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Minireview

Oxidized protein degradation and repair in ageing and oxidative stress

Bertrand Friguet*

Laboratoire de Biologie et Biochimie Cellulaire du Vieillissement (EA 3106/IFR 117), Université Denis Diderot, Paris 7, 2 Place Jussieu, 75005 Paris, France

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Abstract **Cellular ageing is characterized by the accumulation of oxidatively modified proteins which may be due to increased protein damage and/or decreased elimination of oxidized protein. Since the proteasome is in charge of protein turnover and removal of oxidized protein, its fate during ageing and upon oxidative stress has received special attention, and evidence has been provided for an age-related impairment of proteasome function. However, proteins when oxidized at the level of sulfur-containing amino acids can also be repaired. Therefore, the fate of the methionine sulfoxide reductase system during ageing has also been addressed as well as its role in protection against oxidative stress.**

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1. Introduction

Reactive oxygen species-mediated damage to cellular components is believed to be a main contributor to the ageing process [1,2]. Oxidized protein build up represents a hallmark of cellular ageing, a process that results, at least in part from a failure of protein maintenance [3,4]. Indeed, the age-related accumulation of oxidized protein has been proposed to be due to either or both increased protein oxidative damage and decreased oxidized protein degradation and repair (see Fig. 1). In the cytosol and the nucleus, the proteasome has been described as the main intracellular proteolytic pathway implicated in both the degradation of oxidized proteins and the general turnover of proteins [5,6], while the Lon protease has been shown to selectively degrade oxidized proteins within the mitochondrial matrix [7]. More recently, chaperone-mediated autophagy has also been shown to be activated upon oxidative stress [8]. Beside degradation, certain types of protein oxidative damage affecting sulfur-containing amino acids have been found to be reversible, hence leading to the possibility that some oxidized proteins could be repaired [9,10]. Indeed, several oxidation products of methionine and cysteine, that are among the most susceptible amino acids to oxidative modification, can be reversed by dedicated enzymatic systems. Thioredoxin/thioredoxin reductase and glutaredoxin/glutathione/glutathione reductase can reverse the oxidation of disulfide bridges and cysteine sulfenic acids [10], while methionine sulfoxide reductases catalyze the reduction of methionine sulfoxide back to methionine within proteins [9]. An age-related impairment of oxidized protein elimination (i.e., degradation and repair) would therefore be expected to affect intracellular protein homeostasis and to promote accumulation of oxidatively modified protein. In this minireview, the fate of these oxidized protein degradation and repair systems during ageing and upon oxidative stress will be addressed as well as their contribution to protection against oxidative stress. Understanding the mechanisms by which these crucial protein maintenance systems are affected during ageing may open new strategies for delaying ageing and increasing cellular resistance to oxidative stress.

2. Reversible and irreversible protein oxidative damage

Protein oxidation results from the reaction of reactive oxygen species (superoxide, hydrogen peroxide, and hydroxyl radical) and reactive nitrogen species (nitric oxide and peroxynitrite) with both amino acid side chains and peptidic backbone [2]. Such reactive species are produced endogeneously by intracellular aerobic metabolism, specially at the peroxisome and the mitochondria level [1]. In addition, the production of reactive oxygen/nitrogen species can be promoted by different oxidative stress (UV irradiation, inflammation, ischemia–reperfusion, etc). Upon oxidative stress or under physiopathological conditions, an increased production of reactive oxygen/nitrogen species will disrupt the cellular redox homeostasis and provoke damage to cellular components (lipids, nucleic acids, and proteins). Oxidative damage to proteins can virtually affect all amino acids, sulfur-containing amino acids and aromatic amino acids being the most susceptible to oxidation [2] . Interestingly, certain oxidation products of cysteine and methionine are reversible since they can be brought back to the reduced form of the amino acid within proteins by specific enzymatic systems which correspond to oxidized protein repair enzymes [9,10].

Concerning cysteine oxidation products, the thioredoxin/thioredoxin reductase system has been implicated in the reduction of disulfide bridges and cystein sulfenic acid, while both disulfide bridges and low molecular mixed disulfides such as glutathione adducts are reduced by the glutaredoxin/glutathi-

*Fax: +33 0 1 44 27 82 34.
E-mail address: bfriguet@paris7.jussieu.fr (B. Friguet).

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one/glutathione reductase system [10]. Thioredoxin and glutaredoxin are small ubiquitous proteins belonging to the thiol/disulfide oxidoreductase family, the members of which contain a redox active center made of two cysteins that can form a disulfide bridge upon oxidation. Oxidized thioredoxin is then reduced by thioredoxine reductase, a selenocysteine and flavin containing enzyme, in a NADPH dependant manner, while oxidized glutaredoxin is reduced by glutathione reductase also in a NADPH dependent manner. Interestingly, in eukaryotes, both thioredoxin/thioredoxin reductase and glutaredoxin/glutathione/glutathione reductase system are present in the cytosol and the mitochondria and have long been recognized to be involved not only in oxidized protein repair but also in cellular protection against oxidative stress as well as in redox signalling process [10]. Moreover, the oxidative inactivation of thioredoxin-dependent peroxidases, peroxiredoxins, has been shown to be reversible in mammalian cells and the reversion of cysteine-sulfinic acid, resulting from hyperoxidation of peroxiredoxins, back to reduced cystein has been recently documented to be carried out in an ATP- and thioredoxin-dependent manner by specific and yet unexplored enzymes, sulfiredoxin and sestrins [11,12]. Oxidation of methionine residues in proteins has been shown to contribute to the impairment of protein function and in the loss of their activity. In fact methionine residues in proteins can be oxidized in methionine *S* and *R* sulfoxide diastereoisomeric forms that can be catalytically reversed by the peptide methionine sulfoxide enzymes, MsrA and MsrB, respectively, allowing in some cases the recovery of the protein function [13]. In eukaryotes, MsrA is expressed from a single gene and is found in almost all cellular types in the cytosol as well as in the mitochondria [14,15] and in the nucleus of mouse cells [16]. MsrB enzymes are encoded by three different genes and their products are designated as MsrB1 (SelX), a selenoprotein which is present in the nucleus and the cytosol, MsrB2 (Cbs-1) which is localized in the mitochondria and MsrB3A/B, generated by alternative splicing, that are targeted to the reticulum endoplasmic and to the mitochondria, respectively [17]. Msr enzymes, especially MsrA, were described as very important oxidized protein repair systems and as an anti-oxidant enzymes through the catalyzed reversion of oxidized exposed methionines in proteins, hence contributing to the regulation of the cellular redox homeostasis [18].

Irreversible oxidation products of other amino acids are most frequently hydroxylated and carbonylated amino acid derivatives and detection of protein associated carbonyls represents a usual way of assessing protein oxidation after carbonyl derivatization by dinitrophenyl hydrazine [19]. Oxidized proteins are generally less active, less thermostable and are exposing hydrophobic amino acids at their surface. In addition, protein damage can result from protein adduct formation with lipid peroxidation products such 4-hydroxy-2-nonenal and from oxidation of glycation products leading to the formation of glycoxidation adducts such as pentosidine or carboxymethyllysine [2]. These latter modifications often bring carbonyl groups and/or cross-links within the protein. In the cytosol, oxidized proteins have been shown to be preferentially degraded by the 20S proteasome in an ATP- and ubiquitin-independent manner [6,20] although other studies have shown that the ubiquitin-26S proteasome pathway can also be implicated in the degradation of oxidized proteins [21]. Moreover, upon oxidative stress chaperone-mediated autophagy has been recently shown to participate to the accelerated degradation of oxidized proteins carrying a KFERQ motif [8].

The 20S proteasome is a high molecular weight multienzymatic proteolytic complex consisting of a catalytic core that interacts with a variety of regulators such as PA 700 (to form the 26S proteasome) or PA 28 [5,22]. The proteasome, which is present in the archebacteria and in the nucleus and the cytosol of eukaryotic cells, is made up of four stacked rings of seven subunits. The apical rings are formed with α-type subunits while the inner rings are formed with β-type subunits that are carrying the proteolytic activities. Interestingly, in the eukaryotic version of the proteasome only three β-type subunits possess the N-terminal active site threonine [23]. The peptidylglutamyl peptide hydrolase activity, that cleaves after acidic amino acids, is carried by the β1 subunit, the trypsin-like activity, that cleaves after basic amino acids, is carried by the β2 subunit and the chymotrypsin-like activity, that cleaves after hydrophobic and aromatic amino acids, is carried by the β5 subunit. Upon γ-interferon stimulation, these catalytic subunits can be replaced by inducible subunits iβ1, iβ2 and iβ5 to form the so-called immunoproteasome which exhibits higher chymotrypsin-like and trypsin-like activities and lower peptidylglutamyl peptide hydrolase activity [24,25]. Oxidized proteins represent good substrates for the 20S proteasome unless they become heavily oxidized and cross-linked [26]. In such a situation, not only these highly damaged proteins become resistant to proteolysis by the proteasome but they can also act as inhibitors as clearly demonstrated for proteins cross-linked after modification by the lipid peroxidation product 4-hydroxy-2-nonenal [27,28].

Mitochondria are the major source of intracellular reactive oxygen species and they are also one of the main targets for reactive oxygen species induced damage. In the mitochondria, oxidized protein degradation has been shown to be achieved by the ATP-stimulated Lon protease which is homologous to the oligomeric bacterial Lon protease [29]. Indeed, Davies and coworkers have shown that the oxidant-sensitive Krebs cycle enzyme aconitase, when oxidatively damaged, is degraded by the Lon protease in an ATP-stimulated fashion [7]. In the same study, treatment with anti sense oligonucleotides in WI-38 human lung fibroblasts resulted in decreased Lon protease content and activity while causing an accumulation of oxidatively modified aconitase.

3. Impaired removal of oxidized proteins during ageing

Age-related impairment of proteasome function has been evidenced in a wide range or organs or cell types [4]. Such a decline of proteasome activity would therefore be expected to promote the accumulation of oxidized protein with age. Indeed, as first shown by us for the peptidylglutamyl peptide hydrolase activity of proteasome purified from rat liver [30], an age-related decrease of at least certain proteasome peptidase activities, has been since reported for human keratinocytes, human fibroblasts, human eye lens, human lymphocytes, rat liver, rat cardiomycytes, rat brain and rat skeletal muscle [31–42]. Our initial finding of decreased peptidylglutamyl peptide hydrolase activity of proteasome purified from the liver of old rats was in favor of its specific inactivation. Further analyses comparing the 2D gel electrophoresis patterns of proteasome subunits were indicative of either

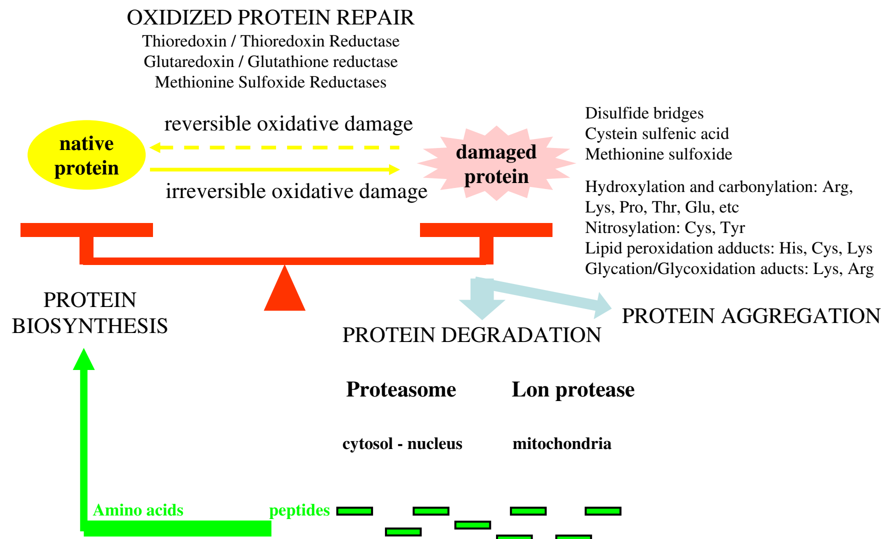

Fig. 1. Intracellular degradation and repair of oxidized proteins. Following protein oxidative damage, oxidized proteins are either degraded or repaired depending on whether the damage is reversible or not. Age-related accumulation of oxidized and aggregated proteins is therefore dependent on the balance between oxidative modification of proteins and oxidized protein elimination through repair and degradation.

subunit replacements and/or post-translational modifications [31,43,44]. More recently, an increased load of modifications by the lipid peroxidation product 4-hydroxy-2-nonenal and the glycoxidation adduct carboxymethyllysine, for specific 20S proteasome subunits was found associated with the age-related decline of 26S proteasome chymotrypsin-like activity purified from human peripheral blood lymphocytes [32]. Increased modification of proteasome β-subunits by 4-hydroxy-2-nonenal was also reported to be associated with decreased proteasome activity in the spinal cord of aged rats [37], while an age-related increased modification of the proteasome by glycoxidation was observed in human lens [42]. In addition to increased modification of proteasome with age, a decreased proteasome subunit expression has been documented in human keratinocytes, human fibroblasts, rat cardiomyocytes and rat spinal cord [31,34,37,39]. Interestingly, fibroblasts from healthy centenarians exhibited a sustained proteasome subunit expression and proteolytic activity which were close to the levels obtained with the young individuals, suggesting that a preserved proteasome function may have contributed to the longevity of these individuals [33]. Transcriptome analyses of both human dermal fibroblasts and mouse skeletal muscle have pointed out an age-related decreased expression of several 20S and 26S proteasome subunits [45,46]. In the mouse model, dietary restriction was shown to reverse this downregulation, implying that the anti ageing effects of dietary restriction could be explained, at least in part, by stimulation of protein turnover and elimination of macromolecular damage [45]. More recently, the expression of only β catalytic 20 S proteasome subunits was found to be decreased in senescent human WI 38 fibroblasts leading to the presence of free α subunits and less assembled proteasome [34]. In the same study, partial inhibition of the proteasome in young WI 38 fibroblasts for two weeks was found to induce a senescent-like phenotype. Finally, in aged rat skeletal muscle, the decreased proteasome activity was attributed to a reduced ability of both PA 28 and PA 700 regulators to bind to the proteasome, while immunoproteasome and constitutive proteasome content were, respectively, increased and decreased [35]. Beside direct inactivation of the proteasome and decreased proteasome subunits expression, highly modified and cross-linked proteins, that have been shown to accumulate with ageing, have been implicated in the inhibition of proteasome activity [27,28,47]. Indeed, a decline of proteasome peptidase activities was observed upon loading of fibroblasts with artificial lipofuscin [48]. In addition, proteasome peptidase activities that were strongly inhibited in heart homogenates from old rats were partially recovered upon purification of the proteasome, strongly suggesting the presence of endogeneous inhibitors in the homogenates that were removed during proteasome purification [31]. Thus, decline of proteasome activity with age is dependant on at least three different inhibition mechanisms: direct inactivation of the proteasome through subunit modification, decreased expression of certain proteasome subunits and increased presence of endogenous inhibitors such as cross-linked proteins.

As for the mitochondrial oxidized protein status and the fate of the Lon protease during ageing, we have reported an age-related accumulation of oxidized and glycoxidized proteins in the liver mitochondrial matrix of rats and a considerable decrease of the ATP-stimulated Lon-like proteolytic activity in 27-month-old rats [49]. This decline of ATP-stimulated Lon-like proteolytic activity was not associated with a concomitant decrease in the level of Lon protein expression suggesting that the Lon protease activity is getting either inhibited or inactivated. However, this decline in Lon protease activity was found to be associated with a decrease in the activity of mitochondrial aconitase [50], an essential Krebs'cycle enzyme known to be very sensitive to oxidative inactivation in mammalian mitochondria. An age-related decline of both Lon gene and protein expression has been documented in mouse skeletal muscle [45,51]. Interestingly, this decline in gene expression was completely prevented when the animals were subjected to dietary restriction, the only intervention known to retard ageing in mammals [45]. Finally, contrary to what we observed in the liver and what was previously observed in mouse skeletal muscle, the ATP-stimulated protease activity was found to re-

main constant in the heart mitochondrial matrix during ageing, and the levels of expression of the Lon protease increased in the older animals in comparison with the younger ones [50]. These results indicate that matrix proteins such as the critical enzymes aconitase and Lon protease are getting inactivated with ageing and that the effects of ageing on the Lon protease vary from one organ to another.

Concerning oxidized repair systems, we have shown that, in rat organs in which MsrA is particularly abundant, i.e., liver, kidney, and brain, peptide methionine sulfoxide reductase activity is notably reduced during ageing [52]. This is related to a decrease in MsrA gene expression in liver and kidney. In contrast, in the brain, the differences in gene expression were not statistically significant and the MsrA protein level was reduced only in 26-month-old rats at the very end of the life of the animals. This intriguing observation may now be explained in light of the existence, in addition to MsrA, of several MsrB genes encoding at least four proteins in mammalian cells. Although MsrB2 (Cbs-1) is not abundant in brain, the other MsrB could be responsible for the alteration in peptide methionine sulfoxide reductase activities determined during brain ageing. The status of the peptide methionine sulfoxide reductases system was also analyzed in WI-38 human fibroblasts during cellular senescence [53]. Total peptide methionine sulfoxide reductase activity was tested by monitoring the reduction of the synthetic substrate *N*-acetyl(^{3}H)Met*R*,*S*(O) to *N*-acetyl(^{3}H)Met*R*,*S*, which can be reduced by either MsrA or MsrB. A decreased peptide methionine sulfoxide reductase activity was observed in senescent cells, that correlated with a decrease in both MsrA and MsrB2 (Cbs-1) gene expression. Impaired expression and/or activity of the peptide methionine sulfoxide reductases system may therefore explain an accumulation of unrepaired proteins, and might contribute to the age-related accumulation of oxidized proteins.

4. Protein maintenance and protection against oxidative stress

To gain better insights in the relationship between oxidized protein removal and cellular resistance against oxidative stress, the effects of oxidative stress on repair and degradation systems have been investigated as well as the influence of increased protein degradation and repair on cellular protection against oxidative stress. Thus, the proteasome has been evidenced as a target for modification by oxidation and related pathways in vivo. Following the demonstration that the proteasome can be inactivated by iron catalyzed oxidation in vitro, treatment of FAO hepatoma cells with iron and ascorbate resulted in the impairment of proteasome peptidylglutamyl peptide hydrolase and trypsin-like activities [54]. Interestingly, overexpression of chaperone proteins such as Hsp 90 or HDJ-1 was found to exert protection against inactivation of the proteasome following oxidative insults to FAO hepatoma cells and neural SH-SY5Y cells, respectively [54,55]. Moreover, treatment of kidney with ferryl nitriloacetate and brain ischemia–reperfusion were associated with decreased proteasome function and modification of the proteasome by the lipid peroxidation product 4-hydroxy-2-nonenal [56,57]. Specific modification of three proteasome subunits by 4-hydroxy-2-nonenal was also associated with inactivation of the trypsin-like activity upon cardiac ischemia–reperfusion [58]. In addition, we have recently found that in vitro treatment of proteasome purified from rat heart results in the preferential inactivation of the trypsin-like activity and the modification of restricted set of proteasome subunits that include those previously reported to be targeted upon ischemia–reperfusion, hence making 4-hydroxy-2-nonenal modification of proteasome as a likely mechanism for proteasome inactivation in vivo [59]. UV irradiation of human keratinocyte also results in a decline of proteasome peptidase activities that has been primarily associated with the build-up of damaged proteins, including proteins modified by 4-hydroxy-2-nonenal [47]. Since low molecular weight compounds such as certain fatty acids have been shown to stimulate proteasome activity in vitro, we have assayed plant and algae extracts for their ability to stimulate proteasome activity in keratinocyte cultures. We have found that a fatty acid-rich extract from the algae *Phaeodactylum tricornutum* was able to stimulate the proteasome peptidase activities both in vitro and in cultured keratinocytes and to reduce the level of oxidized proteins after UV irradiation [60]. Therefore, treatment with such a proteasome stimulating agent may represent not only an efficient strategy for an increased protection against oxidative stress but also for developing anti-ageing strategies. Interestingly, Chondrogianni et al. have recently found that stable overexpression of either β1 or β5 in SV40T/WI 38 fibroblasts and HL 60 resulted in an increased ability of these cells to cope with different oxidative stress through increased rates of proteolysis and decreased oxidized protein content [61]. Moreover, normal IMR 90 fibroblasts transfected by the β5 proteasome subunit exhibited an increased lifespan as demonstrated by an extension of their the replicative potential of 4–5 population doublings [61].

The peptide methionine sulfoxide reductases system has also been implicated in increased longevity and resistance to oxidative stress. Using knockout mice and transgenic Drosophila respectively, Stadtman and Hoshi laboratories [62,63] have addressed the role of MsrA in lifespan regulation related to the antioxidant properties of the enzyme. In transgenic *Drosophila*, overexpression of MsrA extended the mean lifespan by up to 70% and the flies exhibited higher resistance to paraquat-induced oxidative stress [63]. In contrast, mice lacking the msrA gene were more sensitive to oxidative stress, showed an accumulation of oxidized proteins and had a 40% shorter lifespan [62]. We have reported that both MsrA and MsrB (Cbs-1) are upregulated in WI 38 fibroblasts when challenged with low concentration of hydrogen peroxide, hence suggesting an important role of the methionine sulfoxide reductase system in cellular stress defense [53]. In PC 12 neuronal cells, overexpression of MsrA was found to exert a protective effect against oxidative injury by reducing the level of reactive oxygen species after hypoxia/reoxygenation [64]. The same behaviour was observed in MsrA overexpressing human lens epithelial cells when exposed with hydrogen peroxide while MsrA gene silencing was associated with an increased sensitivity to oxidative stress [65]. Such a protective role against oxidative stress was also observed for MsrB in human lens cells since the silencing of one or all MsrB genes was accompanied with increased oxidative stress-induced cell death [66]. We have recently shown that overexpression of MsrA in SV40T/WI 38 fibroblasts led to an increased protection against cell death induced by hydrogen peroxide mediated oxidative stress and found that this increased protection was resulting from a decreased level of intracellular reactive oxygen species and an almost total protection against formation of protein oxidative damage [67].

For the Lon mitochondrial protease, as already stated above, treatment with anti sense oligonucleotides in WI-38 human lung fibroblasts resulted in decreased Lon protease content and activity while causing an accumulation of oxidatively modified aconitase [7]. Moreover, using transgenic mice under-expressing mitochondrial superoxide dismutase as a model of oxidatively challenged animals, Bota et al. reported a decreased Lon protease protein level and this Lon deficiency was associated with increased levels of oxidized proteins [51]. More recently, Bota et al. have also shown that down regulation of the human Lon protease results in the impairment of mitochondrial structure and function and causes cell death, with the majority of cells undergoing caspase 3 activation and apoptosis within four days [68]. Electron microscopy performed on Lon-deficient cells revealed aberrant mitochondrial morphology and the presence electron dense inclusion bodies in the mitochondrial matrix thought to be caused by aggregated proteins. Although to our knowledge, the effects of overexpression of the Lon protease in mammalian cells have not yet been documented, an activation of the ATP-stimulated mitochondrial protease has been recently reported following cardiac ischemia–reperfusion [69], suggesting an important role of the Lon protease in the removal of oxidized proteins and mitochondrial protein homeostasis.

5. Concluding remarks

Protein maintenance and especially oxidized protein degradation and repair systems appears to play a critical role in cell survival and homeostasis, as demonstrated by the impact of improved protein degradation and repair on longevity and/or resistance to oxidative stress. Conversely, decreased protein degradation and repair has been associated to an increased susceptibility to oxidative stress. On the other hand, as illustrated by the now well-established impairment of proteasome function with age and by more recent data on the age-related decline of other protein degradation and repair systems such as the mitochondrial Lon protease and the repair enzymes methionine sulfoxide reductases, protein maintenance failure represents a characteristic feature of cellular ageing that participates to the age-related intracellular build-up of oxidized and aggregated proteins. Understanding the implication and the complex interactions of these systems in cellular protein and redox homeostasis represents an obligatory step as to whether they can be used as valuable handle for slowing down ageing. Importantly, deciphering the molecular mechanisms that contribute to the age-related impairment of protein maintenance systems is likely to provide valuable information that ultimately may lead to the development of anti-ageing strategies aimed at promoting healthy ageing.

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