63). In slow twitch skeletal muscle PLB is expressed in lower amounts, i.e., equimolar to SERCA2a compared with five to seven-fold excess to SERCA2a in the heart, thus providing a diminished capacity for regulation in skeletal muscle (64). An age-dependent loss of function results from the selective nitration of tyrosines in SERCA2a (30). Likewise, the selective nitration of tyrosines in SERCA2a is observed upon its *in vitro* exposure to peroxynitrite, while the homologous SERCA1 isoform of the Ca-ATPase whose sequence around the sites of nitration is identical to that of SERCA2a is not modified (27, 30). These results suggest that these isoforms of the Ca-ATPase adopt unique conformations that may result from the co-expression of the SERCA2a isoform with PLB.

3.3. CaM-Dependent Regulation of Calcium Channel Function

CaM has been shown to associate with a wide range of different calcium channels, including the ryanodine receptor (RyR) and IP_3 receptors located within the endoplasmic reticulum and a range of different calcium channels in the plasma membrane (65-68). In all cases, calcium entry through the respective channels serves to activate CaM, which functions to inhibit calcium release. In this manner the calcium-dependent activation of CaM functions to self-regulate intracellular calcium, functioning as a feedback inhibitor of calcium release. While CaM binding to these various receptors has been documented, the nature of the binding interaction has been most thoroughly investigated in the case of the RyR.

3.3.1. Ryanodine Receptors (RyR)

The RyR isoform, RyR1, expressed in skeletal muscle functions as a homotetramer of 565 kDa (5037 amino acids) subunits while in heart the RyR2 isoform (4969 amino acids), with 67% sequence identity, also functions to mediate excitation-contraction coupling. From the amino acid sequence twelve transmembrane helices are predicted per monomer (69). These large monomers form larger complexes that can be observed by electron microscopy to have substantial cytoplasmic domains and visible pores (70). RyR1 releases lumenal calcium via a conformational coupling, involving direct protein-protein interactions, with the sarcolemmal DHPR. The latter receptor contains a voltage sensor within its sequence that induces conformational changes in response to depolarization at the cell surface. In contrast, the RyR2 in the heart is stimulated by local release of calcium from the DHPR, a mechanism known as calcium-induced calcium release (CICR). This mechanism permits a graded response of calcium release in direct proportion to the extent of stimulus, unlike skeletal muscle that appears to be only capable of an all-or-none response.

Both RyR isoforms exhibit a similar bell-shaped calcium dependence, with low micromolar calcium concentrations activating, whereas higher calcium concentrations inhibit channel activity. Many of the regulators of this highly regulated receptor act by altering its calcium sensitivity. Physiological regulators of the RyR include the following: (a) CaM, by direct binding and (b) CaM by means of phosphorylation by CaM-kinase II. (c)

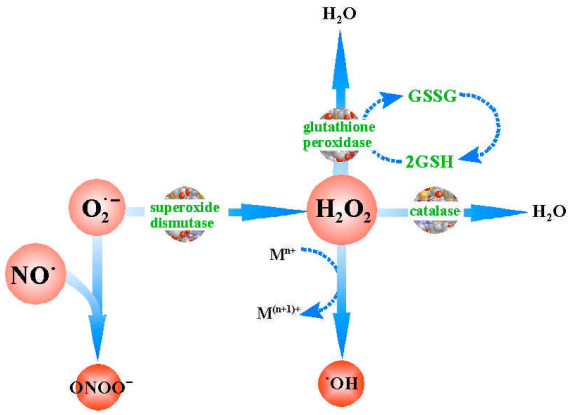

Figure 3*:* Scheme of the Formation and Metabolism of Reactive Oxygen Species. Superoxide ($O_2^{\bullet-}$) produced during normal cellular conditions is dismutated to form hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). H_2O_2 is subsequently degraded to H_2O by antioxidant enzymes such as catalase and glutathione peroxidases. H_2O_2 also can lead to the generation of the highly reactive hydroxyl radical ($^{\bullet}OH$) through the transitional metal (M^{n+}) catalyzed reactions. Cellular messenger nitric oxide ($NO^{\bullet}$) together with excessive $O_2^{\bullet-}$ can form highly reactive peroxynitrite ($ONOO^-$). The reduced and oxidized forms of glutathione are indicated as GSH and GSSG, respectively.

PK A can phosphorylate the RyR as well, but with decreased efficiency relative to CaM kinase (71). (d) RyR2 is regulated by cADP-ribose. (e) ATP, at the millimolar concentrations usually present in cells, allosterically activates calcium release. It is therefore thought that ATP represents a protective mechanism for conditions, such as ischemia-reperfusion, where ATP concentrations are depleted, and RyR activity is decreased to prevent rapid loss of calcium from internal stores and calcium toxicity. (f) Recently, evidence has been presented for post-translational regulation of RyR2 in the heart by nitric oxide ($NO^{\bullet}$), resulting in a reversible S-nitrosylation (-CH2-S-NO) of 10-12 cysteines with concomitant activation of the RyR (72). RyR2 isolated from heart was also observed to be endogenously nitrosylated. These Cys can be oxidized with no effect on channel activity, suggesting that they act as a buffer against oxidation. Meissner and co-workers (72, 73) also demonstrated irreversible activation of the RyR by oxidation of additional thiols (by $ONOO^-$), suggesting that the RyR can differentiate nitrosative from oxidative signals. It was suggested that calcium released at the plasma membrane by the DHPR activates nitric oxide synthase and is thus responsible for cyclic release (in milliseconds) of $NO^{\bullet}$ in the beating heart with activation of the RyR.

On the other hand, in skeletal muscle, the effects of nitric oxide on RyR1 are controversial. A recent study suggests that $NO^{\bullet}$ acts to protect the skeletal muscle RyR from oxidation-induced activation and thus is an inhibitor of the RyR (74, 75). RyR1 contains several hyper-reactive cysteines whose oxidation results in calcium release through the channel. Thus, these results suggest a mechanism for the opposing effects of oxidants (increasing) and $NO^{\bullet}$ (decreasing) on force generation in skeletal muscle, and the fatigue resistance that $NO^{\bullet}$ affords. However, the mechanism, whether by nitrosylation or other means, by which $NO^{\bullet}$ effects changes in the RyR1 has not been elucidated.

Each 565 kDa subunit of the RyR homotetramer binds one mole of CaM is a calcium-independent manner (76). CaM provides activation at low (<0.2 micromolar) calcium concentrations and inhibition at micromolar to millimolar calcium. Based on CaM's binding kinetics, it has been suggested that the role of CaM is to provide time delays in RyR channel activity that allow coordination of calcium release with cytosolic calcium transients. The oxidative sensitivity of CaM, its observed oxidation in aging, and the sensitivity of the RyR to oxidative modifications that prevent its interaction with CaM, all suggests that changes in the redox status of aged muscle may alter the normal ability of the RyR to be regulated by CaM, either through the oxidation of CaM or of the RyR, or both (26, 29, 77). In support of this suggestion, a recent report indicates that the RyR in cardiac SR membranes can be induced to release calcium by exposure to $O_2^{\bullet-}$, and that this release is mediated by endogenous CaM (78). However, there is currently no information with respect to either possible age-related functional alterations of RyR or the impact the age-related accumulation of oxidized CaM has on RyR function.

4. OXIDATIVE STRESS AND POST-TRANSLATIONAL PROTEIN MODIFICATIONS

During normal cellular conditions approximately 1-2% of the total oxygen consumed is reduced to $O_2^{\bullet-}$ (79), which under normal conditions is dismutated to form H_2O_2 by superoxide dismutase (SOD) (Figure 3). H_2O_2 is subsequently degraded to H_2O by catalase, glutathione peroxidases, thiol-specific antioxidant enzymes, and other peroxidases (21, 80). Impairments in mitochondrial oxidative metabolism and cellular peroxidases result in the generation of increased amounts of $O_2^{\bullet-}$, which may exceed the antioxidant capacities of the cell and contribute to the etiology of many age-related diseases as a result of the direct modulation of calcineurin function and through the generation of H_2O_2 , HOCl, and $ONOO^-$ (14, 81-86). H_2O_2, in turn, can lead to the generation of the highly reactive $^{\bullet}OH$ through the Fenton reaction, which is expected to react nonspecifically with a wide range of different amino acids, including Met, Cys, Tyr, Trp, His, Pro, and Lys (87). Reaction of these reactive oxygen and nitrogen species with amino acid side chains is consistent with the loss of function observed for a number of different proteins isolated from senescent animals. Therefore, prior to a discussion of the mechanisms underlying the loss of calcium regulatory protein function, it is of interest to consider some of the more common age-related post-translational modifications reported in proteins isolated from senescent animals.

4.1. Carbonyl Formation

The oxidation of lysine, proline, arginine, and threonine can yield aldehydes and ketones, which are readily assessed using sensitive assays of carbonyl formation (88). In addition, reactive species generated following lipid peroxidation (e.g., 4-hydroxy-2-nonenal) can react with proteins to yield carbonyls (14). Age-related increases in the carbonyl content of proteins isolated from a broad range of tissues have been identified, and it has been estimated that approximately 20-50% of the total protein is oxidized in aged humans (89). The accumulation of protein carbonyls following oxidative stress and during aging suggests an age-related imbalance with respect to the generation of a range of different reactive oxygen species, antioxidant defense mechanisms, and the normal protein turnover mechanisms of the cell. Consistent with this latter view, significant decreases have been reported in the function of the proteasome (90, 91), which has been suggested to represent the major degradative pathway involving oxidized proteins (92-94).

4.2. Methionine Oxidation

Methionine side chains are highly sensitive to oxidation by a range of different ROS, including superoxide, hydrogen peroxide, hydroxyl radicals, peroxynitrite, hypochlorous acid, and chloramines. Oxidation of methioinine can result in the addition of one or two oxygens to form either methionine sulfoxide or sulfone, respectively (95). However, only methionine sulfoxide has been found in biological samples. It should, however, be emphasized that oxidized methionines are not observed following the acid digestion conditions typically used for amino analysis, and the extent of cellular methionine oxidation has probably been underestimated. In a number of instances the specific oxidative modification of cellular proteins under *in vitro* conditions has been shown to inhibit function. For example, the oxidation of Met^{358} in ∀-1-proteinase inhibitor to its sulfoxide prevents the ability to inhibit elastase and has been implicated as a causative factor in the etiology of emphysema (96, 97). Likewise, the *in vitro* oxidation of selected methionines in the *E. coli* L12 ribosomal protein inhibits function and results in decreased rates of protein synthesis (98). In addition, age-dependent increases in cellular levels of methionine sulfoxide are observed within the extracellular matrix and the lens of the eye and following ischemia in the heart (95). These results indicate that methionine sulfoxide is a cellular marker of oxidative stress. However, while it is clear that a range of proteins are functionally sensitive to in vitro oxidative modification and that methionine sulfoxide is a product of oxidative stress, it remains unclear to what extent the oxidation of methionine is responsible for alterations in cellular function. In this latter respect, it is important to point out that in some instances methionine oxidation has been suggested to serve a protective role (99). For example, glutamine synthetase from E. coli contains eight methionine residues that surround the active site which scavage reactive oxygen species to prevent the oxidation of metals within the catalytic site (100). Thus, while the number of examples in which the functional consequences resulting from specific sites of methionine oxidation have been identified is limited, it is tempting to speculate that methionine oxidation may play a regulatory role in cases where methionines play critical roles in mediating protein-protein interactions, while in other cases where methionines play no functional or structural roles their oxidation may represent a mechanism associated with antioxidant defense.

4.3. Cysteine Oxidation

Cysteines are highly sensitive to the reducing environment of the cell, and many examples are now available in which the reversible formation of protein disulfides regulates enzyme function following alterations in cellular redox conditions (101). Reduced glutathione normally functions to maintain reduced thiols on proteins. During aging and under conditions of oxidative stress the ratio of reduced to oxidized glutathione levels declines (102, 103), and critical cysteines on a range of intracellular proteins become oxidized. Ultimately, the maintenance of cellular function involves the NADPH-dependent reduction of oxidized glutathione by glutathione reductase, which is dependent on intracellular respiration. Thus, reduced glutathione functions both as an antioxidant and as a signaling molecule that functions to modulate intracellular metabolism through the regulation of disulfide linkages. An important example of how cysteine oxidation can modulate calcium homeostasis involves the recent identification that oxidation of Cys349 within the SERCA1 isoform of the Ca-ATPase modulates transport activity (104). These results suggest that cysteine oxidation has the potential to regulate muscle function through alterations in the rate of calcium resequestration. Importantly, glutathione modification of the Ca-ATPase protects this cysteine against oxidation (104), suggesting that the covalent modification of critical cysteines may function to modulate cell function in a redox-dependent manner. Likewise, the formation of intermolecular disulfide cross-links has been implicated in the age-dependent loss-of-function of phosphoglycerate kinase (105-107). Additional modulation of calcium homeostasis can involve S-nitrosylation of cysteines, which has been demonstrated to modulate the calcium release kinetics of the RyR (72, 78).

4.4. Tyrosine Nitration

Conditions of oxidative stress results in the widespread nitration of intracellular proteins, which is generally thought to result from the diffusion mediated formation of peroxynitrite (ONOO−) from nitric oxide (NO•) and hydroxyl ions (•OH) (108, 109). The formation of nitrotyrosine can interfere with intracellular signaling pathways by preventing the phosphorylation of tyrosines (110). Likewise, nitrotyrosine formation within structural proteins (e.g., actin and neurofilaments) can interfere with their polymerization and result is the disruption of cytoskeletal elements (111, 112). In addition, the selective nitration of specific tyrosines on transport proteins (i.e., the Ca-ATPase) during biological aging has been shown to result in decreased function that is consistent with the longer calcium transients observed in senescent muscle (27, 30).

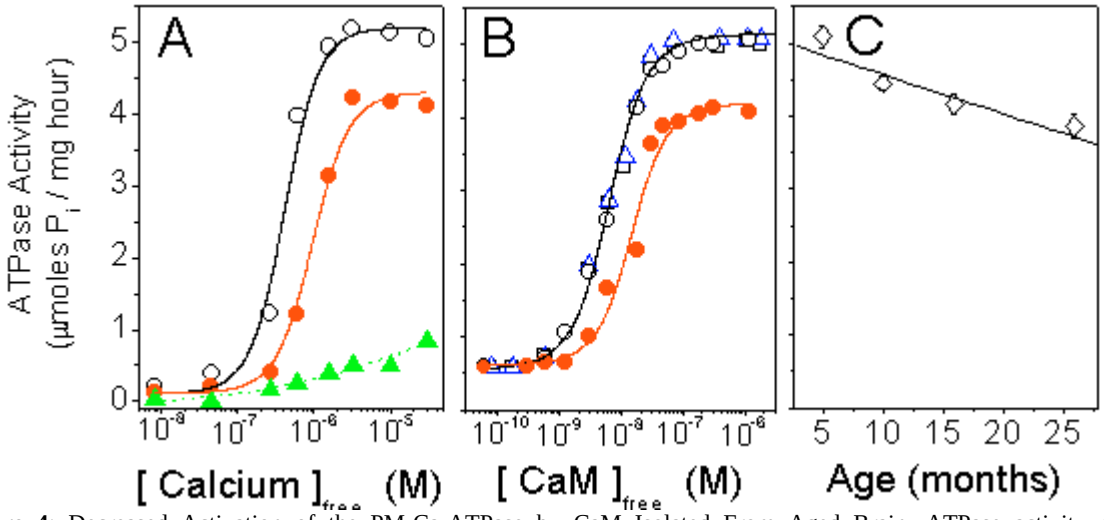

Figure 4: Decreased Activation of the PM-Ca-ATPase by CaM Isolated From Aged Brain. ATPase activity of porcine erythrocyte ghost PM-Ca-ATPase measured in the absence (▲) and presence of CaM isolated from the brains of 6 month (❍) and 26 month (●) Fischer 344 rats in the presence of either 0.3 :M CaM (panel A) or 30 :M free calcium (panel B). For comparison, the CaM-dependent activation by CaM isolated from bovine testes (□) or wheat germ ()) is shown. Maximal activation (V_{max}) of the Ca-ATPase by CaM isolated from brains of variable age Fischer 344 rats are shown (◊) in panel C.

4.5 Deamidation Reactions

The age-related deamidation of asparagine to form isoaspartate and D-aspartate has been reported for a range of different proteins isolated from tissues in which protein turnover is minimal, and can result in a decreased protein stability that is associated with decreased enzymatic activity (113). The rate of deamidation depends on the surface exposure of asparagines within the protein and the identity of the surrounding amino acids, and occurs at selected sites within the calcium regulatory protein CaM upon *in vitro* storage (114-116). However, observed rates of deamidation are sufficiently slow (i.e., 0.14% per year) that in most cells where protein turnover occurs it is expected that the extent of protein deamidation will not be significant (113).

5. STRUCTURAL AND FUNCTIONAL CONSEQUENCES OF CALMODULIN OXIDATION

5.1. Oxidative Modifications to Calmodulin During Aging

There is a progressive age-dependent decline in the ability of CaM isolated from the brains of Fischer 344 rats to activate the PM-Ca-ATPase (Figure 4), which normally functions to maintain the low intracellular calcium levels characteristic of healthy cells. Since the age-dependent loss-of-function is progressive with age and occurs long before the onset of age-related pathologies (Figure 5), observed decreases in CaM function are a manifestation of the aging process per se and are not the result of pathological alterations in metabolism that may accompany the end-stage of the aging process (117). In the presence of saturating concentrations of CaM, there is a shift in the calcium-dependent activation of the PM-Ca-ATPase toward higher calcium concentrations for CaM isolated from senescent (26 mo.; $[Ca]_{0.5}$ = 0.97 ± 0.07 µM) animals relative to that observed for CaM isolated from young (6 mo.; $[Ca]_{0.5}$ = 0.42 ± 0.01 µM) animals (Figure 4A). These latter results are consistent with the reduced calcium affinity of CaM isolated from senescent animals, and suggest that observed increases in cytosolic calcium levels in senescent animals are, in part, the result of CaM oxidation (26). The inability to recover the full activation of the PM-Ca-ATPase by increasing the concentration of CaM isolated from senescent brain indicates that all binding sites on the PM-Ca-ATPase are saturated, and that a sub-population of CaM species isolated from senescent brain binds, but is unable to fully activate, the PM-Ca-ATPase. These latter results suggest that alterations to CaM in aged cells can have a major impact on intracellular signaling and metabolism through their ability to block normal regulation of critical intracellular targets such as the PM-Ca-ATPase.

SDS-PAGE, amino acid analysis, and electrospary ionization mass spectrometry (ESI-MS) were used to identify age-related alterations in the structure of CaM (26). Approximately two methionines are oxidatively modified to methionine sulfoxide in CaM isolated from senescent brain. No oxidation of any other amino acids was observed, suggesting that methionines within CaM are selectively oxidized during normal biological aging. The observed increase in oxidation involves essentially all CaM molecules, where a distribution of CaM oxiforms is observed that contains one, two and three methionine sulfoxides per CaM (Figure 6). The pattern of oxidation is similar to that observed following *in vitro* oxidation using hydrogen peroxide, suggesting a physiological role for this ROS in the oxidative modification of CaM (Figure 7). In contrast, while $ONOO^-$ and HOCl also react selectively

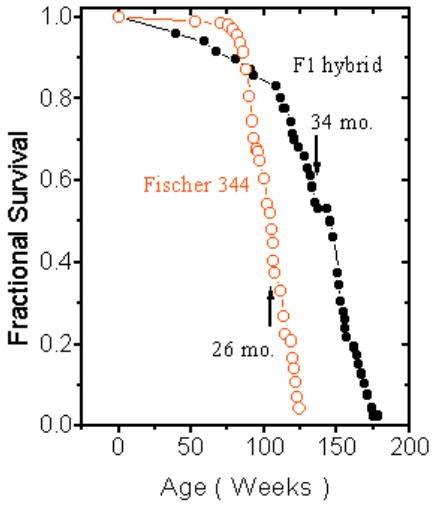

Figure 5: Survival Curves. Comparison of life-spans for Fischer 344 (❍) and F344/BNF1 hybrid (●) rats. Data obtained from Vivian Williams at the National Institutes of Aging

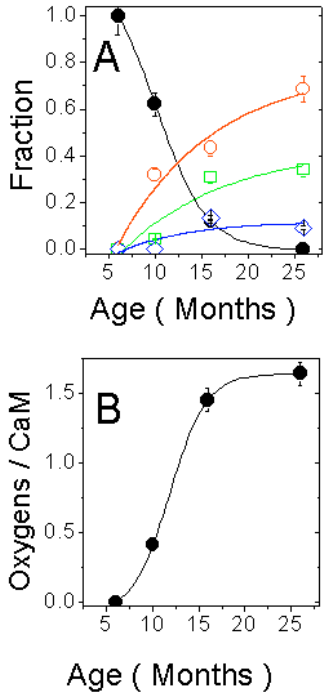

Figure 6: Distribution of Oxidatively Modified Oxiforms of CaM. Fractional contribution of each species of CaM associated with native CaM (●) and oxidatively modified species containing one additional oxygen (❍), two additional oxygens (□), and three additional oxygens (◊) obtained from electrospray mass spectroscopic data following correction for charge-induced dissociation (29). The average number of oxygens incorporated into each CaM species is shown in panel B, which was calculated as the sum of all CaM oxiforms multipled by the number of oxygens present. Lines are drawn for visual clarity, and do not represent a physical model to describe the data.

with methionines in CaM under *in vitro* conditions (118), the resulting pattern of methionine oxidation is different from that observed in CaM isolated from senescent brain.

In contrast to the results obtained using the Fischer 344 rats, CaM isolated from the brains of the long-lived Fischer344/BNF1 hybrid is not oxidatively modified in animals that are 34 months old (56). These results are consistent with the hypothesis that the oxidative modification of calcium regulatory proteins correlate with life-span. Consistent with the accumulation of oxidized calmodulin in the Fischer 344 brain, there are large decreases in the function of the 20S proteasome purified from senescent Fischer 344 rats (90, 91). Since *in vitro* measurements indicate that the 20S proteasome selectively degrades oxidized CaM (119), these results suggest that the accumulation of oxidized calmodulin in Fischer 344 rats may be the result of decreases in proteasome function. It should, however, be emphasized that these results do indicate that the accumulation of oxidatively modified CaM is not a biomarker for aging; rather, the rate at which reactive oxygen species are generated in the vicinity of CaM relative to the rates of repair and degradation of CaM appears to be important in defining successful aging.

5.2. Structural Consequences of Methionine Oxidation

A determination of the mechanisms responsible for the loss-of-function to CaM during aging requires that a causative linkage be identified between the oxidation of specific methionines in CaM and alterations in function or structure. However, an understanding of the functional and structural effects associated with the oxidative modification of specific methionines to their corresponding methionine sulfoxides has been complicated by the difficulty in obtaining a homogeneous sample in which individual methionines are selectively oxidized. Separation methods to isolate individual mono-oxidized species in which specific methionines have been oxidized have been unsuccessful because methionine sulfoxide formation in CaM results in i) no change in net charge, ii) only small changes in calcium affinity, and iii) subtle changes in the secondary and tertiary structure of CaM. Likewise, it has not been possible to simulate structural changes in CaM_{ox} through the use of standard methods of site-directed mutagenesis by substituting individual methionines with naturally occurring amino acids that have a similar polarity and size as methionine sulfoxide, such as glutamine (120). These latter results indicate that the polarity of the methionine sulfoxide is not the cause of reduced CaM function, and suggest that oxidation-induced structural changes result in the decreased ability of CaM isolated from senescent brain to activate the PM Ca-ATPase. Spectroscopic measurements using circular dichroism (**CD**) and fluorescence spectroscopy indicate that while oxidative modification of a limited number of methionines results in minimal alterations in the secondary structural features of CaM_{ox}, there is an altered thermal stability of CaM_{ox} relative to native CaM (28, 29). Large alterations in the lifetime properties of covalently bound fluorophores upon methionine oxidation suggest that the oxidation of methionines in the carboxyl-terminal domain results in

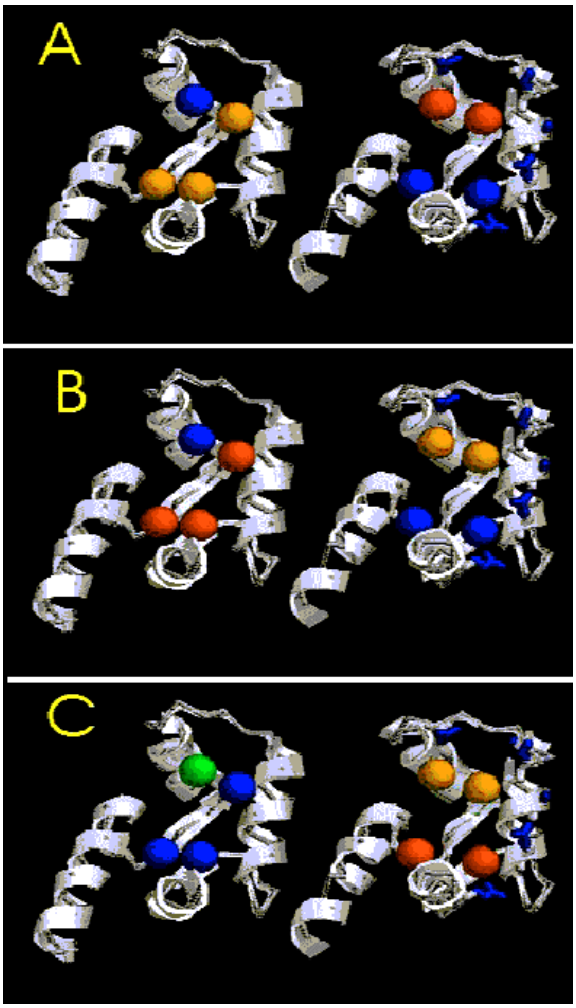

Figure 7: Representation of Patterns of Methionine Oxidation. Colored spheres represent the relative sensitivities to oxidative modification of sulfur atoms in amino-terminal (left) and carboxyl-terminal (right) domains of CaM isolated from senescent brain (A) or obtained following exposure to either H_2O_2 (B) or $ONOO^-$ (C). Colors denote extent of oxidative modification, where red > yellow > green > blue. Glutamic acid side chains within 7 D of individual sulfur atoms are indicated as blue tubes, since they function in the activation of peroxynitrite (187). Structures drawn with ribbons represent the ∀-helical backbone of CaM.

global structural changes involving both the amino- and carboxyl-terminal domains. Since there are no large changes in the average spatial separation between the opposing globular domains of CaM, these results indicate that concerted structural changes within the amino-terminal domain that results from the oxidative modification of methionines in the carboxyl-terminal domain probably involve the central helix and suggest that oxidative modification of selected methionines in CaM_{ox} disrupt specific noncovalent interactions that normally stabilize the structure of native CaM (e.g., the salt linkage between Tyr^{138} and Glu^{82}; see Figure 2).

A functional role for specific methionines located near the carboxyl-terminus was identified using H_2O_2 to oxidize CaM isoforms *in vitro* (121). Large differences in the sensitivities of a pair of methionines near the carboxyl-terminus of CaM isoforms to oxidative modification correlates with the loss-of-function. In contrast, there is no loss-of-function associated with the oxidation of all seven remaining methionines in a CaM mutant in which Met^{144} and Met^{145} were replaced with the nonoxidizable amino acid glutamine. Therefore, while oxidative modification of the majority of the methionines in CaM results in a reduced affinity with respect to binding to the PM-Ca-ATPase, the oxidative modification of methionines near the carboxyl-terminus results in the disruption of the central helix in CaM and the associated inability to fully activate the PM-Ca-ATPase. Consistent with the role of methionines in binding, the apparent affinities of the majority of these mutants for the PM-Ca-ATPase are reduced (Figure 8). However, in all cases these mutants were able to fully activate the PM-Ca-ATPase in the presence of saturating CaM concentrations, irrespective of the number of glutamine substitutions in each domain of CaM (120). Thus, oxidation of a carboxyl-terminal methionine in CaM results in a diminished ability to activate the PM-Ca-ATPase because of global structural changes that result in the nonproductive binding between oxidized CaM and the CaM-binding sequence of the PM-Ca-ATPase.

The mechanism responsible for the observed global structural changes resulting from oxidation of Met^{144} or Met^{145} was identified using fluorescence spectroscopy to investigate the average structure and extent of conformational heterogeneity associated with the central helix in calmodulin (CaM), a sequence that contributes to calcium binding sites two and three and connects the amino- and carboxyl-terminal globular domains. Using site-directed mutagenesis, a double mutant was constructed involving conservative substitution of Tyr^{99} ➜ Trp^{99} and Leu^{69} ➜ Cys^{69} with no significant effect on the secondary structure of CaM (Figure 9). These mutation sites are near the opposite ends of the central helix. Trp^{99} acts as a fluorescence resonance energy transfer (FRET) donor in distance measurements to probe the conformation of the central helix. Cys^{69} provides a reactive group for the covalent attachment of the FRET acceptor 5-((((2-iodacetyl)amino)ethyl) amino)naphthalene-1-sulfonic acid (IAEDANS). AEDANS-modified CaM fully activates the plasma membrane (PM) Ca-ATPase, indicating that the native structure is retained following site-directed mutagenesis and chemical modification. The average spatial separation between Trp^{99} and AEDANS covalently bound to Cys^{69} decreases by approximately 7 ∀ 2 Δ upon calcium binding (47). However, irrespective of calcium binding, there is little change in the conformational heterogeneity associated with the central helix under physiologically relevant conditions (i.e., pH 7.5, 0.1 M KCl). These results indicate that calcium activation alters the spatial arrangement of the opposing globular domains between two defined conformations. In contrast, under conditions of low ionic strength or pH the structure of CaM is altered and the conformational heterogeneity of the central helix is decreased upon calcium activation,

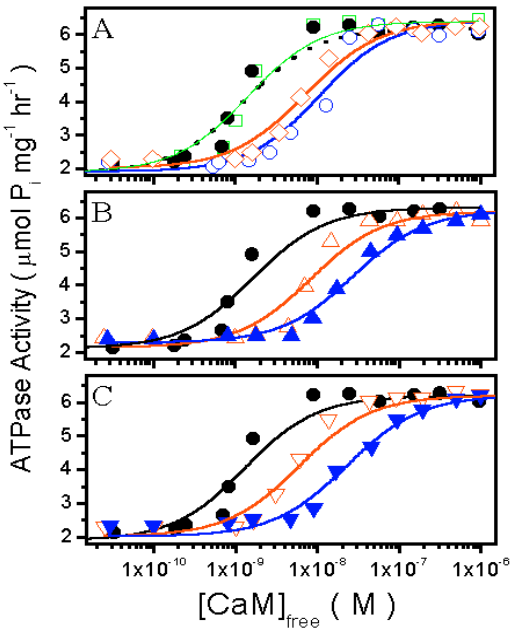

Figure 8: Calmodulin-Dependent Activation of the PM-Ca-ATPase. ATPase activity for wild-type CaM (●) is compared with mutant CaMs involving: (**A**) single methionine substitutions in C-terminus of M124Q (◊), M144Q (○), and M145Q (□); (**B**) multiple methionine substitutions in the carboxyl-terminal domain involving the substitution of Gln for Met[144] and Met[145] (i.e., C-2Q;)) or Met[124], Met[144], and Met[145] (i.e., C-3Q; ▲); and (**C**) multiple methionine substitutions in the amino-terminal domain involving the substitution of Gln for Met[71] and Met[72] (N-2Q; ∇) or Met[36], Met[51], Met[71], and Met[72] (i.e., N-4Q; ▼).

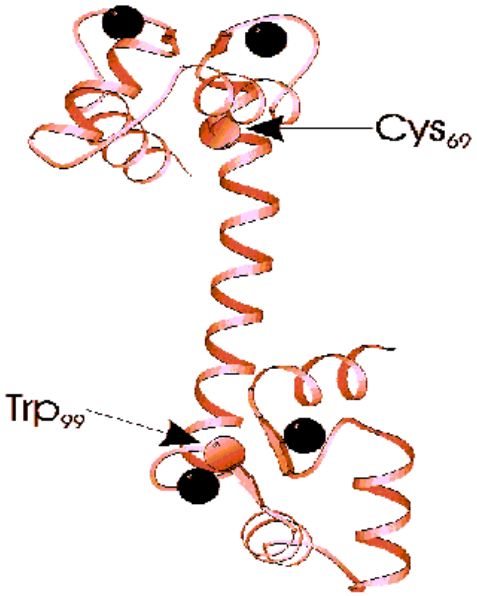

Figure 9: Illustration of Chromophores Used in Fluorescence Measurements of Intramolecular Distances. Positions of Trp[99] and Cys[69] (red spheres) relative to the four high affinity calcium binding sites (black spheres).

consistent with earlier NMR results (122). These results suggest the presence of important ionizable groups that affect the structure of the central helix, which may play an important role in mediating the ability of CaM to bind rapidly and activate target proteins. The reasons underlying the sensitivity of the structure of the central helix to changes in ionic strength or pH can be understood upon consideration of the structure observed using X-ray crystallography (Figure 10). Consistent with the large amount of conformational disorder observed for apo-CaM under conditions of low ionic strength, the proximity of the many acidic amino acid side chains (i.e., Asp[80], Glu[82], Glu[83], Glu[84], Glu[87], Asp[93], and Asp[95]) within the central helix has the potential to destabilize the formation of ∀-helical secondary structural elements because of the proximal negatively charged carboxylic acid groups. Thus, upon increasing the ionic strength repulsive interactions between these negatively charged carboxylates may be minimized, resulting in a highly ordered ∀-helical structure stabilized by a range of specific electrostatic interactions that includes short range hydrogen bonds between ∀-helical backbone elements. Consistent with this interpretation, scanning calorimetry results have demonstrated that when [82]EEE[84] is replaced by [82]KKK[84], that there is a decrease in ∀-helicity that alters the conformational flexibility of CaM (123). This result supports previous suggestions that the hydrogen bond between Tyr[138] and Glu[82] may play an important role in stabilizing the central helix (29, 36, 124). In agreement with this latter suggestion, the destabilization of the carboxyl-terminus or site-directed replacement of Tyr[138] with amino acids incapable of serving as a hydrogen bond donors results in a decreased conformational stability of both apo- and calcium saturated CaM (29, 125). Thus, under normal physiological conditions the association between the high-affinity carboxyl-terminal domain of CaM and target proteins may abolish the hydrogen bond between Tyr[138] and Glu[82] (37-39), thereby destabilizing the central helix and facilitating the rapid association of the amino-terminal domain with the proximal binding site on the target protein.

5.3. Mechanism of Inhibition of the PM-Ca-ATPase by CaM_{ox}

Oxidatively modified CaM binds to the PM-Ca-ATPase, but does not induce the formation of the ∀-helical structure within the CaM-binding sequence normally associated with enzyme activation (26, 28, 29). Previous measurements have demonstrated that this is the result of altered binding interactions between CaM and the CaM-binding sequence on the PM-Ca-ATPase that result from global structural changes involving the oxidative modification of a carboxyl-terminal methionine (29). Recent frequency-domain fluorescence measurements indicate that there is essentially independent rotational dynamics of the opposing globular domains of CaM_{ox} bound to the PM-Ca-ATPase relative to that observed for native CaM, and a much larger amount of conformational heterogeneity between the opposing globular domains that are consistent with CD measurements that indicate that the CaM-binding sequence does not adopt an ∀-helical secondary structure. These results strongly suggest that secondary structural changes within the CaM-binding

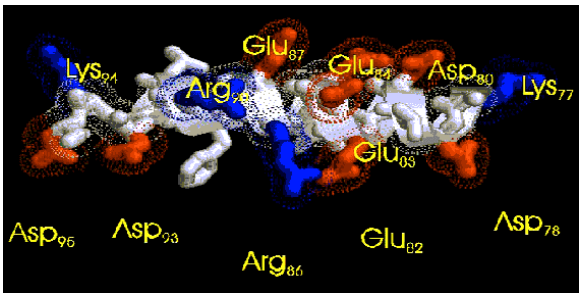

Figure 10: Structure of the Central Helix in CaM. Acidic (red) and basic (blue) residues within the central helix are depicted within the structure of calcium-saturated CaM (36). Illustration was prepared using the coordinates 1cll.pdb and the program RASMOL (188).

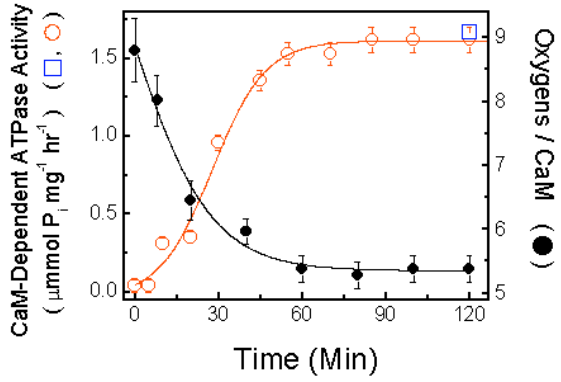

Figure 11: Recovery of the CaM-Dependent Activation of the PM Ca-ATPase Upon Repair of Methionine Sulfoxides in CaM_{ox} by MsrA. The CaM-dependent ATPase activity of PM-Ca-ATPase was measured in the presence of native CaM (~) or CaM_{ox}, whose nine methionines were oxidized to methionine sulfoxide (❍), at various time intervals following the incubation of MsrA. The average number of oxygen atoms in each CaM molecule (●) was determined by ESI-MS

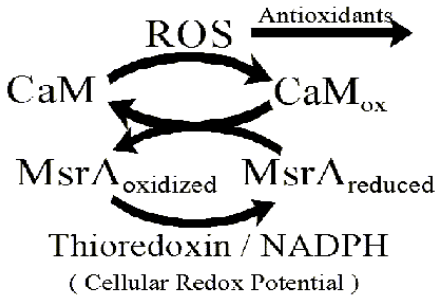

Figure 12: Modulation of CaM Functional Activity by MsrA. Multiple methionines are oxidatively modified in CaM_{ox} when reactive oxygen species (ROS) exceed antioxidant defense mechanisms, impairing the ability of CaM to activate target proteins. In the presence of NADPH, thioredoxin reductase can use thioredoxin to reduce MsrA, which can repair methionine sulfoxides in CaM and restore function.

sequence following CaM binding are essential for enzyme activation.

5.4. Restoration of the Function of Oxidized CaM by Methionine Sulfoxide Reductase

Under *in vitro* conditions, the intracellular protein methionine sulfoxide reductase (MsrA) can repair (i.e., reduce) methionine sulfoxides in oxidatively modified CaM and restore the ability of oxidized CaM to fully activate the PM-Ca-ATPase (126), irrespective of whether CaM_{ox} is isolated from senescent brain or obtained using *in-vitro* conditions that oxidize all nine methionines in CaM (Figure 11). The recovery of CaM function by MsrA is an indication that MsrA may play a pivotal role in maintaining CaM function following conditions of oxidative stress. Restoration of CaM function by MsrA following oxidative modification of all nine methionines results in a distribution of CaM oxiforms containing from three to eight methionine sulfoxides. These remaining methionine sulfoxides at any of the nine positions in CaM_{ox} become inaccessible to repair by MsrA (126). There are corresponding increases in peptide ellipticity observed using circular dichroism spectroscopy accompanying reactivation, suggesting that repaired CaM_{ox} assumes a native-like structure. These results suggest that MsrA does not recognize methionine sulfoxides within native-like ∀-helical secondary structures in functionally active CaM, and provides a possible reason for the presence of methionine sulfoxides in CaM isolated from senescent brain.

The ability of MsrA to reduce (i.e., repair) methionine sulfoxides on physiologically relevant time-scales (i.e., minutes) has the potential to modulate intracellular metabolism, and may enhance the probability that individual neurons survive ischemic episodes (126, 127). Therefore, the oxidative modification of methionine may be unique in that the resulting sulfoxide can be rapidly and selectively repaired (Figure 12), and thus can act as an intracellular signaling mechanism analogous to other regulatory post-translational modifications involving phosphorylation (95). The decreased function of CaM_{ox} may serve an adaptive role that enhances cellular survival under conditions of oxidative stress by decreasing the activity of Ca-ATPases and other energy transducing systems, which would function to reduce the rate of cellular ATP hydrolysis and thereby minimize the energy needs of the cell. The expression of MsrA results in a high resistance to oxidative stress in *E. coli*, *Sarccharomyces cerevisiae* and human T cells in culture (128-130). MsrA is expressed in all mammalian tissues, and immunocytochemical staining indicates that MsrA levels are particularly high in specific brain regions, including the cerebellum (131, 132). Given that the brain has a high metabolic rate and limited antioxidant defense mechanisms in comparison to other tissues (133, 134), the high level of expression of MsrA may play a critical role in the modulation of the activity of intracellular proteins that become functionally impaired upon oxidative modification (95). Consistent with the hypotheses that there are age-related alterations in the function of MsrA, the accumulation of oxidatively modified ∃-amyloid peptide containing methionine sulfoxide has been suggested to

correlate with the pathology associated with Alzheimer's disease (135).

5.5. Degradation of Oxidized CaM and Proposed Role for the Proteasome

CaM is a relatively long-lived signaling protein in comparison to the short half-lives of many transcription factors and other cellular regulatory molecules, with a half-life of approximately 18 hours in rat brain (136). Expression levels of CaM are under tight regulation; large increases in the amount of CaM mRNA results in little or no change in the total amounts of cellular CaM (137). These results suggest that rates of CaM turnover are tightly regulated to maintain constant amounts of expressed CaM, and are important to the maintenance of CaM function. However, little is currently known regarding the regulation of CaM gene expression or the normal degradative pathways that function to maintain normal intracellular concentrations of functionally active CaM.

The 20S proteasome core has been suggested to be largely responsible for the degradation of oxidized proteins in cellular homogenates (92-94). Consistent with this suggestion, oxidized CaM is selectively degraded by the 20S proteasome (119). Recognition of oxidized CaM appears to involve changes in the secondary structure of CaM, which correlates with the rate of CaM_{ox} degradation. Thus, the accumulation of CaM_{ox} during aging is consistent with previously reported reductions in the activity of the proteasome using fluorogenic peptides (90, 91).

6. CONFORMATIONAL AND FUNCTIONAL MODIFICATIONS OF THE SARCOPLASMIC RETICULUM Ca-ATPase.

6.1 Aging in Skeletal Muscle

In both humans and animals, aging is associated with changes in skeletal muscle that include the loss of both muscle mass and strength. Senile atrophy has been estimated to account for almost one-third of the age-related loss of muscle strength (138). However, even before the onset of atrophy, increases in the duration of the isometric twitch are apparent, i.e., in both the initial contraction time and the relaxation time (139-143). Contraction in muscle is generally dependent on the coupled responses to motor neuronal stimulation by L-type calcium channels, dihydropyridine receptors (DHPR) in the sarcolemma, and ryanodine receptor (RyR) calcium channels in the SR. RyRs release sufficient calcium from the SR lumen into the cytosol to bind to troponin C and initiate conformational changes within the contractile fibers, a process collectively known as muscle contraction (144). Thus, alterations in the properties of any of these components may contribute to alteration in contraction times. On the other hand, muscle relaxation is mediated primarily by the rate-limiting active transport of calcium across the SR membrane by the SR Ca-ATPase. Calcium indicator measurements indicate that age-related defects in contractile parameters are specifically related to altered intracellular calcium transients (142). In addition, resting levels of intracellular free calcium have been found to increase almost two-fold in senescent animals (145).

The mechanisms underlying the observed increases in contraction times in aged muscle are not presently well understood, but may relate to defects in DHPR or RyR function or expression. The few studies that have addressed possible alterations in expression levels of both RyR and DHPRs in senescent skeletal muscle report no age-related changes in receptor density based on either antibody detection by Western blots or binding of radioactive ligand, i.e., ryanodine or the dihydropyridine, PN-200-110 (143, 146, 147). However, another study suggested a preferential loss of DHPR density that resulted in an overall loss of DHPR: RyR coupling (148). In addition, an increased sensitivity of RyR to caffeine in aging has been reported, suggesting age-related alterations in channel regulation (143).

In contrast, the involvement of the SR Ca-ATPase in age-related defects in muscle contraction has been more extensively documented in both animal models and humans. For example, a loss in the rate of calcium uptake activity (30-50%) in SR enriched vesicles has been observed that correlates with the extent (40%) of increase in relaxation times (147, 149-153). Although age-related changes in contractile parameters differ both qualitatively and quantitatively for different muscle groups, the loss of Ca-ATPase activity with aging correlates well with a similar loss in isometric twitch duration in slow twitch fibers. For example, Narayanan and co-workers have shown that the half-time of relaxation (RT1/2) is unchanged in fast twitch gastrocnemius muscles with aging as is SR Ca-ATPase activity in microsomes isolated from this muscle (147). In contrast, the RT1/2 is prolonged for the slow twitch soleus muscle, correlating with a similar loss of SR Ca-ATPase activity in the corresponding isolated membranes. Similarly, using SR membranes isolated from several muscle groups of the hind limb muscle of the Fischer 344 rat, calcium transport activity and calcium-dependent ATP hydrolytic activity are substantially diminished in SR isolated from senescent muscles selected from slow twitch muscles, whereas, SR from predominantly fast twitch muscles exhibits no significant differences in Ca-ATPase activity with aging as illustrated in Figure 13 (30, 151). This latter result suggests that the slow-twitch isoform of the Ca-ATPase (SERCA2a) is differentially affected during aging in comparison to SERCA1, the isoform exclusively expressed in slow twitch fibers (58). With an abundance of 40-50% of SERCA2a in the SR prepared from muscles with high levels of slow twitch fibers, the functional deficit accounted for by SERCA2a, itself, would correspond to approximately 40% relative to the activity in young adults.

These decreases in specific activity relate to decreased catalytic rates rather than loss of Ca-ATPase protein levels, based on both immunoblots and protein staining using SDS-PAGE.

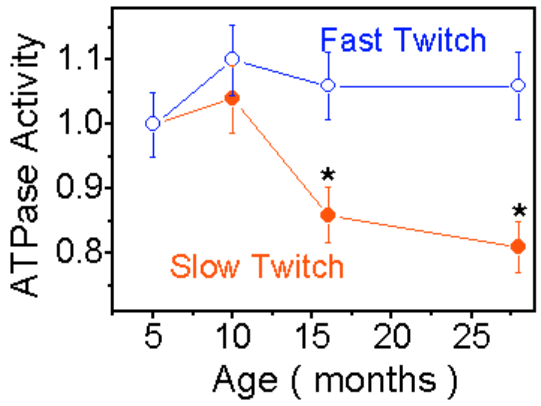

Figure 13: Age-Dependence of Ca-ATPase Function. Calcium dependent ATPase activity measured from fast-twitch SR (❍) and slow-twitch SR (●) isolated from Fischer 344 rats (n=6 for each age). Activities are normalized relative to that of 5 mo samples, which are 3.2 and 2.2 I.U. for fast- and slow-SR, respectively. * represents values statistically different ($p<0.05$) than 5 mo samples.

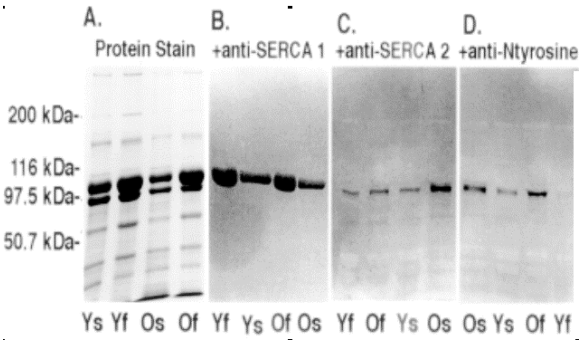

Figure 14: Detection of Nitration of the Ca-ATPase. SR from fast (f)- and slow(s)-twitch enriched skeletal muscle of 5 mo (Y) and 28 mo (O) Fischer 344 strain rats. SR proteins were separated on SDS-PAGE and visualized by: Coomassie blue protein stain (panel A), or immunostaining with anti-SERCA1 (B), with anti-SERCA2a (C), or with antinitrotyrosine (D) antibodies.

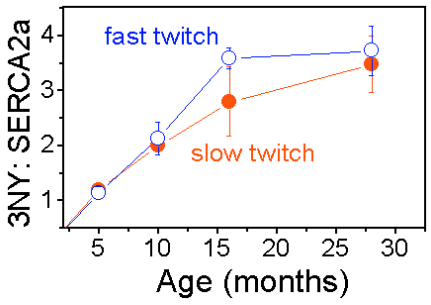

Figure 15: Age-Dependent Increase in Nitrotyrosine. Molar ratio of 3NY per SERCA2a based on amino acid analysis using an authentic 3NY standard. Data obtained from slow-twitch (●) and fast twitch (❍)-enriched SR (n=6 per age group).

The sensitivity of Ca-ATPase activity to the physical properties of its surrounding lipids suggests that the numerous age-related changes in the degree of fatty acyl chain unsaturation and cholesterol content of the SR may be responsible for its altered functional properties with aging (154-157). However, direct spectroscopic measurements have demonstrated that both the overall bilayer fluidity and that of lipids immediately surrounding the Ca-ATPase are not sufficiently altered to explain the age-related inactivation of the Ca-ATPase (158).

6.2. Identification of Sites of Tyrosine Nitration

Loss of heat stability, a characteristic of both oxidized proteins and age-modified Ca-ATPase, has suggested a focus by several investigators in exploring the accumulation of oxidative modifications to this calcium regulatory protein (27, 150-152). Perhaps the most remarkable finding has been the accumulation of 3-nitrotyrosine (3NY) modification specific to the SERCA2a isoform in senescent skeletal muscle to the exclusion of the SERCA1 isoform. These studies take advantage of high-affinity antibodies raised against nitrotyrosine (159). Immunoblots of isolated SR membranes using antibody raised against 3NY-modified protein demonstrate that the only SR protein with detectable 3NY modifications is one which migrates on SDS-PAGE as SERCA2a, but not as SERCA1 (Figure 14). Amino acid analysis of these protein bands has confirmed that the nitrated protein is SERCA2a (30). Quantitation of the levels of nitration of the SERCA2a isoform indicates that SERCA2a is nitrated in young adult (5 mo) animals at approximately one mol per mol SERCA2a and progressively increases with age up to almost four mol of nitrotyrosine per mol of Ca-ATPase in senescent (28 mo.) animals (Figure 15). No amino-acids other than cysteine are appreciably oxidized (at stoichiometric amounts) within either SERCA1 or SERCA2a.

Three of the four sites of 3NY modification within SERCA2a in aging skeletal muscle have been identified following isolation of NEM-alkylated tryptic peptides of the SERCA2a isoform of the Ca-ATPase isolated from young and senescent animals through the use of electrospray ionization mass spectrometry after separation by reversed-phase HPLC (30). With this approach, 84% of the SERCA2a sequence has been identified. For samples from senescent rats, most tryptic peptides containing tyrosines are entirely unmodified. Molecular masses 90 amu (atomic mass units) higher than for the unmodified peptides were detected for peptides G263-R297 and G291-R297, suggesting nitration of Y294 and Y295 (or dinitration of one of these). In addition, both the native peptide A751-R761 and a peak with molecular mass 45 amu higher were detected for samples isolated from both young and aged animals, suggesting the modification of a fraction of Y753 sites. The presence of 3NY in candidate peaks was confirmed by its characteristic absorbance at 430 nm.

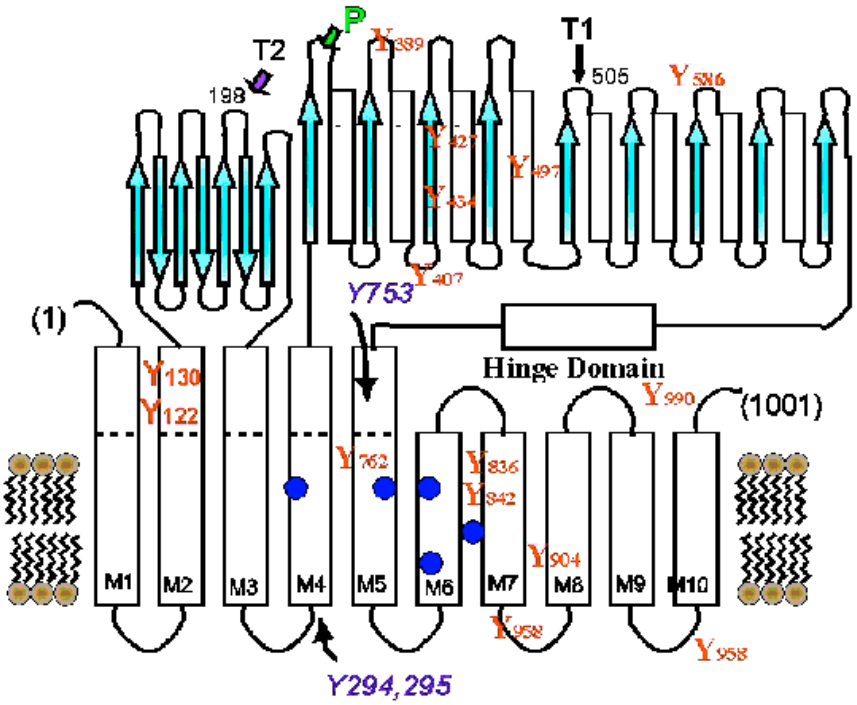

Figure 16: Predicted Structure of the Ca-ATPase. Structural diagram displayng the location (Y) of the 18 Tyr residues within the predicted secondary structure including beta-sheets (arrow) and alpha-helices (boxes) in the cytosolic domain and ten (M1-M10) membrane-spanning helices. Initial tryptic cleavage sites (T1 at R505 & T2 at R198) and the enzyme phosphorylation site (P) at D351 are also shown. Blue dots indicate putative calcium transport sites.

Thus Y753 appears to be the most sensitive site on the SERCA2a isoform of the Ca-ATPase to nitration, being nitrated already in the young animal. This tyrosine is located within the putative stalk helix, S5 (Figure 16). The vicinal tyrosines Y294 and Y295 that are modified with age are localized just adjacent, and on the lumenal side, of the transmembrane helix, M4. As this putative helix both contains one residue involved in high affinity calcium binding and is co-linear with the cytoplasmic phosphorylation site, it has been suggested to be critical for the structural coupling between calcium and nucleotide binding sites required for active calcium transport (53).

6.3. Cysteine Oxidation in Aging.

Modification of cysteines in the Ca-ATPase during aging was determined from radioactivity associated with the 110 kDa Ca-ATPase protein band on SDS-PAGE after reaction of SR with [^{14}C]-N-ethyl maleimide (30). Data obtained from SR membranes enriched with different amounts of SERCA1 and SERCA2a isoforms indicates that approximately one and six free thiols, respectively, are lost with aging. While the loss of thiols may result from protein conformational changes that render some of the 24-26 cysteines unreactive to NEM, cysteine oxidation is more likely, in view of the oxidative sensitivity of Ca-ATPase cysteines (104). This loss of reactive sulfhydryls is not recoverable through chemical reduction with DTT, indicating that disulfides are not a major product of cysteine modifications. More importantly, the loss of free thiols occurs by the age of 10 months, with no further loss at later ages. In contrast, the observed age-related inactivation of the Ca-ATPase does not appear until the animals reach 16 months of age. That the total age-related loss of thiols occurs before inactivation occurs provides a compelling argument that cysteine modifications do not play a major role in the age-related decreases in ATP hydrolytic and calcium transport activities.

6.4. Identification of Physiological Reactive Oxygen Species Through *In vitro* Oxidative Modification of the Ca-ATPase

6.4.1. By peroxynitrite

It has been suggested that the reactive species responsible for *in vivo* 3NY formation on many proteins is peroxynitrite ($ONOO^-$), which is formed by the diffusion-limited reaction of nitric oxide with superoxide ($O_2^{\bullet -}$) (83, 160, 161). Nitric oxide synthase (NOS) is abundant in both skeletal and cardiac muscle, and $O_2^{\bullet -}$ has been shown to be produced in contracting muscle (162-164). These results suggest the potential formation of $ONOO^-$ in muscle. *In vitro* exposure of rat skeletal muscle SR to $ONOO^-$ (or SIN-1 which simultaneously releases $O_2^{\bullet -}$ and $NO^{\bullet}$) results in specific nitration of SERCA2a to the exclusion of SERCA1. That SERCA1 is not nitratable by $ONOO^-$ is confirmed by findings from *in vitro* exposure of rabbit fast-twitch skeletal SR (which consists of SERCA1 alone), where there is no detectable nitration, but instead, significant cysteine oxidation (165). These findings suggest intrinsic differences in the reactivity of the SERCA1 and SERCA2a isoforms of the Ca-ATPase.

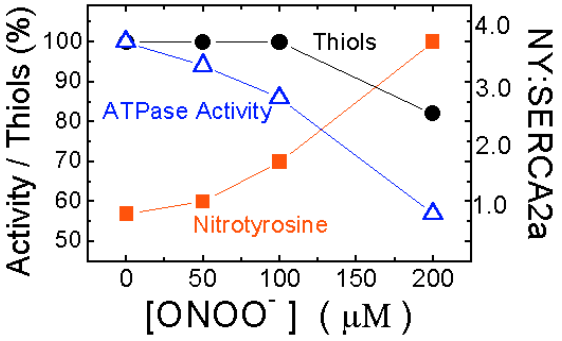

Figure 17: *In-Vitro* Exposure of the Ca-ATPse to $ONOO^-$. Effects of $ONOO^-$ on the calcium-dependent ATPase activity (%) ()), free thiol content (%) (●), and 3NY:SERCA2a content (mol/mol) (¦).

While the molecular mechanisms for these observed differences is not clear, these differences may be the result of alterations in Ca-ATPase structure resulting from the co-expression of the SERCA2a isoform of the Ca-ATPase with the regulatory protein PLB (166, 167).

In vitro exposure of SR (isolated from young rat skeletal muscle) to $ONOO^-$ results in progressive formation of 3-nitrotyrosine, which is directly reflected by inactivation of the Ca-ATPase; some Cys oxidation occurs at high $ONOO^-$ concentrations (Figure 17) (30). In contrast, exposure of SR to the same concentrations of the $NO^\bullet$ generator, diethylamine nonoate, does not result in any 3NY formation. These results demonstrate the requirement for superoxide along with $NO^\bullet$ as the cellular reactive oxygen species. Notably, at the same extent of nitration, the loss of Ca-ATPase activity from *in vitro* exposure to $ONOO^-$ is about two-fold greater than that observed *in vivo* with aging. This observation suggests that aspects of the cellular environment are important in modulating the steady state oxidation products. For example, cellular repair and degradation processes might remove or alter specific oxiforms of the Ca-ATPase that result in large structural and functional effects. Moreover, cellular ROS may undergo different chemical reactions with the Ca-ATPase in the cell as compared with an aqueous solution of vesicles. Alternatively, the *in vivo* nitrating species may not be $ONOO^-$. For example, nitrite in the presence of H_2O_2 and peroxidase has been proposed as an alternative nitrating species (168). In fact, 0.5 mM nitrite and H_2O_2 in the presence of 1 :M horseradish peroxidase is also capable of selectively nitrating SERCA2a in rat skeletal muscle derived SR (30).

Additional effects on a nitrated protein, or other associated biomolecules, has been suggested from the demonstration that a cellular reductase can enzymatically reduce 3NY on proteins to the corresponding nitro anion radical ($RNO_2^{\bullet-}$) which, in turn, is spontaneously oxidized by molecular oxygen to superoxide, regenerating nitrotyrosine (169). Thus, once formed *in vivo*, nitrotyosine may act to promote oxidative stress by means of repetitive redox cycling.

6.4.2. By water-soluble and lipid soluble radicals

Characterization of the oxidation products associated with the Ca-ATPase resulting from *in vitro* oxidation with defined ROS have the potential to identify the potential involvement, in aging, of ROS other than $ONOO^-$. For example, the thermolabile water-soluble azo initiator 2,2' azobis (2-amidinopropane) dihydrochloride (AAPH) has been utilized in order to expose SR membranes to peroxyl and alkoxyl radicals, which are common cellular ROS (152, 170-172). Exposure of SR to AAPH results in a dose-dependent inactivation of the Ca-ATPase and a pattern of oxidation that is similar to that from another oxidant, H_2O_2, but quite distinct from that of aging, particularly with respect to the bityrosine crosslinking resulting from *in vitro* oxidation. In contrast, very mild oxidation with low concentrations of AAPH results in no loss of activity, but a diminished heat stability, increased tendency for aggregation, and increased exposure of buried groups to the solvent. These are common properties of the age-modified Ca-ATPase (151). However, an analysis of oxidized peptides demonstrates that even mild oxidation with AAPH-derived radicals produces a similar, but distinct pattern of oxidation from that of aging (172, 173).

Metal catalyzed oxidation, involving the Fenton reaction ($Fe + H_2O_2 \rightarrow {}^\bullet OH + {}^\bullet OH + Fe^{3+}$), has been shown to occur on proteins at sites of metal binding (87, 89). This reaction has also been shown to induce inactivation of the Ca-ATPase (174, 175). However, this treatment results in protein fragmentation without thiol modification, unlike the forms of the Ca-ATPase that have been observed to accumulate during aging.

Additional studies have used the lipid soluble photosensitizer, benzophenone, that allows the controlled initiation of free radical species in order to expose Ca-ATPase membrane-spanning peptides to oxidation (176). Water soluble spin traps demonstrated that radicals generated within the bilayer were not released into the aqueous media. The resulting selective loss of calcium transport activity suggests that sites within the bilayer are modified, rather than sites that compromise extra-membranous sites of ATP binding or hydrolysis. Thus comparison of characteristic oxidation products from several specific oxidants *in vitro* with the oxidative footprint of aging has provided critical insights that suggest that peroxynitrite to be the cellular ROS most relevant to aging.

6.5. Structural Alterations of the Ca-ATPase

Identification of age-related structural changes of the Ca-ATPase provides an additional dimension in characterization of aged proteins, as well as a means for rationalizing the mechanism of functional inactivation (113). The Ca-ATPase from senescent animals exhibits an increased susceptibility to both tryptic digestion and to aggregation, suggesting conformational changes that involve partial unfolding of the protein structure (151). These changes have been more precisely defined with the use of site-directed spectroscopic probes (31). For example, in aging a 20% increase is observed in the solvent

exposure of the fluorescent label, FITC, bound at Lys^{515} within the nucleotide binding site, while IAEDANS at Cys^{670} is unaffected. Fluorescence resonance energy transfer measurements between these sites indicates an increased conformational heterogeneity of the protein structure with senescence. Thus these localized conformational changes at the nucleotide site of the Ca-ATPase provides a structural footprint of aging that suggests regions of modifications that may explain the loss of conformational stability with age.

6.6. Cellular Defects That Result in the Accumulation of Oxidatively Modified Ca-ATPase

The accumulation of oxidatively modified proteins in the cell can result from increases in cellular concentrations of reactive oxygen and nitrogen species (including losses in antioxidant concentrations or efficiencies) and defects in pathways that repair or degrade oxidized protein. Reliable measurements of ROS concentrations in cells are difficult to obtain. However, numerous measurements of antioxidant concentrations and activities in senescent skeletal muscle have suggested alterations, but not a comprehensive view of the antioxidant status of senescent muscle, possibly confounded by observations that some antioxidant concentrations respond to different types of exercise (177-179).

Slower Ca-ATPase protein turnover times in senescent skeletal muscle of the Fischer 344 rat suggests that deficits in protein turnover may contribute to the accumulation of oxidized proteins (64). However, the specific pathways involved in degradation of Ca-ATPases, which may be defective in the senescent animal, have not been ascertained. Nor is there much specific knowledge regarding the pathways that are responsible for degradation of oxidized Ca-ATPase. It has been shown that a number of soluble proteins are degraded by the 20S subunit of the proteasome, and that oxidative stress induces increased activity of this enzyme, possibly working in conjunction with the heat-shock protein Hsp 90 (92, 180-183). Moreover, the proteasome isolated from senescent liver exhibits lower proteolytic activity relative to that isolated from young animals, suggesting a rationale for the accumulation of oxidized protein in senescent cells (91, 184, 185). However, it is not clear whether the proteasome constitutes all or part of a pathway for degradation of oxidized Ca-ATPase.

An additional mechanism for repair of nitrated proteins has been suggested, i.e., a putative denitrase enzyme (186). This suggestion is based on the description of an activity in cellular homogenates of spleen and lung that diminishes the binding of anti-nitrotyrosine antibody to a nitrated protein substrate on immunoblots. While this activity is consistent with a denitration reaction, cleavage of the nitro group from tyrosine has not been demonstrated.

7. CONCLUSIONS AND PERSPECTIVES

In muscle, the SERCA2a isoform of the Ca-ATPase undergoes extensive nitration by $ONOO^-$ that results in an age-dependent loss-of-function that probably is responsible for the longer calcium transients observed in senescent muscle. The selective nitration of SERCA2a in aging further suggests its susceptibility to more acute oxidative stress, such as ischemia-reperfusion, as well as the possible sensitivity of the alternate-spliced smooth muscle isoform (SERCA2b) in pathological conditions of oxidative stress. Ultimately, understanding the chronic oxidative stress of aging in the myocyte will require an ability to simulate aging, by *in vitro* reconstitution of the pathway, exposure of the Ca-ATPase to the most relevant ROS, to form initial oxidized protein followed by exposure to any repair and degradative enzymes that will convert the initially oxidized protein to the final steady state oxiforms found in the senescent cell.

In brain, the progressive age-dependent oxidative modification of multiple methionines has been observed in the calcium regulatory protein CaM that results in a loss-of-function with respect to the activation of the PM-Ca-ATPase. Following calcium activation, the *in vitro* exposure of isolated CaM to H_2O_2 results in a similar pattern of oxidative modification to the nine methionines in CaM to that observed for CaM isolated from senescent brain, consistent with a physiological role for H_2O_2 in the oxidative modification to CaM. Oxidized CaM has a diminished ability to activate the PM-Ca-ATPase, and functions as an antagonist with respect to the activation of the PM-Ca-ATPase by unoxidized CaM. These latter results indicate that the oxidative modification of CaM may have profound effects on cell function. Site directed substitution of essentially all methionines in either the carboxyl- or amino-terminal domains of CaM with polar glutamine does not affect the ability of mutant CaM to fully activate the PM-Ca-ATPase in the presence of saturating concentrations of CaM, indicating that the altered polarity resulting from methionine oxidation is not the cause of the inability of CaM_{ox} to fully activate the PM-Ca-ATPase. In contrast, oxidative modification of a carboxyl-terminal methionine results in secondary and tertiary structural changes that alter the spatial relationships between the opposing globular domains of CaM. Thus, the oxidative modification of methionines near the carboxyl-terminus of calmodulin (i.e., Met^{144} and Met^{145}) induce global conformational changes that result in an inability to activate the PM Ca-ATPase and other target proteins, resulting in the loss of calcium homeostasis and an increased sensitivity of the cell to oxidative stress. These results are consistent with the disruption of the structural coupling between the opposing globular domains of CaM upon destabilization of the salt-bridge between Tyr^{138} near the carboxyl-terminus and Glu^{82} within the central helix of CaM. In addition to demonstrating age-dependent changes in the function of CaM, substantial decreases in the activity of the PM-Ca-ATPase in synaptic vesicles occur during aging. There are no corresponding alterations in the structure or dynamics of the membrane phospholipids, indicating that the decrease in calcium transport function arises as a result of a decrease in the function of the Ca-ATPase. Thus, post-translational modifications or incorrectly folded calcium pumps accumulate in senescent brain. Taken together, these results indicate that structural and functional changes to the calcium regulatory proteins CaM and the PM-Ca-ATPase,

which normally function to coordinately regulate intracellular signaling mechanisms in all eukaryotic cells, are consistent with observed decreases in calcium regulation in senescent animals. Under *in vitro* conditions, the intracellular protein MsrA is able to repair (i.e., reduce) methionine sulfoxides in oxidatively modified CaM and restore the ability of oxidized CaM to fully activate the PM-Ca-ATPase. Recent work likewise indicates that under *in vitro* conditions that oxidized and nonfunctional CaM is selectively degraded by the 20S proteasome. Thus, the accumulation of oxidized and nonfunctional CaM (and the Ca-ATPase) are consistent with an age-related decrease in the function of MsrA and the proteasome (or other degradative systems).

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