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

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Abstract

Bottom-up mass spectrometry studies typically employ a reduction and alkylation step that eliminates a class of post-translational modification, 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. 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 post-mortem 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 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.

Keywords

S-thiolation; top down mass spectrometry; artifacts; Cu/Zn-superoxide dismutase; post-translation modification

Posttranslational modifications (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 exponentially increased over the number of genes and their analysis has become one of the most active areas of research in mass spectrometry. Bottom-up proteomics has become a staple method for identifying posttranslational modification, but struggles with combinatorial modifications[1-3], and the methodology commonly employs reductive sample preparation that eliminates reductively-labile PTMs. Top-down mass spectrometry, 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]) (supplemental figure 1 and 2). S-thiolation can be mediated enzymatically, for example 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: (1) oxygen-independent thiol-disulfide exchange between a thiolate and disulfide, and (2) 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 pKa and reactivity and eliminate 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 pKa. This low pKa 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 post mortem

intervals (PMI) (estimated 7-17 hours), flash frozen in liquid nitrogen, and stored at -80 °C. Frozen tissue was homogenized in lysis buffer (1X PBS + protease inhibitor tablet (Sigma Chemical Co. St. Louis MO)) at 4 °C followed by centrifugation for 10 minutes 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 minutes 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 mass spectrometry 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 reverse-phase liquid chromatography and Fourier transform mass spectrometry [14] or ESI-ion trap mass spectrometry [11] as previously described. Briefly, reversed-phase liquid chromatography was performed using an Eksigent two-dimensional high performance liquid chromatography (HPLC), a self-packed 12 cm, 150 µm ID column with 5 µm C₁₈ beads (unpacked from a larger Targa column). Following analysis of the respective SOD1 sample, the column was washed with 70 % isopropanol using a 15 minute saw tooth gradient; SOD1 carry-over was less than 5 % via TIC area after the isopropanol wash. Samples were introduced via a nanospray ion source (CaptiveSpray) with a dual ion funnel (solariX) into a 12.0 tesla hybrid quadrupole Fourier transform ion cyclotron resonance (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.

Cysteinylolation 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) (Figure 1 A, E and Figure 2 A, B). In fact, the majority of the samples of human cortical tissue analyzed were cysteinylated (Figure 1 G) with one cysteine per dimer (cysteinylolation of one CYS111 blocks cysteinylolation of the second CYS111). Next, we addressed the possibility that the cysteinylolation detected was tissue-specific.

Cysteinylolation 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 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 (anti-coagulant) and centrifuged at 2000 RPM for 4 minutes 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, cysteinylolation was the most prevalent posttranslational modification observed in SOD1 purified from human spinal cord of various PMIs (7-17 hours) (Figure 1 C, F) and mouse brain (Figure 1 B). In addition, no cysteinylolation was observed in human blood (Figure 1 D) or mouse liver (data not shown). Notably, previous studies have identified glutathionylation and cysteinylolation of SOD1 purified from blood [15-17]. The lack of similar modifications in this study is attributed to separation of red blood cells from plasma, which contains free and protein-bound cysteine and glutathione, by washing of red blood cells prior to homogenization. Next, the effects of variables in tissue preparation on cysteinylolation were addressed, including time taken for purification and presence or absence of thiolate scavengers and molecular oxygen.

Cysteinylolation was reduced 1.5-fold in a cortical sample purified anaerobically. Post-mortem 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 post-mortem intervals based upon the time from death to placing the body in a refrigerator, 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 hours 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 with oxygen levels below 10 ppm monitored by diethyl zinc. Oxygen levels were monitored: (1) prior to conducting experiments, (2) once during experiments, and (3) 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 hours. In addition, all protein work was conducted using reagents and materials brought to 0°C using ice (degassed by 4 vacuum cycles and kept in plastic bags to keep air stored in the ice from escape during thawing). Samples were sealed in autosampler vials within the glovebox to ensure purified samples were not exposed to oxygen prior to LC-FTMS analysis.

Cysteinylolation of SOD1 was again consistently the most prevalent PTM (Figure 2 C, D). However, the amount of cysteinylated SOD1 was reduced by approximately 1.5-fold compared to the aerobic purification (Figure 2 A, B). Given this result, oxygen (from the air) can catalyze cysteinylolation 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, for example “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 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 methanethiosulfanate (MMTS) (0.5 mM) to block any unreacted cysteine residues of SOD1 and to 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 MMTS.

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, Figure 2 A, B). The amount of cysteinylation (molecular mass of 15963.38 Da) observed was approximately 50-fold less than that observed in the aerobic preparation (Figure 2 E; 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 (supplemental figure 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 cysteinylated (one cysteinylation per dimer) when purified from both mouse and human nervous tissue, but not blood, using standard aerobic protocols. Previous reports of SOD1 cysteinylation 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 central nervous system 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 (PRMT1) was depleted from motor neuronal nuclei within 6 hrs delay in post mortem 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 cysteinylolation 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 cysteinylolation of numerous proteins in *Salmonella* Typhimurium processed under aerobic conditions. Their observation of cysteinylolation of buried cysteines, as opposed to more accessible surface cysteines, is therefore consistent with in vivo cysteinylolation, 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 *E. coli* do not contain post-translational modifications. 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 glutathionylation 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. Whereas GSH/GSSG are more predominant intracellularly, Cys/Cystine are more predominant extracellularly [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 endoprotease digestion in bottom-up experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Ansong C, Wu S, Meng D, Liu X, et al. Top-down proteomics reveals a unique protein S-thiolation switch in *Salmonella Typhimurium* in response to infection-like conditions. *P Natl Acad Sci USA*. 2013; 110:10153–10158.
2. Jungblut PR, Holzhutter HG, Apweiler R, Schluter H. The speciation of the proteome. *Chemistry Central journal*. 2008; 2:16. [PubMed: 18638390]
3. Schluter H, Apweiler R, Holzhutter HG, Jungblut PR. Finding one's way in proteomics: a protein species nomenclature. *Chemistry Central journal*. 2009; 3:11. [PubMed: 19740416]
4. Kleinova M, Belgacem O, Pock K, Rizzi A, et al. Characterization of cysteinylolation of pharmaceutical-grade human serum albumin by electrospray ionization mass spectrometry and low-energy collision-induced dissociation tandem mass spectrometry. *Rapid communications in mass spectrometry : RCM*. 2005; 19:2965–2973. [PubMed: 16178042]
5. Lim A, Wally J, Walsh MT, Skinner M, Costello CE. Identification and location of a cysteinyl posttranslational modification in an amyloidogenic kappa1 light chain protein by electrospray ionization and matrix-assisted laser desorption/ionization mass spectrometry. *Anal Biochem*. 2001; 295:45–56. [PubMed: 11476544]
6. Dalle-Donne I, Rossi R, Giustarini D, Colombo R, Milzani A. S-glutathionylation in protein redox regulation. *Free radical biology & medicine*. 2007; 43:883–898. [PubMed: 17697933]
7. Coppo L, Ghezzi P. Thiol regulation of pro-inflammatory cytokines and innate immunity: protein S-thiolation as a novel molecular mechanism. *Biochem Soc Trans*. 2011; 39:1268–1272. [PubMed: 21936800]
8. Jacob C, Battaglia E, Burkholz T, Peng D, et al. Control of oxidative posttranslational cysteine modifications: from intricate chemistry to widespread biological and medical applications. *Chem Res Toxicol*. 2012; 25:588–604. [PubMed: 22106817]
9. Sevier CS, Kaiser CA. Formation and transfer of disulphide bonds in living cells. *Nat Rev Mol Cell Biol*. 2002; 3:836–847. [PubMed: 12415301]
10. Auclair JR, Brodtkin HR, D'Aquino JA, Petsko GA, et al. Structural Consequences of Cysteinylolation of Cu/Zn-Superoxide Dismutase. *Biochemistry*. 2013; 52:6145–6150. [PubMed: 23919400]
11. Auclair JR, Johnson JJ, Liu Q, Salisbury JP, et al. Post-Translational Modification by Cysteine Protects Cu/Zn-Superoxide Dismutase from Oxidative Damage. *Biochemistry*. 2013; 52:6137–6144. [PubMed: 23927036]
12. You Z, Cao X, Taylor AB, Hart PJ, Levine RL. Characterization of a covalent polysulfane bridge in copper-zinc superoxide dismutase. *Biochemistry*. 2010; 49:1191–1198. [PubMed: 20052996]
13. Taylor DM, Gibbs BF, Kabashi E, Minotti S, et al. Tryptophan 32 potentiates aggregation and cytotoxicity of a copper/zinc superoxide dismutase mutant associated with familial amyotrophic lateral sclerosis. *J Biol Chem*. 2007; 282:16329–16335. [PubMed: 17389599]
14. Bosco DA, Morfini G, Karabacak NM, Song Y, et al. Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. *Nat Neurosci*. 2010; 13:1396–1403. [PubMed: 20953194]
15. Nakanishi T, Kishikawa M, Miyazaki A, Shimizu A, et al. Simple and defined method to detect the SOD-1 mutants from patients with familial amyotrophic lateral sclerosis by mass spectrometry. *J Neurosci Methods*. 1998; 81:41–44. [PubMed: 9696308]
16. Shimizu A, Nakanishi T, Miyazaki A. Detection and characterization of variant and modified structures of proteins in blood and tissues by mass spectrometry. *Mass spectrometry reviews*. 2006; 25:686–712. [PubMed: 16565975]
17. Wilcox KC, Zhou L, Jordon JK, Huang Y, et al. Modifications of superoxide dismutase (SOD1) in human erythrocytes: a possible role in amyotrophic lateral sclerosis. *J Biol Chem*. 2009; 284:13940–13947. [PubMed: 19299510]
18. Kabashi E, Agar JN, Strong MJ, Durham HD. Impaired proteasome function in sporadic amyotrophic lateral sclerosis. *Amyotroph Lateral Scler*. 2012; 13:367–371. [PubMed: 22632443]

19. Furukawa Y, O'Halloran TV. Amyotrophic lateral sclerosis mutations have the greatest destabilizing effect on the apo-and reduced form of SOD1, leading to unfolding and oxidative aggregation. *J Biol Chem.* 2005; 280:17266–17274. [PubMed: 15691826]
20. McAlary L, Yerbury JJ, Aquilina JA. Glutathionylation potentiates benign superoxide dismutase 1 variants to the toxic forms associated with amyotrophic lateral sclerosis. *Scientific reports.* 2013; 3:3275. [PubMed: 24253732]
21. Redler RL, Wilcox KC, Proctor EA, Fee L, et al. Glutathionylation at Cys-111 induces dissociation of wild type and FALS mutant SOD1 dimers. *Biochemistry.* 2011; 50:7057–7066. [PubMed: 21739997]
22. Weerapana E, Wang C, Simon GM, Richter F, et al. Quantitative reactivity profiling predicts functional cysteines in proteomes. *Nature.* 2010; 468:790–795. [PubMed: 21085121]

Abbreviations

PTM	post-translational modification
SOD1	Cu/Zn-superoxide dismutase
HPLC	high performance liquid chromatography
FT-MS	fourier transform mass spectrometry
FTICR	fourier transform ion cyclotron mass spectrometry
PMI	post-mortem interval
RBC	red blood cell
LC	liquid chromatography
MMTS	S-methyl methanethiosulfate
PRMT1	protein arginine methyl transferase
ESI	electrospray ionization
TIC	total ion chromatogram

Figure 1. SOD1 is fully cysteinylated when purified from human cerebral cortical tissue using standard protocols

The spectrum in panels A-D were deconvoluted using maximum entropy. FTMS data of the high PMI human cortical tissue (A.) and the human spinal cord tissue (C.) showing cystenylation of SOD1 (only space for one cystenylation 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 cystenylation of SOD1. The 15+ charge state of cysteinylated SOD1 purified from high PMI human cortical tissue (E.) and from human spinal cord (F.), showing cystenylation of SOD1 in raw data. Note: 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 cystenylation 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-FTMS or ESI-ion trap MS. The data presented here for

human spinal tissue is representative of 8 samples analyzed, for human cortical tissue for 4 samples analyzed, for mouse brain for 2 samples analyzed, and for human blood for 2 samples analyzed. In addition, it should be noted that we routinely detect cysteinylolation in similar tissues. In LC-MS experiments, buffer A consisted of 0.1 % formic acid (vol/vol) in HPLC grade water and buffer B consisted of 0.1 % formic acid (vol/vol) in 100 % HPLC grade acetonitrile (vol/vol). Samples were manually injected and eluted at $2.5 \mu\text{L min}^{-1}$ using a 32 minute gradient: 5 % B for 6 minutes, 5-40 % B for 8 minutes, 40-100 % B for 2 minutes, 100 % B for 5 minutes, 100-2 % B for 1 minute and 2 % B for 10 minutes. 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, time of flight = 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 cysteinylatation 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-FTMS. (**B, D, F**) Spectra were averaged (7 total) from 0.1 seconds of the TIC 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 cysteinylatation. The 15+ charge state of cysteinylated SOD1 purified aerobically (**A.**), anaerobically (**C.**), and anaerobically in the presence of alkylating agents (**E.**). The most cysteinylatation was observed in the aerobically prepared SOD1 (**B**), approximately a 1.5-fold reduction in cysteinylatation was observed in the anaerobically prepared SOD1 (**D.**), and approximately a 50-fold reduction in cysteinylatation 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.

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