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PROTEOMIC IDENTIFICATION OF CARBOXYLATED PROTEINS AND THEIR OXIDATION SITES

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Abstract

Excessive oxidative stress leaves a protein carbonylation fingerprint in biological systems. Carbonylation is an irreversible post translational modification (PTM) that often leads to the loss of protein function and can be a component of multiple diseases. Protein carbonyl groups can be generated directly (by amino acids oxidation and the α -amidation pathway) or indirectly by forming adducts with lipid peroxidation products or glycation and advanced glycation end-products. Studies of oxidative stress are complicated by the low concentration of oxidation products and wide array of routes by which proteins are carbonylated. The development of new selection and enrichment techniques coupled with advances in mass spectrometry are allowing identification of hundreds of new carbonylated protein products from a broad range of proteins located at many sites in biological systems. The focus of this review is on the use of proteomics tools and methods to identify oxidized proteins along with specific sites of oxidative damage and the consequences of protein oxidation.

Keywords

carbonylation; oxidative stress; redox proteomics; reactive oxygen species; advanced glycation end products and Lipid peroxidation adducts

INTRODUCTION

Redox regulation is a subject of broad interest in regulatory biology^{1–4}. Oxidation and reduction of amino acid side chains in proteins is a normal part of redox regulation in cells where slight surges in reactive oxygen species (ROS) are generally dealt with by oxidation of sulfhydryl groups to mixed disulfides. After an oxidative stress (OS) episode has passed, these disulfides are reduced back to sulfhydryls and the normal redox potential of the cell is restored. An array of enzymes has evolved in aerobic organisms specifically for repairing oxidative modifications produced in proteins during such events. Proteins that are too seriously damaged for repair are destroyed by proteasomes and lysosomes.⁵

There are however, cases where these coping mechanisms are exceeded. Excessive levels of ROS from either the environment or aberrations in electron transport can produce such high levels of OS that large amounts of proteins can be damaged. Some of these oxidation products can be repaired (e.g. methionine oxidation) but the major fraction are irreparably altered. In the process proteasomes and lysosomes themselves can be altered to the point that their ability to degrade proteins is compromised⁶. Under chronic OS damaged proteins can accumulate to toxic levels, often causing cell death as in OS diseases.^{3,7}

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Pathological levels of OS have been implicated in a plethora of diseases ranging from diabetes mellitus⁸ and neurodegenerative diseases⁹ to inflammatory diseases,¹⁰ atherosclerosis,¹¹ cancer,¹² and even aging^{13,14}. Clearly OS impacts the health of several hundred million people, if not everyone at some point in life. Although all of these diseases have been widely studied, understanding of the protein chemistry involved is relatively primitive. Until very recently protein carbonylation from pathological OS has been accessed with the dinitrophenylhydrazine (DNPH) colorimetric test for carbonyl groups. The proteins involved, potential changes in their structure and function, sites of oxidation, mechanisms of oxidation, repair or degradation, the long term fate of oxidized proteins that precipitate in cells, and how oxidized proteins cause cell death are issues that need more study to understand how oxidative stress diseases threaten health.

Collectively, proteins can be oxidized in more than 35 ways (Table 1). All of these post-translational modifications occur in three basic ways that are distinguishable by mass spectrometry. One involves oxidative cleavages in either the protein backbone or amino acid side chains^{15–17} in which Pro, Arg, Lys, Thr, Glu or Asp residues are most likely to undergo oxidative cleavage. A second mechanism is by indirect addition of lipid oxidation products such as 4-hydroxy-2-nonenal, 2-propanal or malondialdehyde to proteins.¹⁸ Mass increases with this type of modification and is unique to the appended group. Finally, carbonyl groups are generated in proteins by oxidation of what has come to be known as advanced glycation end (AGE) products. AGE products are common in long lived proteins such as hemoglobin, especially in the case where glucose levels and OS are elevated, as in *diabetes mellitus*. Structures of some carbonylated oxidation products are seen in Table 2. All of these forms of oxidation occur simultaneously. The analytical problem is in recognizing oxidized proteins and differentiating between the various types of oxidative modifications.

The focus in this review will be on i) targeting and isolation of carbonylated proteins, ii) strategies for their analysis, iii) and how to identify oxidation mechanisms.

ISOLATING CARBOXYLATED PROTEINS

Biological fluids such as blood plasma contain thousands of proteins that vary 10¹⁰ or more in concentration, a small portion of which will be oxidatively modified. Current proteomics tools require much simpler mixtures and concentrations of a ng/mL or more for large scale protein identification. As a means of dealing with these problems multiple methods have been described for selection and recognition of carbonylated proteins, all of which exploit the relatively unique property of carbonyl groups to form Schiff bases. Through derivatization of carbonyl groups with a reagent such as dinitrophenylhydrazine (Table 3-A) or biotin hydrazide (Table 3-B), affinity chromatography can be used to select oxidized proteins derivatized with these groups; greatly enriching them or their proteolytic fragments in the process.

a) Dinitrophenylhydrazine

Derivatization of carbonyl groups with dinitrophenylhydrazine (DNPH) has been used for more than half a century as a qualitative analytical method in organic chemistry. With slight modification of this old method, DNPH derivatization was adapted to enhance the isolation, identification, and quantification of carbonylated proteins¹⁹ (Table 3-A) through selection of derivatized proteins with DNPH targeting antibodies^{20,21}. Tryptic digestion of protein mixtures thus derivatized and selected followed by reversed phase chromatography coupled with tandem mass spectrometry (RPC-MS/MS)^{22,23} or ion exchange and reversed phase chromatography coupled to tandem mass spectrometry (IEC/RPC-MS/MS)²⁴ has proven successful in identification^{22–24} and quantification of carbonylated proteins^{22,23}.

b) Biotin Hydrazide

Biotin hydrazide (BHZ) and biocytin hydrazide have been used in a similar fashion. BHZ reacts readily with carbonyl groups, allowing carbonyl groups to be derivatized and the parent proteins to be selected with an immobilized avidin or streptavidin sorbent. Streptavidin and avidin bind biotin with comparable affinity²⁵. Isolation and identification of carbonylated proteins through biotin derivatization has been achieved with a multiple step chromatographic process in which carbonyl groups are first derivatized with BHZ (Table 3-B) to form a Schiff base. The Schiff base is then reduced with sodium cyanoborohydride to prevent reversal of derivatization. Excess BHZ is removed before avidin affinity chromatography by either dialysis or precipitation with trichloroacetic acid (TCA). Because the interaction of biotin with native tetrameric avidin affinity chromatography columns is very difficult to disrupt, monomeric avidin columns are frequently used in affinity chromatography. The binding of biotin to monomeric avidin is still highly specific, but much weaker. Monomeric avidin is also easily immobilized²⁶ and has been used to select oxidized proteins^{27–34}. Elution of biotinylated proteins from monomeric avidin can be affected with 2mM biotin or 0.1M glycine. Biocytin hydrazide is similar to biotin hydrazide in structure and reactivity and has been used with streptavidin to isolate and identify carbonylated proteins from aged mice³⁵. The BHZ approach has known been used to study oxidized proteins in yeast^{29–33}, rats²⁸, and humans³⁴.

Additionally 2-D gel electrophoresis (2DGE) and SDS-PAGE has been used to separate biotinylated proteins after which they were detected with labeled avidin³⁶. The limit of detection in gels using avidin FITC (fluorescein Isothiocyanate) has been reported to be 10 ng. Detection of biotinylated proteins in gels using streptavidin-conjugated peroxidase for amplification is even more sensitive. Twenty four oxidized proteins in yeast cultures stressed with hydrogen peroxide were identified using avidin:FITC detection and MALDI-MS fingerprinting³⁷. In similar fashion, carbonylated proteins were identified by 2DGE-MS in the muscle of diabetic Otsuka Long Evans Tokushima Fatty rats in a comparison with control Long Evans Tokushima Otsuka animals³⁸.

c) Girard's P reagent

Derivatization with Girard's P reagent (GPR), i.e. (1-(2-hydrazino-2-oxoethyl) pyridinium chloride) (Table 3-C), provides another route for the selection of carbonylated peptides. GPR contains i) a hydrazide group that reacts readily with carbonyl groups to form hydrazones and ii) a quaternary amine that can be selected by strong cation exchange (SCX) resin at pH 6.0. Following trypsin digestion of proteins derivatized with GPR, quaternary amine containing peptides are selected from mixtures with a SCX column and then further fractionated and identified by RPC-MS/MS³⁹. Advantages of this approach are that excess derivatizing reagent does not have to be removed before chromatographic analysis and derivatization enhances peptide ionization through quaternization.

d) Oxidation-Dependent Element Coded Affinity Tags (O-ECAT)

Isolation, identification, and quantification of carbonylation sites has also been achieved with ((S)-2-(4-(2-aminooxy)-acetamido)-benzyl)-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid (O-ECAT) (Table 3-D). After derivatization of carbonyl groups in a sample, the O-ECAT moiety is used to chelate a rare earth metal such as Tb (158.92Da) or Ho (164.93 Da). Treating samples with different rare earth metals according to sample origin allows differential coding of samples. Native and oxidized human serum albumin samples were allowed to react with this reagent and after coding and mixing were tryptic digested. Coded peptide fragments were then selected with an immunosorbent column targeting the derivatizing agent. Peptides selected in this manner were analyzed by nanoRPC-FTICR mass spectrometry. Relative intensities of the tagged peptides from the

two samples were used to determine the degree of oxidation, which is independent of the amino acid oxidized.^{40,41}

e) Caveats and conclusions

It is important to recognize with all the carbonyl derivatization methods for isolating and identifying oxidized proteins that it is necessary to use fresh samples. Carbonyl groups in stored samples readily undergo Schiff base formation with lysine residues on proteins, even when stored at -20 to -80 °C and are no longer available for analysis based on Schiff base formation. Over the course of a few weeks storage, major amounts of carbonylated protein are lost, even at low temperatures. This makes archived samples of doubtful value in the study of chronic OS at the protein level. Also, it is not clear that all oxidized protein species are lost at an equal rate in archived samples.

Another caveat is that although other types of oxidation not involving carbonylation are seen with the methods described above, the protein in which this occurs must have contained a carbonyl group to have been captured. Moreover, oxidized proteins will probably not be affinity selected by this process if they do not contain a carbonyl group. This method does not see all oxidized proteins.

The final warning is that the binding of a protein from a biotinylated sample by an avidin column does not prove the protein is oxidized. It is known from the Tandem Affinity Purification (TAP) method that affinity columns bind protein complexes⁴². This means that when a biotinylated member of a complex is bound by an avidin affinity column other non-oxidized members can be captured as well. These non-oxidized members of the complex will show up during subsequent shotgun proteomic analyses. Non-oxidized proteins can also bind non-specifically to the chromatographic support matrix or avidin in addition to naturally biotinylated proteins^{43,44}. Proof that a protein is oxidized comes from identification of the oxidation site.

IDENTIFICATION STRATEGIES

Affinity chromatography coupled with modern proteomics methods is now widely used to study many types of post-translational modification (PTM), among them carbonylation. Proteomic analysis of carbonylated proteins have been achieved in three ways (figure 1).

a) Targeting PTM Bearing Peptides

One route of identification is to tryptic digest biotinylated samples immediately with trypsin or glu-C and select only the carbonylated peptides from samples by avidin affinity chromatography. Carbonylated peptide mixtures thus selected are then analyzed by RPC-MS/MS. Because arginine and lysine residues can be oxidized, trypsin cleavage at these sites will be blocked. This means that some carbonylated tryptic peptides will be larger.⁴⁵ Also some peptides will be selected non-specifically that do not bear the PTM being targeted. Advantages of targeted affinity selection of PTM bearing peptides are that mixtures will be greatly simplified and enriched in the PTM of interest. Also, carbonylation sites will be much easier to identify. A disadvantage is the lack of redundant peptides from a protein. If the peptide bearing a carbonylation site in a protein is missed due to poor ionization, there is no backup. That protein will be missed.

b) Identifying Carbonylated Proteins as a Group

A second approach is to target native proteins. With this method carbonylated proteins are first biotinylated and then selected by avidin affinity chromatography. Following proteolysis, un-oxidized peptide fragments from these proteins can be identified by RPC-

MS/MS methods common to shotgun proteomics. There are several advantages with this approach. One is that both unmodified and PTM bearing peptides are available for identification. When ionization of a carbonylated peptide is suppressed or a PTM bearing peptide does not ionize at all, other peptides from the protein are available for identification. Another advantage is that many carbonylated proteins have also undergone methionine oxidation, sulfhydryl oxidation, and tyrosine nitrosylation, to name a few additional types of protein oxidation. The fact that addition types of oxidation are co-selected with carbonylation is fortuitous. The disadvantage of this strategy is that modification sites will not be identified if PTM bearing peptides are not seen and sequenced. Non-specifically bound proteins could also be mistakenly identified as bearing the PTM if the PTM site is not identified.

c) Multidimensional Fractionation

A third strategy is to further fractionate affinity selected proteins by liquid chromatography before proteolysis and identification of peptide fragments by RPC-MS/MS. Beyond affinity selection in the first dimension of chromatography, fractionation is generally achieved by RPC in the second dimension. Protein fractions collected from the RPC column are trypsin digested and the peptide fragments identified by RPC-MS/MS. Carbonylation sites from 87 yeast proteins have identified in this manner.⁴⁶ Again oxidatively modified and un-modified tryptic peptides from a protein will appear together, facilitating protein identification based on peptide sequence analysis. The un-modified peptides are used to identify the protein parent while labeled carbonylated peptides are used to identify oxidation sites. Proteins sometimes appear in multiple peaks during RPC fractionation. These peaks arise from *in vivo* cleavage, protein:protein cross-linking, and protein:RNA cross-linking. Isoforms of a protein will generally be missed by the other two procedures described above. Advantages of this approach are that it has the highest level of structural discrimination on the separation side and will identify the most oxidized proteins. The disadvantage is that it is lengthy.

This strategy is not restricted to liquid chromatography, 2-DE (two dimensional electrophoresis) can be also used. Unfortunately, DNPH derivatization changes the isoelectric point of proteins and can lead to sample loss during fractionation. One way to deal with this problem is by starting the fractionation with isoelectric focusing, then derivatizing with DNP, and finally going to molecular weight separations⁴⁷. Limitations of 2-DE are low sensitivity, poor reproducibility, and limited dynamic range²⁷.

ANALYSIS OF OXIDATION MECHANISMS

Carbonylation of proteins occurs in at least three ways; by direct oxidation with reactive oxygen species (ROS), through Michael addition of lipid peroxidation products, and through formation of advanced glycation end products (figure 2).

a) ROS Oxidation

Cleavage of amino acid side chains is generally associated with oxidation by ROS. Some of these cleavage products are listed in Table 2. Each is linked to a unique change in mass that can be programmed into most peptide/protein identification software. But even so, the molecular weight of the modified peptide can be very similar to that of an un-modified peptide in the proteome. This is best dealt with by acquiring data with a high mass accuracy analyzer capable of differentiating PTM bearing peptides from un-modified peptides on the basis of mass alone. Through hydrogen peroxide induced oxidative stress in yeast cultures and biotin hydrazide derivatization to select carbonylated proteins, 415 proteins have been identified along with specific sites of oxidation in 87 instances³¹. Thirty two cases were seen

in which proteins appear in multiple, non-adjacent peaks during reversed phase chromatography (RPC)⁴⁵. This generally indicates differences in post-translational modification, fragmented forms of the protein, or some type of cross-linking. Typically gel electrophoresis has been used to study protein fragmentation^{48–51} but RPC works equally well.

Cross-linking is often seen in ROS induced protein oxidation, occurring in at least six different ways^{13,32,52}. Among the more frequent are a) formation of disulphide bonds between proteins through cysteine oxidation, b) Schiff base formation between a carbonyl group on an oxidized amino acid side chain of one protein and a lysine residue on another, c) Schiff base formation between the carbonyl group on an HNE adduct of one protein and a lysine residue on another, d) the same process as with HNE but involving a malondialdehyde adduct on a protein, e) Schiff base formation again but with a carbonylated AGE product and another protein or f) by free radical cross-linking involving carbon centered radicals.

Co-elution in multiple separation systems is a good way to recognize cross-linked proteins.⁴⁵ This approach has been exploited in recognizing cross-linking of ribosomal proteins to rRNA⁴⁵. Proteins from H₂O₂ stress yeast were biotinylated, avidin selected, and then further fractionated by RPC. During RPC under strongly denaturing conditions the same ribosomal proteins were noted to elute in three different peaks. The possibility that these proteins were crosslinked to rRNA was examined by tryptic digestion and further chromatography of the peptides on a borate affinity column that would select species bearing a vicinal diol present in RNA bases. Mass spectral analysis of captured peptides indicated they did indeed have covalently appended nucleotide bases and identified the specific bases involved.⁵³ This procedure was used to identify 37 ribosomal proteins from yeast that were cross-linked to rRNA, along with sites in the proteins and on rRNA at which cross-linking occurred.

Although protein oxidation is non-enzymatic and would be expected to be random, it is not. Oxidation occurred at very specific sites. Many of these new, non-genetically coded oxidation sites conveyed a new biological activity to the oxidized protein and have been named “allotypic active sites”⁵⁴. It is also possible that the type of oxidizing agent can play a role in protein oxidation. It has been shown with metal catalyzed oxidation of human serum albumin *in vitro* that carbonylation occurred at Lys-97 and Lys-186 while with hypochlorous acid, carbonylation arose at 5 sites, Lys-130, Lys-257, Lys-438, Lys-499, and Lys-598

A substantial amount of work on OS has been done in model systems. The problem is that protein oxidation *in vitro* is not always the same as *in vivo*. Metals have long been known to play a role in oxidation by ROS, often leading to primary structure cleavage. When the oxidation of human serum albumin (HSA) was catalyzed with FeEDTA, *in vitro* cleavage was more extensive than that seen *in vivo*⁵⁶. This is probably because fewer proteins are in the *in vitro* mixture and ROS are not depleted as quickly.

b) Lipid Peroxidation Adducts

i- Examining the protein-aldehyde adducts—The first direct proof of lipid conjugation to proteins was in oxidized low density lipoproteins (LDL)⁵⁷. LDL is composed of a single apolipoprotein B-100 (Apo B-100) with adsorbed fatty acids that together are water soluble. Oxidation of LDL makes it susceptible to uptake by scavenger receptors inside the endothelium and leads to the formation of “foam cells”. Accumulation of these cells is the first stage of atherosclerotic plaque formation. It is well known that one of the degradation products resulting from lipid oxidation is 4-hydroxynonenal (HNE)

and that HNE can become attached to proteins through either Michael addition (major) or Schiff base formation (minor). Based on this knowledge, a study was undertaken in which oxidized LDL was reduced with NaBH₄ (to stabilize the Michael adduct formed between HNE and histidine), delipidated to remove non-covalently linked lipid, and digested with trypsin to generate peptides for RPC-MS/MS analysis. Mass spectra of all peptides containing the HNE moiety showed an m/z 268 product ion corresponding to the histidine immonium ion modified by HNE. Product ion scanning of all second dimension mass spectra for this m/z 268 ion was used to locate peptides in the RPC eluent carrying HNE. Peptide sequence and the location of HNE in the peptide were extracted from the spectra of these peptides. Modified residues were found to be located on the surface of LDL⁵⁷.

Although the study above targeted adducts on histidine, Michael addition can occur on lysine and cysteine residues as well. Moreover, direct addition of carbonyl groups from malondialdehyde (MDA) and 4-hydroxynonenal (HNE) onto lysine is possible. The potential for multiple products be formed with MDA and HNE leads to a series of questions. One question is the *in vivo* ratio between these adducts? Another is whether any particular amino acid is favored over others in the formation of Michael adducts? Still another is what fraction of any particular proteins carries HNE or MDA?

A recent *in vitro* study with hemoglobin and β -lactoglobulin under near physiological conditions has shown it is possible to differentiate between Michael addition and Schiff base formation through mass spectrometry. One hundred fifty eight Daltons of additional mass is acquired by Michael addition of HNE while that from Schiff base formation is 138 Da. Based on mass spectral analysis, Michael adduct formation dominated Schiff base formation by a 99:1 ratio⁵⁸. When HNE was adducted to apomyoglobin, addition occurred predominantly at histidine residues⁵⁹. Product ion scanning of immonium ions showed that 3–10 histidine residues were derivatized. In contrast, the adduct ratio of HNE to human serum albumin (HSA) was dependent on the molar ratio of HNE to HSA. Moreover, cysteine, histidine and lysine were all modified⁶⁰. Cytochrome c forms adducts with histidine, lysine and arginine. The importance of this is that cytochrome C binds to complexes III and IV in the electron transport chain through lysine residues. HNE-Lys adduct formation could impact electron transport⁶¹. In another study amyloid peptide was shown to form one or more HNE adducts in the residue 6–16 region of the primary structure.⁶² Addition of reducing agents (NaCNBH₃ or NaBH₄) affect the type of adduct formed as well at the site of modification. Interestingly, if NaCNBH₃ was added early in the incubation process, Schiff base formation was more prominent than Michael addition. In addition, the N-terminal amino acid rather than a histidine residue would be modified. On the other hand, addition of NaBH₄ at the end of the reaction between the protein and HNE resulted in the reduction of the Michael adduct formed.⁶³

An additional question with lipid peroxidation product modification of proteins is whether polypeptide structure affects the process. This question was answered by examining HNE and ONE (4-oxo-2-nonenal) modification sites in apomyoglobin and myoglobin. The degree of modification in apomyoglobin was greater than with myoglobin due to its more open structure⁶⁴.

A variety of mass spectrometers have been used in the analysis of protein oxidation. One of the more important issues is the mass accuracy of the instrument. FTICR-MS was used to characterize HNE modifications in apomyoglobin where it was found that three to nine Michael adducts were formed. Schiff base adducts were observed as well, but with less intensity as expected from the discussion above⁵³. An advantage of high mass accuracy and resolving power is that it allows the resolution of fragment ions of very similar mass. Because peptide sequencing by collision induced dissociation (CID) results in neutral loss

and only partial sequence coverage, electron transfer dissociation (ETD) has been used as an alternative and shown to be superior in the characterization of modification sites due to the production of c and z ions.⁶⁵ FTICR-MS has also been used to characterize HNE adducts of creatine kinase isoforms in brain where it was shown that cysteine and histidine residues were most likely to be derivatized⁶⁶. A new method for the characterization of HNE-protein adducts has also been developed using a hybrid linear ion trap-FTICR mass spectrometer (LTQ-FT). In this method, both the usual data-dependent mode of acquisition and a neutral loss driven MS³ (NL- MS³) data dependent acquisition mode were utilized. The latter depended on the isolation and fragmentation of any ion showing a difference of m/z 78, 52 or 39 from the precursor ion. Twenty four HNE modification sites were observed on fifteen mitochondrial proteins of which 6 were seen using NL- MS³ data dependent acquisition⁶⁷.

ii- Affinity purification of the adducts—It has been shown above that monitoring immonium product ions contain HNE is a powerful tool for recognizing HNE bearing peptides. Unfortunately, cysteine and lysine do not produce intense HNE bearing immonium product ions. Several new methods have been developed with the aim of enriching these adducts to circumvent this problem. The first is based on the use of an anti-HNE immunosorbent in which the antibody was immobilized on CNBr activated Sepharose. This immunosorbent has been employed to enrich adducts formed between HNE and peptides from either a tryptic digest of apomyoglobin or a model peptide (residues 87–99) of myelin basic protein. A unique feature of the antibody chosen for HNE selection was that it was specific for Michael adducts only⁶⁸. Anti-dinitrophenyl antibodies have been used as well to select HNE Michael and malondialdehyde Schiff base adducts derivatized with dinitrophenyl hydrazine (DNP). Enrichment and recovery of DNP derivatized peptides was virtually quantitative⁶⁸.

As noted above, enrichment of biotin adducts through avidin affinity purification is another route. Biotinylated hydroxylamine can react with Michael adducts and has been used for enrichment through avidin affinity chromatography. Forming an oxime rather than hydrazone eliminates the need for the reduction step while still allowing determination of the sites of modification (Table 3-E)^{69,70}. Another way to isolate these adducts is by using biotin hydrazide. This allowed the enrichment of HNE modified peptides from HNE spiked yeast lysate. Mapping the HNE modification sites showed that sixty seven proteins were modified, generally on histidine⁷¹. The first step in identifying HNE-modified proteins from adipose tissues was incubation of biotin hydrazide with adipose tissue from obese mice. Proteins thus biotinylated were captured by avidin affinity chromatography, followed by tryptic digestion, and identification by RPC-MS/MS with online database searches to identify peptides. Among the proteins identified was HNE modified adipocyte fatty acid-binding protein⁷². This protein plays a role in insulin resistance. Additionally, treating yeast lysates *in vitro* with HNE resulted in the identification of 67 different proteins carrying 125 HNE modification sites⁷³. HNE adducts seen in the *in vitro* study were not observed in the *in vivo* study of yeast. A major problem with these two approaches is that both of the aforementioned reagents react with all carbonyl groups, not just on HNE.

c) Oxidation of Advanced Glycation End Products

Reducing sugars add to amines in proteins through the Maillard reaction. Addition products thus formed often undergo an Amadori rearrangement and in the course of doing so form an isomeric mixture^{74,75} of products with long term stability⁷⁶ known as advanced glycation end (AGE) products. With diabetes, renal failure, and aging, AGEs and their oxidation products increase in concentration⁷⁷. A series of methods have been developed to assess the nature of these adducts.

Based on a series of simple analytical methods it is known that advanced glycation end products of proteins formed by sugar addition are highly complex⁷⁸. A number of questions are yet to be answered relating to these AGE products. Among these are i) which functional groups on proteins are most likely to be involved, ii) how are specific proteins modified, iii) how much do proteins differ in the way they are modified, and iv) what conditions affect adduct formation? It is interesting that among all the reducing sugars, glucose is not very reactive in AGE formation⁷⁹. The primary reason for glucose involvement in the formation of so many AGE species is its abundance in biological systems.

One of the concerns with glycation is that it will modify the biological activity of a protein. With glutathione peroxidase for example it was shown that methylglyoxal irreversibly modified residues R184 and R185 and inactivated the enzyme. Loss of this oxidative repair enzyme can lead to rising levels of reactive oxygen species⁸⁰. In another study, RNase was incubated *in vitro* with glucose for 14 days at normal physiologic conditions under both aerobic and anerobic conditions. The enzyme was then trypsin digested and the cleavage fragments examined by LC-ESI-MS to map Amadori adduct formation. Under aerobic conditions the Amadori adduct and carboxymethyl group shared three sites, residues K41, K7 and K37. An Amadori adduct was formed at K1 as well. The experiment was set up to allow Amadori adduct formation under anerobic conditions after which the system was subjected to an aerobic environment. The results of the two experiments were the same. Additionally, incubation of the RNase *in vitro* with external glyoxal modified residue K41. Carboxymethylation and glycation appear to be site specific. Also, carboxymethylation is derived principally from the Amadori adduct rather than autooxidation of glucose (glyoxal)⁸¹. Incubating HSA with increasing concentrations of glucose, methylglyoxal and glyoxylic acid increased the molecular mass (according to MALDI-TOF MS) and AGE specific absorbance at 360 nm⁸².

Recently an MS based method has been described that identified glycation sites in human β 2- microglobulin automatically through a PERL script program. After incubating the protein with glucose it was tryptic digested and the cleavage products analyzed by MALDI-TOF MS. The PERL script program matched the masses of the observed peptides to the masses of the peptide putatively modified by AGEs. When a match occurs, the script sends matched masses and structures to a separate output file⁸³.

The literature indicates that glycation modifies the susceptibility of proteins to proteolysis. This might have biological significance in causing the half-life of a glycated protein to be longer⁸⁴. As seen with HSA, glycation reduces the number of tryptic peptides formed. The same is true with endoproteinase Lys-C digestion⁸⁵. But these enzymes do not normally digest glycated proteins. Proteinase K does a better job and more nearly mimics the AGE-protein degrading enzymes occurring *in vivo*. Whether glycation increases protein half-life or not, glycated proteins are clearly digested *in vivo* and excreted through the kidney⁸⁶.

The studies cited are exciting, but only the beginning. As advances continue in the development of mass spectral techniques, analysis of glycation will become easier with both electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI). One promising advance is the introduction of electron transfer dissociation (ETD) based analysis in addition to collision induced dissociation (CID) in ESI-MS instruments. With ETD a nearly full series of c and z type ions are produced with glycated peptides, allowing easier peptide sequencing. CID in contrast produces lower intensity b and y ions and the spectra are filled with ions corresponding to neutral loss of water and furylium ions⁸⁷. Actually, both forms of fragment ion generation have unique applications. Scanning for CID neutral loss of -162 amu is a powerful tool for recognizing glycated peptides. Using an electrospray inlet on a Q-TOF instrument operated at both low and high collision energies has permitted

the identification of 31 out of 59 lysine residues in HSA that were glycosylated⁸⁸. The mode of ionization is also important in glycosylated- and glycosylated-peptide analysis. In addition to the ESI instrumentation described above, MALDI coupled to tandem TOF/TOF mass spectrometers has proven to be a powerful tool in structure analysis⁸⁹. MALDI-MS of glycosylated peptides is more successful when 2,5 dihydroxybenzoic acid is used as the matrix to initiate laser induced ionization.

Methods for the isolation of AGE products are important as well. Affinity chromatography with meta-amino phenyl boronic acid (mAPBA) (Table 3-F) columns has proven to be a valuable tool in the isolation of diol species. mAPBA forms a reversible covalent ester with 1,2 and 1,3 diols in aqueous media that captures glycosylated peptides and proteins, among a variety of other diol containing species. Glycosylated peptides have been isolated in this manner and analyzed by tandem mass spectrometry using ETD and CID fragmentation. Five times as many peptides were identified by ETD as with CID⁹⁰. Using mAPBA to isolate glycosylated proteins and peptides from the human plasma and erythrocytes and ETD in sequencing it was shown that individuals with impaired glucose tolerance or type 2 diabetes were likely to have slightly more glycosylated peptides than normal subjects⁹¹. AGE studies have also been carried out using mAPBA on MALDI chips with minimal interference from nonspecific binding⁹².

QUANTIFICATION

Stable isotope coding in comparative proteomics

Relative quantification studies have been carried out by stable isotope coding where (¹³C₆)-DNPH was used to differentially code OS samples and the unlabeled form of DNPH to code control samples. After differential derivatization of samples with the DNPH isotopomers according to sample origin, samples were mixed and examined by shotgun proteomics using reversed phase chromatography to separate peptide fragments and electrospray ionization tandem mass spectrometry (ESI-MS/MS) for peptide identification.^{22,23}

As has been seen above, most of the quantification studies to date have involved relative comparisons of concentration between samples involving both staining and stable isotope coding methods. The advantage of stable isotope coding is the relative error in quantification is 6–8%, irrespective of the number of steps involved in the analytical process⁹³. Multiple isotopomers of dinitrophenyl hydrazine, GRP, and O-ECAT have been prepared and used in relative quantification studies of protein carbonylation. The great advantages of *in vitro* coding strategy is that it can be used with small quantities of sample, quantification can be achieved with any biological system after the *in vivo* component of an experiment is completed, and multiple samples can be examined simultaneously. An oxidized sample was split into equal parts and after differential derivatization according to sample origin with d₀-GRP and d₅-GRP, the samples were mixed in a 1:1 ratio and examined by RPC-MS/MS. Carbonylated peptides appeared as doublet clusters of ions separated by 5 Da, or a multiple thereof according to the number of carbonyls in the peptide. The possibility of false positive identification was minimized by doing both RPC-MS/MS and MALDI-MS/MS along with parameter filtering including tag number, retention time, resolution, and the correct concentration ratio⁹⁴. A limitation of this strategy is that derivatization may not be quantitative with low abundance proteins.

Another modification of the biotin hydrazide tag called hydrazide functionalized isotope-coded affinity tag (HICAT) was used to achieve relative quantification of the oxylipid-protein conjugates in the heart mitochondrial proteins⁹⁵. In this method an HNE-peptide adduct is synthesized and derivatized *in vitro* with a ¹³C-label (¹³C₄-HICAT). HNE-peptide adducts from the tryptic digest of a sample were then coded with an isotopically light

version of HICAT. The light and heavy isotopomers of HICAT vary by 4 amu due to the presence of four ^{13}C atoms in the heavy form. After mixing the differential labeled isoforms, HNE-peptide adducts were enriched and further fractionated by RPC before analysis by MALDI-MS/MS analysis. Because proteins can be oxidatively modified at multiple sites, it is important to stress that quantification of a single site oxidative modification can involve multiple isoforms of a protein. It is likely that more than a hundred oxidatively modified isoforms of some proteins may occur *in vivo*.

MRM methods and problems

Even though the relative quantification measurements described above will continue to be useful, there is a great need for absolute quantification to evaluate both the absolute load of oxidized proteins being generated in a cell and the fraction of any particular protein being oxidized in a particular pathway. For many years, absolute quantification has been achieved through the addition of heavy isotope labeled internal standards. With proteins the internal standard can either be a heavy isotope labeled isotopomer of a protein generated biosynthetically or a synthetic ^{13}C -labeled peptides that matches a proteolytic fragment derived from the protein. Use of ^{13}C -labeled peptides precludes the possibility that peptide isotopomers will be separated by chromatographic or electrophoretic methods before quantification in the mass spectrometer. The internal standard method is often referred to as “multiple reaction monitoring” (MRM) when multiple analytes are being quantified in a single analysis⁹⁶. New triple quadrupole mass spectrometers with special MRM friendly software are capable of quantifying more than 100 pairs of peptide isotopomers in a single analysis. The reader is directed to several excellent articles on MRM methods for specific details on how to carry out such an analysis^{97–100}. MRM methods can be used to study OS proteomics in several ways. Although not yet described, one will be to determine the concentration of several non-oxidized peptides from each protein being targeted for absolute quantification. After affinity selection the oxidized protein fraction should be tryptic digested, ^{13}C -labeled internal standard peptides added in known amounts, and the mixture analyzed by RPC-MS/MS to determine the isotope ratio of the targeted peptides. Isotope ratio measurements would then be used to compute the absolute concentration of specific proteins. A problem with this method in multiple sample analysis is that peptide retention times can change over time, drifting out of the time windows set for the analysis of specific peptides as they elute from the RPC column.

Internal standards can also be used to determine the concentration of protein isoforms that are oxidized at a particular site. In one study, a method was developed to determine the absolute quantity of HNE adducts on cysteine and histidine containing peptides. The method was validated using H-Tyr-His-OH as an internal standard for absolute quantification of HNE adducts on glutathione (GSH), carnosine (CAR) and anserine (ANS) using the MRM approach. The validated method was then implemented to quantify HNE-Michael adducts in rat skeletal muscle. CAR-HNE was shown to be elevated in the case of lipid peroxidation of excitable tissues.¹⁰¹

The main problem associated with application of the MRM to the analysis of oxidized proteins is the difficulty of synthesizing the very large number of oxidatively modified peptides needed to monitor so many oxidation sites.

BIOLOGICAL CONSEQUENCES OF PROTEIN OXIDATION

Despite the availability of methods such as those described above, the consequences of OS have not been widely studied. When identification of carbonylation sites is an objective, studies have frequently been done *in vitro*, often on model proteins with hydrogen peroxide, and metal catalysis, or through HNE addition. HNE addition *in vitro* to glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) was shown to occur in a sequential manner, first at His-164 and Cys-281, then on Cys-244, and finally at His-327 and Lys-331¹⁰². All these residues are located on the surface of the enzyme and easily accessible to HNE and ROS. The sequential nature of site modifications in GAPDH suggests a cascade of conformational changes may be necessary for later stage additions. The chaperon activity of Rat Hsp90 is lost after HNE modification of a single cysteine residue at Cys-572¹⁰³, again suggesting HNE addition can cause a conformational change. Carbonylation in adipose tissue of obese insulin resistant mice produced a 10 fold reduction in the affinity of fatty acid-binding protein for fatty acids¹⁰⁴. Fatty acid-binding protein was modified by HNE at Cys-117 *in vitro*. At low levels of metal catalyzed oxidation (MCO) only solvent accessible carbonylation sites were detected. increasing the level of MCO led to detection of previously buried carbonylation sites due to local structural changes¹⁰⁵. Similarly, oxidation of GAPDH caused conformational changes that attenuated its function¹⁰⁶. Beyond conformational changes there is the phenomenon of oxidation spreading to neighboring sites in MCO carbonylation. R, K, P, and T enriched regions in close proximity to an iron binding sites (e.g. E, H, Y, C, D) are more prone to carbonylation¹⁰⁵. A new algorithm has been developed that predicted these sites in *E. coli*¹⁰⁵. Few carbonylation sites were detected *in vivo*. For example, carbonylation of Hsp 70-1 in the cornu Ammonis of the macaque monkey occurred at a single site (Arg469) after transient whole-brain ischemia and reperfusion¹⁰⁷. In another example, ADP/ATP translocase 1 is found in cardiac mitochondrial to be carbonylated by HNE and acrolein at Cys-256¹⁰⁸.

Detection of smaller numbers of carbonylation sites *in vivo* could occur for several reasons. One could be that there are so many isoforms none occurs in a detectable amount. A further complication could be that isoforms are separated in preliminary fractionation and are difficult to locate. Insufficient recovery from gels for mass spectral identification could be another reason. Many of the studies on protein carbonylation have been done with 2-D gel electrophoresis. Difficulty in identifying carbonylated peptides that are biotinylated could be another problem. Fragmentation of biotin causes the introduction of noise peaks which lowers identification scores¹⁰⁹. Another problem is that fragmentation of some peptides during CID sequencing is hard to interpret. Use of electron capture dissociation (ECD) might reduce this problem. The CID and ETD modes of fragmentation are complimentary, facilitating the location of modification sites when combined with the hydrazide purification techniques described above¹¹⁰.

How protein oxidation impacts biological systems is a major issue. Much of the discussion encompassing this subject has focused on bulk phenomena such as the propensity of oxidized proteins to cross-link and precipitate, difficulties in their degradation, and their cellular toxicity. Alterations in the activity of specific enzymes following oxidation are important as well. It is clear from the discussion above that i) some proteins are more likely to oxidized than others and ii) oxidative modifications can alter biological activity. GAPDH is an example in which the activity of an oxidative stress associated enzyme is attenuated by oxidation¹⁰⁶. Creatine kinase and carbonic anhydrase are others¹¹¹. Carbonylation of these enzymes under oxidative stress in the vastus lateralis muscle of patients with COPD leads to a reduction in their activity¹¹¹. Sepsis induced by injecting *E. coli* lipopolysaccharides into the diaphragm of rats produced another example. Enolase 3b, triphosphosphate isomerase 1, aldolase, creatine kinase, aconitase 2, dihydrolipoamide dehydrogenase, carbonic anhydrase III and electron transfer flavoprotein all underwent elevation of HNE addition during treatment. *In vitro* incubation of the enolase with HNE following the *in vivo* experiment showed a significant reduction of its activity¹¹². Oxidized proteins can also be immunogenic as has been seen in systemic lupus erythematosus (SLE)¹¹³.

It is possible that oxidized proteins will at some time in the future be used as disease markers. For this to happen it will be necessary to catalog oxidized proteins from microorganisms, pathogens, disease free human subjects, and animal models. That will be a major undertaking that is just beginning. Sixty two carbonylated proteins have been identified from *E. coli* under nitrogen starvation, carbon starvation and phosphate starvation¹¹⁴. Interestingly, carbonylated proteins in *E. coli* were found mainly to be aggregated and resistant to degradation¹¹⁵. The impact of aging, diseases, and drugs on the oxidative stress profile of organs is being initiated as well. More than 100 carbonylated proteins were identified in the mouse brain including both high abundance (e.g. cytosolic proteins) and low abundance proteins (e.g. receptor proteins)¹¹⁶. Carbonylation of three proteins increased significantly in aged SAMP8 mouse brain while the level of 3 oxidized proteins is reduced by treatment with lipoic acid¹¹⁷. Enolase and triosephosphate isomerase were highly modified with HNE in human retinal pigment epithelial cells in culture (ARPE19) and human donor eyes¹¹⁸. Comparative proteomics studies on carbonylated proteins in the muscle of diabetic and control rats is beginning^{119,120}. It has been shown that creatine kinase BB, glutamine synthase, and ubiquitin carboxy-terminal hydrolase L-1 are differentially oxidized in the Alzheimer's disease (AD) brain¹²¹. Perhaps even more proteins experience a similar fate. Incubation of synaptosomes with amyloid beta peptide 1–42 resulted in significant oxidation of actin, glial fibrillary acidic protein, and dihydropyrimidinase-related protein-2¹²². Additionally, eleven HNE-modified proteins were elevated in mild cognitive impairment (MCI) subjects. This leads to neuronal death and dysregulation of protein activity that may lead to the progression of MCI patients on to AD¹²³. Finally, several HNE modified proteins were identified in the hippocampus and inferior parietal lobule brain regions of subjects with AD^{124–135}. Friedreich's ataxia (FA) is another example. FA is an oxidative stress diseases in which failure to sequester mitochondrial iron leads to protein oxidation. Model system studies in yeast showed increased mitochondrial protein oxidation under chronic OS.¹³⁶

The incidence of many disease increases with age. A question is the mechanism, if any by which aging is connected to elevated oxidative stress diseases^{14,137–143}. Data is accumulating that oxidative stress in general increases in multiple organs with age. For example, twelve carbonylated proteins increased significantly in aged mouse liver tissue¹⁴⁴. Carbonyl of Cu,Zn-superoxide dismutase (Cu,Zn-SOD) in liver (at 3 months) and the hippocampal cholinergic neurostimulating peptide precursor protein (HCNP-pp) in brain (at 9 months) was significantly higher in senescence-prone mouse strain 8 (SAMP8). These changes correlated with age related deterioration in learning and memory¹⁴⁵. An age associated increase in the carbonylation of aconitase and ATP synthase is found in rats as well^{146,147}. In addition, adenine nucleotide translocator, voltage-dependent anion channel, glutamate oxaloacetate transaminase, and aconitase are more susceptible to oxidative stress induced carbonylation in the mitochondria of aged rat brain¹⁴⁸. Moreover, twenty two proteins increased significantly with age in the in rat skeletal muscle mitochondria¹⁴⁹. In addition, nitrated and carbonylated creatine kinase was found predominantly in the muscle of aged rodents to exist as high molecular weight oligomers and insoluble aggregates that caused the loss of CK function.¹⁵⁰ Carbonyl levels of ten proteins increased significantly in the temporal cortex of aged rats has been observed as well.¹⁵¹

It is well known that small molecules such as rotenone, paraquat, and diquat which uncouple electron transport induce ROS production and concomitantly carbonylation of proteins. But other pesticides can increase protein oxidation as well. Endosulfan increased lipid peroxidation levels and carbonylation of 17 proteins after only 4 days of exposure in black tiger shrimp¹⁵². Additionally, the fungicide propiconazole increased protein oxidation in mice¹⁵³. Even ethanol exposure can increase protein oxidation as seen with betaine-

homocysteine S-methyltransferase carbonylation in rat livers¹⁵⁴. The degree to which electron transport is uncoupled either by environmental or genetically related factors is a major issue in oxidative stress diseases and aging.

Carbonylated proteins have been reported in blood as well.¹⁵⁵ Sixty five high, medium, and low abundance proteins appeared in most of the four human plasma samples.¹⁵⁶ Seven of these proteins carried fifteen carbonylation sites *in vivo*.

CONCLUDING PERSPECTIVES

Although proteins can be oxidized in 35 or more ways, many of these modifications involve some form of carbonylation. This makes carbonylation the most general type of protein oxidation, even though the structures associated with carbonyl groups may differ. Moreover, proteins frequently undergo several types of oxidation simultaneously. This means there is a high probability that i) a single protein molecule can be oxidized at several sites, ii) different forms of oxidation may be found at these sites, iii) multiple isoforms will have been generated during oxidation that differ in their oxidation pattern, and iv) many, but not all of the isoforms will carry a carbonyl group.

During the past decade techniques have evolved that allow the isolation, identification, and chemical characterization of protein carbonylation along with the accompanying types of oxidative modifications noted above. Oxidation site mapping along with identification of the specific oxidative modifications at a site enables the probable mechanism of oxidative modification of specific amino acids in a protein to be narrowed to i) ROS initiated oxidative cleavage of an amino acid side chain, ii) cleave of the primary structure, iii) glycation, or iv) addition of lipid peroxidation products. Site mapping and structure elucidation are greatly enhanced by the use of high mass accuracy mass spectrometers that allow differentiation between post-translationally modified peptides and normal peptides arising from proteolysis of parent proteins. Great care must be taken in the detection of carbonylation sites. For example, oxidation of proline to glutamic semialdehyde has the same mass shift as the oxidation of M, L, W and C (a mass shift of 15.994915). Thus, it is important to inspect MS/MS spectra manually to identify an oxidation site and confirm the amino acid through DNA database derived sequence. Moreover, combined use of CID and ECD for characterization of these modifications will greatly facilitate identification of larger numbers of carbonylation sites. Synthesis of ¹³C-labeled versions of modified peptides to confirm new structures and aid in MRM quantification is likely to become a standard practice as well.

A critical tool in the identification of oxidative stress initiated post-translational modifications is software that rapidly differentiates between an oxidative stress induced post-translational modification and normal, unmodified peptides. Although the Mascot software has been modified to identify PTMs, restrictions on the number of modifications that can be searched in one analysis make it less than ideal^{157–159}. Better software for PTM analysis is needed.

We predict that over the next decade the oxidative stress induced proteome of multiple cell types, organs, pathogens, and diseases will be mapped and correlated with a broad spectrum of biological phenomena. The fact that so many oxidative modifications of proteins are irreversible and proteins thus modified are not rapidly degraded in many cases may provide a short term history of recent oxidative stress events in cells.

Synopsis

Carbonylation is an irreversible post translational modification (PTM) that often leads to the loss of protein function and can be a component of multiple diseases. The focus of this review is on the use of proteomics tools and methods to identify oxidized proteins along with specific sites of oxidative damage and the consequences of protein oxidation.

Abbreviations

PTM	Post translational modifications
DNPH	dinitrophenyl hydrazine
AGE	Advanced glycation end products
ETD	electron transfer dissociation
ECD	electron capture dissociation
DNPH	Dinitrophenylhydrazine
CID	Collision Induced Dissociation

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Figure 1.

A diagram comparing the three approaches used for the identification of carbonylated proteins and their carbonylation sites. The diagram is a modified version of the diagram in reference ⁴⁵

2-Advanced lipid peroxidation end products:

Figure 2.

A general scheme for the different routes for protein carbonylation

Table 1

A list of the different types of oxidative modifications

Amino acid	oxidative modification	Amino acid	oxidative modification
T	2-amino-3-oxo-butanoic acid	M	sulfone
Y	hydroxylation	L	hydroxy Leucine
R	glutamic semialdehyde	K	aminoadipic-semialdehyde
C	cysteic acid (sulfonic acid)	K	Amadori adduct
C	sulfinic acid	K	3-deoxyglucosone adduct
C	sulfenic acid	K	glyoxal adduct
W	formylkynurenin	K	methylglyoxal adduct
W	kynurenin	N	hydroxylation
W	hydroxykynurenin	P	hydroxylation
W	2,4,5,6,7 hydroxylation of tryptophan	P	glutamic semialdehyde
W	oxolactone	P	pyroglutamic
H	4-hydroxy glutamate	P	pyrrolidinone
H	asparagines	F	hydroxylation
H	aspartate	F	dihydroxy phenylalanine
H	2-oxo-histidine	K	hydroxylation
D	hydroxylation	C/H/K	hydroxynonenal (HNE) Michael adduct
M	oxidation (sulfoxide)	K	malondialdehyde

Table 2

Amino acids and the corresponding carbonylation products

Amino acid	The carbonylation product
<p>Prolyl</p>	<p>Glutamic semialdehyde</p> <p>2-Pyrrolidinone</p>
<p>Arginyl</p>	<p>Glutamic semialdehyde</p>
<p>Lysyl</p>	<p>Amino adipic semialdehyde</p>

<p>Threonyl</p>	<p>2- Amino-3-ketobutyric acid</p>
<p>Glutamyl</p>	<p>Pyruvyl</p>
<p>Aspartyl</p>	<p>Pyruvyl</p>
<p>Peptide or protein</p>	<p>α-amidation pathway</p>

<p>P-Lys-NH₂</p>	<p>Amadori product (followed by the formation of AGE-adducts)</p> <p>Advanced lipidation end product (Michael adduct)</p>
<p>P-His-Im-NH</p>	<p>Advanced lipidation end product (Michael adduct)</p>
<p>P-Cys-SH</p>	<p>Advanced lipidation end product (Michael adduct)</p>

Table 3

A list of the reagents used and their enrichment chemistry

	The chemical reagent	The enrichment chemistry
A	<p>Dinitrophenylhydrazine (DNPH)</p>	The hydrazide group reacts with the proteins carbonyl groups forming hydrazones which can be then isolated with DNPH specific antibodies.
B	<p>Biotin hydrazide</p>	The hydrazide group reacts with the proteins carbonyl groups forming hydrazones which can be then isolated with avidin.
C	<p>Girard's P reagent</p>	The hydrazide group reacts with the proteins carbonyl groups forming hydrazones. The quaternary amine can be then selected by strong cation exchange (SCX) at pH 6.0.
D	<p>Oxidation-specific Element-Coded Affinity Tag (OECAT): AOD, (((S)-2-(4-(2-aminoxy)-acetamido)-benzyl)-1,4,7,10-tetraazacyclododecane-N,N',N'',N'''-tetraacetic acid.</p>	The hydroxylamine group reacts with the proteins carbonyl groups forming oximes

	The chemical reagent	The enrichment chemistry
E	(N'-aminooxymethylcarbonylhydrazino D-biotin)	The hydroxylamine group reacts with the proteins carbonyl groups forming oximes which can be then isolated with avidin.
F	Boronic acid	Forms a reversible covalent ester with 1,2 and 1,3 diols in aqueous media that captures glycosylated peptides and proteins