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Forum Review

Redox Modulation of Cellular Metabolism Through Targeted Degradation of Signaling Proteins by the Proteasome

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ABSTRACT

Under conditions of oxidative stress, the 20S proteasome plays a critical role in maintaining cellular homeostasis through the selective degradation of oxidized and damaged proteins. This adaptive stress response is distinct from ubiquitin-dependent pathways in that oxidized proteins are recognized and degraded in an ATP-independent mechanism, which can involve the molecular chaperone Hsp90. Like the regulatory complexes 19S and 11S REG, Hsp90 tightly associates with the 20S proteasome to mediate the recognition of aberrant proteins for degradation. In the case of the calcium signaling protein calmodulin, proteasomal degradation results from the oxidation of a single surface exposed methionine (i.e., Met^{145}); oxidation of the other eight methionines has a minimal effect on the recognition and degradation of calmodulin by the proteasome. Since cellular concentrations of calmodulin are limiting, the targeted degradation of this critical signaling protein under conditions of oxidative stress will result in the downregulation of cellular metabolism, serving as a feedback regulation to diminish the generation of reactive oxygen species. The targeted degradation of critical signaling proteins, such as calmodulin, can function as sensors of oxidative stress to downregulate global rates of metabolism and enhance cellular survival. *Antioxid. Redox Signal.* 8, 217–228.

BACKGROUND

Intracellular proteolysis functions to modulate cellular metabolism in response to environmental changes involving nutrient or hormone levels, resulting in the efficient reallocation of resources to alternate pathways through the targeted degradation of key proteins associated with committed steps of metabolic pathways. In addition, proteolysis plays a critical role in cellular maintenance through the recognition and removal of damaged and nonfunctional proteins. The proteasome plays a critical role in both processes, functioning to mediate the degradation of most cellular proteins (17).

The 20S proteasome is a cylindrical particle in which the catalytic active sites face a buried chamber. Critical to the specificity of protein degradation is the modular nature of the proteasome, which contains a 20S catalytic core and associated regulatory proteins that function in mediating the recognition and degradation of distinct classes of protein substrates (42–44, 93). The 20S core in association with two 19S protein complexes forms the 26S proteasome, which mediates the ubiquitin-dependent degradation of cellular proteins, including cyclins and numerous transcription factors associated with regulation of the cell cycle (23, 34, 93). Likewise, upon association with the 11S REG (PA28) protein complex or PA200, the 20S core has been suggested to function in the degradation of proteins for antigen presentation via the major histocompatibility complex and in processes associated with DNA repair (11, 75, 91, 96). Proteasomes containing both 11S REG and 19S complexes have been identified, and these hybrid complexes may function in concert to recognize and degrade aberrant proteins (11, 43, 74, 96).

Not all protein substrates degraded by the 26S proteasome need be ubiquitylated (e.g., ornithine decarboxylase), and some protein substrates can be directly degraded by the 20S proteasome lacking either the 19S or 11S REG protein complexes. For example, the cyclin-dependent kinase inhibitor p21, cytochrome c, and the calcium regulatory protein calmodulin (CaM) are degraded in a ubiquitin-independent manner by the 20S proteasome (25, 46, 87, 89). In the case of

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CaM, degradation is facilitated through the association of the chaperone Hsp90 with the 20S proteasome (97). Co-chaperones known to associate with Hsp90, such as CHIP (carboxyl terminus of Hsc70 interacting protein), function to promote degradation (60), suggesting that the 20S proteasome in complex with Hsp90 may play a general role in the degradation of many proteins. Other proteasome regulators important for mediating protein degradation include activators such as poly(ADP-ribose) polymerase as well as numerous inhibitors that compete for binding sites on the 20S core (2, 74). Degradation can occur in a nonprocessive manner, indicating a capacity for flexible loop regions of proteins to enter the proteasome for degradation (25, 27, 56). In all known cases, activation of the 20S proteasome involves association with regulatory proteins through the alpha subunits, which functions to promote access into the catalytic core (74). Thus, changes in the association of the 20S proteasome with different regulatory factors in response to cellular signals will modulate the substrate specificity and degradative activity of the proteasome.

STRUCTURE OF THE PROTEASOME

Substrate access to the catalytic core of the 20S proteasome is hindered by the amino-terminal sequences of the α-subunits, which under resting conditions function to close the entrance to the pore and prevent nonspecific proteolysis (36–38). Under these latent conditions the 20S proteasome exists primarily in a closed conformation; however proteasomes are in a dynamic equilibrium between a closed and open conformation, which is slow relative to the time-scale of protein turnover; further, the open form is stabilized by the presence of substrate (69, 70). Binding of the regulatory 11S REG or 19S protein complexes to the α-subunits of the 20S proteasome stabilizes the open conformation and facilitates regulated substrate access (36, 97). The chaperone Hsp90 has also been suggested to bind the α-subunits of the 20S proteasome and to regulate proteasome function in an analogous manner (19, 24, 57). Thus, observed cellular linkages between Hsp90 function and rates of protein degradation probably involve both the stabilization of partially unfolded proteins, as well as a direct modulation of protein degradation by the proteasome (5, 10, 13, 33, 49, 58, 92, 104).

OXIDATIVE STRESS AND PROTEASOME FUNCTION

Declines in proteasome function have been suggested to underlie the accumulation of oxidized and aggregated proteins during biological aging and numerous disease processes (20, 22, 28, 34, 48, 78, 80). While complexes involving the 20S proteasome core in association with 11S REG (i.e., PA28) and 19S (i.e., PA700) modulate the specificity of protein degradation, these larger protein complexes mediate the ATP-dependent degradation of proteins associated with ubiquitin-dependent processes or the formation of MHC Class I antigens. The 20S core complex in association with Hsp90 has been suggested to be responsible for the degradation of many oxidized proteins, which occurs in an ATP-independent manner (25, 40, 77, 78, 97). The high-affinity association between purified Hsp90 and the 20S proteasome (K_d < 100 nM), in conjunction with the routine presence of Hsp90 in purified preparations of the 20S proteasome, indicates a probable functional involvement in modulating protein degradation that was confirmed following reconstitution of this complex (19, 24, 48, 51, 61, 94, 97). These *in vitro* measurements are consistent with cellular assays using functional inhibitors of Hsp90, which were shown to modulate rates of protein degradation and inhibit the activity of the proteasome (19, 33, 58, 82). Thus, observed age-dependent decreases of both the total cellular abundance of Hsp90 and the amount of Hsp90 that copurifies with the 20S proteasome has the potential to profoundly affect cell function (48, 65, 94).

SENSORS OF OXIDATIVE STRESS AND MODULATION OF CELLULAR METABOLISM: IDENTIFICATION OF CONTROL POINTS

The selective oxidation of critical regulatory proteins offers a means to target these proteins for degradation and modify rates of metabolism and the associated generation of reactive oxygen species (ROS) (Fig. 1). Reductions in the rate of ROS generation, in turn, will minimize protein oxidation and facilitate restoration of cellular homeostasis in response to an oxidative insult. Age-dependent increases in the rate of ROS generation or declines in cellular repair or degradation mechanisms will increase the oxidative load on the cell, resulting in corresponding increases in the concentrations of oxidized proteins and the associated formation of protein aggregates. These results suggest that the general mechanisms underlying oxidant-dependent changes in cell function are likely to be relevant to diverse pathologies, and that generalities are possible form a consideration of the aging process concerning regulatory mechanisms that control the balance between metabolic rates and the generation of reactive oxygen species.

The identification of oxidatively sensitive proteins that modulate energy utilization and the associated generation of ROS are, therefore, critical to understanding adaptive cellular responses to oxidative stress. In this latter respect, site-specific oxidant-induced functional losses of the calcium regulatory proteins calmodulin (CaM), phospholamban (PLB), and the sarco/endoplasmic reticulum Ca-ATPase (SERCA) in response to methionine oxidation and tyrosine nitration contribute to the downregulation of cellular metabolism and ATP utilization and the associated generation of ROS associated with replenishing intracellular ATP through oxidative phosphorylation (9). In the case of CaM, the site-specific oxidation of Met^{145} induces global structural changes, which we find to promote the degradation by the proteasome (25, 97). Degradation is absolutely dependent upon association of the 20S proteasome with Hsp90, which recognizes oxidized CaM and promotes its degradation by the 20S proteasome (97). Under these conditions native (unoxidized) CaM is not a sub-

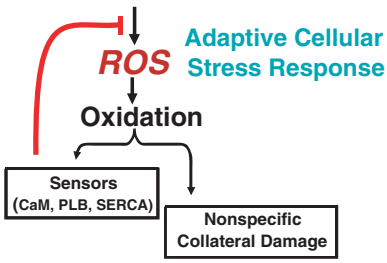

FIG. 1. Critical sensors of ROS determine cellular sensitivity to ROS. The functional sensitivity of key proteins to oxidative damage serves as a detector to balance metabolism, where there is a trade-off between optimal cellular function and the minimization of cellular damage. Oxidative modification of functionally sensitive sites on proteins that modulate calcium signaling and energy utilization will downregulate ATP consumption through respiratory control mechanisms and minimize the generation of ROS and the associated nonspecific oxidative damage to cellular proteins. For example, methionine oxidation in calmodulin (CaM) and phospholamban (PLB) is proposed to stabilize the inhibited state of the Ca-ATPase. Likewise, direct nitration of tyrosines in the Ca-ATPase functions to decrease function. Differential set-points of different organs are determined by the sensitivities of key sensors to oxidative and nitrative modification, which is determined by the reactivity of sensitive sites on regulatory proteins to particular ROS, endogenous antioxidant levels, repair proteins, and rates of protein degradation. Thus, some systems (e.g., heart) have sensitive set-points, which function to minimize nonspecific collateral damage, while others with greater regenerative capacities (e.g., skeletal muscle) tolerate larger amounts of cellular damage, permitting high levels of cellular function under conditions of oxidative stress.

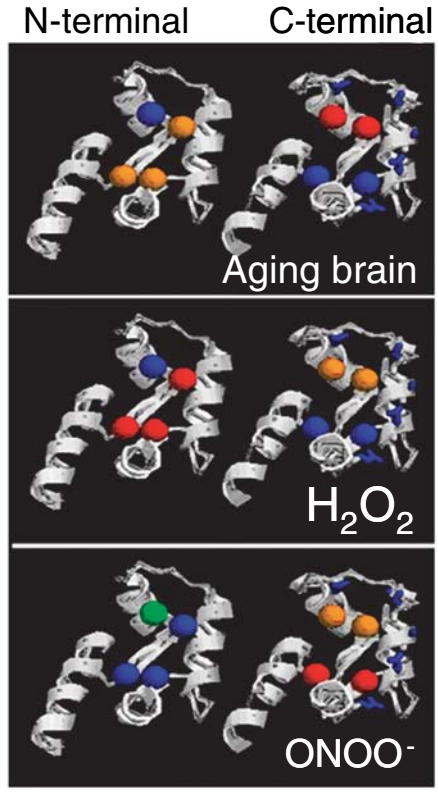

FIG. 2. Representation of patterns of methionine oxidation. *Colored spheres* indicate relative sensitivities of individual methionines to oxidation (*red* > *yellow* > *green* > *blue*) for CaM isolated from senescent brain in comparison to *in vitro* oxidation using either hydrogen peroxide or peroxynitrite (30, 47, 79). Glutamic acid side chains within 7 Å of individual sulfur atoms are indicated as *blue tubes*, since they function in the activation of peroxynitrite (8, 50).

strate for the proteasome. Likewise, Hsp90 has no influence on the rates of fluorogenic peptide cleavage commonly used to assess proteasome function, indicating that Hsp90 specifically promotes the degradation of oxidized CaM through a direct binding interaction that modulates access to the active sites in the 20S proteasome central chamber. The 20S proteasome alone does not degrade native or oxidized CaM (97). Thus, oxidation of multiple methionines in the CaM contributes to the loss of calcium regulation in aged individuals and may be associated with reductions in CaM levels during aging (30, 81).

CaM OXIDATION AND SENSITIVITIES TO DIFFERENT REACTIVE OXYGEN SPECIES

The pattern of methionine oxidation in CaM isolated from senescent brain has the potential to provide information regarding cellular stress mechanisms. Depending on cellular levels of $O_2^{\bullet -}$ and $NO^{\bullet}$, acute stress is possible following the formation of the highly reactive $ONOO^-$ species (6). There are large differences in the sensitivities of methionines in CaM to *in-vitro* oxidative modification (Fig. 2). While Met^{144} or Met^{145} are selectively oxidized by peroxynitrite (red spheres in Fig. 2), these sites are not significantly oxidized upon exposure to hydrogen peroxide (blue spheres in Fig. 2). In all cases oxidation of vicinal methionines near the carboxyl-terminus (i.e., Met^{144} and Met^{145}) correlate with functional inactivation (102). Indeed, a similar decrease in binding affinity and maximal CaM-dependent activation of the plasma membrane Ca-ATPase is observed upon complete oxidation of all nine methionines in comparison to the targeted oxidation of Met^{144} and Met^{145} (Fig. 3). These results indicate that CaM has the potential to function as a sensor for different ROS, such that under acute conditions of stress (associated with the generation of peroxynitrite) that functionally important sites (i.e., Met^{144} and Met^{145}) will be selectively oxidized. In aging brain these sites are not significantly oxidized (30), consistent with the presence of chronic oxidative stress involving relative unreactive species such as hydrogen peroxide, endogenous repair systems involving the methionine sulfoxide reductases, and as a result of targeted degradation following the oxidation of these functionally important sites. The latter involvement of the proteasome in the selective degradation of oxidized CaM is consistent with diminished levels of cellular CaM observed in aged animals.

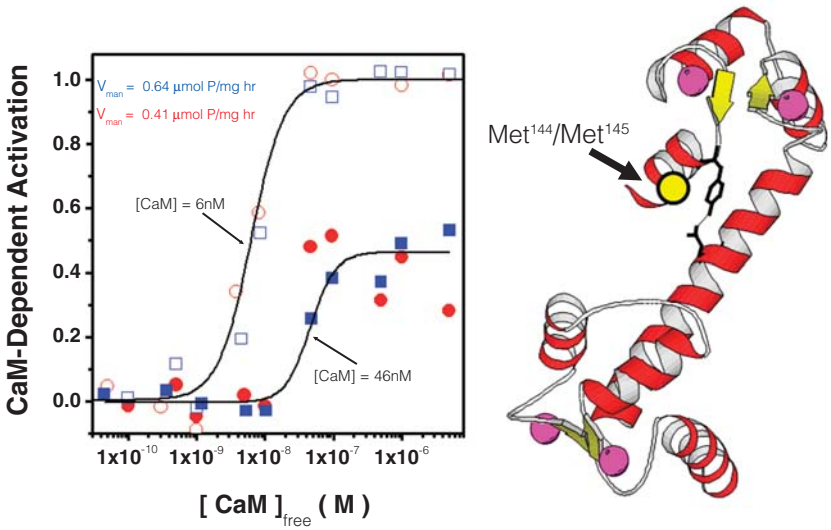

FIG. 3. Oxidant-induced functional loss is result of oxidation of Met[144] and Met[145]. (*Left*) CaM-dependence of the activation of the plasma membrane Ca-ATPase by either unoxidized CaM (○) or following the oxidation of all nine methionines (•) in comparison with a CaM mutant (□) in which site-directed mutagenesis was used to substitute the majority of methionines with leucines, permitting the determination of how the site-specific oxidation of Met[144] and Met[145] affects activation of the Ca-ATPase (■). (*Right*) Structural representation of CaM, depicting the locations of Met[144] and Met[145] in helix H near the C-terminus (*yellow ball*) relative to stabilizing hydrogen bond between Tyr[138] and Glu[82] (31, 86).

SELECTIVE DEGRADATION OF OXIDIZED CaM BY PROTEASOME REQUIRES Hsp90

The role of Hsp90 in mediating the selective degradation of oxidized proteins by the 20S proteasome was determined following the isolation of the 20S proteasome from erythrocytes free of Hsp90, permitting reconstitution of the 20S proteasome with Hsp90 (97). Hsp90 selectively recognizes oxidized CaM and promotes its degradation by the 20S proteasome (Figs. 4 and 5). The rate of CaM_{ox} degradation by the 20S proteasome is proportional to oxidant-induced decreases in secondary structure (Fig. 6A) (25). Cleavage occurs in a nonprocessive manner, such that CaM_{ox} is initially degraded into large fragments (Fig. 6B). The observation of the nonprocessive degradation of CaM_{ox} by the proteasome is consistent with earlier observations in which the degradation of many other protein substrates has been observed to involve the initial cleavage into large protein fragments at early times of digestion that can dissociate and then rebind and be further digested by the proteasome (64, 66, 67, 95, 105).

Native (unoxidized) CaM is not a substrate for the proteasome. Hsp90 has no influence on the rates of fluorogenic peptide cleavage, indicating that Hsp90 specifically promotes the degradation of oxidized CaM through a direct binding interaction that modulates access to the active sites in the 20S proteasome central chamber. Furthermore, 20S proteasome alone does not degrade native or oxidized CaM. These results are consistent with prior measurements of CaM degradation in cell culture, which indicated that CaM is degraded in a ubiquitin-independent mechanism by the proteasome (87). Furthermore, the 20S proteasome tightly binds Hsp90 under physiological conditions and cellular Hsp90 has been shown to play an important role in mediating protein turnover (19, 24, 48, 57, 94, 104). Indeed, from a mechanistic point of view, Hsp90 has been proposed to associate with the 20S proteasome through the same alpha subunits that associate with other regulatory proteins (i.e., 11S and 19S) (19, 36–38). Thus, the proposed role of Hsp90 in mediating the degrada-

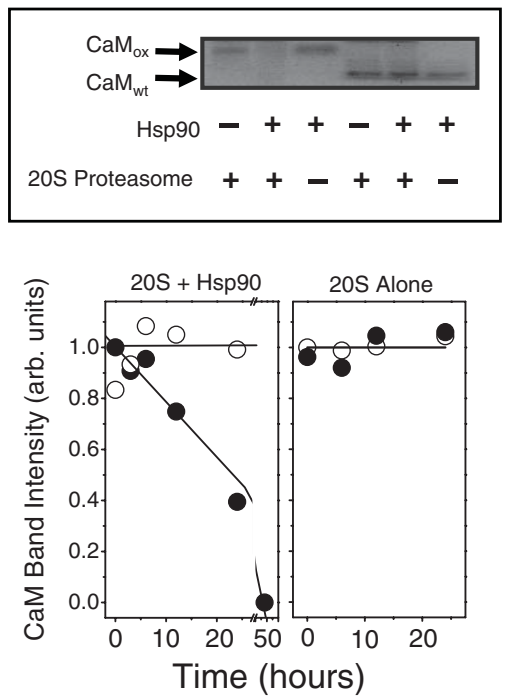

FIG. 4. Selective degradation of oxidized CaM by the 20S proteasome bound to Hsp90. SDS-PAGE following 24 hour incubation (*top panel*) and densitometric analysis of time-dependent changes in band intensity (*bottom panels*) of SDS-PAGE bands corresponding to oxidized (•; *top band in upper panel*) and native unoxidized (○; *bottom band in upper panel*) CaM by the 20S proteasome in the presence (*left panel*) and absence (*right panel*) of Hsp90. Rate of degradation of CaM_{ox} was determined from nonlinear least-squares fits to the data (*solid line*) in the presence of Hsp90 to be 2.2 ± 0.2% per hour, which corresponds to 0.55 ± 0.04 µmol CaM_{ox} degraded/mg 20S proteasome/hour. Experimental conditions involved incubation of 12 µ*M* native or oxidized CaM in the presence of 0.6 µ*M* 20S proteasome, and, when indicated, 2.5 µ*M* Hsp90 in 50 m*M* TRIS (pH 7.5), 0.1 *M* KCl, 10 m*M* $MgCl_2$, and 0.1 m*M* EGTA at 3°C for 24 hours.

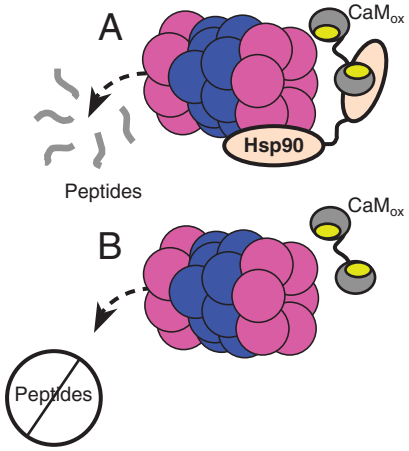

FIG. 5. Model of Hsp90-assisted proteasomal degradation. 20S proteasome protein complex composed of rings of α (*red*) and β (*blue*) subunits binds Hsp90 (*tan*) to selectively recognize and degrade CaM_{ox} (*gray*) (**A**). In the absence of Hsp90, CaM_{ox} is not degraded (**B**). Cartoon does not indicate oligomeric state of Hsp90, which exists primarily as a dimer in solution (32). Furthermore, simultaneous association between Hsp90 complexes at each end of the proteasome is probable, consistent with binding data indicating maximal activation of the 20S proteasome in the presence of a four-fold molar excess of Hsp90 (97).

tion of oxidized proteins is consistent with its association with a binding interface used by other regulators to modulate proteasome activity. Furthermore, since CaM_{ox} is a physiological substrate that is a target of oxidative stress during biological aging that when oxidized functions to tightly bind and block target protein activation (3, 4, 25, 29–31, 62, 87, 99, 100), these results provide a strong indication that critical signaling proteins such as CaM can serve as sensors of oxidative damage to regulate cellular metabolism.

Hsp90 AND CELLULAR STRESS RESPONSES

Hsp90, which accounts for approximately 2% of total cellular protein, is known to associate with approximately 200 different proteins whose intracellular functions are, in general, related to cellular signaling (104). The preferential binding of signaling proteins is consistent with other suggestions that Hsp90 plays a major role in the binding of misfolded proteins to prevent their aggregation and facilitate refolding, and the fact that signaling proteins tend to exhibit greater conformational heterogeneity than the vast majority of house-keeping enzymes whose structures stabilize an active site associated with chemical transformation. In solution, Hsp90 is primarily a dimer, although there is a dynamic equilibrium between

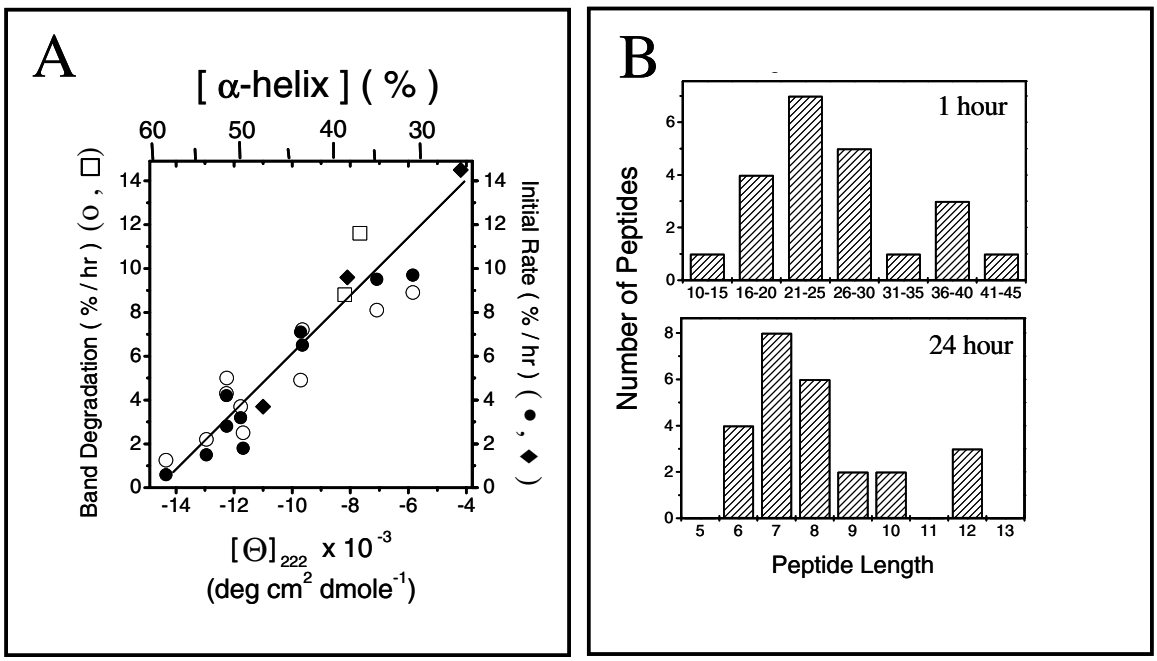

FIG. 6. Nonprocessive cleavage of oxidized CaM in response to disruption of secondary structure. Changes in molar ellipticity ($[\Theta]_{222}$) and calculated α-helical content correlate with rates of CaM degradation measured by the disappearance of the integrated intensity of all CaM bands on SDS-PAGE (○, □) or the initial rates of degradation measured by the reaction of free amines generated by peptide bond cleavage with fluorescamine (●, ■) after 1) variable extents of *in vitro* oxidation (○, ●), 2) calcium binding to oxidized CaM containing 8.2 ± 0.5 methionine sulfoxides/CaM (■), or 3) enzymatic reduction of CaM_{ox} by methionine sulfoxide reductase (□) (**A**). Cleavage occurs in a nonprocessive manner, with initial release of large peptides that subsequently rebind and are further digested (**B**). Adapted from reference (25).

multiple oligomeric states and associations with other co-chaperones (e.g., Hsp70, p23, Hip, and Hop) that is consistent with a large number of cellular roles that are linked to Hsp90 function (32). Like other heat-shock proteins, expression levels of Hsp90 are upregulated under conditions of oxidative stress, and ample evidence has been provided to indicate that Hsp90 plays an important role in mediating the maturation and protein turnover of numerous proteins, including steroid hormone receptors, telomerase, and nitric oxide synthase, as well as buffering mutations during cellular transformation to increase cell viability (12, 41, 71). Thus, given that the 20S proteasome is tightly associated with Hsp90 following ion-exchange and size-exclusion chromatography (18, 19, 61, 94), it is evident that Hsp90 has the potential to function as a physiological regulator of protein degradation. Indeed, under conditions of oxidative stress, such as occurs during biological aging, oxidized and aggregated proteins accumulate and correlate with declines in both the activity of the 20S proteasome and overall cellular function (e.g., calcium regulation and stress responses involving the upregulation of heat-shock proteins) (59, 81). Since oxidative stress both increases cytosolic calcium levels, and induces an upregulation of Hsp90 expression, it seems likely that these conditions promote the formation of an activated proteasome that favors the degradation of oxidized proteins (54).

OXIDATIVE STRESS, PROTEIN AGGREGATION, AND SUSCEPTIBILITY TO PROTEASOMAL DEGRADATION

Oxidative stress can directly inactivate the proteasome, as well as modulate subunit composition to modulate both activity and vulnerability to oxidative stress in accordance with earlier observations that the immunoproteasome is more sensitive to oxidative inactivation by peroxynitrite (1, 88). In addition, while mild conditions of oxidative stress enhance rates of protein turnover, more acute conditions reduce turnover as a result of oxidant-induced increases in protein aggregation (39, 78).

Thus age-dependent increases in the generation of ROS combined with diminished repair and degradative capacities have been suggested to contribute to an age-dependent increase in the rate of formation and accumulation of oxidized proteins that result in the accumulation of oxidized proteins that may underlie a range of human diseases (98). Increases in the fraction of incorrectly folded proteins or decreases in the function of chaperones or proteases (associated with aging) can result in an increase in the fraction of misfolded and damaged proteins, a process associated with the formation of protein aggregates during aging. Protein aggregates, in turn, can disrupt cellular functions and serve as nucleation sites for the aggregation of other unrelated proteins, which can tie up chaperones and proteases in a manner that further enhances the fraction of misfolded proteins in the cell. Critical to understanding this process is an identification of the protein composition within intracellular protein aggregates and associated post-translational modifications that may initiate the process. In this respect, chaperones such as Hsp90 and approximately twenty other signaling proteins are routinely found in protein aggregates (7, 52, 53, 83, 103).

DISTINCT ROLES OF CARBOXYL-TERMINAL METHIONINES IN MODULATING CAM FUNCTION

Oxidation of Met^{144} near the carboxyl-terminus of CaM is primarily responsible for inducing structural changes associated with the nonproductive binding that stabilizes the inhibited state of the plasma membrane Ca-ATPase (4, 29, 31, 68, 100, 102). Oxidation of the remaining methionines has a minimal functional effect, resulting in only modest decreases in calcium affinity and target protein binding (4). This correlation can be appreciated through a consideration of the structure of CaM (Fig. 7), where it is apparent that Met^{144} in Helix H forms contact interactions with neighboring residues on Helix G that have the potential to stabilize the structure of the carboxyl-terminal domain. Since association between CaM and many target proteins, including the plasma membrane Ca-ATPase, involves the initial association between the binding cleft in the carboxyl-terminal domain and a conserved tryptophan residue that functions as a hydrophobic anchor to mediate the ordered and sequential binding of the opposing domains of CaM to mediate enzyme activation (21, 85), these results suggest that modulation of interhelical contacts may function to modulate the structure of the binding site.

In contrast to the functional effects associated with oxidation of Met^{144}, the oxidation of Met^{145} is primarily responsible for the selective degradation of CaM_{ox} by the proteasome, resulting in rates of degradation that are analogous to that observed for fully oxidized CaM (Fig. 8). Oxidation of Met^{144} has been shown to have essentially no effect on rates of CaM_{ox} degradation. Since Met^{145} undergoes contact interactions with Helix E (Fig. 7), which mediates interdomain interactions, these results suggest that disruption of this linkage selectively uncouples the opposing domains of CaM to expose disordered sequences that permit recognition and digestion by the proteasome. Indeed, the disruption of the interaction between Tyr^{138} and Glu^{82} results in the structural uncoupling between the opposing domains of calcium activated CaM (Fig. 9) (86), emphasizing the importance of stabilizing interactions between Helices H and E in mediating interdomain coupling. Further, the degradation of CaM_{ox} by the proteasome involves the cleavage into large pieces that appear to preferentially target the interdomain linker connecting the opposing domain (25). These results indicate that differences in the sensitivities of Met^{144} and Met^{145} to oxidation by difference reactive oxygen species will have important functional consequences. Whereas oxidation of Met^{144} will preferentially alter target protein binding and activation, the oxidation of Met^{145} will promote the recognition and degradation of CaM_{ox} by the proteasome. In conclusion, the distinct roles of Met^{144} and Met^{145} permits their oxidation to function as a general switching mechanism to modulate both target protein function and cellular concentrations of CaM.

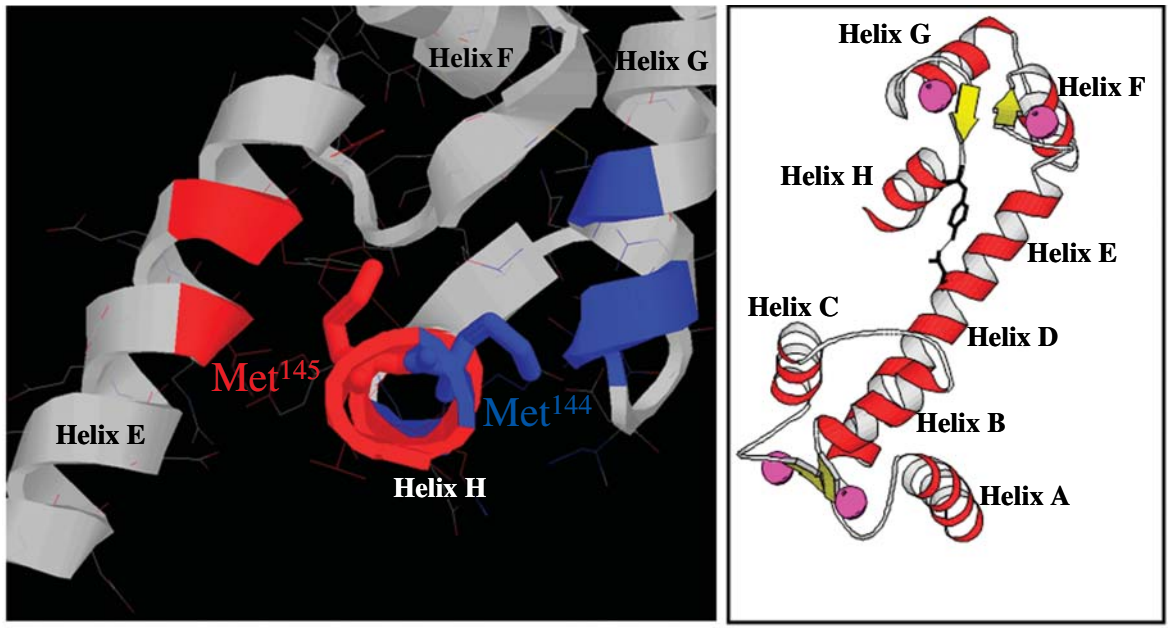

FIG. 7. Spatial relationships of Met^{144} and Met^{145} with respect to global fold of CaM. Protein fold is shown for carboxyl-terminal domain (*left*) and entire protein (*right*) of calcium-activated CaM (1 cll.pdb) (14), demonstrating steric relationships between methionine side chains and contact interactions with proximal helices. **Left panel** shows an end-on view down Helix H (*red*) demonstrating spatial relationships between Met^{144} (*blue*) and Met^{145} (*red*) with respect to sites on Helix G and E in carboxyl-terminal domain of CaM (**Left**). Sites within 8 Å of Met^{144} or Met^{145} are respectively colored either *blue* or *red*. **Right panel** illustrates hydrogen bond between Tyr^{138} in Helix H and Glu^{82} in the interdomain linkage connecting Helix E and Helix D, as previously described (31, 86).

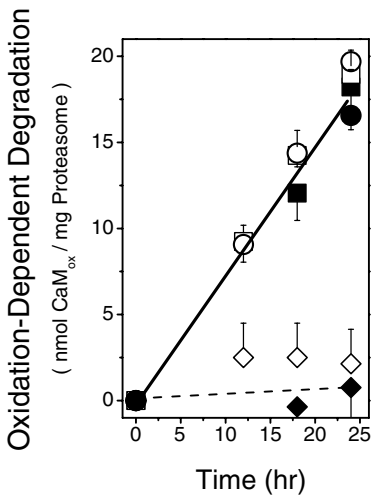

FIG. 8. Targeted degradation of CaM$_{ox}$ by the 20S proteasome upon oxidation of Met^{145}. Extent of proteasomal degradation at indicated time-points is shown upon oxidation of all nine methionines in wild-type CaM (○, ●) and upon the selected oxidation of either Met^{144} (◊, □) or Met^{145} (□, ■) for either apo-CaM (○, ◊, □) or following calcium-activation (●, □, ■).

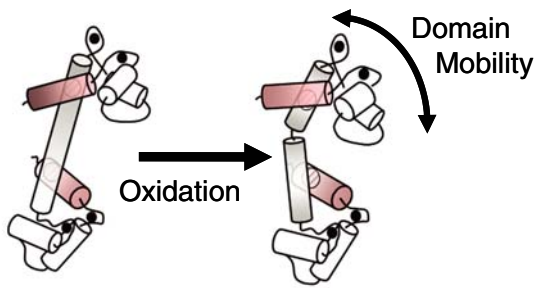

FIG. 9. Methionine oxidation disrupts the structural coupling between the opposing domains of CaM. Methionine oxidation of functionally sensitive methionines (i.e., Met^{144} and Met^{145}) in helix H destabilizes the interhelical interaction between helix H (*pink cylinder*) and Helix E (*gray cylinder*) in the C-terminal domain (*bottom of figure*), depicted as the appearance of an exposed binding site (*light hatched circles*). This structural change results in a less stable interdomain interaction through helices D and E (*gray shaded cylinders*) connecting the opposing domains of CaM, inducing a structural uncoupling between helix A (*pink cylinder*) and helix D (*gray cylinder*) in the N-terminal domain (*top of figure*), resulting in the independent rotational mobility of the opposing domains of CaM. Adapted from references (15, 16).

REDOX COUPLING OF CELLULAR METABOLISM THROUGH CaM OXIDATION

Site-specific post-translational modifications are the hallmark of cellular signaling mechanisms, and identification of a functional correlation between the oxidation of a methionine in CaM to function suggests physiological significance. Thus, the ability of endogenous methionine sulfoxide reductases present in all organisms to reduce (i.e., repair) oxidized methionines or the selective degradation of CaM_{ox} by the proteasome indicates that following an acute oxidative stress that the oxidation of CaM is likely to be transient (25, 35, 45, 55, 63, 76, 84, 101).

The transient decrease in CaM function following oxidation can 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 ATP hydrolysis and thereby minimize the energy needs of the cell. For example, when ROS exceed antioxidant cellular defense mechanisms, cellular NADPH and reduced thioredoxin levels decrease and result in the inactivation of methionine sulfoxide reductases. Following restoration of cellular redox conditions, repair of oxidized methionines occurs with a restoration of protein function. Alternatively, under chronic conditions of oxidative stress degradation of CaM_{ox} by the proteasome will function to lower cellular CaM concentrations with an associated decrease in metabolic rates due to the two-fold excess of CaM-dependent enzymes (72, 73, 90). In conclusion, CaM oxidation represents an important switch point at the first step of the calcium signal to modulate metabolism and orchestrate an adaptive cellular response through transient changes in activity or through the downregulation of CaM levels.

Additional insight regarding the importance of oxidant-induced changes in CaM function is apparent from a consideration of sequence differences near Met^{144} and Met^{145} between species (102). Whereas all CaM sequences have very high levels of sequence identity (91% identity between wheat and vertebrate), most of the sequence divergence is near the carboxyl-terminus (102), suggesting that the sensitivity of CaM to oxidation is dependent on the ecological niche of an organism. Thus, a comparison between the rates of oxidation of CaM isolated from vertebrate and plant sources demonstrated the rate of oxidation of the majority of methionines are essentially identical; however, there is a four-fold greater rate of oxidation of Met^{144} or Met^{145} in plant CaM that correlates with the functional sensitivity of CaM to oxidative stress. These results indicate large species-dependent differences in the functional sensitivities of CaM to oxidative stress.

Given that the oxidation of CaM functions to stabilize the inhibited state of the plasma membrane Ca-ATPase (and presumably other targets as well), these results suggest an optimization of the sensitivity of CaM to oxidative stress to modulate the set point of the system (see Fig. 1). Thus, in the case of vertebrates, larger amounts of nonspecific collateral damage occur prior to the downregulation of CaM function. Markedly different functional sensitivities of CaM to different ROS further modulates function (81). For example, while peroxynitrite selectively oxidizes Met^{144} and Met^{145}, hydrogen peroxide has a minimal effect on these functional sensors of ROS—indicating that the metabolism of different tissues will result in markedly different functional sensitivities of CaM to oxidative modification (30, 79, 102). Likewise, in a comparison of different organ systems, differences in the levels of antioxidants, repair, and degradative enzymes modulate the set point of the system, whereby the accumulation of oxidized CaM functions to downregulate metabolism and minimize the nonspecific oxidative damage to other proteins and biomolecules. Thus, under acute conditions associated with the generation of peroxynitrite, energy metabolism will be transiently downregulated through the reversible oxidation of Met^{144} or Met^{145} in CaM, while under chronic conditions in-

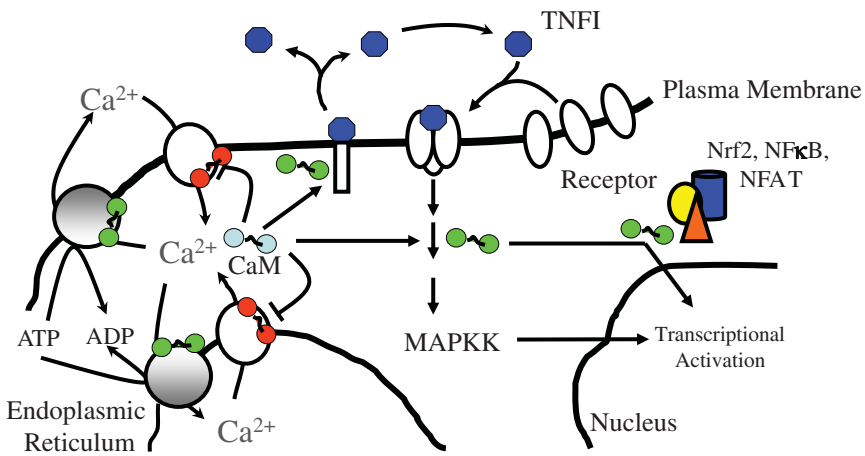

FIG. 10. Depiction of central role for CaM in modulating calcium homeostasis and signal transduction cascades. CaM functions as a central calcium sensor to integrate activating concentrations of calcium through the activation of calcium pumps, inhibition of calcium channels, and coordinated regulation of matrix metalloproteases associated with cellular shedding and cytokine release (e.g., TNFα). Coordinate regulation of the ERK pathways (MAPKK) through modulation of Ras activity and transcriptional activation permits changes in calcium homeostasis to mediate long-term reprogramming of cellular metabolism, consistent with age-dependent changes in growth factor signaling (26).

volving the generation of hydrogen peroxide, minimal levels of oxidation occur at these functionally important sites (79, 81, 102).

The consequences associated with the sensitivity of CaM to oxidative stress is apparent if one considers that cellular levels of CaM are limiting, with an approximately two-fold molar excess of target proteins (72, 73, 90). Given the key role CaM plays in linking calcium signaling to energy metabolism and long-term transcriptional regulation (Fig. 10), diminished CaM levels or function in response to oxidative stress will function to downregulate cellular metabolism. Furthermore, CaM functions both to mediate growth factor and cytokine release and cellular sensing of these bioactive compounds through modulation of receptor function. Additional regulation involves downstream signaling pathways involving the activation of MAP kinases through the regulation of Ras activity. These linked pathways, known to be downregulated under conditions of oxidative stress and during aging (26), suggest that changes in oxidant-induced changes in CaM function or expression will have profound consequences on cellular metabolism to serve as a feedback regulator to minimize nonspecific oxidative damage.

ABBREVIATIONS

CaM, calmodulin; CaM_{ox}, oxidized CaM in which methionines are oxidized to methionine sulfoxide; CaM_{wt}, unoxidized CaM; Hsp90, heat shock protein 90; Msr, methionine sulfoxide reductase; PLB, phospholamban; ROS, reactive oxygen species; SERCA, sarco/endoplasmic reticulum calcium ATPase.

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