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RAPID COMMUNICATION

Artifacts to avoid while taking advantage of top-down mass spectrometry based detection of protein S-thiolation

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Bottom-up MS studies typically employ a reduction and alkylation step that eliminates a class of PTM, S-thiolation. Given that molecular oxygen can mediate S-thiolation from reduced thiols, which are abundant in the reducing intracellular milieu, we investigated the possibility that some S-thiolation modifications are artifacts of protein preparation. Cu/Zn-superoxide dismutase (SOD1) was chosen for this case study as it has a reactive surface cysteine residue, which is readily cysteinylated in vitro. The ability of oxygen to generate S-thiolation artifacts was tested by comparing purification of SOD1 from postmortem human cerebral cortex under aerobic and anaerobic conditions. S-thiolation was ~50% higher in aerobically processed preparations, consistent with oxygen-dependent artifactual S-thiolation. The ability of endogenous small molecule disulfides (e.g. cystine) to participate in artifactual S-thiolation was tested by blocking reactive protein cysteine residues during anaerobic homogenization. A 50-fold reduction in S-thiolation occurred indicating that the majority of S-thiolation observed aerobically was artifact. Tissue-specific artifacts were explored by comparing brain- and blood-derived protein, with remarkably more artifacts observed in brain-derived SOD1. Given the potential for such artifacts, rules of thumb for sample preparation are provided. This study demonstrates that without taking extraordinary precaution, artifactual S-thiolation of highly reactive, surface-exposed, cysteine residues can result.

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Abbreviations: PMI, postmortem interval; **RBC**, red blood cell; **SOD1**, Cu/Zn-superoxide dismutase

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PTMs such as phosphorylation, acetylation, methylation, S-thiolation, and oxidation are key modulators of biological pathways through their impact on protein structure, dynamics, turnover, and interactions. As a result of PTMs, the number of possible proteoforms is expontentially increased over the number of genes and their analysis has become one of the most active areas of research in MS. Bottom-up proteomics has become a staple method for identifying PTM, but struggles with combinatorial modifications [1–3], and the methodology commonly employs reductive sample preparation that eliminates reductively labile PTMs. Top-down MS, on the other hand, does not typically employ a reduction and alkylation step, and therefore detects reductively labile PTMs [4, 5].

One such PTM, protein S-thiolation, occurs when protein cysteine residues form a disulfide bond with low molecular mass disulfides (e.g. cystine) or thiols (e.g. glutathione and cysteine; reviewed in [6]; Supporting Information Figs. 1 and 2). S-thiolation can be mediated enzymatically, e.g. glutathionylation mediated by glutathione S-transferase [7, 8] or can occur nonenzymatically [9], and is affected by redox potential and pH. Organisms take advantage of S-thiolation in sensing and responding to change in redox potential, oxidative stress, and acidity. Nonenzymatic S-thiolation typically occurs through two mechanisms: (i) oxygen-independent thiol-disulfide exchange between a thiolate and disulfide, and (ii) molecular oxygen-dependent disulfide bond formation [9]. Given that both endogenous sulfhydryl-containing molecules and oxygen can lead to artifactual S-thiolation, this possibility was addressed experimentally, in different mammalian organisms and tissue, using a protein with a high propensity for thiolation.

We recently identified protein S-cysteinylation of Cu/Zn-superoxide dismutase (SOD1) and localized the modification to CYS111 using both top-down proteomic approaches and X-ray crystallography [10, 11]. Notably, bottom-up experiments failed to localize the modification due to complete scrambling of cysteinylation, via thiol-disulfide exchange, to each of the four SOD1 cysteine residues. Such scrambling is facilitated by endoproteinase digestion, which tends to normalize cysteine pK_a and reactivity and eliminates kinetic barriers by exposing buried cysteines and removing distance constraints.

CYS111 of SOD1 is a solvent-exposed residue located in the SOD1 homodimer interface and has a relatively low pK_a. This low pK_a leads to deprotonation at physiological pH and the more reactive cysteine thiolate [12]. We analyzed cysteinylation of SOD1 purified from both mouse and human tissue, under aerobic and anaerobic conditions, and in the presence or absence of thiolate scavengers. The results identify precautions that should be taken when interpreting detection of protein S-thiolation, particularly at reactive surface cysteines.

Prevalent cysteinylation of SOD1 was observed using standard protocols for protein purification. Samples of human cerebral cortex were harvested at various postmortem intervals (PMIs; estimated 7–17 h), flash-frozen in liquid nitrogen, and stored at −80°C. Frozen tissue was homogenized in lysis buffer (1× PBS + protease inhibitor tablet (Sigma Chemical Co., St. Louis, MO)) at 4°C followed by centrifugation for 10 min at 14 000 rpm. SOD1 was immunoaffinity-purified as previously described [11, 13]. Briefly, polyclonal rabbit antibodies raised in-house against a mixture of native and modified SOD1 (by both oxygen and sulfur adducts on CYS111) were immobilized upon POROS-AL beads (Applied Biosystems, Framingham, MA). The supernatant of tissue homogenate was applied to these immunoaffinity beads in either batch mode or using home-packed columns. After 20 min of binding at 4°C, samples were washed with ∼20 bed volumes and eluted with 5% acetic acid [13]. Aliquots of these eluates were retained for MS by direct infusion in 50% ACN/water, and the remainder was concentrated and exchanged into 25 mM Tris buffer, pH 7.8, and frozen for further HPLC analysis.

Purified SOD1 protein was analyzed by direct infusion or was solvent-exchanged and further separated using RP-LC and FT-MS [14] or ESI-ion trap MS [11], as previously described. Briefly, RP-LC was performed using an Eksigent 2DHPLC, a self-packed 12 cm, 150 μm id column with 5 μm C18 beads (unpacked from a larger Targa column (The Nest Group, Southborough, MA)). Following analysis of the respective SOD1 sample, the column was washed with 70% isopropanol using a 15-min saw tooth gradient; SOD1 carryover was less than 5% via total ion chromatogram area after the isopropanol wash. Samples were introduced via a nanospray ion source (CaptiveSpray) with a dual ion funnel (solariX) into a 12.0 T hybrid quadrupole FT-ICR, FT-MS mass spectrometer (Bruker Daltonics). Intact protein masses were reconstructed using maximum entropy deconvolution from DataAnalysis (Bruker Daltonics, version 3.4), and peak areas of cysteinylated SOD1 were calculated using the DataAnalysis software (Bruker Daltonics version 3.4). Fold differences were determined by comparing the peak area of the aerobic SOD1-cys peak to the peak areas of the anaerobic or anaerobic with cysteine scavengers peaks, respectively.

Cysteinylation of SOD1 was consistently the most prevalent PTM observed as peaks (e.g. the 15+ charge state at m/z

1165.255) corresponding to a deconvoluted and deisotoped molecular mass of 15963.38 Da, which is 118.9 Da larger than unmodified SOD1 (15844.44 Da; Figs. 1A and E, 2A and B). In fact, the majority of the samples of human cortical tissue analyzed were cysteinylated (Fig. 1G) with one cysteine per dimer (cysteinylation of one CYS111 blocks cysteinylation of the second CYS111). Next, we addressed the possibility that the cysteinlyation detected was tissue-specific.

Cysteinlyation of SOD1 is more prevalent in nervous tissue than blood. Mouse brains were dissected (all experiments approved by Brandeis' Institutional Animal Care and Use Committee), flash-frozen in liquid nitrogen, and stored at −80°C. Human brain and spinal cord were prepared as described above. Blood samples were taken by finger prick from a healthy human volunteer (IRB exempt). Briefly, red blood cells (RBCs) were washed with 7.6% sodium citrate in PBS, pH 7.2 (anticoagulant) and centrifuged at 2000 rpm for 4 min at 4°C (repeated in duplicate). After the final wash, the RBCs were lysed and SOD1 was immunopurified and analyzed as described above.

Similar to SOD1 purified from human cortical tissue of varying PMI, cysteinylation was the most prevalent PTM observed in SOD1 purified from human spinal cord of various PMIs (7–17 h; Fig. 1C and F) and mouse brain (Fig. 1B). In addition, no cysteinlyation was observed in human blood (Fig. 1D) or mouse liver (data not shown). Notably, previous studies have identified glutathionylation and cysteinylation of SOD1 purified from blood [15–17]. The lack of similar modifications in this study is attributed to separation of RBCs from plasma, which contains free and protein-bound cysteine and glutathione, by washing of RBCs prior to homogenization. Next, the effects of variables in tissue preparation on cysteinlyation were addressed, including time taken for purification and presence or absence of thiolate scavengers and molecular oxygen.

Cysteinylation was reduced 1.5-fold in a cortical sample purified anaerobically. Postmortem tissue samples can be taken anywhere from hours to tens of hours following death, and in common with surgically resected tissues and blood samples, can sit for undocumented amounts of time at room temperature before freezing. In addition, the authors have collaborated with tissue banks that report PMIs based upon the time from death to placing the body in a refrigerator,

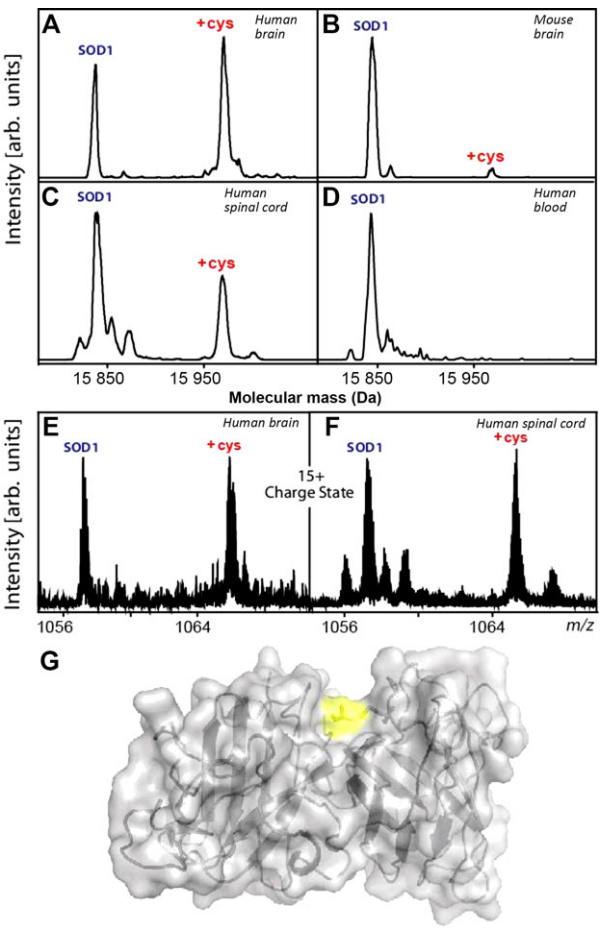

Figure 1. SOD1 is fully cysteinlyated when purified from human cerebral cortical tissue using standard protocols. The spectrum in panels A–D were deconvoluted using maximum entropy. FT-MS data of the high PMI human cortical tissue (A) and the human spinal cord tissue (C) showing cysteinylation of SOD1 (only space for one cysteinylation is available per dimer, see panel G, hence equal intensities of unmodified and modified monomer in panel A). Ion trap data of the mouse brain tissue (B) and the human blood sample (D) showing cysteinylation of SOD1. The 15+ charge state of cysteinlyated SOD1 purified from high PMI human cortical tissue (E) and from human spinal cord (F), showing cysteinylation of SOD1 in raw data. Note that human spinal tissue was not fully cysteinylated in all charge states observed, hence why the maximum entropy deconvoluted spectra (a reconstruction of all charge states) is not fully cysteinylated (panel C versus F). (G) Crystal structure of cysteinylated SOD1 (4FF9; [10]); highlighted in yellow is cysteinylation on CYS111. SOD1 was purified from samples of human cerebral cortex, human spinal cord, and blood, or from mouse brain using standard protocols and analyzed using LC–FT-MS or ESI-ion trap MS. The data presented here for human spinal tissue are representative of eight samples analyzed, for human cortical tissue for four samples analyzed, for mouse brain for two samples analyzed, and for human blood for two samples analyzed. In addition, it should be noted that we routinely detect cysteinylation in similar tissues. In LC-MS experiments, buffer A consisted of 0.1% formic acid (v/v) in HPLC grade water and buffer B consisted of 0.1% formic acid (v/v) in 100% HPLC grade acetonitrile (v/v). Samples were manually injected and eluted at 2.5 μL/min using a 32 min gradient: 5% B for 6 min, 5–40% B for 8 min, 40–100% B for 2 min, 100% B for 5 min, 100–2% B for 1 min and 2% B for 10 min. Important instrument operation parameters include dry gas flow rate 4.0 L/min., neb gas flow rate 1.0 bar, capillary voltage 1555.0 V, source declustering potential = 34 V, source accumulation time = 0.001 s, ion accumulation time = 0.1 s, TOF= 0.001 ms, and sidekick extraction voltages = −1.5 V. Note that as-isolated SOD1 is acetylated and contains an internal disulfide bond, and the solvents and declustering potentials used were such that native metals were not observed.

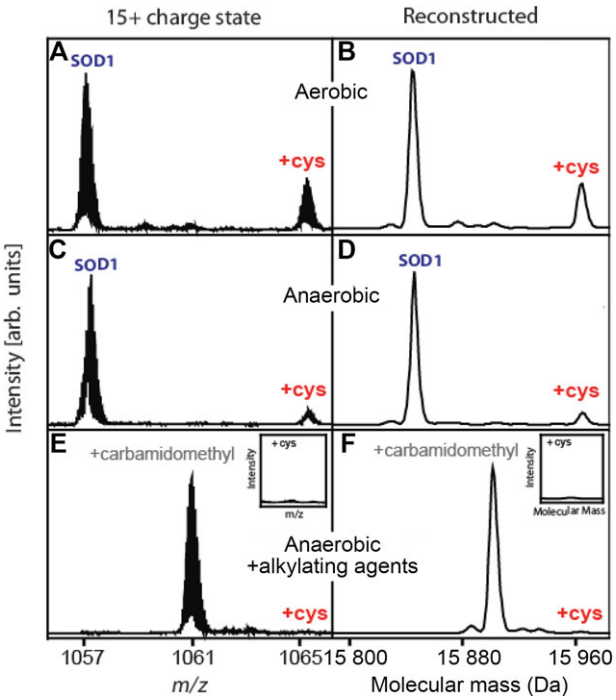

Figure 2. Anaerobic purification reduces that amount of cysteinlyation observed. SOD1 was isolated from a human cerebral cortex using an SOD1-antibody column; purified aerobically, anaerobically, or anaerobically in the presence of cysteine scavengers; and analyzed using LC–FT-MS. (B, D, and F) Spectra were averaged (7 total) from 0.1 s of the total ion chromatogram where cysteinylated SOD1 eluted and maximum entropy deconvoluted data for the entire mass range showing the molecular mass for unmodified and modified SOD1, the delta mass being 118.9 Da, which is consistent with cysteinylation. The 15+ charge state of cysteinlyated SOD1 purified aerobically (A), anaerobically (C), and anaerobically in the presence of alkylating agents (E). The most cysteinylation was observed in the aerobically prepared SOD1 (B), approximately a 1.5-fold reduction in cysteinylation was observed in the anaerobically prepared SOD1 (D), and approximately a 50-fold reduction in cystienylation was observed in the anaerobically prepared SOD1 in the presence of alkylating agents (F). Inset in (E) and (F) is of the region surrounding the region of the +cys mass shift. Note that the solvents and declustering potentials used were such that native metals were not observed.

as opposed to the more common definition of death to dissection. To eliminate this potential source of uncertainty in experimental design, all of the remaining experiments were standardized using brain tissue that had been harvested at a well-defined, relatively low PMI and frozen immediately after dissection (9 h from death to freezing). The tissue was cryosectioned and sections were distributed in a manner that created three approximately equal samples. To assess the possibility that oxygen in addition to "free" endogenous cysteine could result in the artifactual modification of SOD1 during homogenization, SOD1 was then purified from one of these samples anaerobically using an MBraun Unilab glove box (MBraun) with oxygen levels below 10 ppm monitored by diethyl zinc. Oxygen levels were monitored: (i) prior to conducting experiments, (ii) once during experiments, and (iii) at the conclusion of the experiment. All materials, including buffers, antibody beads, and plastics were subject to four cycles vacuum degassing and equilibrated in the glove box for a minimum of 15 h. In addition, all protein work was conducted using reagents and materials at 0°C using ice (degassed by four vacuum cycles and kept in plastic bags to prevent air stored in the ice from escaping while thawing). Samples were sealed in autosampler vials within the glovebox to ensure purified samples were not exposed to oxygen prior to LC–FT-MS analysis.

Cysteinylation of SOD1 was again consistently the most prevalent PTM (Fig. 2C and D). However, the amount of cysteinylated SOD1 was reduced by approximately 1.5-fold compared to the aerobic purification (Fig. 2A and B). Given this result, oxygen (from the air) can catalyze cysteinlyation during homogenization that is an artifact of the purification procedure.

Oxygen is not required for cysteine, or any disulfide-containing compound, to react with CYS111 thiolate, since this reaction proceeds by thiol-disulfide exchange. We therefore addressed the possibility that oxidized thiols, e.g. "free" cystine (two cysteine bound by a disulfide), or bound cystine (part of a protein complex) could react with SOD1 as an artifact of purification, by performing anaerobic homogenization in the presence of thiolate scavengers.

Cysteinylation was reduced 50-fold when SOD1 was purified anaerobically in the presence of alkylating agents. Using the same protocols and tissue as described above, SOD1 was purified from a sample of human cerebral cortex homogenized anaerobically in the presence of iodoacetamide (10 mM), iodoacetic acid (4 mM), and S-methyl methanethiosulfonate (0.5 mM) to block any unreacted cysteine residues of SOD1 and scavenge any free thiolates, respectively. After anaerobic homogenization in the presence of alkylating agents, tissue extract was added to the SOD1-antibody beads and purified aerobically following the above protocol. PBS was used in this purification protocol because amine groups in buffers such as Tris can react with methanethiosulfonate.

Carbamidomethylation of SOD1 due to modification by iodoacetamide was the most prevalent PTM observed as peaks corresponding to a deconvoluted and deisotoped molecular mass of 15901.50 Da, which is 57.1 Da larger than unmodified SOD1 (15844.44 Da, which was not observed, Fig. 2A and B). The amount of cysteinylation (molecular mass of 15963.38 Da) observed was approximately 50-fold less than that observed in the aerobic preparation (Fig. 2E and F). Thus, either the majority of cysteinylation is an artifact of homogenization or these conditions reversed cysteinylation of SOD1. The latter is considered less likely because in control in vitro experiments where SOD1-cys was incubated with the thiol scavengers used here, cysteinylation of SOD1 was not reversed (Supporting Information Fig. 3).

This study utilized cysteinylation of highly reactive surface cysteine, CYS111 of SOD1, as a case study for potential

S-thiolation artifacts in the analysis of protein PTM in tissues. SOD1 was cysteinlyated (one cysteinlyation per dimer) when purified from both mouse and human nervous tissue, but not blood, using standard aerobic protocols. Previous reports of SOD1 cysteinlyation in human blood [15, 16] could not be confirmed here or in our previous studies [13], potentially because blood cells were washed of the cysteine present in plasma prior to homogenization, preventing potential artifacts. In addition, a 1.5-fold reduction in cysteinylation was observed in the anaerobically prepared SOD1, and approximately a 50-fold reduction in SOD1 cysteinylation was observed in tissue homogenized both anaerobically and in the presence of alkylating agents.

S-thiolation was less prevalent in the sample of human cerebral cortex harvested at low PMI compared to higher PMIs; however, due to the limited number of autopsy cases and limited clinical information, we are unable to make conclusions about the influence of PMI on S-thiolation of SOD1 in CNS tissue. We do stress the importance of validating the influence of postmortem harvesting and processing of tissue when conducting proteomic studies with human tissue. In a previous study to evaluate proteasome activity in brain and spinal cord from controls and individuals dying of amyotrophic lateral sclerosis, a study in mice demonstrated stability of proteasomal enzyme activity over typical PMI [18]. In contrast, the protein arginine methyl transferase 1 was depleted from motor neuronal nuclei within 6-h delay in postmortem processing (Tibshirani and Durham, personal communication).

In addition to CYS111 of SOD1 being considered a reactive cysteine [12], it is also surface-exposed. Notably, no cysteinylation of SOD1 CYS6, which is not solvent-exposed, was observed, nor was thiol-disulfide exchange-mediated breaking of the native CYS57-CYS146 disulfide. It is likely that both the solvent exposure of SOD1 CYS111 and this high reactivity make it susceptible to nonspecific S-thiolation. Ansong et al. [1] recently reported detecting cysteinylation of numerous proteins in *Salmonella Typhimurium* processed under aerobic conditions. Their observation of cysteinylation of buried cysteines, as opposed to more accessible surface cysteines, is therefore consistent with in vivo cysteinylation, not artifact of processing.

Another S-thiolation, glutathionylation, of SOD1 has been reported, and has been implicated in dimer dissociation and disease progression in the neurodegenerative disease amyotrophic lateral sclerosis [15–17, 19–21]. Typically, recombinant proteins expressed and purified from *Escherichia coli* do not contain PTMs. Furukawa et al. [19] observed glutathionylation of SOD1 on all four native cysteines (Cys6, Cys57, Cys111, and Cys146) using intact mass analysis (MALDI-TOF). SOD1 was denatured in the process of purification, thus exposing all four native cysteines to solvent, and potentially allowing them to be more readily glutathionylated. This suggests that glutathionylation was facilitated by cell lysis and/or purification. In contrast, McAlary et al. [20] also observed gluathionylation of SOD1 from *E. coli*, however they only observed it on one cysteine. They argue that only one cysteine was modified because SOD1 was not denatured during purification, thus suggesting the glutathionylation observed was due to exogenous GSH/GSSG (where GSSG is glutathione disulfide). Although GSH/GSSG are more predominant intracellularly, cys/cystine are more predominant extracellulary [6], implying that the type of S-thiolation artifacts observed will depend upon biological sample.

In summary, S-thiolation artifacts can occur in tissue sources with endogenous free thiols, especially on highly reactive or solvent-exposed cysteine residues (proteins with highly reactive cysteine residues are highlighted in a recent manuscript by Weerapana et al. [22]). Anaerobic purification and the addition of thiol scavengers during homogenization minimize S-thiolation artifacts, and top-down MS/MS analysis minimizes the additional artifact of S-thiolation scrambling that inevitably occurs following the endoproteinase digestion in bottom-up experiments.

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