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Review

Protein oxidation and 20S proteasome-dependent proteolysis in mammalian cells

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Abstract. The generation of reactive oxygen species is an inevitable aspect of aerobic life. In addition to being exposed to free radicals in the environment, aerobic organisms must also deal with oxygen radicals generated as byproducts of a number of physiological mechanisms – for example, by the mitochondrial and endoplasmic reticulum electron transport chains, and by cells of the immune system. Although most organisms are equipped with several lines of defense against oxidative stress, these defensive mechanisms are not 100% effective, and oxidatively modified forms of proteins accumulate during aging, and in many pathological conditions. Oxidatively modified proteins can form large aggregates due to covalent cross-linking or increased surface hydrophobicity. Unless repaired or removed from cells, these oxidized proteins are often toxic and can threaten cell viability. Mammalian cells exhibit only limited direct repair mechanisms, and oxidatively damaged proteins appear to undergo selective proteolysis, primarily by the major cytosolic proteinase, the proteasome. Interestingly, it appears that the 20S 'core' proteasome conducts the recognition and elimination of oxidized proteins in an ATP-independent and ubiquitin-independent pathway.

Key words. Protein oxidation; free radicals; proteolysis; proteasome; ubiquitin; apoptosis.

Protein oxidation

The accumulation of oxidatively modified forms of proteins that occurs during aging and in many age-related disorders has focused attention on the mechanisms of protein oxidation and the reaction products formed when proteins are exposed to a variety of free radicals. Earlier studies of protein oxidation focused on modification of proteins by reaction with the hydroxyl radical ($^{\cdot}OH$), which was generated by radiolysis of water, and on reactions with hydrogen peroxide (H_2O_2), either added directly or generated by glucose oxidase [1, 6]. The hydroxyl radical and H_2O_2 along with O_2, $O_2^{\cdot-}$ and $HO_2^{\cdot}$, can lead to oxidation of side chains of amino acid residues, formation of protein-protein covalent cross-linkages and protein fragmentation, due to oxidation of the peptide backbone [1–6]. A number of reaction products of protein oxidation have now been extensively characterized [2, 3, 6–8]. Table 1 summarizes some of the amino acid targets of different radical species and some of the major oxidized products formed.

The sulfur-containing amino acids cysteine and methionine are highly susceptible to oxidative damage [6, 9]. Oxidation of cysteine results in the formation of intra- or intermolecular disulfides leading to possible aggregation of proteins or peptides, whereas methionine oxidation primarily forms methionine sulfoxide. Since some of the damage to the sulfur-containing amino acids is often reversible (see below), it has been suggested that oxidation of these amino acids may serve as a first line of antioxi-

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Table 1. Some amino acid modifications [2–8].

Amino acid	Oxidant*	Product
Cysteine	H_2O_2 or $^{\cdot}OH$	Disulfides, cysteic acid
Methionine	H_2O_2 or $^{\cdot}OH$ $ONOO^-$	Methionine sulfoxide
Tyrosine	H_2O_2 or $^{\cdot}OH$ $ONOO^-$ HOCl H_2O_2 or $^{\cdot}OH$ or HOCl	3,4-dihydroxyphenylalanine 3-nitrotyrosine 3-chlorotyrosine dityrosine
Tryptophan	H_2O_2 or $^{\cdot}OH$ $ONOO^-$	N-Formylkynurenine, Kynurenie, 2,4,5,6 and 7-hydroxytryptophan, nitrotryptophan
Phenylalanine	H_2O_2 or $^{\cdot}OH$	2,3-dihydroxyphenylalanine
Histidine	H_2O_2 or $^{\cdot}OH$	2-oxohistidine, aspargine, aspartate
Lysine	H_2O_2 or $^{\cdot}OH$	lysine hydroxides and hydroperoxides α-aminoadipic semialdehyde
Arginine	H_2O_2 or $^{\cdot}OH$	5-hydroxy-2-aminovaleric acid glutamic semialdehyde
Glutamic acid	H_2O_2 or $^{\cdot}OH$	glutamic acid hydroperoxide oxalic acid, pyruvic acid
Proline	H_2O_2 or $^{\cdot}OH$	proline hydroxides and hydroperoxides 5-hydroxy-2-aminovaleric acid 2-pyrrolidone, Pyroglatuamic
Threonine	H_2O_2 or $^{\cdot}OH$	2-amino-3-ketobutyric acid

* Reactive oxygen species: $^{\cdot}OH$, hydroxyl radical; H_2O_2, hydrogen peroxide; $ONOO^-$, peroxynitrite; HOCl, hypochlorous acid.

dant defense against damaging free radicals [6, 10]. Aromatic amino acids are also among preferred targets for attack by reactive oxygen species and are readily oxidized to various hydroxy derivatives [2, 3, 6–8]. Oxidation of several other amino acids such as lysine, arginine, proline or threonine residues may yield carbonyl derivatives as indicated in table 1.

In addition to modification of amino acid side chains, oxidation reactions can also mediate fragmentation of polypeptide chains [2–6, 11]. Protein fragmentation is instigated by withdrawal of the α-hydrogen atom from an amino acid residue by a hydroxyl radical ($^{\cdot}OH$), generating an α-carbon-centered radical. This carbon-centered radical (R-C$^{\cdot}$) can further react with oxygen to form peroxyl species and then a hydroperoxide. Decomposition of such hydroperoxides by the diamide or α-amidation pathway results in peptide bond cleavage, giving rise to peptide fragments with derivatized terminal amino acids [2–7, 11].

Another major consequence of protein oxidation is the formation of large protein aggregates, which are often toxic to cells if allowed to accumulate [4, 5, 6, 11–14]. These aggregates can result from both covalent and non-covalent interactions among oxidized amino acid residues. Some examples of frequently occurring covalent cross-links include the disulfide cross-link and the 2,2′-biphenyl cross-link formed by two tyrosyl radicals [6, 11, 15–25]. Protein cross-links can also be formed when a carbon-centered radical reacts with another carbon-centered radical [6, 11]. Oxidative modification frequently results in increased surface hydrophobicity as a result of partial unfolding of proteins [9, 18, 19]. A good deal of protein aggregation is due to hydrophobic and electrostatic interactions between oxidized residues. Cross-links between proteins can also be generated by products of lipid peroxidation, which act as natural protein cross-linkers. There is substantial evidence documenting the cross-linking action of two abundant lipid-peroxidation products, 4-hydroxynonenal and malondialdehyde [20–22]. Unless moderately oxidized proteins are removed from cells, they tend to aggregate and may form cross-links as mentioned above, eventually leading to the formation of ceroid bodies or inclusion bodies in the cytoplasm, or lipofuscin entrapped within lysosomes. Ceroid, inclusion bodies and lipofuscin are terms for age-related, yellow-brown pigments with a characteristic autofluorescence [12–14]. Although the biochemical composition of these age-related pigments is still being worked out, a number of protein oxidation markers, such as protein carbonyls as well as lipid peroxidation products like 4-hydroxynonenal, have been detected in these intracellular particles [12–14, 20–22, 25]. Accumulation of oxidized protein aggregates can eventually affect cell viability as seen in a number of age-related and neurodegenerative disorders. It is thus necessary to prevent such oxidative modifications from occurring if possible, or to

rapidly eliminate proteins with irreparable damage before they begin to accumulate.

Protein repair mechanisms and cellular antioxidant defenses

Among all the oxidative modifications of amino acid residues mentioned so far, only oxidation products of cysteine and methionine are reversible [6, 10, 11, 23, 24]. Oxidation of sulfhydryl groups in cysteine residues often results in the formation of disulfide bridges that can crosslink proteins. Most mammalian cells contain a number of disulfide reductases, which reverse the formation of such disulfide bridges. Small molecular weight thiols like glutathione, as well as enzyme systems containing redox-active cysteines such as thioredoxin/thioredoxin reductases and protein disulfide isomerases, serve to maintain an overall reducing environment in the cell, and reduce erroneously formed disulfide bridges between proteins [6, 11, 26]. It should be noted that these same enzymes are also responsible for forming or rearranging disulfide bonds – an event necessary for proper protein folding [8, 26]. Methionine is one of the most susceptible amino acids and is readily oxidized to methionine sulfoxide. The enzyme methionine sulfoxide reductase can reverse this oxidation process in free methionines, and a similar, but distinct enzyme – peptide methionine sulfoxide reductase – can reduce oxidized methionine residues in peptides and proteins [6, 9–11].

Further products of methionine oxidation as well as most other amino acid side-chain modifications, however, represent damage that cannot be directly repaired, and the resulting proteins are almost always nonfunctional. If allowed to accumulate, these products can form insoluble aggregates which affect cell function and viability [12–14, 20–25]. Selective recognition and removal of such oxidatively modified proteins therefore constitutes a secondary line of defense against oxidative stress.

Elimination of damaged proteins by proteolysis

Earlier work from our laboratory [2–5, 11, 15, 16, 18, 19, 27–30], as well as that of Rivett [31, 32], Levine [33] and others has shown that oxidative modification of proteins makes them susceptible to proteolysis. We have demonstrated that exposure of Clone9 liver epithelial cells and K562 human hematopoietic cells to different forms of mild oxidative stress significantly increases the intracellular degradation of both ‘short-lived’ and ‘long-lived’ proteins [27–29]. Similarly, moderately oxidized ‘foreign’ proteins, such as hemoglobin and superoxide dismutase, when used as proteolytic substrates in vitro are selectively degraded by various cell lysates and purified proteases [29, 30]. Rivett, and Levine and co-authors have shown that the oxidatively modified, inactive form of glutamine synthetase is preferentially degraded by intracellular proteases as well as some bacterial proteases, in comparison with native glutamine synthetase [31–33]. In cells under oxidative stress, such selective degradation of oxidatively damaged proteins prevents the formation of large aggregates or potentially toxic fragments with derivatized terminal amino acids. Thus, intracellular proteases responsible for the selective degradation of oxidized proteins function as efficient damage removal and repair systems.

Intracellular proteases and role of the proteasome in degradation of oxidized proteins

The major proteases responsible for general protein degradation (fig. 1) in mammalian cells are the lysosomal cathepsins [34], calcium-activated calpains [35], and 20S and 26S proteasomes [36]. Other specialized proteases such as caspases have specific substrates and are beyond the scope of this review. Most extracellular proteins are degraded by the endosomal/lysosomal pathway [37], and some long-lived intracellular proteins are targeted to the lysosomes either by direct transport of cytosolic proteins through the lysosomal membrane [38] or by the processes of microautophagy/macroautophagy [39]. The calpains

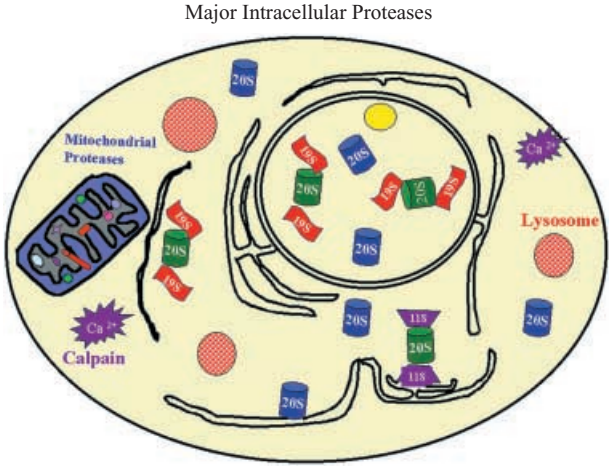

Figure 1. Major intracelluar proteases. Figure 1 schematically represents the major intracellular proteases. Though there are a number of other proteases with very specific substrates (e. g. caspases), this figure represents the major proteases responsible for bulk degradation of proteins localized to different cellular compartments. Proteasomes are responsible for the turnover of most soluble cytosolic and nuclear proteins. Among other major intracellular proteases are the lysosomal cathepsins, which degrade mostly extracellular proteins and certain long-lived cytosolic or organellar proteins. The calpains degrade mostly cytoskeletal proteins upon activation by an increase in intracellular calcium concentrations. A number of proteases within mitochondria are responsible for intramitochondrial protein turnover.

are neutral thiol proteases tightly regulated by intracellular calcium concentrations. Translocation of calpains to the cell membrane followed by their limited autolysis results in calpain activation [40], and activated calpains are generally responsible for partial degradation of membrane and cytoskeletal proteins [35, 41]. In eucaryotic cells, the proteasome is the site for degradation of most soluble intracellular proteins [36, 42, 43]. A vast majority of short-lived regulatory cell proteins as well as most abnormal proteins are degraded by proteasomes.

The proteasome was discovered in the late 1980s by a number of different groups. Isolation of a cation-sensitive neutral endopeptidase by Wilk and Orlowski [44] probably represents the first isolation of a large multicatalytic proteinase complex that corresponds to the proteasome. Rivett purified a high molecular weight, multisubunit, alkaline protease from rat and mouse livers, which specifically degraded the oxidized form of *Escherichia coli* glutamine synthetase [32]. Our laboratory isolated the 20S proteasome as a soluble 670-kDa multisubunit red blood cell proteinase that selectively degraded a variety of oxidized proteins and was initially called 'macroxyproteinase' [43].

Although several purified proteases degrade oxidized proteins more efficiently in vitro, the role of the 20S proteasome in selective recognition and degradation of oxidized proteins within cells has now been well documented. As mentioned above, treatment with hydrogen peroxide greatly increases proteolysis in K562 human hematopoeitic cells. However, when these K562 cells are treated with antisense oligonucleotides to C2, an essential subunit of the proteasome, the hydrogen peroxide-inducible degradation of radiolabeled intracellular proteins, is severely depressed, without affecting baseline proteolysis [11, 27]. Treatment with antisense oligonucleotide against C2 actually causes a decrease in the levels of several subunits of the 20S proteasome, as seen by Western blot analysis [11, 27]. Lysates of K562 cells with decreased proteasome levels are unable to degrade oxidized 'foreign' proteins such as hemoglobin and superoxide dismutase, indicating a major role for the 20S proteasome in degradation of oxidized proteins. Identical results were observed after antisense treatment to C2 in clone 9 liver epithelial cells [28]. The importance of proteasome in oxidized protein turnover was further confirmed by the results of proteasome immunoprecipitation, which causes a dramatic decrease in the ability of cell lysates to degrade oxidized proteins [11, 28]. These studies, as well as inhibitor profiles for the degradation of oxidized proteins, indicate that the proteasome is responsible for ~70–80% of the increased cellular protein degradation after oxidant exposure [18, 19, 27–29]. By selectively recognizing and rapidly degrading oxidized proteins, the proteasome constitutes an important part of cellular antioxidant defenses that prevents the buildup of damaged proteins, and their subsequent aggregation.

Proposed mechanism for the recognition and degradation of oxidized proteins by the 20S proteasome

The proteasome is a multisubunit, multicatalytic complex [36, 44–47] and exists in two major forms – 20S and 26S (fig. 2, table 2). A third but less abundant form of the proteasome, called the immunoproteasome, consists of the core 20S (usually with three substituted subunits) and two copies of the 11S (PA28) regulator. The 20S proteasome is the catalytic core [44–47], whereas the 26S proteasome is formed by complexing of the 20S core proteasome with two 19S regulators, which have subunits for ATP hydrolysis and polyubiquitin recognition [36, 47]. According to our hypothesis, oxidized proteins are degraded by the 20S core proteasome without the requirement for ATP hydrolysis or polyubiquitin conjugation (fig. 3) [11, 18, 19, 27–29, 43]. This hypothesis is based on a number of earlier observations as well as some new evidence in the literature. Earlier work from our laboratory has identified the proteasome as the protease responsible for the selective degradation of oxidatively modified proteins [27, 28, 43]. We now have extensive evidence documenting that purified 20S proteasome preferentially degrades oxidized proteins in vitro without the requirement for ATP or ubiquitin [11, 18, 27–30, 43]. It has not been clear, however, whether the 20S proteasome can function on its own (without the 19S or 11S regulators) independently of ATP and ubiquitin in vivo.

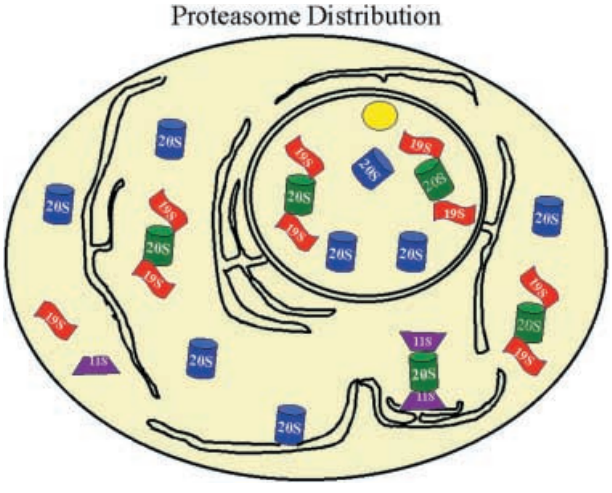

Figure 2. Proteasome distribution. The proteasome can exist as the core 20S proteasome or bound to a pair of either 19S or 11S regulators to form the 26S proteasome or the immunoproteasome, respectively. A limited fraction also appear to exist as hybrid proteasomes consisting of 19S-20S-11S subunits (not shown). Recent studies have shed light on the relative amounts of each of these proteasome forms and their regulators. Stoichiometric evidence from the studies [59–62] suggests that there is a significant excess of free 20S particles over 19S regulators, free or bound to 20S. The cytoplasmic ratio of 20S : 26S particles is about 5:2, whereas the ratio may be slightly lower in the nucleus.

Table 2. Major functional differences between 20S and 26S proteasomes.

20S Proteasome	26S Proteasome
The 20S proteasome has all three catalytic activities: chymotrpsin-like, trypsin-like and peptidylglutamyl-peptide hydrolase [36, 44, 45]	The 26S proteasome contains the 20S catalytic core and has additional subunits for ATP hydrolysis as well as polyubiquitin recognition [36]
Purified 20S proteasome degrades oxidized proteins in vitro without the requirement for ATP or ubiquitin. [2–5, 11, 13–16, 18, 19, 27–30]	Purified 26S requires ATP hydrolysis and degrades mostly ubiquitin-conjugated proteins [36, 47], with a few exceptions, such as ornithine decarboxylase and calmodulin [49, 65, 66]
Hydrophobic patches exposed by oxidatively damaged, unfolded proteins may be recognized by the 20S proteasome [18, 19, 54]	Since proteins degraded by the 26S proteasome are often in their native conformation, hydrophobic patches are conferred by way of polyubiquitin conjugation [52, 53]
The 20S proteasome maintains its activity even when treated with moderate to high concentrations of hydrogen peroxide, and is therefore considered to be fairly resistant to oxidative stress [57, 58]	The 26S proteasome loses its activity (as measured by ATP-dependent proteolysis) when treated with even low concentrations of H_2O_2, and is more suceptible to oxidative stress [57, 58]
Recent studies of subcellular distribution and stoichiometry show that there is two-to three fold molar excess of free 20S particles over 26S particles, i.e. the ratio of 20S to 26S particles within cells is about 5:2 [59–62]	There are far fewer 26S particles compared with 20S particles and hardly any free 19S particles within cells, suggesting a separate function for the 20S complex (59–62)

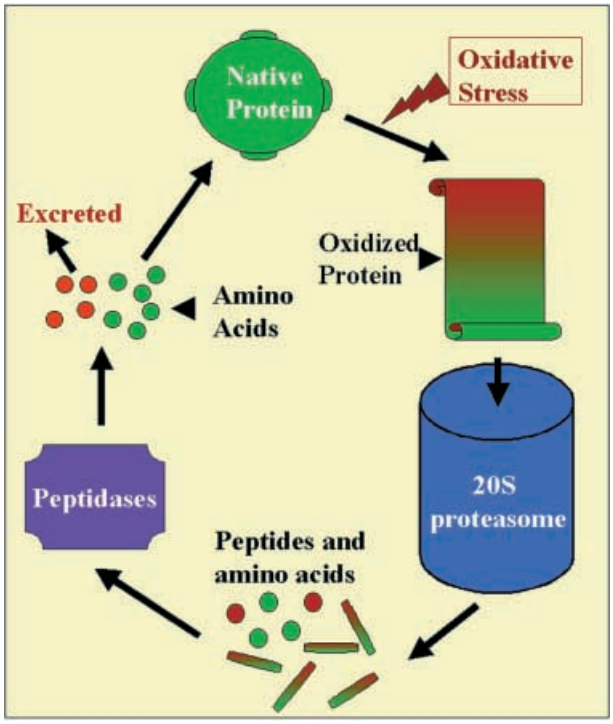

Figure 3. Degradation of oxidized proteins by the 20S proteasome. Following an oxidant attack, most proteins are partially unfolded exposing their inner hydrophobic residues. The 20S core proteasome can recognize such proteins with increased hydrophobicity and degrade them into peptides and amino acids. Such recognition and degradation of oxidatively modified proteins does not require ATP hydrolysis or substrate ubiquitinylation. The peptides may be further broken down into amino acids by other cellular peptidases, and the undamaged amino acids are recycled for protein synthesis.

The 26S proteasome requires ATP hydrolysis for substrate unfolding, and mostly degrades ubiquitin-conjugated proteins [36, 44–47], with a few known exceptions [48–51]. The 19S regulator, which comprises mainly of ATP hydrolases, multiubiquitin chain-binding proteins and deubiquitinating enzymes [36, 47], serves two main functions – (i) recognition of ubiquitin-conjugated proteins followed by their deubiquitinylation and (ii) unfolding the intact protein to allow entry into the catalytic core of the proteasome. Pickart and co-authors have shown that it is primarily the hydrophobic effect which contributes to polyubiquitin chain recognition by the 26S proteasome [52, 53]. Many substrates of the 26S proteasome are short-lived regulatory proteins which are not necessarily damaged or denatured, and therefore have to be tagged with an external hydrophobic patch in the form of a polyubiquitin chain [52, 53]. Also, since these proteins are generally in their native conformation, ATP hydrolysis is required for unfolding the substrate so that it can enter the catalytic core of the proteasome [51].

Oxidative damage to a protein, however, directly results in partial unfolding of that protein, exposing otherwise hidden hydrophobic residues [4, 5, 9, 54]. Therefore, an oxidized protein does not need to be further modified by ubiquitin conjugation to confer a hydrophobic patch, nor does it require energy from ATP hydrolysis as it is already unfolded, though ATP may stimulate or accelerate further unfolding in some cases. An excellent example that confirms this phenomenon is the fact that casein, a protein lacking any secondary structure, can be degraded by the proteasome independent of ubiquitin conjugation [50, 51]. We [18, 19, 43] and others [10, 55] have shown that the 20S proteasome has a distinct preference for hydrophobic and bulky (aromatic) residues. We have there-

fore proposed that the 20S proteasome selectively recognizes oxidatively modified and therefore partially denatured proteins on account of their exposed hydrophobic moieties [5, 11, 18, 19, 29, 43, 54].

Recent (and ongoing) work from our laboratory, with cells incapable of ubiquitinylating protein substrates for the 26S proteasome, indicates that the 20S proteasome does, indeed, conduct the recognition and degradation of oxidatively modified proteins in vivo [56]. We have also recently found that the 20S proteasome is quite resistant to oxidative stress, whereas the 26S proteasome loses its activity (as assessed by ATP-dependent proteolysis) even at low concentrations of hydrogen peroxide [57, 58]. Since the 26S proteasome is so easily oxidatively inactivated, these results indicate that the 20S proteasome is mostly responsible for secondary antioxidant defenses during oxidative stress (fig. 4). Moreover, contrary to earlier belief that the 26S form of the proteasome was the major intracellular proteasome 'species' it is now clear from the literature that there is a significant excess of free (and enzymatically active) 20S particles over 19S regulatory complexes, free or bound to 20S (fig. 2) [59–62]. In fact it now appears that there are approximately five free 20S proteasome complexes for every two 26S complexes in the cytoplasm, and the 20S/26S ratio in the nucleus may be 5/3 [59–62]. Recent work also indicates that hybrid proteasomes consisting of one 19S regulator, a 20S core, and one 11 S regulator also exist in mammalian cells [62], although their function is not yet clear.

Studies of antigen presentation by the proteasome using cell lines incapable of ubiquitin conjugation suggest that antigen presentation may also be a ubiquitin-independent process, similar to oxidized protein degradation [63]. The best-studied example of ubiquitin-independent degradation by the protesome is the degradation of ornithine decarboxylase, a short-lived enzyme involved in polyamine biosynthesis [36, 49]. Although c-Jun degradation in vivo may be a ubiquitin-stimulated process [64], ubiquitinylation is not an absolute requirement for degradation of c-Jun protein by the 26S proteasome in vitro [48]. Recent

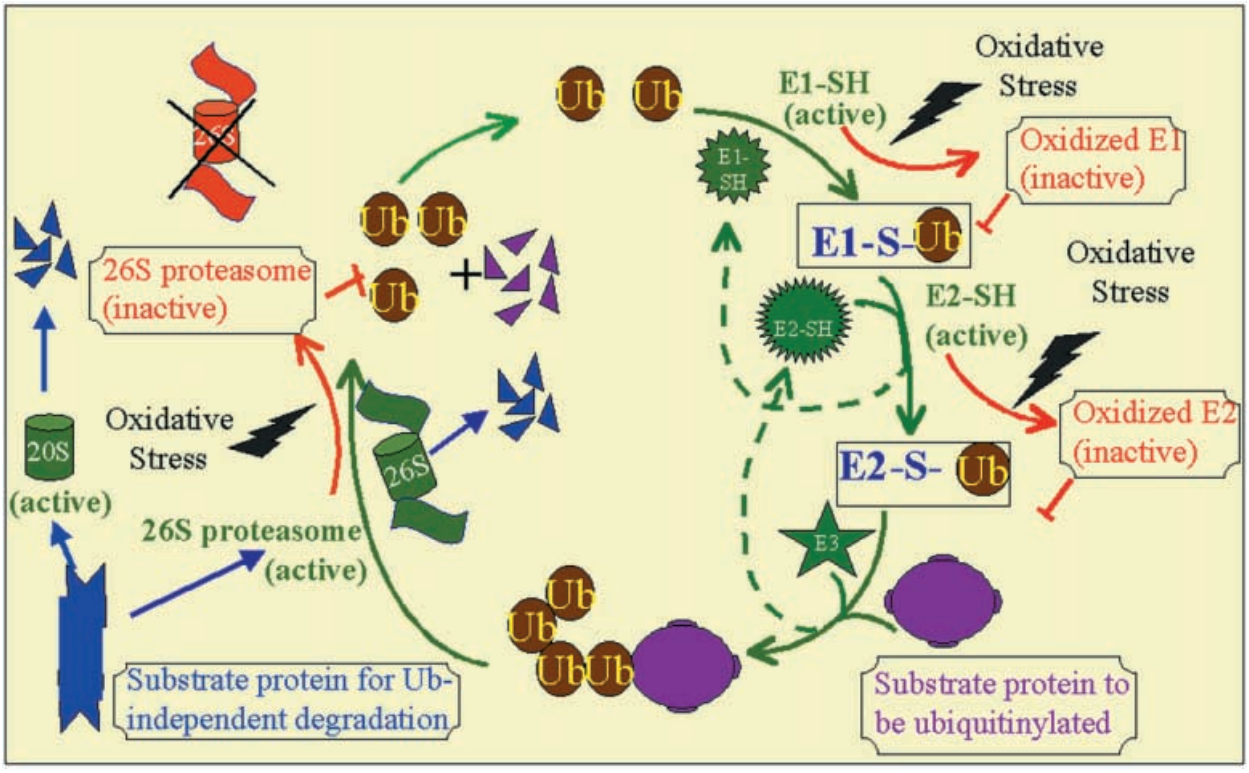

Figure 4. Effect of oxidative stress on the ubiquitin-proteasome (26S) pathway. Many substrates of the 26S proteasome are short-lived regulatory proteins, which are not necessarily damaged and therefore have to be tagged with an external hydrophobic patch in the form of a polyubiquitin chain to ensure their recognition by the 26S proteasome. The ubiquitin-conjugation pathway includes ubiquitin activation by the ubiquitin-activating enzyme. El, followed by transfer of the active ubiquitin moeity to a ubiquitin conjugating enzyme, E2, and finally transfer of this ubiquitin to the destined protein, sometimes via a ubiquitin ligase, E3. The E1 and E2 enzymes have active-site cysteine residues, which have to be in the reduced state for the enzymes to be active. However, oxidation of these sulfahydryl groups inactivates these enzymes, inhibiting protein ubiquitinylation. Also, the 26S proteasome is quite susceptible to oxidative stress, whereas the 20S proteasome is relatively resistant. Since three components of the ubiquitin-proteasome (26S) pathway, specifically, E1 and E2 enzymes and the 26S proteasome itself, are susceptible to oxidative stress, it seems unlikely that this form of the proteasome is involved in the degradation of oxidized proteins. The 20S proteasome, however, is quite resistant to oxidative stress and is available to conduct protein degradation when cells are exposed to oxidants.

studies on the degradation of calmodulin have shown that oxidized calmodulin is selectively recognized and degraded by the 20S proteasome in vitro [65], whereas Ca^{2+}-free calmodulin and calmodulin damaged by in vitro aging are selectively degraded by 26S proteasomes without ubiquitinylation [66]. Thus, with more and more examples for ubiquitin-independent degradation by the proteasome [48, 49, 56, 63–67], it is highly likely that degradation of oxidized proteins in vivo is also a process independent of ubquitin conjugation that is conducted by the 20S proteasome.

Regulation of the proteasome pathway in response to oxidative stress

Since exposure of cells to oxidants greatly increases intracellular proteolysis by the proteasome, we looked at any possible stimulation or upregulation of proteasome in response to oxidative stress. We found no obvious upregulation of any proteasome subunits, or of overall proteolytic capacity, in response to oxidative stress, and we believe that the increased cytoplasmic proteolysis is largely due to increased susceptibility of substrates when oxidized. In contrast, we have recently shown that nuclear proteasome can be activated by poly-ADP ribosylation in nuclei of K562 human hematopoeitic cells [68, 69]. Nuclear 20S proteasome activated by poly-ADP ribosylation can eliminate oxidatively damaged histones more efficiently [68, 69].

It has long been known that low concentrations of denaturing agents, such as 0.1% SDS or mild heat, can greatly activate the proteinase and peptidase activities of the proteasome [36, 70]. It is believed that this stimulation is because of opening up of the complex to allow better substrate access. We have previously demonstrated that such studies must be very carefully performed and interpreted because SDS actually alters both the proteasome and its substrates. For example, in studying the degradation of albumin by red blood cell fraction II (a proteolytic extract containing proteasome), we discovered that SDS both activated proteolytic activity and partially unfolded native hemoglobin, thus making it a better proteolytic substrate. With increasing oxidative modification, however, SDS exhibited a progressively smaller activating effect on both protease and substrate; presumably because the oxidatively modified hemoglobin 'activated' the proteasome to conduct its efficient degradation [19].

Though we have not seen direct stimulation of the proteasome by reaction with hydrogen peroxide, it is possible that other oxidants which may cause a slight dissociation of the complex actually stimulate the proteaosme [71]. In particular, thiol-specific agents have been shown to activate the proteasome [71–73], and it is possible that changes in the thiol content during oxidative stress may affect protelytic activity of the proteasome. Our recent studies show that thiol-specific agents such as GSH, GSSG and cysteine, when used in micromolar concentrations, activate the chymotrypsin-like activity of the proteasome, but actually inhibit it when used at millimolar concentrations [74]. It is therefore apparent that a number of chemical modifications, most of which act to relax the structure of the proteasome, can activate its proteolytic activities.

Our studies of comparative resistance of purified 20S and 26S proteasomes in response to hydrogen peroxide treatment show that the 26S proteasome, the ubiquitin-and-ATP-stimulated form of the proteasome, is much more susceptible to oxidative stress than is the 20 S 'core' proteasome, which can function independent of ATP and ubiquitin conjugation (fig. 4) [57, 58]. Whereas the degradation of the fluorogenic peptide, suc-LLVY-MCA, by the 20S proteasome was inhibited by 50% with 12 μmol of H_2O_2/mg, only 3 μmol of H_2O_2/mg was enough to inhibit ATP-stimulated degradation by the 26S proteasome by 50%. [57]. These results indicate that the 19S regulators are probably more susceptible to oxidative stress, and it is the 20S proteasome which contributes to the degradation of oxidized proteins when cells are exposed to various oxidants.

Taylor and co-authors [75–77] have studied the activity of the ubiquitin-dependent pathway for proteolysis in response to oxidative stress. These authors have reported that the activity of the ubiquitin activating/conjugating system is reversibly depressed during oxidative stress (fig. 4). The mechanism for the reversible oxidation-inducec loss of ATP/ubiquitin-stimulated proteolysis by the 26S proteasome appears to involve glutathiolation of ubiquitin-activating enzyme E1, and the family of ubiquitin-conjugating E2 enzymes by GSSG (levels of which rise during oxidative stress) [75–77]. We have independently found that ATP/ubiquitin-stimulated proteolysis (by the 26S proteasome) is also decreased during oxidative stress, though the activity of the 20S proteasome is largely unaffected. This decrease is also reversible, and the activities are restored during recovery from oxidative stress [57, 58]. Considering that several components of the ATP/ubiquitin-dependent pathway for proteolysis by the 26S proteasome are sensitive to oxidative stress, it seems reasonable to believe that this pathway does not have a major involvement in the elimination of oxidized proteins.

Another regulator of the core 20S proteasome, known as the PA28 or 11S regulator (fig. 2), increases the peptidase activity of the proteasome tremendously and is believed to be a part of the immunoproteasome, which is involved in antigen presentation [36, 47]. The P28 regulator itself does not seem to be affected by treatment with oxidants, as observed by Strack and co-authors [71]. All subunits of the immunoproteasome are upregulated by γ-interferon

stimulation, however, and since some immune responses involve the production of free radicals, one can speculate that the immunoproteasome may also be involved in degradation of oxidized proteins, though this hypothesis needs to be tested. To summarize, more and more evidence suggests that the 20S proteasome is responsible for degradation of oxidized proteins in an ATP/ubiquitin-independent manner that protects cells against aggregation and cross-linking of oxidized proteins.

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