Serial Review: Oxidatively Modified Proteins in Aging and Disease
Guest Editor: Earl Stadtman

CARBONYL MODIFIED PROTEINS IN CELLULAR REGULATION, AGING, AND DISEASE

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Abstract—The oxidative modification of proteins by reactive species is implicated in the etiology or progression of a panoply of disorders and diseases. The level of these modified molecules can be quantitated by measurement of the protein carbonyl content, which has been shown to increase in a variety of diseases and processes, notably during aging. For the most part, oxidatively modified proteins are not repaired and must be removed by proteolytic degradation, a process which normally proceeds very efficiently, from microorganisms to mammals. In eukaryotes, removal is usually carried out by the proteosome, which selectively degrades oxidatively modified proteins, whether they be damaged by reactive oxygen species or specifically oxidized by cellular regulatory processes. The molecular deficiencies that cause accumulation of oxidatively modified proteins are not identified, but regardless of cause, the accumulation is likely to disrupt normal cellular function. Published by Elsevier Science Inc.

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INTRODUCTION

Accumulating experimental evidence supports the proposal that many of the changes that occur during aging and during the evolution of certain diseases are a consequence of oxidative stress and resulting cellular damage [1]. The list of diseases and processes in which oxidative damage is implicated reads like a textbook of pathology and includes atherosclerosis, cancer, neurodegenerative diseases such as Alzheimer and Parkinson, and the aging process. At present the implicating evidence is heavily correlative, with a large number of published studies documenting the close correlation of markers of oxidative stress and the progression of the process under study. Thus the cells of older organisms, be they worms or humans, carry an increased burden of oxidatively damaged macromolecules including nucleic acids, lipids, and proteins. In this Serial Review, we focus on one class of oxidative modification of proteins, those which cause the introduction of a carbonyl group into the protein.

BACKGROUND

A major goal of biological researchers of the last 50 years is the understanding of the mechanisms of regulation of cellular metabolism. By the mid 1970s it was clear that two major mechanisms were the control of the rate of synthesis of specific proteins (gene regulation) and the control of the activity of already synthesized proteins (feedback regulation and covalent modification). Work on the elucidation of these mechanisms left little time to investigate an obvious third mechanism: regulation of the rate of degradation of proteins, although it was well known that the basal rates varied among proteins.

1 In the context of oxidative modification of proteins, carbonyl refers to moieties with the reactivity of aldehydes and ketones—in other words, a subset of the general class of carbonyl groups. Thus, the carbonyl group of the peptide bond is excluded since it does not have the chemical reactivity of an aldehyde or ketone.
and that they changed in response to changes in the cellular environment [2].

Bacterial glutamine synthetase occupies a central position in metabolism and thus is exquisitely regulated by a complex system that includes control of gene expression, reversible covalent modification, and feedback inhibition [3]. It seemed reasonable that the degradation of glutamine synthetase would also be regulated. Preliminary investigation in Escherichia coli and Klebsiella aerogenes established that it was one of the proteins rapidly degraded when the cells were stressed by nitrogen starvation, and the process was likely under metabolic control because it was inhibited by uncouplers of oxidative phosphorylation (dinitrophenol) and accelerated by inhibitors of protein synthesis (chloramphenicol) [4]. Dissection of the time course of the degradative process revealed that glutamine synthetase lost catalytic activity before it was degraded, suggesting a two-step process [5]. Cell-free extracts of the nitrogen-starved cells rapidly inactivated both endogenous glutamine synthetase and exogenously added, purified glutamine synthetase. Thus it appeared that glutamine synthetase was undergoing a modification that “marked” it for subsequent proteolytic degradation. The marking reaction had three requirements: (i) oxygen, (ii) iron, and (iii) reducing equivalents, supplied by NADH or NADPH. These three requirements: (i) oxygen, (ii) iron, and (iii) reducing equivalents proteolytic degradation. The marking reaction had three requirements: (i) oxygen, (ii) iron, and (iii) reducing equivalents, supplied by NADH or NADPH. These are the three necessary and sufficient elements of classical mixed-function oxidation reactions, typified by cytochrome P-450. This recognition led to the demonstration that a purified cytochrome P-450 system as well as an ascorbate model system catalyzed the oxidative inactivation of glutamine synthetase [5] and that this was sufficient to mark the protein for degradation by a purified bacterial protease or the mammalian proteosome [6,7]. This in turn led to a large number of studies from many laboratories on metal-catalyzed oxidation of proteins and its relationship to protein turnover [8].

What was the modification? Was it a conformational change, perhaps induced by the binding of small molecules that were generated by a mixed-function oxidation stimulated by nutrient starvation? Or was it a covalent modification, perhaps mediated by enzymes that were specifically activated by the stress of starvation? Investigators in the seemingly unrelated field of the biology of aging were wrestling with the same questions. By the late 1970s they had established that many enzymes purified from older animals had a lower specific activity than did the same enzyme purified from younger animals [9], and they applied a variety of biochemical and biophysical techniques in the search for conformational or covalent modifications. Of course, even when a conformational change is detected, one does not know whether this is a consequence of an induced change in shape or a consequence of a covalent modification.

Unfortunately, even today the various available techniques may fail to detect conformational differences between proteins with dramatically different functional characteristics. For example, purified glutamine synthetase subjected to metal-catalyzed oxidation completely loses its catalytic activity. Yet neither circular dichroism nor sedimentation velocity measurements detected a difference between the native and the oxidized proteins [7]. Similarly, the failure to detect a covalent modification with available techniques does not rule out the existence of a modification.

Returning to the situation 25 years ago, no covalent modifications had been detected in the modified enzymes purified from older animals, nor in the glutamine synthetase inactivated by exposure to model systems that mimicked the extracts from the Gram-negative bacteria. By default this lack of evidence for a covalent modification led to the favoring of an induced conformational change as the mechanistic explanation for loss of catalytic activity. However, continuing studies revealed a subtle but definite difference in the ultraviolet spectra of the native and inactivated glutamine synthetases (Fig. 5 in [5]). The difference spectra persisted when the proteins were fully denatured, providing strong evidence for the existence of a covalent modification.

Subsequent investigations led to the recognition that the initial step in the degradation of glutamine synthetase was an oxidative modification that introduced carbonyl groups into the protein that reacted with classical carbonyl reagents such as 2,4-dinitrophenylhydrazine [10]. Since the parallel between the modified glutamine synthetase and modified enzymes from older animals had already been recognized, it was logical to determine the carbonyl content of tissues and cells during aging. The initial investigations have been replicated in many laboratories and led to the generalization that the carbonyl content of tissues from a variety of species increases dramatically in the last third of lifespan (Fig. 1).

**TYPES OF OXIDATIVE MODIFICATIONS AND CHOICE OF MARKER**

Reactive species can react directly with the protein or they can react with molecules such as sugars and lipids, generating products that then react with the protein. Within the protein, either the peptide bond or the sidechain may be targeted. Many of the reactions are mediated by free radicals, usually in a site-specific fashion [11]. The reactions are frequently influenced by redox cycling metal cations, especially iron or copper. The protein may be cleaved to yield lower molecular weight products, or it may be cross-linked to give higher molecular weight products. Classification of the oxidative modifications of proteins is usually based on these
characteristics, but there is no generally accepted scheme for classification. At present we find it helpful to separate the reactions into those that oxidize and cleave the peptide bond and those that modify sidechains [12]. The latter includes modification by the oxidation products of reducing sugars and lipid and generates products including pentosidine and the Michael addition products of alkenals such as 4-hydroxy-2-nonenal [12].

The oxidative modifications can also be grouped into those which are quite specific, both in the residue oxidized and the product generated, and those which can alter multiple residues and may give rise to several products. Examples of a specific oxidation are the conversion of phenylalanine residues to o-tyrosine and of tyrosine to dityrosine [13]. As mentioned, carbonyl group introduction into sidechains is an example of a global modification. These can arise from direct oxidation of most residues or from secondary reaction with the primary oxidation products such as 4-hydroxy-2-nonenal. The choice of a specific or global assay may depend on the purpose of the study being undertaken, and in many cases either may be a useful marker for oxidative stress or damage. The specific modifications reported to date affect a tiny fraction of the at-risk residues or proteins while the global modifications often affect a substantial fraction of the proteins in the sample. For example, dityrosine is clearly increased in atherosclerotic regions of human aorta, with the highest level reported in early fatty streaks [13]. However, the actual content of dityrosine was 1 residue for each 3300 tyrosine residues. In contrast, the carbonyl content increases drastically in the last third of lifespan (Fig. 1), reaching a level such that on average 1 out of every 3 protein molecules carries the modification [11]. Since oxidative modifications that give rise to carbonyl groups generally cause loss of catalytic or structural function in the affected proteins, it is likely that the level of oxidatively modified proteins observed during aging will have serious deleterious effects on cellular and organ function.

**TECHNICAL CONSIDERATIONS**

Methods for determination of carbonyl content have been discussed in a number of reviews of the methodology [14]. The most common and reliable method is based on the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine to form a 2,4-dinitrophenylhydrazone, which can then be detected and quantitated spectrophotometrically or immunochemically. Assays have been developed which employ precipitation, solvent extraction, gel filtration, or electrophoresis for removal of excess reagent. Detection by ultraviolet spectrophotometry can be done in standard spectrophotometers, in-line HPLC spectrophotometers, or 96-well plate readers. Since excellent antibodies directed against the dinitrophenyl group are commercially available, Western blot, dot blot [15,16], immunocytochemical [17], and ELISA techniques are accessible [18]. The Western blot technique developed by Shacter and colleagues [19] and independently by Keller and colleagues [20] has emerged as the most popular incarnation of the assay, after either one or two-dimensional blotting [21–24]. This popularity is probably because antibodies are readily available commercially as well as a Western-blotting kit (OxyBlot Protein Oxidation Kit, Intergen, Gaithersburg, MD, USA). A kit for ELISA analysis is also marketed, although there is not yet a sufficient
literature to assess its usefulness and validity (Zentech, Dunedin, New Zealand).

Results from the various assays have been widely published in a variety of journals, from basic to clinical. It is awkward to specifically retrieve these citations since protein carbonylation is not yet an official search term for MEDLINE. However, a recent search using the words protein carbonyl returned over 5000 citations, and a cursory review indicates that hundreds report protein carbonyl determinations under a variety of conditions.

As with any assay performed on a variety of extracts from biological sources, certain pitfalls have been recognized and techniques for avoiding them have been pointed out [25,26]. These will not be detailed here, but the importance of considering them deserves emphasis. Failure to recognize artifacts can naturally lead to erroneous conclusions. For example, a paper entitled “Protein oxidation and aging: Difficulties in measuring reactive protein carbonyls in tissues using 2,4-dinitrophenylhydrazine” was published in 1995 by Cao and Cutler [27]. The authors listed a number of procedures which did not work in their hands, although they had worked in several different laboratories, a point emphasized by Sohal and colleagues in a subsequent publication [28]. Each of the difficulties encountered by Cao and Cutler appears to have been the consequence of a technical pitfall or artifact. For example, their control chromatograms running only buffer blanks showed peaks and dips, which interfered with analysis of the derivatized proteins. We had noted the same problem with columns obtained from the same manufacturer (Zorbax, Agilent Technologies, Wilmington, DE, USA) after a certain manufacturing date. This change in performance was evident because our standard practice is to run a mixture of reference peptides and proteins every day on every column used for HPLC analyses. A phone call to the manufacturer elicited the information that a change in manufacturing procedures had been instituted, causing a serious deterioration in column performance under the denaturing conditions essential for the carbonyl assay!

Switching to columns from other companies solved the problem in our hands [25]. Thus, since the difficulties encountered by Cao and Cutler are avoidable and did not occur in other laboratories, their experience serves as a reminder of the need for attention to technique and not as a criticism of the 2,4-dinitrophenylhydrazine methodology for detection of protein carbonyls.

SELECTED STUDIES

In previous reviews we have included compilations summarizing studies of protein carbonyl during aging and in various disease processes [11]. The tremendous increase in papers reporting such results, noted above, now makes it impossible to continue including such listings. Studies of aging and of neurodegeneration often include measurements of protein carbonyl since increases are frequently observed. Figure 1 shows the typical exponential increase in tissue carbonyls in the last third of lifespan reported in most, but not all, studies of carbonyl during aging. The mechanism causing this accumulation is of obvious interest but has not been established. One or more of several possibilities could cause an increase in the steady state level of oxidatively modified proteins. These include (i) an increase in the rate of oxidizing species, (ii) a decrease in scavenging of those species, (iii) an increased susceptibility of the protein to oxidation, and (iv) a decrease in the rate of removal of oxidized species. Evidence for all of these has been published from many laboratories and compilations of reviews are available [29,30].

Studies by Sohal and his colleagues established that mitochondria from older flies and rodents have an increased rate of generation of reactive oxygen species compared to younger animals [31], providing support for the first mechanism. Nyström and coworkers provide a counterpoint which supports an increased susceptibility of proteins to oxidation [32,33]. Their studies in E. coli establish that transcriptional or translational errors produce proteins that are more susceptible to oxidative modification. Oxidative modification of proteins involved in gene regulation, transcription, and translation could well increase the error rate in proteins, amplifying the entire process. While nucleic acids and proteins are often thought of as chemically unrelated macromolecules, they are clearly intertwined in cellular regulation. Thus alterations in DNA or RNA may affect the level of oxidized proteins and vice versa.

There is as yet no experimentally obtained evidence to establish the mechanism of the marked rate of increase in carbonylated proteins in the last third of lifespan, a pattern that would be expected when the rate of production of modified proteins exceeds the capacity of the degradative system in removing them. And it would occur regardless of whether the imbalance is caused by increased production or by inhibition or inactivation of the degrading machinery. This is a well-established phenomenon in enzymology and pharmacology and can be observed even with a single enzyme that follows Michaelis-Menten kinetics. In this case increasing the input of a substrate will lead to an increased rate of product formation, thus keeping the substrate concentration relatively low. However, when the enzyme reaches its maximum velocity any additional input of substrate will cause the substrate concentration to increase by the amount of additional input. The final result is a pattern similar to that in Fig. 1.

Recently there has been a notable increase in the
number of published studies on noninvasive measurements of protein carbonyl in human disease, a trend of interest since most animal studies have been on tissues. For example, Renke and colleagues reported that children with juvenile rheumatoid arthritis had elevated plasma protein carbonyl content compared to control children (1.36 ± 0.68 versus 0.81 ± 0.16 nmol carbonyl/mg protein, p < .001) [34]. Notably, they also found that the plasma carbonyl content was higher in patients whose disease was active than in those in better control.

Newborns, especially those born prematurely, are at increased risk of pulmonary disorders that may require mechanical ventilation with high concentrations of oxygen. Although this intervention is essential for their survival, toxic side effects are common, particularly a form of emphysema which is termed bronchopulmonary dysplasia. Endotracheal tubes are required for the mechanical ventilation, and routine nursing care includes suctioning of the tubes to remove mucus; the suctioning yields tracheal aspirates that can be analyzed for protein carbonyl content. The earliest study was designed to determine whether it was technically feasible to measure protein carbonyl on these small samples [35]. That study established the feasibility but also noted a significantly higher carbonyl content in babies who required prolonged ventilation, placing them at risk of bronchopulmonary dysplasia. Some clinicians treat early bronchopulmonary dysplasia with corticosteroids, and babies who received such treatment had a statistically significant drop in tracheal protein carbonyl within 24 h of starting treatment. Varsila and colleagues examined the relationship of an early elevated tracheal protein carbonyl to the development of bronchopulmonary dysplasia [36]. Recently, Buss and coauthors confirmed the elevated protein carbonyl in ventilated babies and established a correlation between the protein carbonyl content and myeloperoxidase activity [37]. Neutrophil myeloperoxidase produces hypochlorous acid, known to be a potent oxidizing agent that introduces carbonyl groups into proteins [18,38].

Pre-eclampsia is a systemic disorder of pregnancy which is relatively common and which causes substantial perinatal morbidity and mortality. Zusterzeel and associates found that the plasma protein carbonyl content of pregnant women is significantly higher than that of non-pregnant women. They further found that women with pre-eclampsia had significantly higher levels than their healthy, pregnant controls (Pre-eclampsia: 0.49 ± 0.25; healthy pregnant: 0.28 ± 0.11; healthy nonpregnant: 0.17 ± 0.06 nmol carbonyl/mg protein; p < .001 for both comparisons) [39].

As illustrated by these selected studies, many of the investigations on protein carbonyl are based on a hypothesis which emerged from the initial work summarized in the background section: carbonyl-bearing proteins are oxidatively damaged, either as a byproduct of normal metabolism or as a consequence of disease processes. However, the initial work with bacteria also generated the hypothesis that normal, regulated cellular processes utilize the oxidative modification of specific proteins as a mechanism for triggering their degradation. Recent investigations with mammalian systems support that concept. In mammalian cells, iron regulatory proteins 1 and 2 (IRP1 and IRP2) sense iron levels and regulate expression of genes controlling iron metabolism [40]. In iron-deficient cells, the IRPs bind to specific RNA stem-loop structures, the iron-responsive element. If the binding site is in the 5’ UTR as in the case of the storage protein ferritin, then translation is inhibited. Conversely, if the binding is in the 3’ UTR as in the case of the transferrin receptor, then the mRNA is protected from degradation by nucleases. Thus, coordination of iron metabolism is effected by the presence or absence of functional IRP.

The mechanisms by which IRP1 and IRP2 are regulated are known to be quite different. IRP1 is a bifunctional protein whose cellular levels of IRP1 are stable. When iron is limiting, IRP1 lacks a complete iron-sulfur center and functions as an iron regulatory protein. When iron is sufficient, the iron-sulfur center is restored in IRP1 and it functions as the cytosolic aconitase, providing one of the first examples of moonlighting by proteins. In contrast, IRP2 is selectively but very rapidly degraded in iron-sufficient cells. It is stable and therefore functional only in iron-depleted cells [41,42]. Given that metal-catalyzed oxidation of glutamine synthetase rendered it susceptible to degradation by the proteosome, both in vitro and in vivo [7,43], we hypothesized that a similar metal-catalyzed oxidative modification of IRP2 might trigger its degradation. A series of experiments in collaboration with Tracey Rouault and her colleagues established that the following occur, again both in vitro and in vivo [44]:

1. IRP2 binds iron and undergoes metal-catalyzed oxidative modification in the presence of oxygen, with introduction of carbonyl groups.
2. The oxidatively modified IRP2 is ubiquitinylated.
3. The modified IRP2 is then degraded by the proteosome.

This yields an elegantly simple mechanism for coordinated regulation of cellular iron metabolism. When iron is deficient, IRP2 is stable and active. When iron becomes sufficient it will bind to a specific site on the protein and catalyze an oxidative modification of IRP2. The oxidation converts a cysteine residue [45] to a novel amino acid, aminomalonic acid [46], perhaps via a dehydrocysteine intermediate. This change suffices to trig-
ger degradation of the entire 107 kD protein by the proteosome.

Hypoxia inducible factor 1-α (HIF-1α) is an important transcription factor recently shown to be regulated by essentially the same mechanism, although the oxidative modification is mediated by an enzyme rather than proceeding nonenzymatically [47]. When cells are deficient in oxygen (hypoxic), HIF-1α is stable. When oxygen is sufficient, the protein undergoes an iron-and-oxygen-dependent modification with conversion of a proline residue to hydroxyproline [48,49]. It is then ubiquitinated and degraded [50]. The examples of IRP2 and HIF-1α demonstrate that cells also utilize oxidative modification of specific proteins as a controlled mechanism of marking them for proteolytic degradation, thereby allowing coordinated regulation of metabolic responses.

REFERENCES

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ABBREVIATIONS

HIF—Hypoxia inducible factor
IRP—Iron responsive protein