Ubiquitin Conjugation Is Not Required for the Degradation of Oxidized Proteins by Proteasome*

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Oxidatively modified proteins that accumulate in aging and many diseases can form large aggregates because of covalent cross-linking or increased surface hydrophobicity. Unless repaired or removed from cells, these oxidized proteins are often toxic, and threaten cell viability. Most oxidatively damaged proteins appear to undergo selective proteolysis, primarily by the proteasome. Previous work from our laboratory has shown that purified 20 S proteasome degrades oxidized proteins without ATP or ubiquitin in vitro, but there have been no studies to test this mechanism in vivo. The aim of this study was to determine whether ubiquitin conjugation is necessary for the degradation of oxidized proteins in intact cells. We now show that cells with compromised ubiquitin-conjugating activity still preferentially degrade oxidized intracellular proteins, at near normal rates, and this degradation is still inhibited by proteasome inhibitors. We also show that progressive oxidation of proteins such as lysozyme and ferritin does not increase their ubiquitylation, yet the oxidized forms of both proteins are preferentially degraded by proteasome. Furthermore, rates of oxidized protein degradation by cell lysates are not significantly altered by addition of ATP, excluding the possibility of an energy requirement for this pathway. Contrary to earlier popular belief that most proteasomal degradation is conducted by the 26 S proteasome with ubiquitinylated substrates, our work suggests that oxidized proteins are degraded without ubiquitin conjugation (or ATP hydrolysis) possibly by the 20 S proteasome, or the immunoproteasome, or both.

Accumulation of oxidatively damaged proteins is a characteristic feature of aging cells and a number of age-related pathologies (1–3). In young, healthy cells, moderately oxidized soluble proteins are selectively recognized and rapidly degraded by the proteasome (4–8). We have previously demonstrated that exposure of mammalian cells in culture to moderate oxidative stress significantly increases the degradation of intracellular proteins (5, 9). Proteasome immunoprecipitation, treatment of cells with antisense oligonucleotides to essential proteasome subunits, and proteasome inhibitor profiles (4, 5, 9) all confirm that proteasome is primarily responsible for the degradation of oxidized proteins in mammalian cells. The 20 S proteasome constitutes the catalytic core, whereas the 26 S proteasome is formed by addition of two 19 S regulators, which have subunits for ATP hydrolysis and polyubiquitin recognition (10–12). We have extensive evidence that purified 20 S proteasome preferentially degrades oxidized proteins in vitro in an ATP- and ubiquitin-independent manner (4, 5, 9, 13, 14), however, it has not been clear if proteasome can degrade oxidized proteins without ubiquitin and ATP in vivo.

The 20 S proteasome now appears to be the predominant proteasome species in mammalian cells (15). Furthermore, a number of proteins have now been shown to undergo ubiquitin-independent degradation by the 26 S proteasome (16–19). Our studies on comparative resistance of the 20 S and 26 S forms of the proteasome in response to hydrogen peroxide treatment show that the 26 S proteasome is much more susceptible to oxidative stress than is the 20 S form (20, 21), suggesting that it is probably the 20 S proteasome, which is responsible for the degradation of oxidized proteins when cells are exposed to oxidative stress.

The current study was undertaken to determine whether ubiquitin conjugation is essential for degradation of oxidized proteins by the proteasome in intact cells. For this we utilized the Chinese hamster lung fibroblast cell line, CH E36 and a cell cycle mutant derived from this line, ts20, which harbors a thermolabile ubiquitin-activating enzyme (22–24). These cell lines have been previously used to study the role of ubiquitin conjugation in various processes (24–26). We also tested ubiquitin conjugation of progressively oxidized substrates, to determine whether oxidation actually increases the tendency of a protein to be ubiquitylated.

EXPERIMENTAL PROCEDURES

Cell Culture—CH E36 and clone ts20 cells were a kind gift from Dr. Alan L. Schwartz, Washington University, St. Louis, MO. The parental cell line E36 is a hypoxanthine guanosine-phosphoribosyl transferase-negative mutant (HGPRT−) derived from the Chinese hamster lung fibroblast cell line V-79 (22). Clone ts20 is a cell cycle mutant derived from E36 cells by ethylmethane sulfonate mutagenesis (23), which contains a thermolabile ubiquitin-activating E1 enzyme (24). Both, E36 cells (wild-type for E1) and E1 mutant ts20 cells were grown as monolayers at 30.5 °C on minimal essential medium-a (Invitrogen) supplemented with 10% fetal bovine serum and 4.5 g/liter glucose under 5% CO2. Flasks of both E36 and ts20 cells were shifted to the restrictive temperature, 39.5 °C, for a minimum of 2 h to inactivate E1.

Preparation of Cell Extracts—Cells were washed twice in phosphate-
buffered saline, bathed in cold phosphate-buffered saline containing 1 mM EDTA, and scraped with a rubber policeman. About 10⁶ cells were resuspended in 300 μl of 1× dithiothreitol and incubated at 4°C for 1 h, with vigorous shaking for hypotonic lysis. The crude extracts were centrifuged at 13,000 × g in an Eppendorf centrifuge for 15 min at 4°C, supernatants were collected and stored at −70°C until used. Protein content was determined using the BCA assay (Fierce) or the DC assay (Bio-Rad).

**SDS-PAGE and Immunoblotting**—Total soluble cell protein was separated according to Laemmli (27) by SDS-PAGE using a mini-PROTEAN II electrophoresis cell (Bio-Rad). Following transfer, the nitrocellulose membranes were probed with antibodies to E1 (Calbiochem) or ubiquitin (Chemicon) and visualized using the ECL chemiluminescent system (Amersham Biosciences). Antibodies to ubiquitin conjugation substrates were obtained from Roche Molecular Biochemicals (anti-ubiquitin) or Chemicon (anti-lysozyme).

**Cell Counting and Cell Survival Analysis**—Live, attached cells were harvested by trypsinization and counted in a Z1S Coulter particle counter. Growth curves were performed by seeding equal numbers of cells in 24-well plates and then harvesting and counting cells at various time points. To test if H₂O₂ treatments were toxic, cell survival analysis was performed using neutral red (Sigma) uptake as described previously (28).

**Determination of Activity of the 20 S and 26 S Proteasome**—20 S Proteasome activity was measured at 37°C as described previously (29) by monitoring the release of 7-amido-4-methylcoumaric acid during degradation of the fluorescent substrate Suc-Leu-Leu-Val-Tyr-7-amido-4-methylcoumaric acid by extracts of CH E36 and ts20 cells. Activity of the 26 S proteasome was determined by monitoring ability of cell lysates to degrade pre-formed lysozyme-ubiquitin conjugates. Ubiquitin conjugates were prepared from heat-denatured lysozyme as described below. These conjugates were then incubated either with extracts from ts20 cells at the restrictive temperature or with buffer alone, for 20 min at 39.5°C. The reaction mixtures were separated by 12.5% SDS-PAGE, transferred to nitrocellulose, and the membranes were probed with an anti-lysozyme antibody. The amount of total lysozyme present in each lane was then quantified using NIH Image software to determine the net loss of lysozyme.

**Metabolic Labeling, Exposure of Cells to Oxidative Stress, and Proteolysis Measurements**—Cells were seeded at 2–5 × 10⁵ cells/ml in 24-well plates and grown at 31°C. On day 3, cells were metabolically labeled with 1/25[35S]Cys/Met mixture for 2 h (to generate predominantly “short lived” proteins) or for 16 h (to generate mostly “long lived” proteins) as described (5, 9). Both, CH E36 and ts20 cells were then shifted to the restrictive temperature (39.5°C) for 2 h to inactivate the E1 ubiquitinating enzyme in ts20 cells (cells were maintained at 39.5°C for all further treatments). The monolayers were then exposed to oxidative stress by bolus addition of increasing concentrations (0.1 to 1 mM) of H₂O₂ in PBS, for 30 min. Following oxidative stress, cells were incubated for 2 h with or without proteasome inhibitors, and percent protein degradation was monitored by measuring acid-soluble [35S] counts from previously acid-precipitable [35S]-labeled proteins as described (5, 9).

**Determination of Protein Carbonyls**—A sensitive ELISA method as described by Winterbourn and colleagues (30) with modifications described by Sitte et al. (31) was used for the quantitative determination of protein carbonyls.

**Oxidative Modification and Heat Denaturation of Ferritin/Lysozyme**—Isolated ferritin and lysozyme (Sigma) were oxidized with various concentrations of hydrogen peroxide as described previously (29) with a few modifications. Proteins (1 mg) were exposed to various concentrations of hydrogen peroxide in an Eppendorf thermomixer for 2 h at 30°C in a total volume of 1 ml of phosphate buffer. For heat denaturation, the proteins were incubated in a boiling water bath for 5 min, followed by quick chilling on ice. Untreated proteins, peroxide-treated proteins, and heat-denatured proteins were extensively dialyzed overnight at 4°C against 200 volumes of buffer, with 2 changes, to remove any residual hydrogen peroxide or spontaneously generated fragments.

**Ubiquitin Conjugation of Ferritin/Lysozyme in Vitro**—Ubiquitin conjugation of purified protein substrates was carried out as described by Finley et al. (32), except that in our assay, the F-enolpyruvylpyruvate kinase system was used for ATP regeneration and glutathione S-transferase was used to generate reduced ubiquitin used as the source of ubiquitin. Briefly, the assay mixture contained 20 μl of CH E36 cell lysate (4 mM mg/ml), 50 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, 1 mM dithiothreitol, 3.6 mM F-enolpyruvate, 0.5 μM of pyruvate kinase, 5 mM ATP, 5 μg of glutathione S-transferase-ubiquitin, 0.09 units of inorganic pyrophosphatase (to reduce the build-up of PPI), 50 μM lactoycin (to inhibit proteasomal degradation of Ub conjugates), as well as 1 μM ubiquitin-aldehyde (to inhibit de-ubiquitinating enzymes). The reaction was initiated by adding 10 μg of oxidized or heat-denatured ferritin. For ubiquitin conjugation of lysozyme, the assay was similar except that the widely used creatine phosphate (10 mM)/creatine phosphokinase (100 μg/ml) system was used for ATP regeneration. In some reactions, ATP-depleted lysates were produced by incubation with 10 mM 2-deoxyglucose and 0.5 units of hexokinase. After 30 min at 30°C, the reaction was stopped by addition of SDS-PAGE loading buffer and an aliquot of the mixture was loaded on 12.5% polyacrylamide gels after boiling. After loading, the blots were probed with antibodies to the substrate proteins ferritin (Roche Molecular Biochemicals) or lysozyme (Chemicon).

**Degradation of Ferritin and Lysozyme**—Degradation of ferritin by purified 20 S proteasome was monitored by measuring acid-soluble free amines using fluorescamine, as described (8, 29). To determine the role of ATP, degradation of oxidized [3H]ferritin by cell lysates (60 μl; 2.5 mg/ml) was measured both in the presence and absence of 10 mM ATP. Percent degradation was calculated by measuring acid soluble radioactivity as described previously (14, 29). Lysozyme was labeled with [3H] by reductive methylation (33) and percent degradation was measured by formation of acid-soluble counts, as described (14, 29).

**RESULTS**

**E1 Inactivation Results in Decreased Ubiquitylation and Decreased Basal Proteolysis of Short Lived Proteins**—Incubation of temperature-sensitive ts20 cells for 2 h at the restrictive temperature (39.5°C) resulted in loss of E1. Cells were initially shifted to the restrictive temperature for 2 h, followed by sham treatment with PBS (or treatment with 500 μM hydrogen peroxide) for 30 min. In extracts of E1-mutant ts20 cells incubated at the restrictive temperature for more than 2.5 h, the enzyme could not be detected immunologically with an anti-E1 antibody (Calbiochem) at the expected position of 110 kDa (Fig. 1A, lanes 4 and 6). There was, however, no difference in the levels of E1 in the wild-type CH E36 cells when incubated at the restrictive temperature (Fig. 1A, lanes 3 and 5). Both E36 cells and ts20 cells exhibited comparable amounts of enzyme at the permissive temperature of 30.5°C (Fig. 1A, lanes 1 and 2). Similar results were obtained for cells treated with hydrogen peroxide (data not shown).

To determine whether this was a truly functional inactivation of the enzyme, we looked at the ability of ts20 cells to form high molecular weight ubiquitin conjugates de novo. CH E36 cells and ts20 cells were incubated at 39.5°C for 3 h, after which equal amounts of protein from cell extracts were separated by a 12.5% SDS-PAGE. Following transfer, the membranes were probed with an anti-ubiquitin antibody (Chemicon) to detect protein-ubiquitin conjugates. As seen in Fig. 1B, the steady-state levels of endogenous, high molecular weight ubiquitin conjugates were considerably lower in ts20 cells, than in wild type CH E36 cells, indicating markedly compromised ubiquitin-conjugating activity in ts20 cells at the restrictive temperature. Similar results were obtained by Kulka et al. (24) presumably because there is no de novo synthesis of high molecular weight Ub conjugates in ts20 cells at the restrictive temperature. Although this has been previously seen in ts20 cells (24), we reconfirmed these properties of the temperature-sensitive ts20 mutants in light of recent reports of residual ubiquitin-conjugation activity for a similar, yet distinct ts85 mutant cell line (34).

Most short lived proteins are degraded by the 26 S proteasome in an ATP and ubiquitin-dependent fashion (35). Baseline proteolysis of short lived proteins was reduced by almost 50% in ts20 cells, indicating that ubiquitin-dependent proteolysis was indeed compromised in temperature-sensitive E1 mutants (Fig. 1C). It should be noted that besides ubiquitin-independent proteolysis by the proteasome, proteolysis is also conducted by other proteases. Similar studies have been performed in
E1 Mutants Degrade Oxidized Proteins Despite Compromised Ubiquitinylation—To test whether ubiquitin conjugation is necessary for the degradation of oxidized proteins, we studied the turnover of short lived and long lived proteins after treatment with hydrogen peroxide in ts20 cells with compromised ubiquitinylation. In wild-type CH E36 cells and E1 mutant ts20 cells (Fig. 2C), hydrogen peroxide caused a similar growth arrest in both cell lines (Fig. 2C) as expected from previous work (37, 38). Because the cells were maintained at the restrictive temperature for only 24–26 h, the two cell lines did not show a major difference in cell number. To test whether the ts20 mutants were more susceptible than CH E36 cells to hydrogen peroxide toxicity because of their thermolabile E1, we analyzed cell survival in response to H$_2$O$_2$ treatment in both cell lines by the neutral red uptake assay (Fig. 2, D and E). There were no significant differences in survival over a 24-h period following H$_2$O$_2$ treatment, further indicating that lack of ubiquitin-conjugating activity did not alter the short term ability of ts20 cells to survive an acute oxidative stress.

Because most proteolysis experiments were performed at 39.5 °C, we measured the activity of the 20 S core proteasome in both cell lines, at both temperatures, to determine whether the proteasome was inactivated at the restrictive temperature. As seen in Fig. 2F, the proteasome was not inactivated at the restrictive temperature and loss of proteasome-dependent proteolysis may, therefore, be attributed to defects in ubiquitin conjugation.
mized ubiquitin-conjugating activity. CH E36 and ts20 cells were metabolically labeled for 2 h (Fig. 3A) or 16 h (Fig. 3B) with [35S]Cys/Met to label pools of either short lived (2 h) or mostly long lived (16 h) proteins, respectively. As seen in Fig. 3, intracellular proteolysis was considerably higher in cells treated with 400 μM H₂O₂ than in untreated cells. We have previously reported a similar oxidation-induced increase in overall proteolysis for many different cell types (4, 5, 9, 39) but we can now show that E1 mutant ts20 cells still preferentially degrade oxidized proteins despite the lack of an E1. Because a number of short lived regulatory proteins are known to be degraded by the ubiquitin-proteasome pathway (35, 40), the total turnover of short lived proteins was considerably lower in ts20 cells, as expected. However, the relative increase in degradation because of oxidation was comparable for both cell lines, indicating that the pool of oxidized proteins can still be eliminated in ts20 cells. The degradation of long lived proteins (Fig. 3B) also showed a similar oxidation-induced increase in both CH E36 and E1 mutant ts20 cells as seen previously for other cell types. In fact, it may be noted that the absolute increase in proteolysis because of H₂O₂ is actually higher in the ts20 E1 mutant than in the parent CH E36 cells (Fig. 3B). In earlier studies we reported that moderate oxidation of proteins rapidly increases their degradation, whereas severe oxidation causes a gradual decline in intracellular proteolysis (4, 5, 9, 29). The E1 mutant ts20 cells exhibited exactly this pattern of response to hydrogen peroxide concentration (Fig. 3C), indicating that the E1 mutant ts20 cells utilize the same pathway for degradation of oxidized proteins described previously (4, 5, 9).

The Proteasome Is Largely Responsible for the Degradation of Oxidized Proteins in ts20 Cells—To determine whether the proteasome is still responsible for oxidation-induced protein degradation in ts20 cells with compromised ubiquitin-conjugating activity, we examined the effects of proteasome inhibitors. The selective proteasome inhibitor NLVS (5 μM) strongly inhibited the increase in degradation because of oxidation in both CH E36 control cells and E1 mutant ts20 cells, indicating that the proteasome was still largely responsible for the degradation of oxidized proteins even without the synthesis of ubiquitin conjugates (Fig. 4). Similar results were also obtained with another well known proteasome inhibitor, lactacystin (not shown).

E1 Mutant ts20 Cells Can Eliminate Carbonyl-containing Proteins without Ubiquitin Conjugation—Because the above experiments measured overall proteolysis, we next studied protein carbonyls to address the turnover of oxidized proteins. The total carbonyl content of both wild type CH E36 cells and E1 mutant ts20 cells was increased by H₂O₂ but then dramatically decreased over the following 24 h, whether measured by ELISA or Western blot assays (Fig. 5, A and B), showing that the ts20 cells could still degrade oxidized, carbonyl-containing proteins without the presence of an active ubiquitin-conjugating system.

Progressive Oxidation of Ferritin Does Not Promote Its Ubiquitination in Vitro Yet the Protein Is Degraded by Proteasome without ATP Hydrolysis—Fig. 6A shows an in vitro ubiquitin-conjugation assay for native, heat-denatured, and progressively oxidized ferritin. Native ferritin (lanes 1 and 2) was modified either by heat denaturation (lanes 3 and 4) or increasing concentrations of hydrogen peroxide (lanes 5–10). The protein was then incubated either by itself (lanes 1, 3, 5, 7, and 9) or in the presence of a complete ubiquitin-conjugation system (lanes 2, 4, 6, 8, and 10) for 30 min. at 30 °C. The high molecular weight ferritin-immunoreactive bands represent high mo-
Ubiquitin-independent Proteolysis by the Proteasome

A. Turnover of Short-Lived Proteins

B. Turnover of Long-Lived Proteins

C. Pattern of Proteolysis in ts20 Cells

Fig. 3. E1 mutant ts20 cells degrade oxidized proteins despite compromised ubiquitinylination. CH E36 and E1 mutant ts20 cells were metabolically labeled with [35S]Cys/Met for 2 h (panels A and C) or 16 h (panel B) to label pools of short lived, or mostly long lived, proteins, respectively. After incubation of these cells for 2 h at the restrictive temperature (39.5 °C), the cells were treated with PBS (no H2O2) or increasing concentrations of hydrogen peroxide for 30 min at 39.5 °C, followed by incubation in regular, cold medium for a further 24 h at 39.5 °C. Percent degradation was measured by formation of acid-soluble [35S] counts from previously acid-precipitable [35S]-labeled proteins as described (5, 9). All data points are mean ± S.E. of at least three independent experiments.

Fig. 4. The proteasome inhibitor NLVS inhibits H2O2-induced proteolysis. Proteolysis was measured exactly as described in the legend to Fig. 3A, except that the cells were treated either with 5 μM NLVS or MeSO vehicle alone. MeSO or NLVS was added 2 h prior to treatment with peroxide, and remained in the final incubation medium. All data points are mean ± S.E. of at least three independent experiments.

Fig. 5. E1 mutants can still degrade carbonyl-containing proteins. Panel A, ELISA for the quantitative determination of protein carbonyls using an anti-DNP antibody (31, 32). Cells were incubated at the restrictive temperature for 2 h to inactivate E1, followed by treatment with hydrogen peroxide (or PBS in controls). Protein carbonyls were measured in untreated cells and hydrogen peroxide-treated cells for both the CH E36 and the E1 mutant ts20 cell lines, either immediately after treatment (0 h), or 24 h after treatment (24 h) with 0.5 mM hydrogen peroxide. Panel B, Western blot (anti-DNP antibody) to detect carbonyl content in ts20 cells sham treated with PBS (C), or in ts20 cells treated with hydrogen peroxide (P), at both 0 and 24 h. In both panels cells were treated with H2O2 or PBS for 30 min, and H2O2 was then replaced by dialysis with medium. Results of panel A are mean ± S.E. of at least three independent experiments and the Western blot of panel B was repeated several times with very similar results.

A similar ubiquitin-conjugation assay was performed with the widely used antibody as described under “Experimental Procedures.” This modified ferritin was still preferentially degraded by purified 20 S proteasome in the absence of ATP (Fig. 6C). As described for several proteins in our earlier work (4, 5, 8, 9, 13, 14, 20, 21, 29, 41, 47, 48, 51), moderately oxidized ferritin was preferentially degraded by the 20 S proteasome whereas severely oxidized ferritin tended to be a poorer substrate.

Based on our results, as well as new evidence in the literature (21, 41), we speculated that this degradation of oxidized proteins is most likely conducted by the 20 S proteasome or the immunoproteasome. Because the 26 S proteasome requires ATP hydrolysis, we tested the effect of ATP on degradation of oxidized ferritin by both CH E36 and E1 mutant ts20 cell lysates (Fig. 6D). As seen in Fig. 6D, ATP did not enhance the degradation of oxidized ferritin by the wild-type, CH E36 cell lysates, or E1 mutant ts20 cell lysates, thus excluding any energy requirement for the degradation of oxidized protein substrates.

Progressive Oxidation of Lysozyme Does Not Promote Its Ubiquitinylination in Vitro and Oxidized Lysozyme Is Still Preferentially Degraded by Lysates of ts20 Cells—A similar ubiquitin-conjugation assay was performed with the widely used proteasome substrate, lysozyme. As seen in Fig. 7A, heat-denatured lysozyme showed a large increase in ubiquitin conjugates as seen by the dark smear (lane 12) as compared with native lysozyme (lanes 1 and 2). Neither heat-denatured lysozyme by itself (lane 11), nor heat-denatured lysozyme when incubated with an ATP-depleted extract (lane 13), nor a ts20 extract incubated at 39.5 °C with inactive E1 (lane 14) showed
Radioactivity as described under "Experimental Procedures." We have shown that E1 mutant ts20 cells can eliminate nonspecific ubiquitin conjugation activity, and that this degradation is still catalyzed by the proteasome system. This experiment clearly shows that oxidized heat-denatured lysozyme is degraded by the ubiquitin 26 S proteasome. We (14, 42) and others (53, 54) have shown that the 20 S proteasome has a distinct preference for hydrophobic and bulky (aromatic) residues. We therefore suggest that the 20 S proteasome requires ATP hydrolysis and mostly degrades ubiquitin-conjugated proteins (10–12), with a few known exceptions (17, 43–45). The 19 S regulator is required for recognition and binding of ubiquitin-conjugated proteins, followed by their deubiquitinylation and unfolding to allow entry into the catalytic core (20 S) of the proteasome. Pickart and co-authors (46, 47) have shown that it is primarily the hydrophobic effect that contributes to polyubiquitin chain recognition by the 26 S proteasome. Many substrates of the 26 S proteasome are short lived regulatory proteins that are not necessarily damaged or denatured and, therefore, have to be tagged with an external hydrophobic patch, nor does it require energy from ATP hydrolysis as it is already unfolded. We (14, 42, 52) and others (53, 54) have shown that the 20 S proteasome has a distinct preference for hydrophobic and bulky (aromatic) residues. We therefore suggest that the 20 S proteasome selectively recognizes oxidatively modified, partially denatured proteins because of their exposed hydrophobic moieties. Contrary to earlier belief that the 26 S proteasome is the major intracellular proteasome species, recent literature on proteasome stoichiometry suggests that there is a significant excess of free (and enzymatically active) 20 S proteasome form.
(15, 55–57) in mammalian cells. Several examples of ubiquitin-independent degradation of proteins by the proteasome have now been reported (16–19, 44, 58). We have demonstrated that the 20 S proteasome is resistant to oxidative stress, whereas the 26 S proteasome is quite susceptible. Taylor and co-authors (41, 59, 60) have shown that activity of ubiquitin-activating and -conjugating enzymes (E1 and E2s) is reversibly depressed during oxidative stress, possibly because of glutathiolation of the active site cysteine residues in both E1 and the family of E2 enzymes. Because both the 26 S proteasome and the ubiquitin-conjugation system are inhibited/inactivated by oxidative stress, it is unlikely that the ubiquitin-26 S proteasome pathway is involved in the degradation of oxidized proteins in cells undergoing oxidative stress.

Our studies on ubiquitin conjugation of oxidized substrates in vitro show that progressive oxidation of a protein does not increase its propensity to be ubiquitinylated (Figs. 6 and 7). Conjugation of the polyubiquitin chain occurs via internal lysine residues of the substrate protein and progressive oxidation of a protein actually modifies an increasing number of lysine residues as seen by formation of the oxidation product, 2-aminoadipic semialdehyde. Despite the lack of ubiquitylation, and loss of lysine residues, oxidized proteins such as ferritin and lysozyme are still preferentially degraded in wild-type cells and E1 mutant ts20 cells, and by purified 20 S proteasome. Besides, addition of ATP did not enhance the degradation of oxidized proteins either by wild-type CH E36 or E1 mutant ts20 lysates, suggesting that the 26 S proteasome, which requires ATP hydrolysis, may not be involved in oxidized protein degradation. Our current data, especially when combined with previous reports by us (4, 5, 8, 9, 13, 14, 20, 21, 29, 31, 39, 42, 52), and others (41, 59, 60), appear to rule out any significant involvement of the ubiquitin-26 S proteasome system in the degradation of oxidatively modified proteins. Both this study and previous reports, however, clearly demonstrate that some form of the proteasome is essential for selective intracellular clearance of oxidized proteins.

Careful quantification has now revealed that the free 20 S proteasome is the predominant form of the enzyme in mammalian cells (15, 55, 57). In vitro studies by our group have repeatedly shown that the free 20 S proteasome efficiently and aggressively degrades a wide variety of oxidized proteins (4, 5, 9, 13, 14, 29, 31, 39, 42, 52) and our preliminary studies indicate that the immunoproteasome can also degrade oxidized proteins (data not shown). Although our data does not conclusively rule out involvement of the 26 S proteasome in the degradation of oxidized proteins, it now appears to be less likely, because our results reveal that oxidized proteins are degraded with no requirement for either ATP hydrolysis or ubiquitin conjugation. It will now be important to directly test this hypothesis in intact cells, and to determine the relative contributions of the free 20 S proteasome and the immunoproteasome to the degradation of oxidized proteins.

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