Proteomic Strategies for the Analysis of Carbonyl Groups on Proteins

Verónica Irazusta[§], Armando Moreno-Cermeño, Elisa Cabiscol, Jordi Tamarit and Joaquim Ros*

Departament de Ciències Mèdiques Bàsiques, IRBLleida, Universitat de Lleida, Montserrat Roig 2, 25008-Lleida, Spain

Abstract: Oxidative stress is caused by an imbalance between formation and destruction of reactive oxygen species. Analysis of the reaction products of reactive oxygen species in biomolecules is an indirect way of determining the existence of oxidative stress. In this context, the formation of carbonyl groups in proteins has been one of the most studied oxidative stress markers because of its stability and easy detection. Various proteomic tools offer great potential for the discovery of new proteins susceptible to oxidative stress, determination of quantitative changes in the profile of these modifications under different biological conditions, and characterization of the type of modification a particular protein has suffered. This paper reviews the different approaches used for the detection of protein carbonyls and the proteomic tools that can be used to identify them.

Keywords: Oxidative stress, protein oxidation, carbonyl groups, redox proteomics.

INTRODUCTION

Oxidative stress is described as a situation in which antioxidant defences are insufficient to completely inactivate reactive oxygen species (ROS) [1]. This imbalance between production and destruction of ROS can affect many cell components, including lipids, proteins, carbohydrates and nucleic acids [2-4]. Many of the changes that occur during aging and during the progression of certain diseases are a consequence of oxidative stress [5-7]. Proteins are one of the main targets for oxidative modification by ROS. This leads to structural changes, generally involving the partial or total loss of protein function [4, 8, 9]. In this context, protein oxidation plays an important role in the development of the aging process and in atherosclerosis, cancer, and various neurodegenerative diseases, including Alzheimer and Parkinson [10]. Many amino acid residues of proteins are susceptible to oxidation by various ROS. The protein oxidation products most widely studied in recent years are carbonyl groups because of their stability and easy detection. Therefore, their quantification has become the most accepted method for measuring protein oxidative damage in situations where oxidative stress is involved.

FORMATION OF CARBONYL GROUPS

Oxidative stress may cause reversible or irreversible changes in proteins. Reversible changes, usually in the cysteine residues, can be repaired by specific enzymes such as glutaredoxin or thioredoxin [11, 12]. Similarly, methionine is readily oxidized to methionine sulfoxide, which can be reduced by methionine sulfoxide reductase [13]. Such changes are meant to modulate protein function (redox regulation) or protect against irreversible damage that causes the inactive proteins to accumulate or become degraded. Carbonylation is a good example of irreversible changes [14, 15].

Carbonyl groups can be introduced into proteins at different sites and through different mechanisms. Primarily, carbonyl groups are produced in the side chains of certain amino acids, especially from proline, arginine, lysine and threonine residues oxidized to aldehydes or ketones [2, 16]. Generally, the change results from hydroxyl radical ($^{\bullet}$OH) attack, which can be produced by ionizing radiation or by a Fenton reaction of metal cations with hydrogen peroxide. The majority of protein carbonyls formed by metal catalyzed oxidation are glutamic semialdehyde and aminoadipic semialdehyde, derived from arginine and lysine, respectively Fig. (**1A**) [17]. Carbonyl groups can also be generated by protein proteolysis through the alpha-amidation pathway after $^{\bullet}$OH radical attack or after the oxidation of the side chains of glutamyl residues, resulting in the formation of a peptide in which the N-terminal amino acid is blocked by an alpha-ketoacyl derivative [16]. Other mechanisms that can lead to protein carbonylation include reactions with products generated during lipid peroxidation, such as 4-hydroxynonenal (4-HNE) Fig. (**1B**), 2-propenal (acrolein) and malondialdehyde [18]. This type of reaction, called "Michael Addition", involves the addition of reactive aldehyde groups to the side chains of cysteine, histidine or lysine residues. Finally, reactive carbonyl groups can be generated through the reaction of the amino group of lysine residues with reducing sugars or their oxidation products (glycation / glycoxidation) that results in compounds such as carboxymethyl lysine [2, 3].

MEASURES OF OXIDATIVE DAMAGE TO PROTEINS: CARBONYLS

ROS are generally very reactive and have a very short half life, which makes it difficult to measure them directly. An indirect way of determining the existence of oxidative stress is to measure the products of ROS reaction with biomolecules. As in the case of proteins, carbonyl groups are

*Address correspondence to this author at the Departament de Ciències Mèdiques Bàsiques, IRBLleida, Universitat de Lleida, Montserrat Roig 2, 25008-Lleida, Spain; Tel: (34)9737022275; Fax: (34)973702426;
E-mail: joaquim.ros@cmb.udl.cat

[§]*Present address: PROIMI-CONICET. Av Belgrano y Pje Caseros (CP 4000), Tucumán, Argentina*

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among the products generated and their quantification has become widely accepted as a parameter for analyzing the presence of oxidative damage under stress conditions. The most commonly used method of quantifying carbonyl groups is based on its reaction with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone. In turn, this method is based on the ability of DNPH to bind covalently to the carbonyl groups, forming a Schiff base to allow formation of stable hydrazones Fig. (**1C**). Dinitrophenyl group (DNP) can be detected and quantified spectrophotometrically because it displays a characteristic absorption spectrum with a maximum absorbance at 365-375 nm [8, 19]. Given the excellent commercially available antibodies against dinitrophenyl group [20, 21], the detection of carbonyl groups can be performed by western blot, dot blot, immunohistochemistry or ELISA. Various methods have been developed for derivatization and sample preparation [10, 22-24].

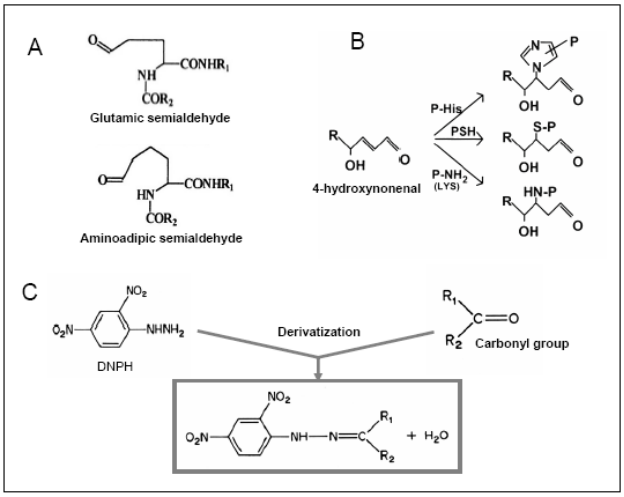

Fig. (1). Formation of carbonyl groups with DNPH derivatization. **A**: products generated by the attack of •OH radical on arginine (glutamic semialdehyde) and lysine side chains (aminoadipic semialdehyde) [17], **B**: the reaction of 4-HNE with the side chains of cysteine, histidine and lysine residues involves the addition of reactive aldehyde groups to proteins [16], **C**: 2,4-dinitrophenylhydrazone formation after reaction of DNPH with carbonyl groups.

However, alternatives to DNPH methods are emerging because some authors question the stability of the 2,4-dinitrophenylhydrazone in alkaline conditions or during prolonged storage. The most commonly accepted alternative uses hydrazide chemistry. Hydrazides react specifically with protein carbonyls (primarily as aldehydes) by forming a Schiff base, which can be stabilized by reduction with cyanoborohydride [25-27]. The advantages of this approach are not only greater stability of the final reaction product but also the presence of a wide range of functional groups coupled to the hydrazide, allowing the design of strategies for the capture or detection of carbonylated proteins [28]. Among these functional groups are biotin, digoxigenin, and various fluorescent compounds. Thus the carbonyl groups can be derivatized with digoxigenin-hydrazide and identified using antibodies against digoxigenin [29]. A similar technique can be used with carbonyl groups derivatized with biotin-hydrazide, using fluorescein-labeled avidin to identify the derivatized proteins [30]. Another compound used for fluorescent detection of carbonyl groups is the fluorescein-5-thiosemicarbazyde. The reactivity of the thiosemicarbazyde group with carbonyl groups is similar to that of hydrazides [31].

Finally, the total carbonyl content in biological samples can be measured by determination of specific products, such as glutamic semialdehyde and aminoadipic semialdehyde, by gas chromatography and mass spectrometry with isotope dilution. This approach, developed by Requena and colleagues, has been used to characterize the nature of the different carbonyl products present in biological samples taken from Alzheimer patients [17, 32].

IDENTIFICATION OF OXIDIZED PROTEINS BY PROTEOMIC TOOLS

"Redox proteomics," defined as the set of proteomic approaches that allow identification of oxidatively modified proteins, offers great potential for discovering new proteins susceptible to oxidative stress, correlating quantitative variations in the profile of these changes with the state of a disease or aging, and characterizing the exact site and type of change by tandem mass spectrometry [3, 33]. This kind of approach can lead to the identification of groups of proteins selectively oxidized under certain conditions and thus help to elucidate the molecular mechanisms involved. To date, proteomic studies have shown that carbonylation is highly selective, because different groups of proteins particularly sensitive to oxidative stress have been identified in different models studied. Here we review some of the strategies used to identify carbonylated proteins, with a summary of the relevant studies in Table **1** and a schematic overview of the main strategies in Fig. (**2**).

Systems based on Two-Dimensional Electrophoresis

Two-dimensional electrophoresis (2DE) has been widely used to identify carbonylated proteins in complex samples. The detection of this oxidative modification can be performed by western blot with antibodies that identify the modifying molecule attached to the protein (e.g., anti-4-HNE) [34], or with anti-DNP antibodies if the sample has been previously derivatized with DNP [35-37]. An example of this type of approach is shown in Fig. (**3**). In other cases, reactive carbonyls are covalently labeled with biotin-hydrazide and detected in the gel by avidin-fluorescein [30] or avidin-peroxidase [25]. The use of two-dimensional gels has proved very useful for the identification of oxidized proteins, especially when comparing two physiological conditions [38-40]. It has the advantage of showing the images obtained after detection of the functional group attached to the carbonyl group as well as those obtained after staining the gels with protein dyes (silver staining, SYPRO Ruby, Coomassie Brilliant blue, etc.). This allows the normalization of the carbonyl signal to the amount of protein. This is an important point because, as in any proteomic analysis, the study of carbonylated proteins is biased in favor of the most abundant proteins. Therefore, the relative level of carbonylation on a particular protein is more informative than the total carbonyl signal observed for that protein. Otherwise, abun-

Table 1: Studies Identifying Carbonylated Proteins

Reference	Organism	Model	Compound	Strategy	Identified Proteins
Systems Based on Two-Dimensional Electrophoresis					
[40]	Yeast	Frataxin Deficiency	DNPH	2DE Oxy-blot	14
[39, 50]	Human Brain	Huntington Disease	DNPH	2DE Oxy-blot	17
[38]	Yeast	Aging	DNPH	2DE Oxy-blot	16
[37]	Human Brain	Alzheimer Disease	DNPH	2DE Oxy-blot	4
[31]	Mouse	Aging	Fluorescein-5-Tiosemicarbazide	2DE/fluorescent detection	12
[30]	Yeast	H_2O_2	Biotin hydrazide	2DE/avidin-fluorescein	20
[25]	Rat	Diabetes	Biotin hydrazide	2DE avidin blot	7
[56]	*Arabidopsis thaliana*	Life Cycle	DNPH	2DE Oxy-blot	5
Gel-Free Approaches					
[28]	Yeast	H_2O_2	Biotin hydrazide	Affinity/LC/proteolysis/MS-MS	99
[26]	Rat Mitochondria	Basal	Biotin hydrazide + iTRAQ	Affinity/proteolysis/iTRAQ/LC-MS-MS	200
[27]	Mouse Brain	Aging	Biotin hydrazide	Affinity/LC/proteolysis/LC-MS-MS	100
[43]	Tumor Cells	Photodynamic Treatment	Biotin hydrazide	Affinity/1DE/proteolysis/ LC-MS-MS	81
[47]	Yeast	H_2O_2	Girard's Reagent	LC-MS/MALDI-MS-MS	36
[44]	Adipose Tissue	Obesity	Biotin hydrazide	Affinity/proteolysis/ LC-MS-MS	37

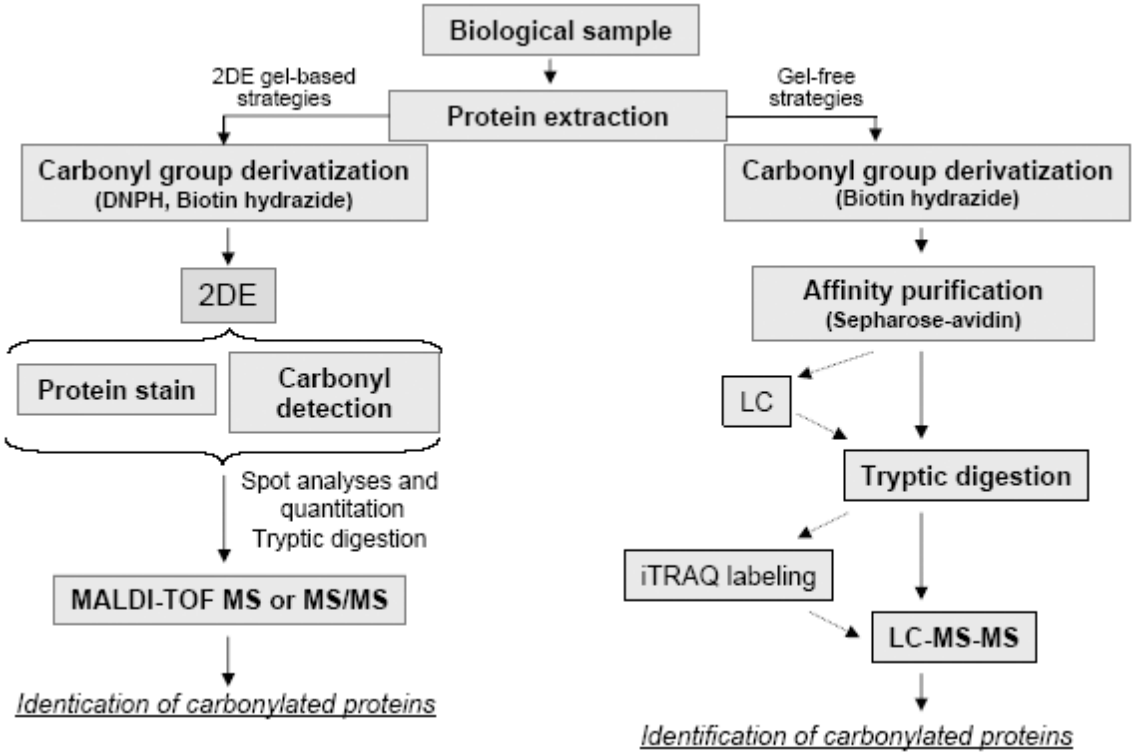

Fig. (2). Outline of various analytical strategies used for the identification and quantification of protein carbonyls.

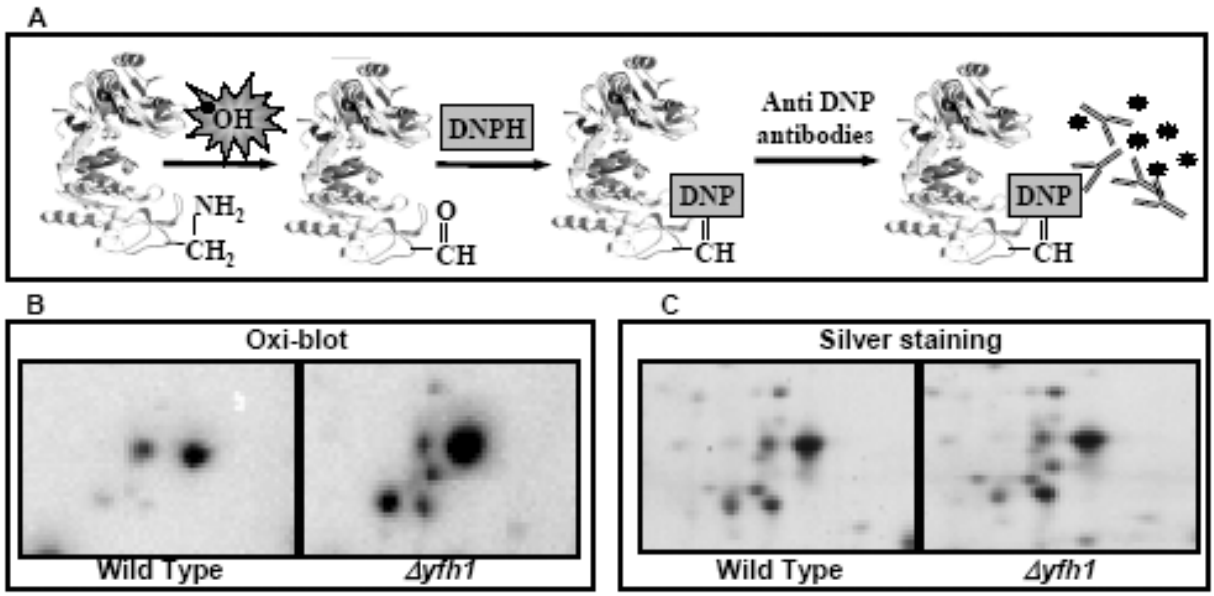

Fig. (3). Formation of carbonyl groups and detection by anti-DNP antibodies (Oxi-Blot)**. A**: The attack of an •OH radical to an amino acid side chain generates a carbonyl group. The DNPH reacts specifically with the carbonyl group, forming a stable hydrazone that can be detected immunologically by anti-DNP antibodies. **B**: Detection of carbonylated proteins in cell extracts of two yeast strains by two-dimensional electrophoresis and Oxi-Blot. **C**: Silver staining of proteins in the same region of two-dimensional gels allows normalization of the carbonylation levels. It can be observed that different proteins have a higher degree of carbonylation in *Δyfh1* strain (for details see ref.40).

dant proteins with low quantities of specific carbonyls may appear as predominant in oxi-blots, while proteins of low abundance but high levels of specific carbonylation may be discarded [10]. In addition, a change in the level of expression of a given protein between two conditions could be erroneously identified as a change in the degree of damage to this protein. It is noteworthy that several studies based on two-dimensional gels have identified low abundance proteins, such as mitochondrial chaperones [40-42], as highly sensitive to oxidative stress.

Despite the extensive information obtained by gel-based proteomic analysis, this type of approach has limitations in large-scale analysis and the identification of membrane or very low abundant proteins. On the other hand, a potential problem with two-dimensional gels is the validation of results. The comparison of images obtained by specific staining (anti-DNP, for example) and total proteins is not always easy, and the choice of criteria in each case is left to the researcher. In theory, dual fluorescent staining allows a better match between the different images obtained. Mass spectrometry analysis of the presence of modified peptides could confirm the damage to a particular protein. However, this strategy is not regularly used because approaches specifically designed for that purpose must be developed before the results would be conclusive. An indirect way of validating the results is to measure the enzymatic activities of proteins identified as oxidized. This approach has been used successfully in several studies [9, 38, 40], although the main limitation is that it can be applied only to enzymes for which an effective method exists to measure their activity.

Gel-Free Systems

Several authors have explored gel-free methods based on affinity enrichment of carbonylated proteins and subsequent detection by mass spectrometry. In theory, these approaches would dispel some of the limitations of 2DE-based systems. However, the disadvantage of these approaches is that they do not allow quantitative analysis of the degree of carbonylation of the identified proteins, which limits their usefulness for studying changes in the pattern of protein oxidation under different biological conditions. The most common system for the enrichment of carbonylated proteins uses avidin affinity columns with carbonyls previously derivatized with biotin-hydrazide. The enriched proteins are digested and the peptides obtained are identified by LC-MS/MS [26-28, 43, 44]. Using this approach in a single experiment with brain homogenates of mice of different ages, Soreghan and colleagues identified 100 carbonylated proteins, including low abundance receptors and proteins involved in mitochondrial energy metabolism and stress response. Moreover, they were able to observe abundant cytoplasmic protein carbonyls previously identified by 2DE [27]. An extension of this technology using iTRAQ™ distinguishes carbonylated proteins from those with affinity to avidin and has led to the identification of 200 carbonylated proteins in extracts of rat muscle mitochondria, showing high susceptibility of these proteins to oxidative stress [26]. Alternatively, proteins derivatized with biotin-hydrazide and purified by avidin affinity chromatography can be separated by liquid chromatography prior to digestion. Using this sort of "top-down" approach, Mirzaei and Regnier identified 99 carbonylated proteins in yeast treated with H_2O_2 [28].

Identification of Carbonylation Site

Recent attempts have been made to develop methodologies to identify the specific amino acids oxidatively modified. This information is important to an in-depth understanding of the functional effects that may result from a

change on a given amino acid in a protein. Most of the analytical strategies for the detection of modified amino acid are based on the observation of variation in the specific molecular mass of a covalently modified amino acid, which is easily measured by mass spectrometry. In some of the studies that used biotin-hydrazide to label protein carbonyls, identification of the biotinylated peptide confirmed the oxidative modification and it even provided information about its location [28, 43]. On the other hand, the reaction of 4-HNE with lysine histidine or cysteine residues can be monitored in intact proteins by detecting products with mass increases of 156 Da. The peptide bearing this specific mass difference can also be identified [3, 34, 45]. Another approach uses MALDI-TOF to identify 4-HNE modified peptides by the reaction of DNP with this lipid peroxidation product. These carbonylated peptides can be identified by the mass difference of 180 Da (corresponding to DNP) observed between derivatized and non derivatized samples. Given the low stability of the hydrazone under conditions typically used in MALDI-TOF analysis, this strategy requires the use of DNPH as a matrix for ionization [46]. Finally, Mirzaei and Regnier used two isotopic forms of Girard reagent (containing a hydrazide group) to characterize carbonylation sites in proteins using yeast extracts treated with H_2O_2 [47].

SELECTIVITY OF PROTEIN CARBONYLATION

A final aspect to consider is whether or not we can define groups of proteins that will be preferentially damaged under conditions of oxidative stress. After studying oxidative modifications using several cell models and different causes of oxidative stress, we realized that targeted proteins show some specificity. This was based on special characteristics of the protein: presence of metal ions, molecular chaperones or the high reactivity of thiol groups with lipid-peroxidation products.

Given that carbonyl groups can be generated by metal catalyzed oxidation, notably through the reaction of iron with H_2O_2, the presence of metal ions attached to the protein contributes to specificity. Thus, iron-binding proteins are prone to be oxidatively modified, as initially observed in iron-dependent alcohol dehydrogenases from *E. coli, Z. mobilis, S. typhimuirium* and *S. marcescens* [9, 48]. More evidences about the general role of iron in promoting damage were obtained studying protein oxidative damage in mutants of *S. cerevisiae* lacking yeast frataxin homolog (*YFH1*), where accumulation of iron is paradigmatic. Among the consequences observed was the modification of proteins that bind to magnesium ions either directly or through nucleotides. These proteins were pyruvate kinase, phosphoglycerate kinase, F_1F_O ATP synthase, adenylate kinase and elongation factor EF1alpha [40]. The enzymatic activities of F_1F_O ATP synthase and pyruvate kinase (in which magnesium ions are chelated by amino acid side chains) were more markedly affected than those of phosphoglycerate kinase or adenylate kinase in *Δyfh1* cells. These results concur with previous studies performed in *E. coli* cells challenged with hydrogen peroxide or superoxide-generating compounds resulting in carbonylation of elongation factor G (EF-G), or the beta subunit of F_1F_OATP synthase [9]. When *S. cerevisiae* was used as a model, pyruvate and alpha-ketoglutarate dehydrogenases were identified as targets for oxidative modification [49].

Moreover, in human brain samples of patients affected with Huntington disease, the identified targets have common traits with those cited above: endoplasmic reticulum ATPase (VCP), pyridoxal kinase, pyruvate kinase isoenzymes M1 and M2, creatine kinase and ATP5A1 (alpha subunit of ATP synthase) [50]. What do all these identified targets have in common? The most important shared trait is that they bind to molecules such as magnesium or nucleotides (or thiamine pyrophosphate) which can facilitate iron binding at specific loci and, as a consequence, promote metal-catalyzed oxidation at specific sites. This suggests that enzymes containing magnesium binding sites in their active centers would be sensitive to iron-mediated oxidative stress. The notion that magnesium- and nucleotide-binding proteins could be specific targets of oxidative stress or iron overload has been previously suggested by several authors. This sensitivity has also been attributed to the ability of Fe(II) to replace magnesium ions bound to the phosphate groups of the nucleotides; their reaction with H_2O_2 or oxygen would then trigger the formation of reactive oxygen species [51]. Indeed, oxidation of ATP synthase *in vitro* has been shown to be mediated by iron binding to magnesium-binding sites. The presence of ATP promoted further oxidation and fragmentation of the protein [52]. On the other hand, metal-binding sites that cannot be occupied by iron are not prooxidant, as in the case of zinc-binding proteins [53].

Molecular chaperones are also prone to oxidative modification. In fact, identification of DnaK in *E. coli* or heat shock proteins such as Hsp60, Hsc71, Hsp78 in *S. cerevisiae* were detected after several stresses. These proteins also bind specific nucleotides that could promote iron binding and carbonylation. Nevertheless, oxidative modification of chaperones can be explained by their interaction with damaged proteins produced under oxidative stress. It has been suggested that oxidized proteins contain reactive species that can, in turn, damage other proteins [54].

Some specificity may be attributed to the high reactivity of thiol groups with lipid-peroxidation products such as malondialdehyde or 4-hydroxynonenal. This could also explain the carbonylation of glyceraldehyde-3-phosphate dehydrogenase [55] and the modification of pyruvate and alpha ketoglutarate dehydrogenases [9, 49], as they both contain a dithiol group in their lipoic acid moiety.

It is clear that proteomic techniques are helping to deepen the detection, identification and understanding of mechanisms underlying oxidative modifications of proteins. In turn, this contributes to a better understanding of the significance of these changes at the cellular level and will undoubtedly lead to the next challenge: understanding their impact on cell physiology, that is, how the metabolome is affected as a result of the impaired function of certain proteins.

ABBREVIATIONS

LC = Liquid Chromatography

MS = Mass spectrometry

MS/MS = Tandem mass spectrometry

2DE = Two-dimensional gel electrophoresis

1DE = One-dimensional gel electrophoresis

DNPH = 2,4-dinitrophenylhydrazine; 4-HNE, 4-hydroxynonenal

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