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Articles

Secondary Reactions and Strategies To Improve Quantitative Protein Footprinting

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Hydroxyl radical-mediated footprinting permits detailed examination of structure and dynamic processes of proteins and large biological assemblies, as changes in the rate of reaction of radicals with target peptides are governed by changes in the solvent accessibility of the side-chain probe residues. The precise and accurate determination of peptide reaction rates is essential to successfully probing protein structure using footprinting. In this study, we specifically examine the magnitude and mechanisms of secondary oxidation occurring after radiolytic exposure and prior to mass spectrometric analysis. Secondary oxidation results from hydrogen peroxide and other oxidative species generated during radiolysis, significantly impacting the oxidation of Met and Cys but not aromatic or other reactive residues. Secondary oxidation of Met with formation of sulfoxide degrades data reproducibility and inflates the perceived solvent accessibility of Met-containing peptides. It can be suppressed by adding trace amounts of catalase or millimolar Met-NH_2 (or Met-OH) buffer immediately after irradiation; this leads to greatly improved adherence to first-order kinetics and more precise observed oxidation rates. The strategy is shown to suppress secondary oxidation in model peptides and improve data quality in examining the reactivity of peptides within the Arp2/3 protein complex. Cysteine is also subject to secondary oxidation generating disulfide as the principal product. The disulfides can be reduced before mass spectrometric analysis by reducing agents such as TCEP, while methionine sulfoxide is refractory to reduction by this reagent under typical reducing conditions.

Hydroxyl radical-mediated protein footprinting with mass spectrometry provides a novel avenue toward examining the structure and dynamic processes of proteins and large biological complexes that are difficult to study due to size limitations in NMR or difficulties of crystallization.[1−8] Technically, aqueous protein solutions are irradiated using X-rays or γ-rays or exposed to hydroxyl radicals generated by other means such as Fenton reagent or photochemical oxidation.[9,10] The solvent-accessible and reactive amino acid side chains undergo stable oxidative modification; peptides are generated by proteolysis, and quantitative LC/MS is used to determine the oxidation extents and rates through dose−response analysis while LC/MS/MS analysis is used to determine the site(s) of oxidation. The binding interfaces of protein complexes and structural allostery of proteins are visualized via the alternation of accessibility of local structure as monitored by the relative reactivity of peptides to hydroxyl radicals.

Extensive research has been carried out on the fundamental chemistry of radiolytic and metal-catalyzed oxidation of amino acid side chains.[11−19] Under typical conditions used for radiolytic footprinting, the side chains of both hydrophobic and hydrophilic amino acid residues including Cys, Met, Trp, Tyr, Phe, Leu, Ile,

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Val, Pro, His, Arg, Lys, Glu, and Asp are reactive and represent useful probes of protein structure.[15−19] Radiolytic oxidation leads to peptides modified with mass additions of +16 or +14 Da due to the formation of hydroxyl or carbonyl groups in the side chains for aliphatic and aromatic amino acid residues; multiple modifications of this type are also common.[11−15] Notable exceptions include the characteristic −43 Da mass change for Arg as result of oxidative elimination of the guanidino group,[16] a −30 Da mass change for Asp and Glu as result of oxidative decarboxylation of the side chain carboxyl groups,[17] +32 and +48 Da mass shifts for Cys due to formation of sulfinic and sulfonic acids,[19] and a number of mixed products with mass changes of −22, −10, +5, and +16 Da for His as a consequence of ring opening and addition.[16] Understanding the common and characteristic modifications of amino acid side chains is a prerequisite for successful footprinting research.

Accurately determining the value of the fraction modified for peptides analyzed in LC/MS experiments is the quantitative basis for reliably determining reactivity changes as a function of ligand binding or macromolecular assembly. Oxidations not due to the primary radiolysis event may have unanticipated consequences for determining the modified fraction. Methionine in particular has been observed to have reactivity in footprinting experiments that does not always correlate well with the expected solvent accessibility.[9,20] Although exposed Met residues are generally observed to be reactive as would be expected, buried Met residues are occasionally seen to be reactive.[20] Although this has not negated the successful use of Met oxidation to probe structure changes, the lower precision of data for Met-containing peptides necessitates caution in analysis.[2,20] Cys and Met are both reactive to mild oxidizing agents such as hydrogen peroxide[21,22] and other oxidizing species;[23,24] these species can be generated during aerobic radiolysis. It has also been reported that methionine sulfoxide, the primary oxidation product of Met, may be reduced by dithiothreitol (dTT), which is used to cleave disulfide bonds during protein denaturation and proteolysis.[25−27] Even though these reactions are relatively slow under ambient conditions, they may significantly alter observed oxidation extents during sample storage and handling prior to analytical workup. In this study, we carefully examine the possible secondary reactions after irradiation and demonstrate strategies to control them.

EXPERIMENTAL SECTION

Materials. Peptides Gln-Asn-Cys-Pro-Arg-Gly-NH_2 (QNCPRG-NH_2), His-Asp-Met-Asn-Lys-Val-Leu-Asp-Leu (Antiflammin-2: HDMNKVLDL), Leu-Trp-Met-Arg-Phe-Ala (LWMRFA), Ac-Lys-Ile-Phe-Met-Lys-NH_2 (Ac-KIFMK-NH_2), Cys-Ile-Ile-Arg-Asn-Cys-Pro-Lys-Gly-NH_2 (CIIRNCPKG-NH_2, internal disulfide), Gly-Cys-Gly (GCG), Gly-Trp-Gly (GWG), Gly-Tyr-Gly (GYG), Gly-Phe-Gly (GFG), methionine (Met-OH), and methionine amide (Met-NH_2) were purchased from Bachem California Inc. (Torrance, CA). All peptides had a purity at least of 90% and were used without further purification. Met(O)-antiflammin-2 with the methionine residue oxidized to sulfoxide was prepared according to previously published precedures.[19] dTT was purchased from Roche Diagnostics Inc. (Indianapolis, IN). Trialkylphosphine tris(2-carboxyethyl) phosphine (TCEP) was from Pierce Biotechnology Inc. (Rockford, IL). Sodium cacodylate and catalase from *Micrococcus lysodeikticus* (138 700 units/mL) were purchased from Fluka. Sequencing grade modified trypsin was bought from Promega Biosciences (Madison, WI). B & J Brand high-purity acetonitrile was purchased from Honeywell International Inc. (Muskegon, MI). Peptide samples of 20−40 μM were prepared in Nanopure water (generated by a Millipore Ultrapure water system) or in 10 mM sodium cacodylate buffer (pH 7.0).

Sample Preparation, Irradiation, and Analysis of Peptides. *Reduction of Met(O)-antiflammin-2 and CIIRNCPKG-NH_2 (Internal Disulfide).* In three 0.2-mL Eppendorf tubes, 16 μL of sample at 20 μM concentration for each of the two peptides in 10 mM sodium cacodylate pH 7.0 buffer was added, mixed with 4.0 μL of 50 mM dTT or 50 mM TCEP, or buffer as control, heated at 95°C for 5 min, diluted with 80 μL of buffer, kept at 37°C for 15 h and then analyzed by LC/MS.

Exposure to Radiation. Peptide solutions were exposed to a cesium-137 γ-ray source (1300 rads/min) at the Albert Einstein College of Medicine. A portion of 40 μL of peptide solution in 10 mM sodium cacodylate pH 7.0 buffer loaded in a 1.5-mL Eppendorf tube was exposed for 0.5−20 min,[16] and 1.0 μL of 0.4 M Met-NH_2 (pH 7.0 adjusted using NaOH) was added to the tube immediately after irradiation to suppress the secondary oxidation or the same amount of buffer was added as a control. To test the secondary oxidation of peptides after irradiation and the quenching efficiency of catalase and Met-NH_2, 39 μL of water or sodium cacodylate buffer in a 1.5-mL tube was irradiated for specific time intervals, and then 1.0 μL of peptide stock solution (1.0 mM) along with 1 μL of catalase (0.14 unit/μL) or Met-NH_2 (0.4 M) was added to the tube. All samples were frozen in dry ice after exposure and then stored at −20°C until analysis.

Mass Spectrometric Analysis. Peptide samples prepared in pure water were analyzed directly by electrospray (ESI) mass spectrometry without chromatographic separation, while those prepared in 10 mM sodium cacodylate buffer were analyzed by HPLC/ESI-MS. For direct infusion ESI-MS, the peptide concentrations were adjusted to 10 μM with acetonitrile and were infused directly into the ESI-MS at a flow rate of 3 μL/min. Mass spectra were acquired on a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Electron Inc., San Jose, CA) with a mass accuracy of ±0.2 amu and unit resolution. The needle voltage was set at 4.5 kV. The instrument was tuned using the known masses of the unmodified peptides, and the spectra were recorded in the profile mode as indicated in the results. The sites of amino acid oxidation were identified by the low-energy collision-induced dissociation (CID) MS/MS spectra for selected ions. For LC/MS analysis, 100 pmol of peptides was injected onto a Vydac C18 0.1 × 150 mm column. The mobile phases consist of 5 and 95% of acetonitrile with 0.05% TFA, and a linear gradient of 10−80% organic phase for 70 min was used. The column flow was set at 50 μL/min and directed to the mass spectrometer. The amounts

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of peptides were measured by the total ion chromatograph (TIC) of corresponding mass spectroscopic signals.

Sample Preparation, Irradiation, and Analysis of Proteins. *Preparation*. Bovine Arp 2/3 complex (provided by Tom Pollard, Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520) was dialyzed against a pH 7.0 buffer containing 10 mM sodium cacodylate, 1.0 mM ATP, and 1.0 mM $MgCl_2$ at 4 °C overnight with three changes of buffer. The protein concentration was adjusted to 1.0 μM for the dose−response experiments. Each protein solution was dispensed individually as 5.0-μL portions into 0.2-mL microcentrifuge tubes for radiolysis. All experiments were performed at ambient temperature.

Exposure to Radiation. Protein samples were irradiated by synchrotron X-rays at the X-28C beamline of NSLS at Brookhaven National Laboratory (Long Island, NY). 5.0-μL samples portions in 0.2-mL Eppendorf tubes were irradiated for 10−80 ms at a beam current ranging between 195 and 225 mA using a stand and shutter according to published procedures.[28,29] 10 mM Met-NH_2 buffer (pH 7.0) was added immediately after exposure to quench secondary oxidation. Three separate dose−response experiments were carried out to verify the reproducibility of the results.

Proteolysis and Mass Spectroscopic Analysis. The irradiated protein solutions were subjected to proteolysis using trypsin at an enzyme-to-protein ratio of 1:50 at 37 °C for 12 h. The digested samples were analyzed by using LCQ DecaXP Plus quadrupole ion trap mass spectrometer (Thermo Electron Inc., San Jose, CA) equipped with a nanospray ion source coupled with an UltiMate capillary-HPLC system (LC Packings). The 1.0-pmol sample was injected into a PepMap reversed-phase C18 (75 μm i.d. × 15 cm) capillary column (LC Packings). The mobile phases consisted of 5 and 95% of acetonitrile with 0.1% formic acid, the column flow was set at 200 nL/min, and a linear gradient of 10−80% organic phase for 70 min was used. The fraction of unmodified peptide (y) was calculated as the ratio of the peak area under the TIC signal for the unmodified peptide to the sum of those for the unoxidized peptide and the radiolytic products (no corrections were made for potential ionization efficiency differences between unoxidized and oxidized products). The rate constant (k) was obtained by fitting the fraction unmodified data (y) and exposure time (t) into the first-order reaction equation $y = \mathrm{Exp}(-kt)$.[16] Tandem MS/MS spectra were acquired to identify sites of oxidation as previously described.

RESULTS AND DISCUSSION

Secondary Oxidations of Amino Acid Side Chains after Radiolytic Exposure. Highly reactive amino acid residues are susceptible to further oxidation after radiolytic exposure by those oxidative species generated during the radiolysis of protein solutions. In addition to reactive species with a short half-life, such as hydroxyl radicals ($HO^{\bullet}$), solvated electrons (e_{aq}^-), and hydrogen radicals ($H^{\bullet}$),[30] radiolysis of aqueous solutions also gives rise to relatively stable oxidative species, including hydrogen peroxide along with peroxides and superoxides on proteins due to the free-radical oxidation of amino acid residues in the presence of oxygen.[19] H_2O_2 can be formed possibly via three routes, and the oxygen atoms in H_2O_2 originate from both water and dissolved O_2. Hydroxyl radicals may self-quench by dimerization to form H_2O_2; e_{aq}^- and $H^{\bullet}$ may react with O_2 leading to superoxide radical anion ($O_2^{\bullet-}$) and hydroperoxyl radical ($HO_2^{\bullet}$) and finally H_2O_2, and oxidation of amino acids may generate H_2O_2 as side products.[19] These oxidative species are able to attack highly reactive amino acid side chains, particularly those containing sulfur.[21−24] Even though the reactions are relatively slow, the time period of several days between irradiation and sample analysis in typical footprinting experiments may allow secondary reactions to accumulate.

Methionine. A typical methionine-containing peptide, antiflammin-2 (HDMNKVLDL), was selected to demonstrate the vulnerability of methionine residues to secondary oxidation after irradiation. The peptide solution at 40 μM concentration was exposed to γ-rays for 12 min. The samples were analyzed by ESI-MS within 30 min and also after freezing while being maintained at −20 °C for 44 h, a time comparable to that typically seen in the laboratory. The corresponding positive ESI-MS spectra are displayed in Figure 1A and B, respectively. The peak observed at m/z 1084.3 corresponds to the unmodified peptide. The peak at m/z 1100.3 corresponds to the primary product with +16 Da mass addition resulting from the oxidation of methionine to methionine sulfoxide, while the peak at m/z 1052.3 is likely due to the further oxidation of sulfoxide leading to the cleavage of sulfur moiety and formation of an aldehyde group at the γ-carbon.[19] In Figure 1A, when the sample was analyzed soon after irradiation, the signal from the +16 Da oxidation product at m/z 1100.3 is less than half of that of unmodified peptide. However, as shown in Figure 1B, the +16 Da species dominates after the irradiated sample was frozen and maintained at −20 °C for 44 h. The data indicate significant secondary oxidation that takes place after irradiation in the case of methionine-containing peptides.

To confirm the secondary oxidation and determine the site of modification, the peptide was added to 40 μL of water *that had been first irradiated for 8 min* (to a final concentration of 40 μM), was frozen and kept at −20 °C for 44 h, and then was analyzed by ESI-MS. The positive ESI-MS spectrum is shown in Figure 1C. Only one product with +16 Da mass addition at m/z 1100.3 was generated. Low-energy CID tandem mass spectrometry was performed, and the MS/MS spectrum of m/z 550.7 corresponding to the doubly charged ion of the +16 Da product is exhibited in Figure 1D. The peak values are labeled, and fragment ions are assigned clearly in the figure. One striking characteristic of the +16 Da modified fragment ions is that each of them is accompanied by a fragment with −64 Da mass shift as shown by the arrows in Figure 1D. A doubly charged signal corresponding to −64 Da mass shift from the parent ion is also found at m/z 518.7. The unique pattern of fragmentation is a consequence of characteristic elimination of methanesulfenic acid (CH_3SOH, 64 Da) from the side chain of methionine sulfoxide[31,32] and is a signature of methionine sulfoxide. The spectrum explicitly points to the Met residue as the target of the secondary oxidation.

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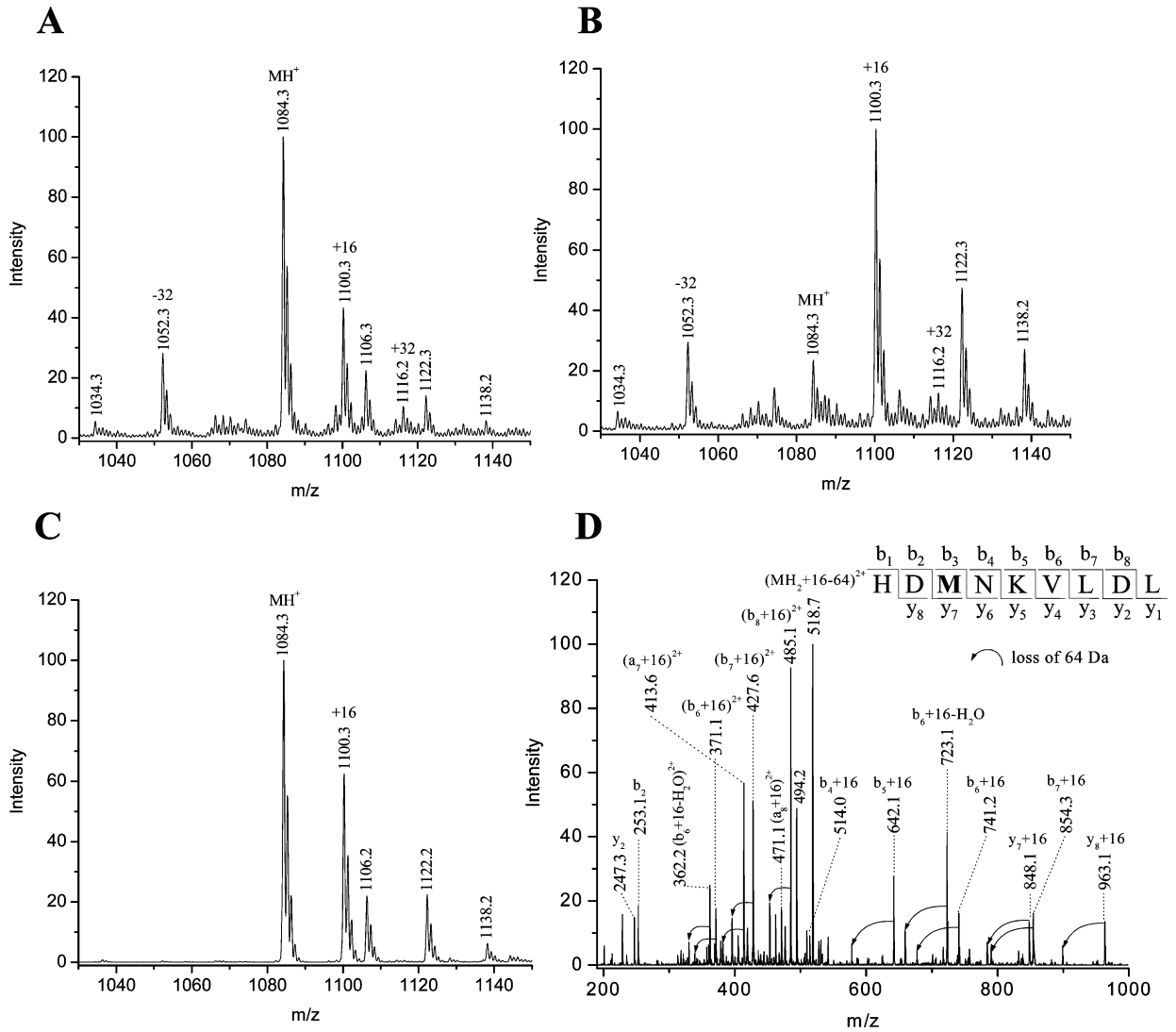

Figure 1. Positive ESI-MS spectra of 40 μM antiflammin-2 (HDMNKVLDL) in water exposed to γ-rays for 12 min, MS run within 30 min after irradiation (A) and run after freezing at −20 °C and storing for 44 h (B). Positive ESI-MS spectrum of 40 μM antiflammin-2 added to water that was first exposed for 8 min and frozen at −20 °C for 44 h (C). MS/MS spectrum of $(MH_2 + 16)^{2+}$ ion observed at *m/z* 550.2 (D).

The secondary oxidation of methionine was also demonstrated for peptides LWMRFA and Ac-KIFMK-NH_2, which were added to irradiated water, frozen, kept at −20 °C for 1 day, and analyzed by mass spectroscopy (data not shown). Similar to the case of antiflammin-2, the only significant oxidation product is the +16 Da species, and the low-energy CID MS/MS spectra indicate that the oxidation is exclusively located at Met residue. Even though aromatic residues such as Trp are highly reactive, secondary oxidation[33] was not found to any significant degree for reactive residues such as Trp, Tyr, Phe, and Leu.

Cysteine. Cys is another reactive residue susceptible to oxidation by mild oxidizing agents. Peptide QNCPRG-NH_2 was exposed to γ-rays for 8 min. One sample was analyzed by ESI-MS within 30 min after exposure, and the positive ESI-MS spectrum is displayed in Figure 2A. Another sample was frozen and kept at −20 °C for 44 h and then analyzed by ESI-MS, and the positive ESI-MS is shown in Figure 2B. The peak at *m/z* 673.2 corresponds to the unmodified peptide, while the peak at *m/z* 672.3 belongs to the doubly charged peptide dimer generated via the formation of a disulfide bond. The peaks at *m/z* 705.2 and 721.1 correspond to oxidation products with +32 and +48 Da due to oxidation of Cys to sulfinic and sulfonic acids,[19] In addition, the peaks at *m/z* 687.2, 689.2, 703.2, 735.1, and 737.0 corresponding to +14, +16, +30, +62, and +64 Da adducts, are likely due to the additional oxidation of other residues such as Pro or Arg. Compared to the sample examined soon after irradiation, the sample analyzed after 44 h at −20 °C contained more dimer product as shown in the spectrum in Figure 2B. The data indicate that significant secondary reactions of Cys take place after irradiation leading to the formation of disulfide bonds.

The secondary reactions of Cys are also demonstrated by simply adding the same peptide to water first irradiated by γ-rays for 8 min. The positive ESI-MS spectra of the sample kept at −20 °C for 44 h is shown in Figure 2C. Strong signals of doubly charged peptide dimer due to formation of disulfide bond were found, but only trace amounts of +32 and +48 Da signals due to the formation of cysteine sulfinic and sulfonic acids were present. The formation of the negatively charged sulfinic and sulfonic acids at the

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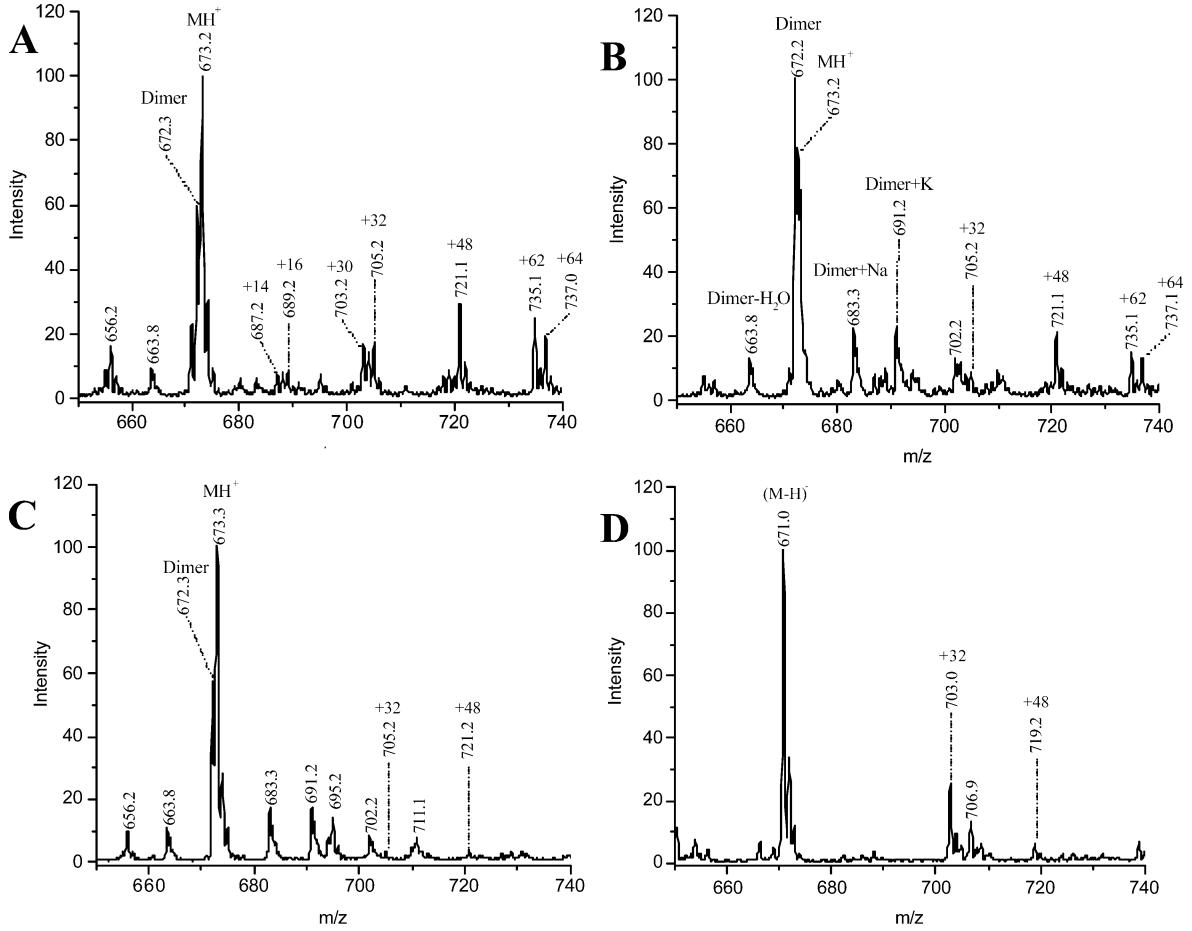

Figure 2. Positive ESI-MS spectra of 40 μM QNCPRG-NH_2 in water exposed to γ-rays for 8 min, MS run within 30 min after irradiation (A) and run after freezing at −20 °C and storing for 44 h (B). Positive (C) and negative (D) ESI-MS spectra of 40 μM QNCPRG added to water first exposed for 8 min and frozen at −20 °C for 44 h.

cysteine side chains is expected to decrease the ionization efficiency in positive electrospray; however, it will significantly increase the ionization efficiency in negative electrospray. Therefore, the sample was also analyzed by negative ESI-MS, and the spectrum is shown in Figure 2D. In the negative spectrum, the peptide dimer is not present here because it was seen only in its singly charged state. The two ions observed at m/z 703.0 and 719.2 correspond to +32 and +48 Da mass shifts from the oxidation of Cys to sulfinic and sulfonic acids; this is supported by the MS/MS data (not shown). The data indicate that Cys is susceptible to secondary oxidation after irradiation generating disulfide bonds as primary products as well as sulfinic and sulfonic acids as minor products.

The secondary oxidation of Cys to form disulfides is sensitive to sequence, steric limitations, sample concentration, O_2 concentration, pH, and temperature. A tripeptide, GCG, was added to water irradiated by γ-rays for 12 min, frozen, and kept at −20 °C for 2 days, and then analyzed by negative ESI-MS (data not shown). Significant amounts of peptide dimer due to the formation of disulfide bond were found, but no +32 and +48 Da oxidation products corresponding to sulfinic and sulfonic acids were observed (even in negative ESI-MS). The data further confirmed that the disulfide is the primary product for the secondary oxidation of Cys.

A peptide containing an internal disulfide bond, CIIRNCPKG-NH_2, was tested for secondary oxidation by addition to irradiated water; the sample was kept at −20 °C for 2 days and then analyzed by ESI-MS (data not shown). No oxidation was found.

Other Residues. Other amino acid residues including Trp, Tyr, Phe, His, and Leu were also tested for any possible secondary oxidations. Small peptides, including YPFVEPI, GWG, GYG, GFG, GHG, and GLG were added individually into water irradiated by γ-rays for 8−18 min, frozen, and kept at −20 °C for one to several days and then analyzed by ESI-MS. No oxidation was found for these peptides. The data suggest that these residues are not susceptible to secondary oxidation to any measurable degree in typical footprinting conditions, even though some of them could be oxidized by H_2O_2 under harsher condition.

Elimination of Secondary Oxidation of Methionine Residues by Adding Catalase or Methionine Buffer. As discussed above, the primary targets for the secondary oxidation are Met and Cys residues, and the primary oxidizing species is expected to be H_2O_2. The result is consistent with previous research on oxidation with H_2O_2,[21,22] where methionine was seen to be more reactive than any other residues to H_2O_2 and aromatic residues are resistant when present in peptides. One plausible approach is to remove peroxides and other mild oxidizing species using reversed-phase extraction (i.e., Zip-Tips or Sep-Paks) immediately after radiolysis. However, this will inevitably lead to loss of sample, and radical species generated on proteins will not be removed by such a procedure.

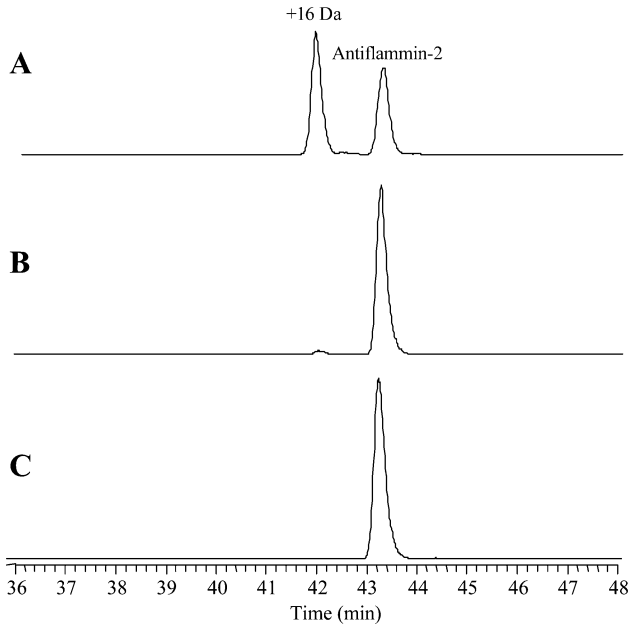

Figure 3. TIC of antiflammin-2 added to 10 mM sodium cacodylate pH 7.0 buffer first irradiated by γ-rays for 8 min, frozen, and kept at −20 °C for 2 days: (A) control; (B) 0.14 unit of catalase added with peptide; (C) 10 mM Met-NH_2 added with peptide.

There are at least two additional approaches to reduce or prevent secondary oxidation. One is to add catalase, an extremely efficient enzyme that rapidly decomposes H_2O_2 at neutral pH and room temperature.[34] A disadvantage is that it introduces an extraneous protein into the sample. Another method to prevent peptide oxidation would be to add a reductant after irradiation and maintain a sufficient concentration throughout sample treatment and storage to compete for most of the oxidative species. We hypothesized that methionine in amino acid form might be a suitable reductant for consuming the oxidizing agents in the sample solution. Either methionine (Met-OH) or methionine amide (Met-NH_2) can be used, the only difference is that Met-NH_2 solution is easier to prepare than Met-OH because of its higher solubility in water.

The efficiency of catalase and methionine to suppress the secondary oxidation was tested using antiflammin-2 as model peptide. A stock solution of 200 mM methinoine amide (Met-NH_2) was prepared, and the pH was adjusted to 7.0 using NaOH. The peptide along with Met-NH_2 or catalase was added to sodium cacodylate buffer (10 mM, pH 7.0) first irradiated by γ-rays for 8 min to a final concentration of 40 μM peptide with 10 mM Met-NH_2 or 0.14 unit of catalase. One unit of catalase is able to decompose 1 μmol of H_2O_2 per minute at pH 7.0 and 25 °C. The samples were frozen and kept at −20 °C for 2 days and then analyzed by LC/MS. The control sample was subjected to an identical procedure with the same amount of buffer instead of Met-NH_2 or catalase added. The TIC curves are displayed in Figure 3A−C for the control and the samples with catalase and Met-NH_2 added, respectively. The peaks with retention time at 43.2 and 41.9 min correspond to the unmodified peptide and +16 Da oxidation products, respectively. From an analysis of the TIC peak areas, ~56% of the peptide sample was oxidized (with +16 Da mass shift) if no Met-NH_2 buffer or catalase was added as shown in Figure 3A. However, in the presence of 0.14 unit of catalase as shown in Figure 3B, the oxidation was significantly reduced such that only 2.5% peptide was oxidized. This result supports the above prediction that H_2O_2 is the primary species responsible for the secondary oxidation; catalase can efficiently suppress most of the secondary oxidation of Met-containing peptides. An even better suppression of secondary oxidation is observed by adding Met-NH_2; in this case, no secondary oxidation was detected. Met-NH_2 or Met-OH is particularly preferred to catalase such that catalase peptides will not be introduced into the LC/MS analysis of protein samples.

The effect of secondary oxidation on the quantitative analysis of peptide oxidation rates and the efficiency of methionine buffer for suppressing the secondary oxidation products were demonstrated using antiflammin-2 (HDMNKVLDL) as a model peptide. Three different dose−response experiments were carried out using γ-ray exposure. In the first, a sample containing 39.0 μL of 25.6 μM peptide in 10 mM sodium cacodylate buffer was exposed for times ranging from 1 to 16 min, and then 1.0 μL of 0.4 M Met-NH_2 buffer was added immediately after irradiation to quench the secondary oxidations. In the second experiment, the same amount of buffer was added as a control. Meanwhile, a third dose−response experiment was carried out by exposing 39.0 μL of buffer for same time interval, and then 1.0 μL of 1.0 mM of peptide stock solution was added. Thus, the final peptide concentration for all samples was 25 μM. All samples were frozen in dry ice, kept at −20°C for several days, and then analyzed in parallel by LC/MS. The dose−response curves are presented in Figure 4A, and the oxidation rate constants are listed in Table 1. When secondary oxidation was quenched by adding Met-NH_2, compared to controls, the rate constant of peptide oxidation rate decreases ~86% from 0.10 to 0.013 s^{-1}. For the peptide added to the irradiated water, the oxidation rate constant is ~0.09 s^{-1}, close to that for direct exposure without adding Met-NH_2 for quenching. Moreover, in the presence of secondary oxidation, the data clearly deviate from pseudo-first-order reaction kinetics. However, once the secondary reaction was suppressed by Met-NH_2 after sample exposure, the dose−response curve is first order within the data precision.

The same experiment was performed for another peptide LWMRFA, and the results were shown in Figure 4B and Table 1. Similar to antiflammin-2, the secondary reaction contributed significantly to the total oxidation of the peptide. Elimination of secondary reaction decreased the rate constant of the peptide from 0.025 to 0.015 s^{-1} it also significantly improved the linearity of the dose−response curve increasing both the accuracy and the precision of the observed rate constant.

Reduction of Disulfides, Prevention of Mixed Disulfide Formation, and Reduction of Methionine Sulfoxide. In footprinting research, the irradiated protein samples are typically subjected to proteolysis before analysis by LC/MS and LC/MS/MS. For proteins containing disulfide bonds, the disulfide bonds need to be cleaved for efficient enzymatic digestion and mass spectrometric analysis. For proteins containing only non-disulfide-bound cysteine residues, disulfide bonds are unlikely to be generated by radiolytic oxidation because of steric hindrance. However, during sample treatment (i.e., proteolysis) and storage,

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Table 1. Rate Constants along with R^2 (Coefficient of Determination) of Regression Corresponding to the Two Peptides in Figure 4 and the Four Peptides from Arp2/3 Complex in Figure 5[a]

peptide	no Met-NH_2 rate (s^{-1})	no Met-NH_2 R^2	10 mM Met-NH_2 rate (s^{-1})	10 mM Met-NH_2 R^2	added to exposed buffer rate (s^{-1})	added to exposed buffer R^2
Two Individual Peptides						
HDMNKVLDL	0.10 ± 0.02	0.91	0.0134 ± 0.0005	0.99	0.09 ± 0.01	0.93
LWMRFA	0.025 ± 0.002	0.96	0.0145 ± 0.0004	0.99	0.016 ± 0.001	0.94
Four Peptides from Protein Arp2/3 Complex						
54−65	16 ± 3	0.27	1.4 ± 0.1	0.92		
300−316	18 ± 4	0.31	4.8 ± 0.4	0.85		
79−106	1.1 ± 0.1	0.94	1.2 ± 0.1	0.93		
199−209	1.5 ± 0.1	0.94	1.3 ± 0.1	0.89		

[a] The data were obtained as described in the Experimental Section.

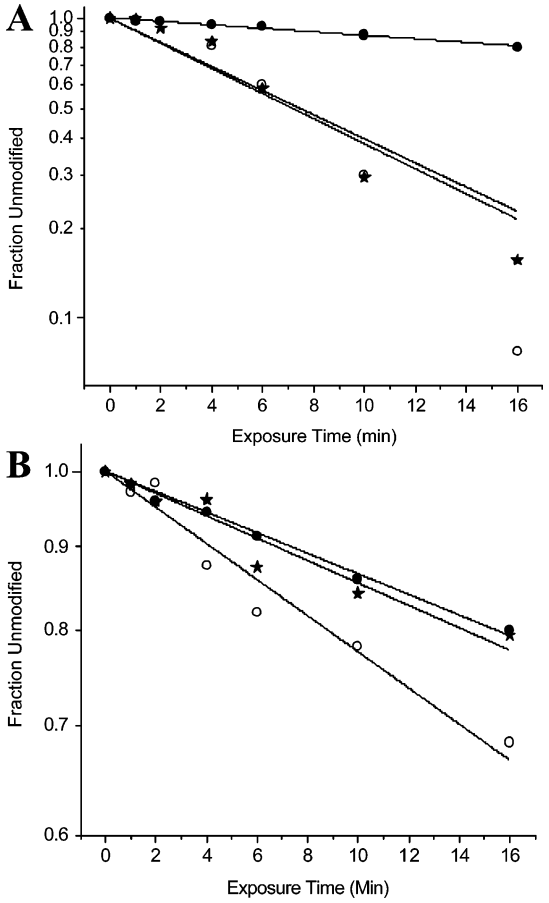

Figure 4. Three different dose−response experiments for peptides antiflammin-2 (A) and LWMRFA (B) carried out in 10 mM sodium cacodylate pH 7.0 buffer. Peptides exposed to γ-rays for different time intervals with (solid circles) or without (open circles) addition of 10 mM Met-NH_2 immediately after exposure. Peptides were also added to buffer first irradiated for different time intervals (stars). Peptide concentration was 25 μM for all samples. The results of fitting to first-order reaction kinetics are listed in Table 1.

cysteine residues in different peptides may cross react, allowing the formation of disulfides and thiol−disulfide exchange to take place. The neutral or slightly basic pH used for enzymatic digestion also allows thiol−disulfide exchange via thiolate displacement to occur easily.[35] As a result, those cysteine-containing peptides expected from the enzymatic cleavage could be missed.

Table 2. Reduction of Mixed Peptides Containing CIIRNCPKG-NH_2 (Internal Disulfide) and Met(O)-antiflammin-2 (HDM(O)NKVLDL) by dTT and TCEP[a]

	antiflammin-2 (reduced)	CIIRNCPKG-NH_2 (reduced)
no reducing agent	0.9	0.0
10 mM dTT	0.9	82.8
10 mM TCEP	0.8	98.7

[a] Shown as percentages of the reduced forms observed.

A logical approach to simplify the analysis is to thoroughly reduce any disulfides using a sufficient amount of reducing agent and then alkylate all of the cysteine residues.

A typical sulfhydryl-containing reducing agent, dTT, and a nonsulfhydryl reducing agent, TCEP, were tested for their efficiency to reduce the disulfide bonds. In three Eppendorf tubes, 20 μL of mixed peptides solution containing 40 μM Met(O)-antiflammin-2 and 40 μM CIIRNCPKG-NH_2 (internal disulfide) in 10 mM pH 7.0 sodium cacodylate buffer, with 10 mM dTT or TCEP added or nothing added for control, was heated at 95°C for 5 min and then diluted 5-fold with buffer and kept at 37 °C for 15 h. The treated samples were then analyzed by LC/MS, and the percentages of reduced antiflammin-2 and reduced CIIRNCPKG-NH_2 were calculated by TIC of related mass spectroscopic signals and shown in Table 2.

The peptide with an internal disulfide, CIIRNCPKG-NH_2, had 99% of disulfide reduced by TCEP, while only ~83% was reduced by dTT. The data indicate that TCEP is more efficient than dTT for reducing disulfide bonds at neutral pH. The reducing efficiency of thiols is pH dependent and generally low at pH values well below the pK_a of thiol (pK_a ~9.3 for dTT), because thiols react as thiolate anions.[36] TCEP is also preferred to dTT and other thiol-containing reducing agents as it is nonvolatile, odorless, and of low toxicity; it is highly selective for disulfides and unreactive toward alkylating reagents, so reductions can be carried out simultaneously with alkylation; it is stable in solid state in air for several months, and its dilute solution in air shows no appreciable oxidation up to at least 72 h at pH <7.7. Thiol reductants react

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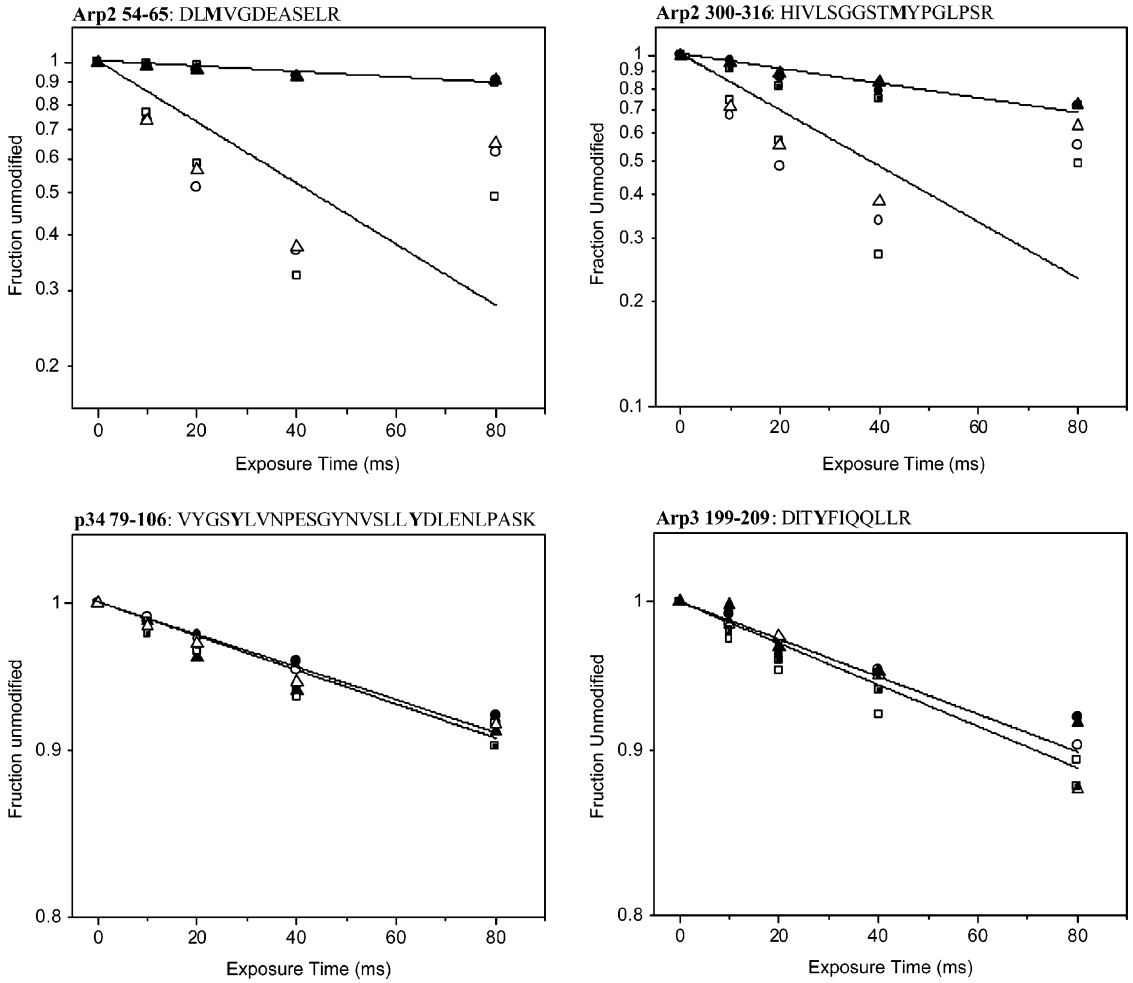

Figure 5. Effect of secondary oxidation to four selected peptides in synchrotron footprinting of protein complex Arp2/3. The four peptides are (A) Arp2 54−65 (DLMVGDEASELR), (B) Arp2 300−316 (HIVLSGGSTMYPGLPSR), (C) p34 79−106 (VYGSYLVNPESGYNVSLLYDLENLPASK), and (D) Arp3 199−209 (DITYFIQQLLR). The experiment was carried out with (solid symbols) and without (open symbols) adding 10 mM Met-NH_2 immediately after exposure, and three replicates were performed for each exposure time. The results of global fitting of the three replicates to first-order equation are listed in Table 1.

with air and alkylating agents, so a higher concentration is needed. TCEP also obviates any possible disulfide formation between sample and reducing agents.[37]

Oxidation of Met to sulfone is irreversible; however, it was reported that methionine sulfoxide can be reduced back to methionine by methionine sulfoxide reductase as well as strong reducing agents.[25−27] In footprinting experiments, the irradiated protein samples are subjected to enzymatic digestion before analysis by LC/MS, and strong reducing agents such as dTT or TCEP are used to reduce the disulfide bonds and denature the proteins. As shown in Table 2, neither dTT nor TCEP reduced Met(O)-antiflammin-2 back to antiflammin-2 to a measurable extent. Appreciable reduction of sulfoxide demands strong reducing agents at high concentration and with prolonged treatment such as 0.75 M dTT for 72 h.[26] It is concluded that methionine sulfoxide is refractory to reducing agents such as dTT and TCEP in the typical conditions used for protein denaturation and enzymatic digestion.

Enhancement of Quantitative Protein Footprinting of Arp2/3. The Arp2/3 complex is a stable assembly of two actin-related proteins (Arp2, Arp3) and five additional protein subunits (ARPC1 p40, ARPC2 p34, ARPC3 p21, ARPC4 p20. ARPC5 p16). It nucleates branches in actin filaments,[38,39] and is a central player in controlling eukaryotic cell motility.[40] The complex is activated by binding activated WASp/Scar proteins or other nucleation-promoting factors and mediated by preexisting actin filaments. Since crystallographic data are available only for the inactive Arp2/3 complex,[41] we have used footprinting to probe the structure and dynamics of the activated assembly. More than 200 tryptic peptides, covering 80% of the sequence in Arp2/3 complex, were examined by nanospray LC/MS analysis. Since there are 50 methionine residues present in the complex, Met represents a large fraction of our initial probes, and quantification of methionine oxidation is thus crucial for the success of the investigation.

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The impact of secondary oxidation to the quantitative analysis of the peptide modification and the successful suppression by methionine buffer to improve the quantitative footprinting of Arp 2/3 complex are demonstrated using four peptides. Two peptides, Arp2 54−65 (DLMVGDEASELR) and Arp2 300−316 (HIVLSGGSTMYPGLPSR), contain Met residues, while another two peptides, p34 79−106 (VYGSYLVNPESGYNVSLLYDLENLPASK) and Arp3 199−209 (DITYFIQQLLR), do not contain Met but have other highly reactive residues such as Tyr and Phe. Other Met-containing and non-Met-containing peptides behave similarly to these four typical peptides and their data are not presented here. Experiments in triplicate were performed for the cases of adding or not adding Met-NH_2 immediately after exposure. The dose−response curves are displayed in Figure 5, and the rate constants along with the R^2 value are listed in Table 1. The three open symbols represent the data points without adding Met-NH_2 buffer after exposure, while the three solid symbols represent the data points with Met-NH_2 added after exposure.

Two distinct phenomena are evident for the Met-containing peptides, indicating that the elimination of secondary oxidation results in significantly improved quantitation. First, addition of Met-NH_2 improves the data linearity, significantly improving the regression coefficient. In the case of no Met-NH_2 added, the apparent reaction does not follow the pseudo-first-order kinetics; in fact for the 80-ms data point, a *reduction* in the extent of modification is seen with increasing exposure. Once the secondary reactions are quenched by Met-NH_2, the dose−response curves reflect only the limited primary radiolytic oxidation events and thus are adequately fit with first-order kinetic equations. The improved regression is reflected clearly in the error of rate constants, which decreases from ±3 to ±0.1 s^{-1} for Arp2 peptide 54−65, which has an error of 19% in the first case and 7% in the second case. The error is reduced from ±4 (22%) to ±0.4 s^{-1} (8%) for Arp2 peptide 300−316. The R^2 value of the fitting increases from 0.27 to 0.92 for peptide Arp2 54−65 and from 0.31 to 0.85 for peptide Arp2 300−316 in the presence of Met-NH_2 quencher. Second, the addition of Met-NH_2 quencher suppresses the overall oxidation rate as the rate constants decrease from 16 to 1.4 s^{-1} for Arp2 54−65 and from 18 to 4.8 s^{-1} for Arp2 300−316.

The two non-methionine-containing peptides, p34 79−106 and Arp3 199−209, do not vary in their observed oxidation rates or regression statistics by virtue of the addition of Met-NH_2, as shown in Figure 5C and D and Table 1. The results confirm the peptide data that the other highly reactive residues, including Trp, Tyr, Phe, His, and Leu, are generally not susceptible to secondary oxidation after irradiation. Overall, the relative reactivity of the peptide oxidation data for Arp2/3 is consistent with the crystallographic data for the inactive complex; the data in the presence of quencher more accurately reflect the solvent-accessibility differences.

CONCLUSIONS

We have developed a strategy to eliminate the effects of secondary oxidation of sulfur-containing amino acid residues that can take place after irradiation; this greatly improves quantitative footprinting for proteins with sulfur-containing residues. Met is converted to methionine sulfoxide by secondary oxidation, giving rise to misleading information about the solvent accessibility of methionine-containing peptides. The secondary oxidation of Met residues can be suppressed by adding catalase, Met-NH_2, or Met-OH buffer immediately after irradiation. Met buffer is preferred to catalase for not introducing any extraneous protein into the sample. Cysteine residues are primarily converted to disulfide as a result of secondary oxidation. The disulfides can be reduced before mass spectroscopic analysis by reducing agents such as TCEP, while methionine sulfoxide is refractory to reduction under typical reducing conditions. Elimination of secondary reactions significantly improves data accuracy, precision, and reproducibility for determining oxidation rates of sulfur-containing peptides in quantitative protein footprinting experiments.

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