Hsp90 Enhances Degradation of Oxidized Calmodulin
by the 20S Proteasome

Jennifer E. Whittier*, Yijia Xiong*, Martin C. Rechsteiner†, and Thomas C. Squier*

* Cell Biology Group, Biological Sciences Division, Pacific Northwest National Laboratory, Richland, WA 99352 and †Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, UT 84132.

Keywords: Oxidative Stress, Protein Degradation, Calcium Signaling Proteins, Heat Shock Proteins

¶This work was supported by a grant from the National Institutes of Health (AG17996).

Correspondence should be addressed to Thomas C. Squier, Pacific Northwest National Laboratory; P.O. Box 999; Mail Stop P7-53; Richland, WA 99352; email: thomas.squier@pnl.gov; Tel: (509) 376-2218; FAX: (509) 372-1632.
Running Title: Oxidative Stress and Protein Degradation
SUMMARY

The 20S proteasome has been suggested to play a critical role in mediating the degradation of abnormal proteins under conditions of oxidative stress, and has been found in tight association with the molecular chaperone Hsp90. To elucidate the role of Hsp90 in promoting the degradation of oxidized calmodulin (CaM\textsubscript{ox}), we have purified red blood cell 20S proteasomes free of Hsp90 and assessed their ability to degrade CaM\textsubscript{ox} in the absence or presence of Hsp90. Purified 20S proteasome does not degrade CaM\textsubscript{ox} unless Hsp90 is added. CaM\textsubscript{ox} degradation is sensitive to both proteasome and Hsp90-specific inhibitors, and is further enhanced in the presence of 2 mM ATP. Irrespective of the presence of Hsp90, we find that unoxidized CaM is not significantly degraded. Direct binding measurements demonstrate that Hsp90 selectively associates with CaM\textsubscript{ox}; essentially no binding is observed between Hsp90 and unoxidized CaM. These results indicate that Hsp90 in association with the 20S proteasome can selectively associate with oxidized and partially unfolded CaM to promote degradation by the proteasome.
INTRODUCTION

The age-dependent decline in proteasome function has been suggested to underlie the accumulation of oxidized and aggregated proteins during biological aging (1-7). The 20S proteasome is a cylindrical particle in which the catalytic active sites face a buried chamber. The 20S proteasome associates with other protein complexes such as 11S REG (i.e., PA28) and/or 19S (i.e., PA700) protein complexes that modulate the specificity of protein degradation (8-11). While 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 has been suggested to be largely responsible for the degradation of oxidized proteins, which occurs in an ATP-independent manner (5, 12-14).

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 (15-17). 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 (18, 19). 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 (16, 20). The chaperone Hsp90 has also been suggested to bind the α-subunits of the 20S proteasome and to function as a regulator of proteasome function (21-23). Thus, observed cellular linkages between Hsp90 function and rates of protein degradation may involve both the stabilization of partially unfolded proteins, as well as a direct modulation of protein degradation by the proteasome (24-31).

The high-affinity association between purified Hsp90 and the 20S proteasome (Kd < 100 nM), in conjunction with the routine presence of Hsp90 in purified preparations of the 20S proteasome, suggests a possible functional involvement in modulating protein degradation (7, 21, 23, 32-34). Indeed, functional inhibitors of Hsp90 modulate rates of protein degradation and inhibit the activity of the proteasome in cellular assays (21, 25, 28, 35). 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 (7, 32, 36).
Understanding the mechanism underlying observed cellular responses to modulators of Hsp90 function is compounded by the multiple cellular functions ascribed to Hsp90. These functions include, in addition to the minimization of irreversible protein aggregation between unstructured proteins, associations with other chaperones (e.g., Hsp70 and p50) to assist in protein folding as well as stabilizing approximately 200 different signaling proteins (26, 35, 37, 38). The association between Hsp90 and the 20S proteasome has, furthermore, been implicated in modulating the dynamic exchange of the 11S REG and 19S regulatory subunits to affect proteasome function, as an allosteric activator of the proteasome, and to play a critical role in protecting the 20S proteasome against oxidative inactivation (2, 22, 39). Thus, there is expected to be an important interplay between oxidative modification and subsequent degradation of key signaling proteins by the proteasome, and the oxidative-inactivation of the proteasome itself.

In this latter respect, conditions of oxidative stress are known to result in the selective oxidative modification of calcium regulatory proteins to modulate signal transduction and cellular energy metabolism (40). For example, multiple methionines in the calcium regulatory protein calmodulin (CaM) are selectively oxidized to their corresponding methionine sulfoxides during aging, resulting in a reduced ability to activate a range of different target proteins, including the plasma membrane Ca-ATPase, nitric oxide synthase, and the ryanodine receptor calcium release channel (41-44). Restoration of cell function requires the repair or degradation of CaM and other signaling proteins. Given the known role of Hsp90 in modulating the stability and function of approximately 200 different signaling proteins, and the role of the 20S proteasome in the selective degradation of oxidized proteins, we have investigated the possible functional involvement of Hsp90 in facilitating the degradation of oxidized CaM by the 20S proteasome. This work is, in part, motivated by the fact that the vast majority of previous measurements of 20S proteolytic activity used fluorogenic peptides or extensively alkylated and unfolded proteins as substrates. These artificial substrates may not reflect proteasome function vis-a-vis normal physiological substrates (40, 45).

To assess the possible role of Hsp90 in mediating the selective degradation of oxidized proteins by the 20S proteasome, we have isolated the 20S proteasome from erythrocytes free of Hsp90, and measured the ability of the proteasome to recognize and degrade native and oxidized CaM. CaM was chosen both because of its key role in intracellular signal transduction and its functional sensitivity to oxidative stress (41, 42, 46). Furthermore, CaM has previously been shown to be degraded in a ubiquitin-independent mechanism by the proteasome (47). However, Hsp90 selectively recognizes oxidized CaM and promotes its degradation by the 20S proteasome. Under these conditions native (unoxidized) CaM is not a substrate for the proteasome. Likewise, 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. We find that 20S proteasomes alone do not degrade
native or oxidized CaM to any appreciable extent.
EXPERIMENTAL PROCEDURES

**Materials.** ATP, AMP-PNP, bovine brain Hsp90α/β, carboxbenzox-Leu-Leu-Glu-β-napthylamine (Cbz-LLE-Na), fluorescamine, geldanamycin, MES, MG132, MOPS, novobiocin, N-succinyl-Leu-Leu-Val-Tyr-7 amido-4-methyl coumarin (Suc-AAF-Amc), N-tBoc-Gly-Lys-Arg-7 amido-4-methyl coumarin (tBoc-GKR-Amc), PIPES, and Tris were purchased from Sigma (St. Louis, MO). Glycine, SDS, precast gels, and Gel Code Blue stain were obtained from Bio-Rad (Hercules, CA). Benchmark protein ladder molecular weight markers were from Life Technologies, Inc (Grand Island, NY). Calmodulin (CaM), bovine 20S proteasome, and 11S REG proteasome regulator were purified, essentially as described previously (46, 48-50). Isolated 20S proteasome was shown to be free of Hsp90 by Western immunoblotting, in agreement with earlier observations (50). Protein concentration was measured using a micro-BCA assay reagent kit (Pierce, Rockford, IL) using either CaM or bovine serum albumin as protein standards. In the case of the CaM protein standard, the concentration was determined using the published molar extinction coefficient (ε_{277nm} = 3029 M⁻¹ cm⁻¹) for calcium saturated CaM (51).

**CaM Oxidation.** Oxidation of all nine methionines in wild-type CaM (CaM_{wt}) to their corresponding sulfoxides in oxidized (CaM_{ox}) was accomplished by incubating 60 µM CaM_{wt} in 50 mM HOMOPIPES (pH 5.0), 0.1M KCl, 1 mM MgCl₂, and 0.1 mM CaCl₂ with 50 mM H₂O₂ for 24 hours at 25 °C. The concentration of H₂O₂ was determined by using the published extinction coefficient, ε_{240nm} = 39.4 ± 0.2 M⁻¹ cm⁻¹(52). Following incubation, the reaction was stopped by dialyzing sample against multiple changes of distilled water (3 × 5 liters) buffered with sodium bicarbonate at 4 °C.

**Mass Spectrometric Analysis.** CaM samples were injected onto the column (C₄, 5 cm × 1 mm i.d., Micro-Tech Scientific) equilibrated with 40 % ethanol in 0.1% aqueous TFA and eluted with a linear gradient (3 % ethanol/min) developed with an Ultra Plus II MicroLC system (Micro-Tech Scientific, Vista, CA) at a flow rate 50 µl/min. For routine analyses in which the total CaM oxiform distribution was to be determined a hand packed C₁₈ trap column (1 cm × 1 mm i.d.) was used with elution by 60 % ethanol in 0.1% aqueous TFA. The HPLC or trap column was coupled to the electrospray source of a Q-Tof-2™ mass spectrometer (Micromass Ltd, UK). Mass spectra were acquired with the instrument cone voltage at 60 eV and dry gas nitrogen heated to 150° C. The experiment was set up such that ESI-MS and ESI-CID spectra were acquired in alternating 5 sec scans. Mass spectra in the ESI-MS mode were acquired in the mass range 1000-3000. ESI-CID mass spectra...
were acquired on ions in 14+ charge state clusters in the mass range 100-3000. Precursor ions were selected with a window 11 Da wide in order to cover all possible oxiforms in the 14+ cluster for oxidized CaM. The collision energy was 40 eV. To obtain whole protein CaM mass spectra, charge deconvolution of all major charge states (15+ through 6+) was performed.

**CaM Proteolysis by the 20S Proteasome.** Rates of proteolysis using oxidized CaM as a substrate for the proteasome were determined using two different assays, essentially as described previously (13, 50). Briefly, these assays involved i) monitoring the disappearance of the integrated intensity of CaM bands on SDS-polyacrylamide gels and 2) measurement of the initial release of peptides generated by proteasome cleavage using fluorescamine, which forms a fluorescent adduct with the amino termini of peptides (13, 53). Identical experimental conditions were used for both assays, which involved the incubation of native or oxidized CaM (12 µM) with the 20S proteasome (0.6 µM) in the absence and presence of Hsp90 (2.5 µM) at 37 °C in 0.1M KCl, 10mM MgCl2, 0.1 mM EGTA and 50 mM of one of the following buffers: MES (pH 6.0), PIPES (pH 6.5), MOPS (pH 7.0), or TRIS (pH 7.5). In all cases the 20S proteasome in the absence or presence of Hsp90 was incubated at 37 °C for one hour prior to substrate addition, permitting an efficient functional reconstitution. Purity of individual proteins was demonstrated by SDS-PAGE (Figure 1). Oxidation of CaM results in a mobility change on SDS-PAGE gels, with CaMox running at a significantly higher apparent mass in comparison with unoxidized CaM (13).

**Fluorescent Labeling of CaM.** CaM was reacted with Alexa Fluor 532 using a protein labeling kit purchased from Molecular Probes (Eugene, OR) and separated from unreacted probe using a G-25 Sephadex column. The final molar stoichiometry was approximately one mole of fluorophore per mole of CaM.

**FCS Measurements.** Fluorescence correlation spectra were obtained using a Nikon TE300 inverted microscope modified for these measurements, where excitation was from a Coherent Verdi laser (532 nm) focused by a 100× objective lens (S Fluo100, Nikon, Japan). The fluorescence was collected using the same objective, separated by a 550DCLP dichroic mirror (Chroma Technology, Brattleboro, VT), split by a cube beam splitter (Thorlabs, Newton, NJ), and then filtered using an HQ560 emission filter (Chroma Technologies). The resulting fluorescence was detected by a pair of SPCM-AQR-14 avalanche photodiodes (Perkin-Elmer Optoelectronics, Vaudreuil, Canada). The output was detected by a Flex01-05D multi-tau correlator (Correlator.com, Bridgewater, NJ), run under dual-cross mode for 300 seconds. Evaluation of the curves was carried out by Origin’s nonlinear fitting function (MicroCal software, Northampton, MA).
RESULTS

Oxidized CaM as a Substrate for the 20S Proteasome. A quantitative assessment of the extent of CaM oxidation was obtained using ESI-MS. Before oxidative modification, CaM exhibits a single major ESI-MS peak corresponding to a mass of 16,706 ± 3 Da (Figure 2), in close agreement with the theoretical average mass of vertebrate CaM expressed and purified from *E. coli* (16,705.4 Da). An additional peak at 16,688 ± 3 Da corresponds to a dehydration artifact associated with ionization in the mass spectrometer (54). Overnight exposure of CaM<sub>wt</sub> to H<sub>2</sub>O<sub>2</sub> resulted in a 144 Da mass shift of the ESI-MS spectrum, with a major peak at 16,850 ± 3 Da corresponding to the oxidative modification of all nine methionines to their corresponding methionine sulfoxides in CaM<sub>ox</sub>.

Hsp90 Mediates the Selective Degradation of CaM<sub>ox</sub> by the 20S Proteasome. We have investigated the possible involvement of Hsp90 in the degradation of oxidized CaM by the 20S proteasome. In the absence of added Hsp90, neither native nor oxidized CaM is significantly degraded over the 24 hour time-course of the experiment, as determined following a densitometric analysis of the SDS-PAGE gels (Figure 3). These results suggest that Hsp90, which co-purified with 20S proteasome isolated from liver homogenates, may have been a key factor in the selective degradation of CaM<sub>ox</sub> previously attributed to the 20S proteasome alone (13). To investigate this possibility, we have reconstituted the 20S proteasome with Hsp90. Following addition of a four-fold molar excess of Hsp90 to the 20S proteasome, the selective degradation of CaM<sub>ox</sub> is observed. Under these conditions the rate of degradation (i.e., 0.55 ± 0.04 µmol CaM<sub>ox</sub> degraded / mg 20S proteasome / hour) is virtually identical to that previously observed for 20S proteasome purified from liver, which contains tightly associated Hsp90 (13). In contrast, native CaM is not significantly degraded over this time-span, irrespective of the presence or absence of Hsp90. These results demonstrate that association of Hsp90 with the 20S proteasome facilitates the preferential degradation of CaM<sub>ox</sub>.

Hsp90-Dependence of CaM<sub>ox</sub> Degradation by 20S Proteasome. An appreciation of the molar stoichiometry of Hsp90 for maximal activation of the 20S proteasome is necessary to understand the underlying mechanism of CaM<sub>ox</sub> degradation. We have, therefore, assessed the ability of the 20S proteasome to degrade CaM<sub>ox</sub> in the presence of variable amounts of Hsp90. One observes a linear increase in the rates of CaM<sub>ox</sub> degradation upon increasing the molar stoichiometry from zero to four moles of Hsp90 per mole of 20S proteasome, with no additional increase in activity upon the addition of more Hsp90 (Figure 4). Given the high-affinity association between Hsp90 and the 20S proteasome (*K<sub>d</sub> < 100 nM*) (21, 23, 32-34), the maximal activation of 20S proteasome degradative
activity in the presence of four Hsp90 molecules suggests occupancy of all regulatory sites on the 20S proteasome. The lack of any additional activation in the presence of excess Hsp90 argues against an indirect role, where Hsp90 binding to CaMox would, for example, expose specific binding sites on CaMox that result in targeted protein degradation by the 20S proteasome. Further, since Hsp90 normally exists as a dimer in solution (55), these results are consistent with a model where maximal activation of the 20S proteasome involves association with two Hsp90 dimers.

**Minimal Effect of Hsp90 on Fluorogenic Peptide Hydrolysis by 20S Proteasome.** The underlying mechanism responsible for the Hsp90-mediated degradation of CaMox was investigated using the fluorogenic peptide Cbz-LLE-Na, which is commonly used to access the peptidylglutamyl or caspase-like activity of the proteasome. We find that the rate of peptide cleavage for the 20S proteasome alone is comparable with those previously reported, and is inhibited by the proteasome-specific inhibitor MG132 (Figure 5) (7, 56). However, in contrast to the Hsp90-stimulated rate of degradation of CaMox, there was no significant increase in the rate of peptide hydrolysis in the presence of Hsp90. A similar insensitivity of the 20S proteasome to the presence of Hsp90 is observed using the fluorogenic peptides Suc-LLVY-Amc and tBoc-GKR-Amc, which respectively measure the chymotrypsin-like and trypsin-like activities of the proteasome (Table 1, Figure 6). Thus, the mechanism underlying degradation of fluorogenic peptides by 20S proteasome is fundamentally different from that underlying the selective degradation of oxidized proteins mediated by Hsp90. These latter results are consistent with earlier observations that the catalytic mechanism underlying the cleavage of fluorogenic peptide is distinct to that associated with protein substrates (45).

**Time-Dependent Activation of 20S Proteasome.** Irrespective of the presence of Hsp90, the 20S proteasome remains catalytically inactive for approximately ten to fifteen minutes upon incubation at 37 °C (57) (Figure 6). At 25 °C, longer times are required for the appearance of the latent enzyme activity of the 20S proteasome. These results are consistent with a requirement for temperature-induced conformational rearrangements that enhance the probability of channel opening to promote the degradation of small peptides by the 20S proteasome (57). Further, while the peptidolytic activity of the 20S proteasome for different fluorogenic peptides are known to have very different pH optima (58), rates of peptidase activity are independent of Hsp90 under slightly acidic conditions (i.e., pH 6.5) (Table 1). These results indicate that while pH-dependent conformational changes differentially affect the proximity of active sites in the proteasome that are primarily associated with the caspase-like, chymotrypsin-like, and trypsin-like activities, that Hsp90 does not function to modulate these allosteric interactions to affect enzyme activity.
Optimal Rates of CaM<sub>ox</sub> Degradation Under Conditions Favoring Association with Hsp90.

Additional understanding of how the specificity of Hsp90 binding to the 20S proteasome affects catalytic function was assessed from a consideration of the pH-dependence of CaM<sub>ox</sub> degradation by the 20S proteasome (Figure 7). Previously, Angeletti and co-workers reported that optimal binding of Hsp90 to the 20S proteasome occurred under slightly acidic conditions near pH 6.5 (23). In these measurements, initial rates of protein degradation were measured, where the overall rates of protein degradation are similar to that observed using SDS-PAGE. Consistent with the proposed role of Hsp90 in mediating protein degradation by the 20S proteasome, we find a similar trend, with an optimal activity for CaM<sub>ox</sub> degradation by the 20S proteasome at pH 6.5 (Figure 7B). Equivalent levels of inhibition that approached the activity observed for 20S proteasome alone (no added Hsp90) were observed using inhibitors specific against either the 20S proteasome (i.e., MG132 and epoxomicin) or those against either the amino- or carboxyl-terminal domains of Hsp90 (i.e., geldanamycin and novobiocin) (59, 60) (Figure 7C). These latter results are in contrast to those associated with the peptidase activity against fluorogenic peptides, where Hsp90 had not affect on the rates of hydrolysis (Table 1). Thus, the selective degradation of CaM<sub>ox</sub> by the 20S proteasome in the presence of Hsp90 involves a distinct mechanism to that associated with peptide recognition and hydrolysis by the 20S proteasome. These latter results strongly support a direct role for Hsp90 in mediating the degradation of CaM<sub>ox</sub> by the 20S proteasome.

ATP Hydrolysis Stimulates Degradation of CaM<sub>ox</sub>. ATP hydrolysis has been implicated in the ability of Hsp90 to mediate protein folding (26, 61), and the ability of the nucleotide antagonist geldanamycin to inhibit the degradation of CaM<sub>ox</sub> by the 20S proteasome in the presence of Hsp90 suggests a possible role for nucleotide binding or hydrolysis in mediating protein degradation. To assess the possible role of nucleotide hydrolysis, we have investigated the rate of CaM<sub>ox</sub> degradation in the presence of either ATP or the nonhydrolyzable ATP analog AMPPNP. Addition of ATP results in an approximate 30% increase in the rate of protein degradation (data not shown). In contrast, addition of AMPPNP does not affect the rate of CaM<sub>ox</sub> degradation relative to that associated with Hsp90 alone. These results indicate that ATP hydrolysis facilitates Hsp90 function under steady-state conditions.

Hsp90 Selectively Associates with CaM<sub>ox</sub>. To determine whether Hsp90 selectively recognizes and binds oxidized CaM, we have used fluorescence correlation spectroscopy (FCS) to monitor the association between CaM and Hsp90 (Figure 8). This technique monitors the diffusion times for individual molecules across a diffraction limited spot, permitting the use of nanomolar concentrations of proteins to directly access high-affinity binding interactions with dissociation constants in the nanomolar range (62, 63). The raw data monitors the time it takes for individual fluorescently labeled
molecules to traverse through the illuminated region, and is analyzed by autocorrelating time-dependent changes in the signal intensity. The half-time associated with the autocorrelation function is a measure of the apparent translational diffusion coefficient, which is directly related to the size of the diffusing protein complex.

The sensitivity of the FCS measurement to protein association is clear from a consideration of antibody binding to an Alexa-Fluor 532 labeled CaM. Under these conditions the half-point of the autocorrelation function shifts by 0.13 ms toward longer times upon association with an IgG antibody (Stokes radius of 5.5 nm) (64) (Figure 8A). The dilute concentrations of CaM (1 nM) and antibody (5 nM) indicate a high affinity binding interaction. A similar shift of 0.10 ms toward longer times is observed upon incubation of CaMox with Hsp90 (Stokes radius of 6.5 nm) (55) (Figure 8C), consistent with a high affinity interaction between CaM and Hsp90. Further, the comparable shift is the autocorrelation function to that observed upon antibody binding is consistent with earlier observations that Hsp90 self-associates to form oligomeric complexes with a comparable Stokes radius to IgG. In contrast, there is no significant change in the autocorrelation function associated with native CaM upon incubation with Hsp90 (Figure 8D). These latter results indicate a selective high-affinity association between CaMox and Hsp90.

Under the conditions used for the above measurements, Hsp90 selectively associates with the 20S proteasome, as indicated by the shift in the autocorrelation function between Alexa-Fluor 532 labeled Hsp90 following incubation with increasing amounts of 20S proteasome (Stokes radius equals 7.6 nm) (65). These latter results are consistent with earlier measurements of the high affinity association between Hsp90 and the 20S proteasome (21, 23) (Figure 5), suggesting that prior to degradation by the 20S proteasome that CaMox selectively associates with Hsp90 (Figure 9).
DISCUSSION

Summary. We have demonstrated an important role for Hsp90, in association with the 20S proteasome, in mediating the selective degradation of an oxidized signaling protein (i.e., CaM). Significant degradation of CaMox by the 20S proteasome occurs only in the presence of Hsp90 (Figure 3), indicating that the commonly observed co-purification of 20S proteasome with Hsp90 from tissue is likely to be a reflection of their close association in vivo. Maximal rates of CaMox degradation by the 20S proteasome are observed upon addition of a four-fold molar excess of Hsp90 (Figure 4), consistent with a high-affinity association between two Hsp90 dimers and the proteasome. Based on FCS measurements, CaMox selectively associates with Hsp90 (Figure 8). Thus, CaMox degradation is the result of a specific association between oxidized protein and Hsp90. In addition, Hsp90 does not facilitate the degradation of fluorogenic peptides (Figures 5 and 6; Table 1), as would be expected if Hsp90 binding were simply to enhance the population of 20S proteasomes with open structures that permit ready access of small substrates to the catalytic core. Further, ATP hydrolysis enhances the steady-state rate of CaMox degradation, in a manner that is directly modulated by Hsp90; antagonists that selectively associate with the nucleotide binding sites in Hsp90 block protein degradation (Figure 7C). These results indicate that the Hsp90-mediated recognition of CaMox and other proteins with altered structures prior to their degradation by the 20S proteasome is likely to play an important physiological role in mediating adaptive cellular responses to oxidative stress.

Structure of the Proteasome and Functional Roles of Regulatory Complexes. The current results, demonstrating an important role for Hsp90 in the degradation of oxidized CaM by the 20S proteasome, can be understood in the context of other measurements, which also suggest a critical role for key regulatory subunits in modulating substrate accessibility into the catalytic core of the 20S proteasome complex. In this respect, physiological regulation of proteasome activity appears to be modulated by a dynamic equilibrium between different forms, in which the catalytic 20S core proteasome is present alone or in complex with other protein complexes, including 19S (PA700), 11S REG (PA28), and Hsp90. Indeed, prior measurements have emphasized that the binding of regulatory proteins to the 20S proteasome is necessary for degradative activity (6, 7). In this respect, maximal accessibility of substrates into the catalytic chamber of the 20S proteasome is, of necessity, controlled by association with regulatory complexes such as 11S REG, the 19S regulatory complex, and PA200 to mediate regulated protein degradation (66). However, the function of the 20S proteasome complexed with Hsp90 has not been fully appreciated. In part, this is because Hsp90 routinely
copurifies with the 20S proteasome, and most previous measurements considered nonphysiological substrates (i.e., fluorogenic peptides) whose rate of hydrolysis is not affected by the association of Hsp90 with the 20S proteasome. The use of CaM$_{ox}$ as a physiological substrate, in conjunction with the purification of the 20S proteasome that contains no bound Hsp90, has permitted an understanding of Hsp90 in mediating the degradation of oxidized CaM by the 20S proteasome. Since the loss of secondary structure correlates with the rate of CaM$_{ox}$ degradation (13), these results suggest that oxidant-induces disruption of the secondary structures of other proteins will also target them for degradation.

The relative abundance of different complexes between the 20S proteasome and regulatory factors has previously been shown to influence the type of degradation mediated by the 20S proteasome. For instance, upon association of 19S (PA700) with the 20S proteasome, there is degradation of polyubiquitylated proteins in an ATP-dependent manner (6, 9). In contrast, binding of 11S REG (PA28) to the 20S proteasome induces the degradation of small peptides in an ATP-independent manner (20, 67). Hybrid proteasomes, containing the 20S proteasome complex in association with both 19S (PA700) and 11S REG (PA28) regulators are present as well (8, 68). However, in addition to these well-characterized complexes, the 20S proteasome also tightly binds Hsp90 under physiological conditions (7, 21-23, 26, 32). Further, cellular Hsp90 has been shown to play an important role in mediating protein turnover (26), consistent with the observed Hsp90 mediated degradation of CaM$_{ox}$ (Figure 3). In contrast, Hsp90 association with the 20S proteasome did not affect the rate of degradation of fluorogenic peptides (Figures 5 and 6; Table 1), suggesting that Hsp90 plays a specific role in the degradation of oxidized CaM and other proteins. This latter result is consistent with other measurements of 20S activity, where the mechanism and the regulation of rates of cleavage have been demonstrated to be very different for fluorogenic peptides relative to that associated with proteins (45).

Few measurements of proteasome degradation have been made using physiological substrates (i.e., protein targets of oxidative stress); rather, extensively alkylated or exhaustively denatured protein substrates are commonly used that are unlikely to recapitulate physiological mechanisms of degradation. In contrast, this study utilizes a physiological substrate (i.e., CaM$_{ox}$) that i) is known to be degraded in a ubiquitin-independent mechanism, ii) is a target of oxidative stress during biological aging, and iii) when oxidized functions to tightly bind and block target protein activation (13, 41-44, 47, 54, 70). Further, prior measurements indicate that the rate of degradation by the 20S proteasome is proportional to modest decreases in secondary structure, and that cleavage occurs in a nonprocessive manner (13). Since these earlier preparations contained tightly associated Hsp90, and exhibited a
similar catalytic turnover to the current preparation following reconstitution with Hsp90, these results suggest an important physiological role for Hsp90 in complex with the 20S proteasome in the degradation of oxidized proteins. 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) (15-17, 21). Thus, the proposed role of Hsp90 in mediating the degradation of oxidized proteins is consistent with its association with a binding interface used by other regulators to modulate proteasome activity, albeit Hsp90 does not induce the same conformational changes in the 20S proteasome that function to enhance access of fluorogenic peptides into the catalytic chamber.

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 (26). 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 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 (55). 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 (59, 71, 72). Thus, given that the 20S proteasome is tightly associated with Hsp90 following ion-exchange and size-exclusion chromatography (21, 32, 33, 73), 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 up-regulation of heat-shock proteins) (40, 74). 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 (75).
Conclusions and Future Directions. We have demonstrated a key role for Hsp90 in mediating the energy-independent degradation of oxidized CaM by the 20S proteasome, and we propose a mechanism of regulation whereby Hsp90 binding facilitates substrate entry into the proteasome through a mechanism that involves the selective recognition and delivery of CaM_{ox} into the catalytic core of the 20S proteasome (Figure 9). Thus, like other regulatory protein complexes (e.g., 11S REG and 19S), Hsp90 functions as a regulator of 20S proteasome function to mediate selected protein degradation. Future measurements should investigate the dynamic regulation of other signaling proteins through degradation by 20S proteasome-Hsp90 complexes, and the importance of this degradative pathway on cell function.
REFERENCES


FOOTNOTES

1 Abbreviations used include: CaM, calmodulin; CaMox, oxidized CaM in which each of the nine methionines are oxidized to methionine sulfoxide; CaMwt, unoxidized CaM; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; FCS, fluorescence correlation spectroscopy; HOMOPIPES, homopiperazine-1,4-bis-(2-ethane-sulfonic acid); Hsp90, heat shock protein 90; Cbz-LLE-Na, carboxbenzoxyl-Leu-Leu-Glu-β-napthylamime; MES, 2-(N-morpholino)ethane-sulfonic acid; MG132, benxyloxy carbonyl-leucyl-leucyl-leucinal; MOPS, 3-(N-morpholino)propane-sulfonic acid; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
ACKNOWLEDGMENTS

We thank Drs. Nadezhda A. Galeva and Todd D. Williams for collecting ESI-MS data and Diana Bigelow for helpful discussions.
FIGURE LEGENDS

**Figure 1: SDS-PAGE of Purified Proteins and 20S Proteasome.** SDS-PAGE visualized with Gel Code Blue staining showing mobility differences between unoxidized wild-type CaM (CaM_{wt}) and oxidized CaM (CaM_{ox}) (lane 1), 20S proteasome subunits (lane 2), and Hsp90 (lane 3). Experimentally 12 µg of CaM_{wt} and CaM_{ox}, 35 µg of 20S proteasome, and 18 µg of Hsp90 were loaded onto a 4-20% SDS-PAGE gradient gel (76), and visualized with Coomassie Blue staining.

**Figure 2: Electrospray Ionization Mass Spectra of Native and Oxidized CaM After Deconvolution of Multiply Charged Ions.** Spectra correspond to CaM prior to or following overnight exposure to 50 mM H_{2}O_{2}, where experimental masses of whole proteins (indicated in figure) were determined within 3 atomic mass units. Theoretical masses of expressed CaM and following oxidation of nine methionines to their sulfoxides are 16,705 Da and 16,849 Da, respectively. Peaks corresponding to dehydration artifacts characteristic of the ionization conditions are apparent to the left of the major peak, as previously described (54).

**Figure 3: Kinetics of Proteolytic Degradation.** 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 uM native or oxidized CaM in the presence of 0.6 uM 20S proteasome, and, when indicated, 2.5 uM Hsp90 in 50 mM TRIS (pH 7.5), 0.1 M KCl, 10 mM MgCl_{2}, and 0.1 mM EGTA at 37 °C for 24 h.

**Figure 4: Hsp90-Dependence of CaM_{ox} Degradation by 20S Proteasome.** Rates of degradation of CaM_{ox} by 20S proteasome was measured in the presence of variable amounts of Hsp90 using SDS-PAGE, where extent of degradation was measured using a densitometric analysis of changes in band intensity, as described in the legend to Figure 3. Line represents expected dependency of proteasome
activity as a function of Hsp90 concentration assuming activation involves a high-affinity association with two Hsp90 dimers per proteasome.

**Figure 5: Fluorogenic Peptide Cleavage by 20S Proteasome.** Kinetic data (A) and calculated rates per hour (B) of catalytic activity of the 20S proteasome (100 nM) in the absence (○; dashed line) or presence of either 2.5 µM Hsp90 (●; solid line) or 40 nM MG132 (■; dotted line). Catalysis was measured against 100 µM carboxbenzoxo-Leu-Leu-Glu-β-napthylamine (Cbz-LLE-Na), a fluorogenic peptide specific for the caspase-like activity of the proteasome in 50 mM Tris (pH 7.5), 0.1 M KCl, 10 mM MgCl₂, and 0.1 mM EGTA at 37 °C. Absolute rates were measured in conjunction with a fluorescence standard curve using napthylamine (data not shown).

**Figure 6: Time-Dependent Activation of 20S Proteasome.** Time-dependent cleavage of 100 µM N-tBoc-Gly-Lys-Arg-7 amido-4-methyl coumarin (tBoc-GKR-Amc) (A) or 100 µM N-succinyl-Leu-Leu-Val-Tyr-7 amido-4-methyl coumarin (Suc-AAF-Amc) (B) upon incubation of 20S proteasome (100 nM) in the absence (○, □; dashed lines) or presence of 2.5 µM Hsp90 (●, ■; solid lines) at either 37 °C (○, ●) or 25 °C (□, ■). Absolute rates were measured in conjunction with a fluorescence standard curve using 7 amido-4-methyl coumarin.

**Figure 7: Initial Rates and Selectivity of Protein Degradation by 20S Proteasome.** Following incubation of CaMoxid with 20S proteasome in the absence (○) and presence (●) of Hsp90, initial rates of CaMoxid degradation were followed by reaction of free amines generated by peptide bond cleavage with fluorescamine, where reaction was linear for three hours at pH 6.5 (panel A). Calculated rates of reaction indicate a pH optimum (panel B); catalytic activity is diminished following 30 minute incubations with specific inhibitors of either the 20S proteasome (i.e., 40 nM MG132 or 2 mM epoxomicin) or Hsp90 (36 µM geldanamycin or 10 mM novobiocin)(panel C). In all cases, experimental conditions are as indicated in the legend to Figure 3, using the following buffers: 50 mM MES (pH 6.0), 50 mM PIPES (pH 6.5), 50 mM MOPS (pH 7.0), and 50 mM TRIS (pH 7.5). Peptide concentrations were determined by reacting fluorescamine with known concentrations of glycine to generate a standard curve.

**Figure 8: Fluorescence Correlation Spectroscopy Measurements of Protein-Protein Interactions.** Normalized autocorrelation data depicting antibody binding to CaM (panel A), Hsp90 binding to 20S proteasome (panel B), and the selective interaction between Hsp90 and CaMoxid (panel C) relative to
that observed for native CaM (panel D). Experimental conditions involve 1 nM Alexa-532-labeled native unoxidized CaM (black lines; panels A and D) or oxidized CaM (black line; panel C) in the presence of 5.0 nM polyclonal antibody (red line, panel A) or 50 nM Hsp90 (red lines, panels C and D). Alternatively, 0.5 nM Alexa-532 labeled Hsp90 (panel B) is incubated in the absence (black line) or presence of 2.5 nM (red line) or 10 nM (blue line) 20S proteasome. Temperature was 25 °C.

**Figure 9: 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$_{ox}$ (grey) (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 (55). Furthermore, simultaneous association between Hsp90 complexes at each end of the proteasome is probable, consistent with the binding data in Figure 4 indicating maximal activation of the 20S proteasome in the presence of a four-fold molar excess of Hsp90.
Table 1: Rates of Peptide Hydrolysis by 20S Proteasome in Absence and Presence of Hsp90.

<table>
<thead>
<tr>
<th>Peptide Substrate</th>
<th>Hsp90</th>
<th>Catalytic Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(µmol substrate cleaved / mg 20S / hour)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pH 6.5</td>
</tr>
<tr>
<td>Cbz-LLE-Na</td>
<td>–</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Suc-LLVY-Amc</td>
<td>–</td>
<td>52 ± 3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>tBoc-GKR-Amc</td>
<td>–</td>
<td>10 ± 3</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>14 ± 3</td>
</tr>
</tbody>
</table>

Rates of reaction were measured from a nonlinear least squares fit to the data collected over one hour essentially as described in the legend to Figure 7 using 100 µM fluorogenic peptide substrates in the presence of 0.6 µM 20S proteasome in the absence and presence of 2.5 µM Hsp90 in either 50 mM TRIS (pH 7.5) or 50 mM PIPES (pH 6.5), 0.1 M KCl, 10 mM MgCl₂, and 0.1 mM EGTA at 37 °C.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
Figure 9

A

Hsp90

CaM<sub>ox</sub>

Peptides

B

Hsp90

CaM<sub>ox</sub>

Peptides

Figure 9
Hsp90 enhances degradation of oxidized calmodulin by the 20S proteasome
Jennifer E. Whittier, Yijia E. Xiong, Martin C. Rechsteiner and Thomas C. Squier

J. Biol. Chem. published online August 19, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406048200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2004/08/19/jbc.M406048200.citation.full.html#ref-list-1