Ca\textsuperscript{2+}-free Calmodulin and Calmodulin Damaged by \textit{in Vitro} Aging Are Selectively Degraded by 26 S Proteasomes without Ubiquitination*

The ubiquitin-proteasome pathway is believed to selectively degrade post-synthetically damaged proteins in eukaryotic cells. To study this process we used calmodulin (CaM) as a substrate because of its importance in cell regulation and because it acquires isoaaspartyl residues in its Ca\textsuperscript{2+}-binding regions both \textit{in vivo} and \textit{after in vitro “aging”} (incubation for 2 weeks without Ca\textsuperscript{2+}). When microinjected into \textit{Xenopus} oocytes, \textit{in vitro} aged CaM was degraded much faster than native CaM by a proteasome-dependent process. Similarly, in HeLa cell extracts aged CaM was degraded at a higher rate, even though it was not conjugated to ubiquitin more rapidly than the native species. Ca\textsuperscript{2+} stimulated the ubiquitination of both species, but inhibited their degradation. Thus, for CaM, ubiquitination and proteolysis appear to be dissociated. Accordingly, purified muscle 26 S proteasomes could degrade aged CaM and native Ca\textsuperscript{2+}-free (apo) CaM without ubiquitination. Addition of Ca\textsuperscript{2+} dramatically reduced degradation of the native molecules but only slightly reduced the breakdown of the aged species. Thus, upon Ca\textsuperscript{2+} binding, native CaM assumes a non-degradable conformation, which most of the age-damaged species cannot assume. Thus, flexible conformations, as may arise from age-induced damage or the absence of ligands, can promote degradation directly by the proteasome without ubiquitination.

One function of the ubiquitin (Ub)-proteasome pathway is the selective degradation of abnormal proteins, as may arise by mutation or post-synthetic damage (1, 2). Although proteins can undergo various types of chemical modifications with time, the intracellular fate of such damaged proteins has not been systematically studied. We chose to use CaM to investigate this process, because it plays an important role in cell regulation (3), therefore factors influencing its function and degradation can have important physiological consequences. Moreover, its tertiary structure is known in detail (4, 5) and spontaneous chemical modifications that occur in CaM upon aging (6, 7) have been shown to promote its degradation (8).

CaM has been reported to have a half-life of 18–25 h (1–2%/h) (9, 10), which resembles the half-life of the bulk of cell proteins (11). Over time, such long-lived proteins are susceptible to a variety of spontaneous chemical modifications, including the deamidation of asparaginyl residues and the isomerization of aspartyl residues (7, 12). These modifications arise through slow, non-enzymatic rearrangements that yield isoaspartyl residues and also some racemized aspartyl residues (13). The appearance of these residues has been detected by enzymatic carboxyl methylation using protein-I-isoaspartate (D-aspartate) O-methyltransferase (14), and has been demonstrated to occur in several proteins \textit{in vivo} as well as \textit{in vitro} upon prolonged incubation at physiological pH and temperatures (15, 16).

When CaM was incubated for 2 weeks at pH 7.4 and 37 °C in the presence of Ca\textsuperscript{2+}, small amounts of isoaspartyl residues were formed on two aspartates at flexible regions of CaM, which do not participate in Ca\textsuperscript{2+} binding (6). Isoaspartates were found at these same positions in CaM isolated from bovine brain (17). When Ca\textsuperscript{2+} was not present during the 2-week incubation, there was a dramatic increase in the isoaspartyl content of CaM, due to the deamidation of several asparaginyl residues and the isomerization of additional aspartates (6, 12). Because these isoaspartates were in positions implicated in Ca\textsuperscript{2+} binding, their appearance correlated with a significant loss of Ca\textsuperscript{2+}-dependent regulatory activity (18). Interestingly, in CaM isolated from human erythrocytes, the principal sites of isoaspartyl residues were also in these Ca\textsuperscript{2+}-binding regions (7). Thus, preincubation of CaM in the absence of Ca\textsuperscript{2+} may serve as an \textit{in vitro} model for protein “aging” in intact cells (12).

Age-induced chemical modifications, damage by oxygen-free radicals and other post-translational damage are believed to trigger rapid degradation of proteins \textit{in vivo} (19). In previous studies, we demonstrated that \textit{in vitro aged} (deamidated) CaM was rapidly degraded following microinjection into \textit{Xenopus} oocytes (8). By contrast recently isolated CaM, as well as CaM preincubated for 2 weeks in the presence of Ca\textsuperscript{2+}, were stable for several hours. In the present studies we sought to uncover the molecular basis for the differential degradation of age-damaged CaM and to define the role of the Ub-proteasome pathway in this process.

In the Ub-proteasome pathway, regulatory proteins or proteins with abnormal conformation are marked for rapid degradation by a series of enzymatic reactions leading to the attachment of a chain of ubiquitin molecules to lysine residues on the...
substrate (2). These Ub-conjugated proteins are then digested rapidly to small peptides by the 26 S proteasome, a 2.4-MDa complex containing multiple proteolytic sites within its 20 S core particle (20, 21). Recognition of short-lived regulatory proteins by the ubiquitin-protein ligases (E3s) can depend on the presence of particular sequences (e.g. the destruction box of mitotic cyclins or substrate phosphorylation) (2), but the mode of recognition of substrates with abnormal conformations is unknown. It has been suggested that the appearance of modified amino acids leads directly to the proteins recognition and rapid degradation (19). Alternatively, these modified residues may have an indirect effect by disrupting the proteins normal conformation, thus leading to its selective degradation. It is assumed that the Ub-protein ligases (E3s) are the recognition elements, or that ubiquitination of abnormal proteins may follow their selective binding to molecular chaperones (22). Although Ub conjugation is essential for the rapid elimination of many regulatory and mutant polypeptides (2, 23) and for accelerated proteolysis under certain physiological conditions (24–26), proteasome-mediated degradation of some proteins can occur without ubiquitination (27–29). While ubiquitination of proteins generally enhances their breakdown (30, 31), in vitro certain unfolded proteins and the short lived enzyme, ornithine decarboxylase, can be hydrolyzed by proteasomes rapidly in an ATP-dependent manner in the absence of ubiquitination (27, 32). It remains uncertain to what extent this Ub-independent process occurs in vivo.

In this study we have used oocytes, HeLa cells lysates, and purified 26 S proteasomes to explore the biochemical mechanisms leading to the rapid destruction of the in vitro aged CaM. We demonstrate here that aged CaM and the apo-form of native CaM are degraded rapidly by 26 S proteasomes, apparently without ubiquitination. Interestingly, upon Ca\(^{2+}\) binding, native CaM assumes a conformation that prevents its degradation by the proteasome, even though Ca\(^{2+}\) binding promotes CaM ubiquitination. Furthermore, the chemical modifications that occur during aging, which make CaM less able to bind Ca\(^{2+}\), lead to its rapid degradation directly by the proteasomes, even in the presence of Ca\(^{2+}\).

**EXPERIMENTAL PROCEDURES**

**Purification of CaM—Recombinant chicken hemagglutinin-tagged CaM (HA-CaM) and metabolically labeled CaM ([\(^{35}\)S]CaM) were purified from P4830 Escherichia coli cells that had been transformed with the pcAM4 plasmid containing the chicken CaM gene under the control of the \(\lambda\) phage left promoter. The P4830 host *E. coli* (Amer sham Pharmacia Biotech) contains a temperature-sensitive variant of the cl repressor.**

For HA-CaM purification the *E. coli* cells were grown in LB medium, and for \([^{35}\)S]CaM purification in M9 medium (33) supplemented with 0.1% glucose, 1 \(\mu\)g/ml thiamine, and 0.2 mM each of the amino acids, excluding methionine. Both cultures were grown at 30 °C until the culture density reached an \(A_{600}\) of 0.4 and CaM synthesis was induced by shifting the cultures to 41 °C. For \([^{35}\)S]CaM preparation, \([^{35}\)S]Express \(^{35}\)SS-tag labeling solution (1175 Ci/mMol, NEN Life Science Products Inc.) was added to the cultures at the time of the temperature shift to give a concentration of 10 \(\mu\)Ci/ml. After 2 hours the cells were harvested, and CaM was purified as described (34, 35). Approximately 80 \(\mu\)g of \([^{35}\)S]CaM was obtained from a 100-ml culture (specific activity: 200,000 cpm/\(\mu\)g).

**Preparation and Analysis of Aged CaM—HA-CaM and \([^{35}\)S]CaM (100 \(\mu\)M) were incubated for 2 weeks at 37 °C in 50 mM Na-HEPES or K-HEPES, pH 7.4, containing 1 mM EGTA as described (18). Control (“native”) samples were incubated for the same time in 50 mM Na-HEPES or K-HEPES, pH 7.4, containing 5 mM Ca\(_{\text{Cl}_2}\). Native and aged CaM had the same mobility on SDS-PAGE (36); therefore they were routinely analyzed by nondenaturing PAGE, in the presence of 2 mM EDTA (18). For two-dimensional separations, strips containing the different CaMs were cut from nondenaturing gels containing EDTA and equilibrated for 10 min in sample buffer containing 2 mM EDTA or 5 mM Ca\(_{\text{Cl}_2}\). These strips were then layered across the top of a second native gel that contained either 2 mM EDTA or 5 mM Ca\(_{\text{Cl}_2}\) in the gel solutions and running buffer.

**Microinjection of HA-CaM into Xenopus Oocytes—Xenopus oocytes were first injected with 47 nl of a solution containing 10 pmol of lactacystin in 10 mM sodium phosphate, pH 6.8, using a Nanoject injection apparatus.** The lactacystin was diluted from a 10 mM stock in dimethyl sulfoxide immediately before injection. A control group was injected with a similar dilution of dimethyl sulfoxide alone. Thirty minutes later, oocytes were injected with 2.5 ng of aged HA-CaM in 47 nl. The oocytes were incubated at 20 °C, and at indicated times, HA-CaM levels were measured in extracts prepared from groups of 10 oocytes using the immunoblotting method described previously (8).

**Degradation of CaM in HeLa Cell Extract—** HeLa cells were harvested, and CaM was purified as described (34, 35). Approximately 150–200 \(\mu\)g of CaM was used. A degradation mixture containing 500 \(\mu\)g of HeLa cell extract supplemented with an ATP-regenerating system (10 mM creatine phosphate, 0.2 mg/ml creatine kinase) and protease inhibitors (50 \(\mu\)M E64, 1 mM phenylmethylsulfonyl fluoride, 50 \(\mu\)M chymostatin, and 20 \(\mu\)M AEBSF) was dialyzed against 20 mM Tris-Cl, pH 7.6, 20 mM KCl, 5 mM MgCl\(_{2}\), 1 mM DTT, 20% glycerol at 4 °C for 48 h, and stored at -80 °C.

**Degradation of CaM by 26 S Proteasomes—** To assay ATP-dependent proteolysis, the ATP present in the 26 S proteasome mixture was removed by preincubating the proteasomes with 2 mM EDTA for 5 min at 30 °C, then removing the EDTA by centrifugation and osmotically lysing by the addition of 5 volumes of hypotonic buffer (10 mM Tris-Cl, pH 7.9, 1.5 mM MgCl\(_{2}\), 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT). The mixture was incubated on ice for 10 min, then homogenized with 12 strokes of a Dounce homogenizer. After centrifugation for 15 min at 10,000 \(\times\) g, the supernatant was ultracentrifuged at 100,000 \(\times\) g for 30 min. This supernatant was concentrated by the addition of solid (NH\(_{4}\))\(_2\)SO\(_4\) to 80% saturation, stirred at 4 °C for 30 min, and centrifuged for 15 min at 20,000 \(\times\) g. The precipitate was resuspended in 1/2 volume of 20 mM Tris-Cl, pH 7.6, 20 mM KCl, 5 mM MgCl\(_{2}\), 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 50 \(\mu\)M chymostatin, and 20 \(\mu\)M AEBSF, and dialyzed against 20 mM Tris-Cl, pH 7.6, 20 mM KCl, 5 mM MgCl\(_{2}\), 1 mM DTT, 20% glycerol at 4 °C for 48 h, and stored at -80 °C.

**Ubiquitin-independent Degradation of Calmodulin by Proteasomes—** We demonstrate here that aged CaM and the apo-form of native CaM are degraded rapidly by 26 S proteasomes, apparently without ubiquitination. Interestingly, upon Ca\(^{2+}\) binding, native CaM assumes a conformation that prevents its degradation by the proteasome, even though Ca\(^{2+}\) binding promotes CaM ubiquitination. Furthermore, the chemical modifications that occur during aging, which make CaM less able to bind Ca\(^{2+}\), lead to its rapid degradation directly by the proteasomes, even in the presence of Ca\(^{2+}\).
Aged and Native CaM Differ in Conformation and Ca$^{2+}$ Binding—Conformational changes accompanying Ca$^{2+}$ binding and deamidation of CaM have been demonstrated using nondenaturing polyacrylamide gels (18, 36). To compare the conformational changes that occur upon Ca$^{2+}$ binding in native CaM and in the in vitro aged species (incubated for 2 weeks at 37 °C and pH 7.4 in the absence of Ca$^{2+}$), we extended this approach, using two-dimensional gels that were run in the presence of EDTA in the first dimension and in the presence of either EDTA or Ca$^{2+}$ in the second dimension. In the first dimension, in the presence of EDTA, native CaM migrated as one predominant species plus two minor, slower variants. By contrast, aged CaM was resolved into at least four major and two minor variants, most of which showed a lower electrophoretic mobility than the predominant form of native CaM (data not shown). These differences were most clear when electrophoresis was carried out in the second dimension in the presence of EDTA, when the different aged species were distributed along the diagonal (Fig. 1). The reduced electrophoretic mobility of aged CaM variants on native gels has been attributed to their having a more extended conformation (18), since on the basis of charge alone, the deamidation occurring during aging would be expected to increase the proteins negative charge and electrophoretic mobility. The low amounts of slowly migrating variants in native CaM are due to isomerization of aspartyl residues at positions outside the Ca$^{2+}$-binding regions (6, 17).

When electrophoresis was performed in the presence of Ca$^{2+}$ (5 mM) in the second dimension, the native CaM (including the minor variants) showed a large decrease in electrophoretic mobility, which is characteristic of Ca$^{2+}$-liganded CaM. By contrast, most of the species present in the aged CaM preparation, when separated in the presence of Ca$^{2+}$, showed a smaller mobility shift away from the diagonal than was seen with the native CaM. This mobility difference may reflect decreased Ca$^{2+}$ binding by the aged species and/or a reduced ability of the bound Ca$^{2+}$ to induce the same conformational changes in the aged CaM as in the native form. In addition, a small amount of the aged CaM showed an electrophoretic mobility shift, which was similar to that observed for the native species. Thus, some unmodified CaM appears to persist even after 2 weeks of in vitro aging. These findings support the prior observations that the presence of isoaspartyl residues in the Ca$^{2+}$-binding regions of aged CaM alters its conformation and may interfere with Ca$^{2+}$-binding or Ca$^{2+}$-induced conformational changes (6).

Proteasomes Mediate the Degradation of Aged CaM in Oocytes—Following in vitro aging, hemagglutinin-tagged CaM (HA-CaM) is rapidly degraded upon microinjection into Xenopus oocytes (8). By contrast, HA-CaM that was freshly isolated or was preincubated for 2 weeks in the presence of Ca$^{2+}$ was stable for several hours after microinjection (8). To determine if proteasomes catalyze this selective degradation of the aged CaM, the oocytes were injected with the specific proteasome inhibitor, lactacystin (39), 30 min prior to CaM injection. In the untreated oocytes, the aged HA-CaM had a half-life of approximately 40 min (Fig. 2, upper panel). However, when lactacystin was injected in amounts that should give a final intracellular concentration found maximally effective in cultured cells (~10 μM) (39), it prevented the degradation of the aged HA-CaM (Fig. 2, lower panel). These results clearly implicate the proteasomes in the degradation of aged HA-CaM in oocytes. However, no accumulation of ubiquitinated derivatives of HA-CaM (i.e., higher molecular weight forms) could be detected in either the presence or absence of lactacystin (see below).

Aged CaM Is Degraded More Rapidly Than Native CaM in Cell Extracts—To study the mechanisms responsible for the rapid degradation of aged CaM, we investigated this process in HeLa cell extracts, which have previously been shown to carry out both the ubiquitination and the degradation of many intracellular proteins (37). Degradation and ubiquitination of native and aged [35S]CaM were studied both in the presence and absence of Ca$^{2+}$, because the data in Fig. 1 suggested major structural differences between native and aged CaM upon Ca$^{2+}$ binding. In extracts containing EGTA to chelate Ca$^{2+}$, aged [35S]CaM was degraded 8 times more rapidly than native [35S]CaM (Table I). The addition of high concentrations of Ca$^{2+}$ (5 mM) reduced the rate of hydrolysis of native [35S]CaM to almost half of that observed in the absence of Ca$^{2+}$, while the rate of degradation of aged [35S]CaM was also reduced by the addition of Ca$^{2+}$, but the relative effect was smaller (~26%). Thus, in the presence of Ca$^{2+}$, the difference in the rate of degradation between aged and native [35S]CaM was even more pronounced; the aged species was degraded 11 times faster. These results are in good agreement with the findings in oocytes (Fig. 2 and Ref. 8) demonstrating that aged CaM is degraded much more rapidly than the native molecule.

To analyze how Ca$^{2+}$ and aging influence degradation by the proteasomes, we focused on the component of the proteolysis...
that is sensitive to the proteasome inhibitor, MG-132 (Table I).

In the absence of Ca^{2+}, the proteasome-mediated degradation of aged \(^{35}\text{S}\)CaM (i.e., the MG-132 sensitive component) was 11 times larger than that of native \(^{35}\text{S}\)CaM. The addition of Ca^{2+} reduced the proteasomal degradation of native \(^{35}\text{S}\)CaM by over 50% and that of aged CaM by about 30%. Therefore, in the presence of Ca^{2+} the proteasome-mediated degradation of aged \(^{35}\text{S}\)CaM was actually 16 times that of native CaM. MG-132 reduced total proteolysis in these crude extracts by 50–60%, however, these measurements most probably underestimate the contribution of proteasomes to CaM degradation, since these peptide aldehyde inhibitors are less effective in crude extracts than in intact cells (40). Although additional proteases may also contribute to the degradation of CaM in these extracts, the application of inhibitors of serine and cysteine proteases (chymostatin, phenylmethylsulfonyl fluoride, E64) and incubation at pH 7.4 should have minimized the contribution of lysosomal proteases or calpains. (In fact, the addition of Ca^{2+}, which usually activates calpains, inhibited the degradation of CaM.)

The results obtained in the extracts are consistent with the \textit{in vivo} data indicating a major role for the proteasomes in the degradation of aged CaM. In addition, the degradation of native CaM by proteasomes appears to be very sensitive to Ca^{2+}-induced conformational changes, and these effects appear less pronounced with the aged species.

\textbf{Aging Does Not Promote CaM Ubiquitination—Ubiquitin conjugation to CaM was low} (Fig. 3a), which is consistent with its being a relatively stable protein \textit{in vivo} (9, 10). The addition of Ca^{2+} to the HeLa extracts stimulated the formation of high molecular weight adducts of \(^{35}\text{S}\)CaM (Fig. 3a, lane 2 versus 3), as was also found in reticulocyte lysates (42). To prove that these adducts were indeed ubiquitin-CaM conjugates, Me-Ub was added to the reactions to inhibit the formation of poly-Ub chains (38). As expected, the addition of Me-Ub resulted in the disappearance of the high molecular weight \(^{35}\text{S}\)CaM adducts from the top of the gel, and in an increase in the amount of the monoubiquitinated form (Fig. 3a, lane 4), indicating that the high molecular weight species were \(^{35}\text{S}\)CaM conjugates with Ub chains of different lengths. The effect of Ca^{2+} was specific for the ubiquitination of CaM, since overall ubiquitination in the extracts, as determined by \(^{125}\text{I}\)-Ub conjugation to endogenous substrates, was not influenced by the presence of Ca^{2+} (Fig. 36).

When the ubiquitination of aged CaM was compared with
that of the native molecule, no major differences were found in the extent of Ub conjugation (Fig. 3c), despite the much faster degradation of the aged species. In the absence of Ca²⁺ (i.e. with EGTA present), only a small amount of monoubiquitinated [³⁵S]CaM was observed with both the native and aged substrates, even though the degradation of both proteins was rapid under these conditions. The presence of Ca²⁺, while inhibiting CaM degradation, stimulated the ubiquitination of both aged and native forms. In some experiments, as shown in Fig. 3c, the extent of ubiquitination of the aged molecule was actually lower than that of native [³⁵S]CaM. Thus, the ubiquitination of the aged and native CaM does not correlate with its rate of degradation (Table I).

Because we had anticipated greater ubiquitination of the more rapidly degraded aged CaM, the rates of ubiquitination of these proteins were also compared in extracts prepared from Xenopus eggs, rabbit reticulocytes, and rabbit skeletal muscle. In all extracts, Ub conjugation to [³⁵S]CaM was stimulated by Ca²⁺, and the degree of Ub conjugation to aged and native [³⁵S]CaM was similar (data not shown). Clearly, while aged [³⁵S]CaM is degraded much more rapidly than the native form, it is ubiquitinated to a similar or lesser extent. These various observations suggested that the age-damaged CaM may be degraded by the proteasome in a primarily Ub-independent manner.

**Native and Aged [³⁵S]CaM Are Degraded by Pure 26 S Proteasomes and Ca²⁺ Inhibits this Process—Using 26 S proteasomes purified from rabbit skeletal muscles (32), we tested the possibility that the aged [³⁵S]CaM was degraded directly by the proteasome without prior ubiquitination. No ubiquitin or ubiquitinating enzymes were present in these reactions. As expected from the above experiments, aged [³⁵S]CaM was rapidly degraded by the 26 S proteasomes in the absence of ubiquitination (Fig. 4). Surprisingly, however, native apo-CaM was also degraded at a significant rate by the 26 S proteasomes, but not as rapidly as the aged species (Fig. 4, left panel). The degradation of both native and aged [³⁵S]CaM was stimulated by ATP (1 mM) by about 3-fold (Table II), indicating that the degradation was catalyzed primarily (or exclusively) by the 26 S form of the proteasome.

In the absence of Ca²⁺, both aged and native [³⁵S]CaM were rapidly degraded, and the rate of degradation of the aged species was about twice that observed for the native molecule. The addition of Ca²⁺ was found to reduce the degradation of both native and aged [³⁵S]CaM by the 26 S proteasome, but the magnitude of this inhibition was very different for the two species. In the presence of Ca²⁺, native [³⁵S]CaM degradation was almost completely blocked, while the breakdown of aged [³⁵S]CaM was decreased by only about 15%. This dramatic effect of Ca²⁺ on the degradation of the native protein is most likely due to the change in CaM's conformation and cannot be attributed to an effect of Ca²⁺ on the functional properties of the 26 S proteasome, because the degradation of other proteins was not inhibited by the addition of Ca²⁺, but instead appeared to be stimulated (Table III). Similarly, Ca²⁺ enhancement (by 21%) of the cleavage of the specific tetrapeptide substrate, succinyl-LLVY-aminomethyl coumarin (100 µM). These observations indicate that upon Ca²⁺ binding, native CaM assumes a conformation that cannot be degraded by the 26 S proteasome. However, the chemical modifications, which occur during aging, appears to interfere with Ca²⁺-binding or with Ca²⁺-induced conformational changes, allowing the aged CaM to be rapidly degraded by the 26 S proteasome regardless of the Ca²⁺ levels.

**DISCUSSION**

**Ubiquitin-independent Degradation of Native CaM in Vitro—**Although our initial goal was to identify the mechanism for the selective degradation of age-damaged proteins using CaM as a model substrate, these studies have uncovered unexpected findings regarding the pathway for the degradation of the native molecule. Rapid degradation of proteins in vivo by the 26 S proteasome usually requires their conjugation with a chain of ubiquitin molecules (2). The generality of this ubiquitin requirement, however, has been very difficult to establish in vivo due to lack of specific inhibitors of ubiquitination, and because of the transient nature of the Ub-protein conjugates. The only clear exception to this Ub requirement for proteolysis in vivo is the enzyme ornithine decarboxylase, which is targeted for degradation by the 26 S proteasome through its association with the specific regulatory protein, antizyme (27).
Although both native and aged CaM can be conjugated with ubiquitin (Fig. 3c), this step did not correlate at all with proteolysis and was not required for degradation by the purified 26 S proteasomes (Fig. 4). Ubiquitination was markedly stimulated by Ca\(^{2+}\) binding, while proteolysis was greatly reduced (Table I). Thus, for CaM, and presumably some other proteins, ubiquitination and degradation can be uncoupled (31, 43).

CaM is one of the few clear examples of an intracellular protein that can be degraded rapidly by the 26 S proteasome in the absence of ubiquitination (28–30). Recently, the Cdk inhibitor p21 has also been shown to be degraded in vitro by proteasomes without ubiquitination, and it was proposed that the lack of a “well-defined tertiary structure” triggers its recognition by the proteasomes (44). CaM without bound Ca\(^{2+}\), however, has a specific tertiary structure although it differs from that of the Ca\(^{2+}\)-bound form (5). Therefore other factors, e.g. increased flexibility, are more likely to contribute to recognition and degradation by the proteasomes. Indeed, NMR studies have shown that native CaM, with four Ca\(^{2+}\) ions bound, is significantly less flexible than the Ca\(^{2+}\)-free, apo-form (5). In the Ca\(^{2+}\)-liganded form, the Ca\(^{2+}\)-binding loops are highly structured, and the only flexible regions are in the N terminus and in the middle region between the two globular domains (4). In the absence of Ca\(^{2+}\), there is a major change in helix packing leading to a large increase in the mobility of the Ca\(^{2+}\)-binding loops (5). Presumably, the rigidity of the Ca\(^{2+}\)-liganded form accounts for its resistance to degradation by the 26 S proteasome, while the greater flexibility of the apo-form allows it to be readily unfolded and translocated into the 20 S core particle.

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Ubiquitin-independent Degradation of Aged CaM—In vitro aging results in the formation of isoaspartyl residues predominately in the Ca\(^{2+}\)-binding loops of CaM (12), which presumably alters its conformation, increases its flexibility, and reduces its Ca\(^{2+}\) binding, as suggested by Fig. 1. Although aged CaM was selectively degraded in intact oocytes and cell lysates by proteasomes, the altered conformation of the aged CaM did not increase its rate of ubiquitination. On the contrary, in several experiments, Ub conjugation to the aged CaM was actually reduced (e.g. Fig. 3c), even though it was degraded many times faster than the native molecule (Table I). Since purified 26 S proteasomes were consistently able to degrade the aged species without ubiquitination, the structural modifications during aging must directly target the damaged protein to this degradative complex. These findings do not support the widespread belief that Ub-protein ligases (E3s) are responsible for the recognition of substrates with abnormal conformation and their channeling to the proteasome via ubiquitination. Instead, components of the 26 S proteasome must be able to selectively bind the aged molecules. Recognition is most probably mediated by the 19 S regulatory particle, which has recently been found to preferentially bind denatured proteins (45). More specifically, substrate binding might occur through the six ATPase subunits of the 19 S particle, since the homologous protease-regulating ATPase complexes in prokaryotes (HsIV, ClpA, and PAN) are all critical in substrate recognition and preferentially bind unfolded polypeptides (46, 47).

It seems quite unlikely that the proteasomes directly recognize the isoaspartyl residues or other amino acid modifications in the aged protein, since after Ca\(^{2+}\) removal, the native CaM was degraded almost as fast as the aged molecule. More likely, the increased conformational flexibility arising from chemical damage or ligand-removal enhances a generally Ub-independent degradation by the 26 S proteasome. It is noteworthy that a number of unfolded proteins have been shown both to be degraded in extracts without ubiquitination (48) and also to be digested by isolated proteasomes in an ATP-dependent manner (32). It remains unclear to what extent other polypeptides may be degraded by the proteasome without ubiquitin conjugation in vivo.

The Effect of Ligand (Ca\(^{2+}\)) Binding on CaM Ubiquitination and Degradation—As noted, the ubiquitination and degradation of CaM paradoxically show opposite sensitivities to Ca\(^{2+}\) (Table I). In the absence of Ca\(^{2+}\), some monoubiquitination of CaM occurred (Fig. 3c), although most data suggest that the attachment of one Ub to a substrate does not generally promote its degradation (31, 49). The monoubiquitination of CaM probably involves Lys-115, a residue only available for ubiquitination in bacterially expressed CaMs (as used here) and in Dicyostelium discoideum (50). In other species, Lys-115 is blocked by post-translational trimethylation, therefore the functional importance, if any, of CaM monoubiquitination is unclear. On the other hand, the formation of mult ubiquitin conjugates in these extracts required the presence of Ca\(^{2+}\) (Fig. 3g), as was also found in reticulocyte lysates by Laub and co-workers (51) who showed that Ub is conjugated to Lys-21 in the first Ca\(^{2+}\)-binding loop of CaM. Although the formation of mult ubiquitin conjugates would be expected to promote proteolysis (31, 52), the degradation of CaM is clearly decreased in the extract in the presence of Ca\(^{2+}\) (Table I) (53). It is possible that the mult ubiquitin conjugates retain sufficient Ca\(^{2+}\) binding ability to maintain a structure too rigid to be unfolded and translocated into the proteasome. Whether ligand binding can prevent the degradation of other substrates by the 26 S proteasome remains to be investigated. Another example in which ligand binding prevented proteasomal degradation is dihydrofolate reductase, whose degradation, after ubiquitination, was inhibited by binding of its ligands folic acid or methotrexate (31, 43).

CaM Degradation in Vivo—Our data clearly demonstrate that aged and native apo-CaM can be degraded by purified proteasomes without prior ubiquitination in vitro. Since CaM in cells is generally in the Ca\(^{2+}\)-free form, Ub-independent degradation may also represent a major pathway for CaM degradation in vivo. However, we cannot exclude the possibility that ubiquitination may still influence CaM degradation. Perhaps when cytosolic Ca\(^{2+}\) levels rise (e.g. in contracting muscle), Ub conjugation to CaM is temporarily stimulated, and subsequently, when Ca\(^{2+}\) levels return to normal, and Ca\(^{2+}\) dissociates from CaM, these ubiquitinated apo-species are degraded by the proteasomes.

In addition to influencing proteasomal degradation directly, the greater flexibility of the apo-CaM also makes it more susceptible to the age-related reactions that generate isoaspartyl residues (12), thereby further enhancing its proteasomal degradation. Since the degradation of the aged CaM was much less

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<tr>
<th>Substrate</th>
<th>Amount degraded</th>
<th>Effect of Ca(^{2+}) addition</th>
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<tbody>
<tr>
<td>Native calmodulin</td>
<td>1.56 ± 0.1</td>
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<tr>
<td>Aged calmodulin</td>
<td>3.61 ± 0.2</td>
<td>-25%</td>
</tr>
<tr>
<td>β-Casein</td>
<td>2.02 ± 0.2</td>
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<tr>
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<td>1.65 ± 0.5</td>
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inhibited by the presence of Ca\textsuperscript{2+}, these damaged species are probably eliminated continuously, without ubiquitination, regardless of the Ca\textsuperscript{2+} level in the cell. However, these damaged species can have alternative fates. Enzymatic carboxyl methylation of isoaspartyl residues in aged CaM was shown to reduce the degradation of the aged molecule in Xenopus oocytes (8). This protective effect of methylation may be due to enzymatic repair and refolding of the damaged molecule. In fact the enzyme protein-isoaspartyl methyltransferase has been shown to catalyze the formation of a new electrophoretic variant from the repair and refolding of the damaged molecule. This protective effect of methylation may be due to enzymatic carboxyl methylation of isoaspartyl residues in aged CaM was shown to reduce the selective hydrolysis by the proteasome and enzymatic repair and refolding of the damaged molecule. In the fact the enzyme protein-isoaspartyl methyltransferase has been shown to catalyze the formation of a new electrophoretic variant from the repair and refolding of the damaged molecule. In fact the enzyme protein-isoaspartyl methyltransferase has been shown to catalyze the formation of a new electrophoretic variant from the repair and refolding of the damaged molecule. In fact the enzyme protein-isoaspartyl methyltransferase has been shown to catalyze the formation of a new electrophoretic variant from the repair and refolding of the damaged molecule. In fact the enzyme protein-isoaspartyl methyltransferase has been shown to catalyze the formation of a new electrophoretic variant from the repair and refolding of the damaged molecule. In fact the enzyme protein-isoaspartyl methyltransferase has been shown to catalyze the formation of a new electrophoretic variant from the repair and refolding of the damaged molecule.

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