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A Robust Analytical Approach for the Identification of Specific Protein Carbonylation Sites: Metal-Catalyzed Oxidations of Human Serum Albumin

Zafer Ugur and Scott Gronert*

Department of Chemistry, Virginia Commonwealth University, Richmond, VA 23284 USA

Abstract

The formation of protein carbonyls in the metal-catalyzed oxidation of human serum albumin (HSA) is characterized using a new analytical approach that involves tagging the modification site with multiple hydrazide reagents. Protein carbonyl formation at lysine and arginine residues was catalyzed with copper and iron ions, and the resulting oxidation patterns in HSA are contrasted. A total of 18 modification sites were identified with iron ion catalysis and 14 with copper ion catalysis. However, with the more stringent requirement of identification with at least two tagging reagents, the number of validated modification sites drops to 10 for iron and 9 for copper. Of the 14 total validated sites, there were only five in common for the two metal ions. The results illustrate the value of using multiple tagging agents and highlight the selective and specific nature of metal-catalyzed protein oxidations.

INTRODUCTION

Oxidative damage by reactive oxygen species (ROS) is associated with aging and age-related disease (Dalle-Donne et al. 2006; Stadtman 2004; Stadtman and Levine 2000). The production of ROS is an inevitable result of aerobic metabolism (Sastre et al. 2003) and ROS can oxidatively modify a wide array of biomolecules such as proteins, nucleic acids, sugars, and lipids (Stadtman and Levine 2000). Proteins are a major target for oxidation, and because much of the cellular work is done by proteins, oxidative modifications can result in serious damage to a living organism. Numerous reports support the notion that metal catalyzed oxidation (MCO) is the most important mechanism of protein oxidative damage and results in polypeptide backbone cleavage, cross linking, and modification of amino acid side chains, all of which can result in structural alteration and loss of protein functions (Maisonneuve et al. 2009; Shacter 2000). Proteins can undergo various types of oxidative modifications, but protein carbonylation is often used to monitor oxidative damage due to its irreversible and irreparable nature, as well as the fact that an aldehyde or ketone functional group allows for selective labeling techniques. Protein carbonyls can be formed by direct MCO attack on the side chains of amino acids such as arginine (Arg), lysine (Lys), threonine (Thr), or proline (Pro). Protein carbonyls are also formed by adduction with reactive α , β -unsaturated aldehydes. These aldehydes are derived from oxidation of lipids or

*Corresponding Author. sgronert@vcu.edu.

carbohydrates and can add to nucleophilic residues such as Lys, Cys, His, and Arg (Requena et al. 2001, 2003).

It has been reported that approximately 10% of the proteome is vulnerable to protein carbonylation (Maisonneuve et al. 2009). A number of studies have reported that protein oxidation is selective and only certain proteins are oxidized in the systems under oxidative stress (Aksenov et al. 2001; Cabiscol et al. 2000, 2014; Colzani et al. 2013; Das et al. 2001; Halliwell and Whiteman 2004; Jana et al. 2002; Sohal et al. 1993; Yan et al. 1997, 2000). The specificity is likely driven in part by the proximity of the oxidant to the protein because the most reactive oxidants have short half-lives in biological systems. However, the molecular basis for the observed specificity is not clear. Interestingly, carbonylation sites show a very strong tendency to cluster in the protein's primary sequence suggesting some kind of distinguishable mechanism (Moller et al. 2011).

Different physiological and non-physiological processes can generate ROS that result in protein oxidation. Various methods can be employed to generate ROS for experimental purposes such as X-ray or UV irradiation, or chemical oxidants. The most physiologically relevant mechanism of protein carbonylation in living cells appears to be MCO (Requena et al. 2001; Moller et al. 2011; Yuan et al. 2007). MCO generally happens when reduced metal ions such as Fe^{+2} and Cu^{+1} engage in Fenton reactions and generate extremely reactive hydroxyl radicals.

Despite extensive research on protein carbonylation over several decades (Aldini et al. 2006; Colzani et al. 2013; Curtis et al. 2012; Feng et al. 2008; Kim et al. 2010; Madian and Regnier 2010; Madian et al. 2011; Mirzaei and Regnier 2006a, 2006b), a full understanding of the carbonylation process is still lacking. One area of interest is how the nature of the oxidant correlates with the type of damage. In the present study, we investigate how the identity of the metal affects the selectivity of the MCO process. For this work, we have chosen human serum albumin (HSA) as a model protein and subjected it to MCO with copper and iron salts. We have previously examined HSA with iron (Temple et al. 2006), but here we apply a more sensitive and reliable approach. Vachet and others have shown that copper coordination can direct the oxidation of histidine residues in proteins (Bridgewater and Vachet 2005; Bridgewater et al. 2006a, 2006b; Lim and Vachet 2003, 2004; Vachet et al. 2001). However, correlations between protein carbonylation and copper coordination have not been fully established although it has recently been shown that the oxidation state of the free cysteine in HSA affects the carbonylation process. (Penezic et al. 2015). HSA is known to bind transition metals and binding sites have been identified near the N-terminus (involving His-27). His-91, His-129, His-130, and His-271 have also been implicated as potential metal binding sites (Azzellini et al. 2010; Bal et al., 1998; Giroux and Schoun, 1981; Glennon and Sarkar, 1982; Laussac and Sarkar, 1984; Rozga et al. 2007). Here we examine whether protein carbonylation selectivity can be correlated with the known metal binding properties of this model protein.

METHODS AND MATERIALS

Materials

Human serum albumin (HSA), 2,4-dinitrophenylhydrazine (2, 4-DNPH), and sodium cyanoborohydride (5 M solution in aqueous 1 M sodium hydroxide) were purchased from Sigma (St. Louis, MO). Sequencing grade, modified trypsin was obtained from Promega (Madison, WI). The hydrazide reagents were obtained from ThermoFisher Scientific (Waltham, MA). All other chemicals and solvents were obtained from commercial sources and used without further purification.

MCO of HSA

HSA was dissolved at a final concentration of 25 mM in a solution of 50 mM HEPES, (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) at pH 7.4. The solution also contained 100 mM KCl and 10 mM MgCl₂. For oxidations, ascorbic acid and the metal ions (Fe and Cu) were added to give final concentrations of 15 mM and 1 mM, respectively. The mixture was incubated for 24 h at 37 °. The oxidation was terminated by the addition of EDTA (ethylenediaminetetraacetic acid) to a final concentration of 1 mM.

Labeling the Modified Protein

Labeling of the carbonyl groups of oxidized HSA was performed by reaction with biotin hydrazide using an approach based on previous work by Yoo and Regnier (2004). In summary, the oxidized protein samples were mixed with standard, extended-chain, and PEG4-linked biotin hydrazide to a final concentration of 5 mM using a 50 mM stock solution. The samples were incubated at room temperature for 2 h with shaking and then cooled to 0 °C. To stabilize the imine bond the hydrazide, an equal volume of 30 mM sodium cyanoborohydride in PBS buffer was added. The modified HSA was precipitated with 20% trichloroacetic acid for 1 hour at 0° C. The resulting pellets were then washed with ethanol/ethyl acetate (1:1) three times and finally dissolved in ammonium bicarbonate buffer for the enzymatic digestion.

Enzymatic Digestion

Protein samples at 1 mg/ml in 50 mM ammonium bicarbonate buffer were reduced with 30 mM dithiothreitol at 50 °C for 20 min and then alkylated with 55 mM iodoacetamide in the dark at room temperature for 30 min. The protein was separated from excess dithiothreitol and iodoacetamide using a Microcon YM-30 filter at 8000 g for 15 min. The proteins were redissolved in 50 mM ammonium bicarbonate, and digested with trypsin (using a substrate/enzyme ratio of 40/1, wt/wt, (37 °C for 24 h). To terminate the digest, 0.1% formic acid (120 µL) was added.

LC/ESI-MS/MS

The digested HSA samples were analyzed using a capillary HPLC system (Thermo Finnigan, San Jose, CA) connected to an LTQ ion trap mass spectrometer (Thermo Finnigan, San Jose, CA). Peptides were separated with a C18 reverse-phase column (C18 Thermo, 5µm particle size, 300Å, 100×0.18 mm) using a split flow system. The split is approximately

100:1 with the master flow rate set at 50 $\mu\text{l}/\text{min}$ (corresponding to approximately 0.5 $\mu\text{l}/\text{min}$ on the C18 column). The samples were eluted using two mobile phases, A (0.1% formic acid in water) and B (0.1% formic acid in methanol). The initial gradient was 2% B. It was increased to 15% B over 15 min, then to 80% B over 70 min, and to 95% B over 25 min. The peptides (singly, doubly, or triply-charged ions) were analyzed with a data-dependent scan procedure. A dynamic exclusion feature allowed a particular m/z value to be processed no more than twice in a 2 min window. The MS/MS spectra were searched against the NCBI non-redundant human protein database (GI: 4502027) using the Finnigan Bioworks v.3.2 (Sequest) software. Only peptides within the mass range 500–4000 Da were included in the search. Two missed cleavage sites were allowed in the search. Cysteines were defined as fixed modifications (carbamidomethylation, +57 Da). To search for oxidized peptides, the dynamic modifications of methionine, histidine, lysine, and arginine were set to +16, +16, +241, and +199 Da, respectively for standard biotin hydrazide, +16, +16, +354, and +312 Da, for extended-chain hydrazide, +16, +16, +488, and +445 Da, for PEG4-linked biotin hydrazide. The Yates Rule was applied and the Xcorr threshold was set to 1.9, 2.2 and 3.75 for +1, +2 and +3 charged peptides, respectively (Washburn et al. 2001). C was set to 0.1 and the number of top matches was set to 1.

RESULTS

HSA (SwissProt P02768) is a 66 kDa protein with 609 amino acids including 59 lysine and 24 arginine residues in its secreted form. The first 24 amino acids are signal and propeptide portions that are not observed in the transcribed, translated, and transported protein. HSA was oxidized under MCO conditions and we focused specifically on protein carbonylation at lysine and arginine (Scheme 1). The resulting aldehydes were labeled with the three biotin hydrazides. Labeling protein carbonyls with a hydrazide (including reduction) converts it to a stable functional group and results in predictable increases in the masses of the modified amino acid residues. Here, three labels were used in the proteomic analysis in order to offer more confident assignments. The rationale for the use of three labels is 2-fold. First, coincident masses are possible and can lead to false positives. For example, the combination of a lysine and leucine (or isoleucine) provides the same nominal mass shift as carbonylation and labeling of a lysine with biotin hydrazide. Second, we have shown that some combinations of modified peptides and labeling agents can lead to poor detectability and as a result, some modifications could be missed if a single label is used. For the purposes of this study, we required evidence with two hydrazide labels to confirm a modification site. The labeling process is illustrated in Scheme 2. The mass shifts caused by the labels are listed in the methods section. After labeling, the HSA samples were subjected to a standard trypsin digest and after work-up, the resulting peptides were analyzed by LC-MS and identified with the SEQUEST algorithm in the Bioworks software package.

Analysis of HSA Subjected to MCO(Fe)

When HSA was oxidized under MCO(Fe) conditions and labeled with the three biotin hydrazides, the overall sequence coverage from the LC-MS analysis was approximately 88%. As noted above, an advantage of using three labels is that detection sites are confirmed in multiple experiments involving different mass shifts, which reduces the possibility of false

positives due to coincident masses as well as reducing the possibility of missing a modification due to poor detectability with a specific labeling agent. Table 1 lists a number of MS/MS spectra supporting the oxidation sites that were identified when HSA is subjected to MCO(Fe) conditions.

MCO oxidation of HSA with iron ions results in the identification of ten modification sites validated with at least two labels: Arg-34, Lys-97, Arg-105, Arg-210, Lys-223, Lys-236, Lys-249, Lys-469, Arg-490 and Lys-549. Eight additional sites were supported with data from a single label. In general, these sites had only a few supporting spectra with the one label. No false positives were discovered in the control sample. In previous work (Temple et al. 2006), we had only identified Lys-97 from this set (it provided the greatest number of spectra with biotin hydrazide in this work) and had tentative data for Lys-186, which was not identified here. The superiority of the current methodology is clear from this result. Figure 1 shows spectra for peptides modified at Lys-97 (AA 89–105) and labeled with the three different biotin hydrazide labels. The modified lysine in these peptides was a missed cleavage site in the tryptic digests. The mass shifts corresponding to the labels are observed in the appropriate b and y fragment ions for all of the cases. For the sake of simplicity, the losses of water or ammonia from b and y ions were not annotated in the spectra. The effect of the varying labels on the MS/MS spectra is best illustrated with the doubly-charged b_{13} fragment. It is well represented in each spectrum and appears at m/z 857.8, 914.0, and 981.2 for each of the hydrazide labels. However, the tagging agents have an impact on the fragmentation patterns of the peptides and although they have many fragment ions in common, there are distinct differences in relative intensities.

Analysis of HSA Subjected to MCO(Cu)

HSA was treated under MCO(Cu) conditions and labeled with the three biotin hydrazides. The overall sequence coverage was approximately 86%. Table 2 lists the number of MS/MS spectra supporting the oxidation sites.

MCO oxidation of HSA with copper ions resulted in the identification of nine modification sites that were supported with data from two labels: Lys-97, Lys-161, Arg-169, Lys-198, Lys-223, Lys-236, Lys-375, Lys-490, and Lys-549. Five other sites were supported by data from a single label. From this group, K-257 stands out because it provided a significant number of modified spectra (seven) with the extended chain biotin label. This modification was also only identified with the extended chain biotin under the MCO(Fe) conditions.

DISCUSSION

For each metal, there is roughly an even mix between unique and shared sites that were confirmed with data from two labels. The sites are spread throughout the protein sequence for both of the oxidation conditions. There is no indication of “hot” regions in the protein. In oxidation processes, surface accessibility might also play a role. This can be examined with a simple solvent accessible surface area (SASA) calculation for each of the residues. Even though it may not be a perfect measure of the environment, it gives some insight. Side chain and backbone SASAs for the lysines and arginines in HSA are plotted in Figures 2 and 3 based on estimates from the GETAREA program. As we have shown in the past with

hydroxynonenal modifications (Liu et al. 2012), surface accessibility is not a strong driver in the modification process. In this case the modifications tend to be in sites with moderate surface accessibility.

More insight can be gained by looking at the number of spectral counts for each modification. Under the chosen conditions, the overall number of spectra associated with the modification sites (sum of all labels) was similar for oxidations with iron (113 spectra) and copper (118 spectra). However there are a number of notable differences in the patterns of the oxidation sites. This is illustrated in Figure 4. First, the distribution between oxidation at lysine and arginine depends on the identity of the metal. Although lysine oxidation is preferred over arginine oxidation with copper and iron, it is much more highly skewed with copper (19:1 vs. 2.5:1 for Cu and Fe, respectively). The ratio with iron is close to the lysine/arginine ratio in the protein so it appears that the MCO(Fe) process is relatively indiscriminate with respect to lysine versus arginine. In contrast, the copper system is strongly biased towards lysine oxidation and this process accounts for about 95% of the spectra with modifications. There is only a single arginine site definitively identified for MCO(Cu) and interestingly, this is a unique site that was not identified with MCO(Fe). Second, the patterns for the two metals are very different, suggesting that specific, local interactions are driving the selectivity of the process. In this respect, Lys-97 and Lys-549 offer the greatest contrast in terms to their reactivity with iron vs. copper. Lys-97 provided 30 spectra with modifications under MCO(Fe) conditions, yet only 3 under MCO(Cu) conditions, whereas the situation is reversed with Lys-549 (9 under MCO(Fe) conditions and 38 under MCO(Cu) conditions).

The Vachet group has extensively studied MCO conditions with copper and investigated a number of proteins (Bridgewater and Vachet 2005; Bridgewater et al. 2006a, 2006b; Lim and Vachet 2003, 2004; Vachet et al. 2001). This group and a number of others have reported that histidine residues bind copper and direct oxidation processes, particularly oxidation of histidine to 2-oxohistidine. As a result, we were expecting that MCO(Cu) conditions would result in modification sites that are close to histidine residues. Temple et al. have suggested that MCO(Fe) conditions could result in modification sites that are near acidic residues, which can serve as a metal binding site for iron (Temple et al. 2006). The crystal structure of HSA shows that the modification sites we have identified with MCO(Cu) generally have at least one histidine residue within 10 Å (Lys-375 is the sole exception). All of the modification sites that we have identified for MCO(Fe) are in regions with multiple acidic residues where the iron could potentially bind. Human serum albumin is known to bind transition metals at the N-terminus. In addition, His-27, His-91, His-129, His-170, and His-271 also have been reported be metal binding sites for human serum albumin (Bal et al., 1998; Glennon and Sarkar, 1982; Laussac and Sarkar, 1984; Giroux and Schoun, 1981; Rozga et al. 2007; Azzellini et al. 2010). In the current study, the modifications at Lys-97, Lys-161, Lys-169, Arg-169, and Lys-490 are very close to these reported metal-binding sites. However, there do not appear to be discernable, direct correlations between the potential metal-binding and the modification sites that could be used to predict reactivity. The ability of the MCO-generated hydroxyl radicals to diffuse short distances probably allows them to explore residues relatively far from the metal coordination site in protein sequence space; therefore complicating analysis of the selectivity of the processes.

CONCLUSIONS

In this study, specific carbonyl modification sites in HSA subjected to and MCO were identified. The MCO(Cu) condition resulted in 9 modifications whereas the MCO(Fe) condition gave rise to 10 modification sites. These sites are generally close to residues that could bind a metal ion. There are 5 sites that have been identified with both MCO conditions, suggesting that there are some similarities between the two oxidation mechanisms; however, each of the MCO conditions had roughly an equal number of unique sites. Comparisons to previous data involving acrolein modifications of lysines also highlight differences in selectivity - three of the five major lysine modification sites found with acrolein treatment (Ugur et al. 2012) were not modified by either of the MCO conditions. Overall, the results support the idea that these post-translational oxidation processes are intrinsically selective and not controlled by surface accessibility or other general properties.

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#18332-18332 NL: 2.28E4

#18423-18423 NL: 1.69E5

(b)

#18446-18446 NL: 2.45E4

(c)

Figure 1. MS/MS spectrum of the (a) biotin hydrazide, m/z 725.31, (b) extended-chain biotin, m/z 763.24, and (c) PEG4-linked biotin, m/z 807.94 labeled peptides containing AA# 89–105. Fragmentation patterns indicate modification at Lys-97.

Figure 2.

Solvent accessible surface area (SASA) in $\text{\AA}^2$ for the lysines of HSA. Light gray bars are the backbones, and dark gray bars are the side chains. Black arrows indicate the sites that were modified with metal-catalyzed oxidation with iron (MCO(Fe)). Grey arrows indicate the sites that were modified with MCO(Cu). White arrows indicate the sites that modified with both conditions. In the graph, the data are too extensive to include residue numbers, but modified sites are labeled.

Figure 3.

Solvent accessible surface area (SASA) in $\text{\AA}^2$ for the arginines of HSA. Light gray bars are the backbones, and dark gray bars are the side chains. Black arrows indicate sites that were modified with metal-catalyzed oxidation with iron (MCO(Fe)). Grey arrows indicate sites that were modified with MCO(Cu). X-axis shows the residue numbers.

Figure 4.

Number of spectrum matches for modified peptides from human serum albumin (HAS) subjected to metal-catalyzed oxidation (MCO) and labeled with hydrazide reagents. Data are a composite from all labels. K/R represents the ratio of the total number of lysine/arginine MCO-modified peptides that were identified for each metal.

Scheme 1.

Oxidation of lysine and arginine residues to protein carbonyls.

Scheme 2.
Labeling with hydrazide reagents.

Table 1

Modified sites identified in MCO(Fe) oxidation of HSA. Biotin hyrazide, extended-chain hydrazide, and PEG4-linked hydrazide were used and the total number of spectra supporting the identification of each modification is listed (data from three replicates). The control is HSA that was not oxidized nor labeled with the biotin reagents.

MODIFICATION SITES	STANDARD BIOTIN	EXTENDED-CHAIN BIOTIN	PEG4-LINKED BIOTIN	CONTROL
R-34	2	4	0	0
K-97	15	9	6	0
R-105	1	5	1	0
R-141	0	3	0	0
K-161	3	0	0	0
R-210	0	3	3	0
K-223	0	8	1	0
R-233	1	0	0	0
K-236	1	4	0	0
K-249	2	6	0	0
K-257	0	3	0	0
K-375	0	1	0	0
K-402	0	2	0	0
R-469	5	7	1	0
K-490	5	6	5	0
K-499	0	0	4	0
R-496	0	4	0	0
K-549	6	3	0	0

Table 2

Modified sites identified in MCO(Cu) oxidation of HSA. Biotin hyrazide, extended-chain hydrazide, and PEG4-linked hydrazide were used and the total number of spectra supporting the identification of each modification is listed (data from three replicates). The control is HSA that was not oxidized nor labeled with the biotin reagents.

MODIFIED SITES	STANDARD BIOTIN	EXTENDED-CHAIN BIOTIN	PEG4 LINKED BIOTIN	CONTROL
K-46	1	0	0	0
K-97	0	1	2	0
K-161	4	2	0	0
R-169	0	1	2	0
K-198	3	5	2	0
R-210	0	2	0	0
K-223	2	6	5	0
K-236	5	4	1	0
R-246	0	1	0	0
K-257	0	7	0	0
K-375	5	1	0	0
R-469	1	0	0	0
K-490	9	7	10	0
K-549	18	15	5	0