Rapid Online Oxidation of Proteins and Peptides via Electrospray-Accelerated Ozonation

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ABSTRACT: When combined with mass spectrometry, solution-phase labeling such as oxidative footprinting, hydrogen/deuterium exchange, and covalent labeling are powerful tools for the analysis of protein conformation. The throughput of these workflows, however, is frequently limited due to their reliance on slow labeling reactions, proteolysis, and chromatographic separations to be fully realized. Here, we present an ozone-driven oxidation reaction that occurs on the microsecond time-scale during the electrospray ionization (ESI) process. Selective oxidation of methionine and tryptophan residues in peptides and proteins occurs spontaneously upon the introduction of ozone into the ESI chamber. Trp and Met residues are frequently buried in folded proteins and thus, when applied to natively folded cytochrome C and carbonic anhydrase, little oxidation is observed. When these proteins are denatured and ozonated, a dramatic increase in the number of oxidation events and yield is measured. This methodology’s applicability to any instrument equipped with an ESI source, facile interpretation of results, limited sample handling, high-throughput nature, and rapid labeling reaction makes this technique a promising new tool for the analysis of protein folding.

Mass spectrometry (MS) has become one of the prevalent methods for profiling protein identity and post-translational modification; but, as typically implemented is ignorant of protein conformation. An approach developed to overcome this limitation leverages solution-phase labeling of protein residues to encode the solvent accessibility and higher-order structure of the protein into its mass.1–7 MS is then utilized to assign the location of the modification. Due to their low sample requirements, rapid implementation, and applicability to proteins in membranes, disordered states, and complexes, these chemical labeling techniques have found a niche alongside the modern high-resolution structural techniques and have been widely utilized to address a diverse set of protein systems.8–19

The reagents utilized in these workflows and hence amino acid selectivity, reaction rates, and necessary considerations can vary widely.1,2,16–18,20–22,3,8,12,13 A concern of many labeling-based technologies is the influence derivatization has on the conformation of the protein. This is especially pertinent with these technologies, as many implementations generate multiply derivatized species and utilize reaction durations in the second to minute timescales. When multiple sequential reactions occur over times greater than protein conformational rearrangements (low millisecond), subsequent labeling events may reflect nonnative conformations. Consequently, for these relatively slow reaction times, great care must be taken to ensure that labeling is reflective of native structure.7 To bypass this limitation, rapid labeling technologies have been developed that can assess protein conformation in sub-millisecond timescales.13,23,24 The most frequently implemented version of this technology utilizes hydroxyl radicals generated via the homolysis of hydrogen peroxide. While powerful, these approaches require complex microfluidic setups, an electromagnetic radiation source, and low concentrations of hydrogen peroxide in solution, potentially perturbing the protein prior to analysis. These constraints hinder the widespread implementation of these techniques, and thus, the development of new rapid labeling technologies is needed.

In this work, we demonstrate that when introduced into an electrospray ionization (ESI) source, ozone promotes the oxidation of proteins and peptides. This oxidative labeling is selective towards tryptophan and methionine residues. We also find that the labeling patterns are selective to protein conformation. This is particularly significant as the labeling must occur prior to ion desolvation and the desolvation of protein ions is thought to occur in the μs timescale.25 This rapid chemical labeling strategy requires no complicated sample handling, lasers, or microfluidic systems and represents a facile alternative to other rapid labeling technologies.

Experimental Section

Materials and Reagents

Mass spectrometry-grade methanol, water, formic acid, ammonium acetate, collagen binding fragment (CBF, CQDSETRTFY), and substance P (RPKQQFFGLM-NH2) were all acquired from Fisher Scientific (Waltham, MA). Carbonic anhydrase, glu-fibrinopeptide B (EGVNDNEEGFFSAR), and cytochrome C were procured from Sigma Aldrich (St. Louis, MO). angiotensin I (DRVYIHPFHL), ACTH 1-14 (SYSMEHFRWKPGV), amyloid β 10-20 (YEHHQKLVFF), and melittin
(GIGAVLKEFTLGALISW1KRRQGQ-NH₂) were attained from BaChem (Bubendorf, Switzerland).

Mass Spectrometry

All experiments were performed on a Bruker timsTOF (Billerica, MA). Peptide and protein samples were diluted to between 1 and 5 μM in either 49.5/49.5/1 Methanol/water/formic acid or 100 mM ammonium acetate and water prior to ionization via an electrospray ionization source. The sample was directly infused into the source at 3-5 μl/min. The capillary voltage nebulizing gas, dry gas, and dry gas temperature were set to 4000 V, 400 V, 0.9 bar, 4 l/min, and 200 °C, respectively. Tunnel-in pressure was set between 2.5 and 1.5 mbar and the funnel RF was set to 350 Vpp. Tandem mass spectrometry experiments were conducted using a 5 Th isolation window and analyzed manually using Protein Prospector (https://prospector.ucsf.edu/prospector/mshome.htm). CIU experiments were performed with a tunnel-in pressure of 1.5 mbar and an accumulation time of 70 ms. For carbonic anhydrase a D6 value of 90 V was chosen to remove interference from the acetate-bound species and adjusted to 150 V to induce unfolding.

Ozonation

Ozone was generated with an A2Z ozone (Louisville, KY) A2ZS-6GLAB and directly introduced into the ionization chamber orthogonally to the nebulizing gas (Scheme S1). The source 99.5% oxygen tank was set to 2 bar and the flowrate was controlled via the included the flow meter to 1 l/min. This generator is equipped with a variable ozone control dial that controls the field strength applied to the corona discharge tube and is adjustable from 0-100%. Ozone is toxic and thus, several precautions were taken. A custom fitting was fabricated enabling direct fastening to the ESI spray chamber. The source chamber was thoroughly sealed and all excess nebulizing gas and ozone was directed through an ozone destruct (Simply 03, Grand Ledge, MI) prior to being vented from the room. Additionally, Moldex N95 AirWave respirators were acquired from Fisher Scientific for all researchers.

Accessibility of individual residues was calculated using GETAREA (http://curie.utmb.edu/getarea.html) using a probe radius of 1.5 Å.26 PDB accession codes 1HRC and 1V9E were used for cytochrome C and carbonic anhydrase, respectively.

Results and Discussion

Ozonation of peptides and proteins is typically implemented in aqueous solvents over long reaction times. These studies have identified ozone as a relatively promiscuous labeling reagent and oxidation of cysteine, tryptophan, methionine, histidine, tyrosine, and phenylalanine residues have been achieved.27-28 Contrastingly, gas-phase ozone reactions are typically restricted to alkenes and the interconversion of lipid double bonds29-30 as only deprotonated cysteine residues have been documented to have any reactivity with gaseous ozone.31 Recent work by Kim et al. demonstrated oxidation of peptides on the second timescale within a suspended droplet in a field-induced droplet ionization (FIDI) source.32 In this previous work, a droplet was suspended from a capillary, exposed to ozone for 0-30 s, and then an electric field was pulsed to induce ionization. This droplet-based labeling demonstrated selectivity towards tryptophan (Trp) and methionine (Met) residues but was unable to demonstrate peptide labeling in reaction times less than 5 seconds.32

An electrospray ionization (ESI) source has several advantages that may accelerate this droplet-based labeling. These advantages predominately stem from the fact that ESI ionization methods progress through much smaller, droplets. The increase in number and reduced diameter of these droplets plays two important roles, 1) the surface area by which ozone can traverse the air/liquid interface is increased compared to the single large-volume droplet found in a FIDI source; 2) droplet volume rapidly decreases with radius, and thus, ozone or its degradation products must navigate shorter distances to encounter a reaction partner. Lastly, the increased proton concentration found in positive-ion mode electrospray droplets may reinforce the selectivity in the FIDI source as selectivity towards Trp and Met in bulk solution has been shown to increase at low pH.33

Ozonation of Peptides

Ozone output is determined by two major factors, the source gas (99.5% oxygen) flow rate and the electric field strength applied to the corona discharge reactor. To examine if the addition of ozone was sufficient to promote the oxidation of peptides, we coupled the ozone generator directly to the electrospray source of a Bruker timsTOF (Scheme S1). The oxygen flow rate was set to 1 l/min and directed orthogonally to the nebulizing gas. For all experiments the capillary voltage, nebulizing gas, and dry gas flow rate of the electrospray source were set to 4000 V, 0.9 bar, and 4 l/min, respectively. A series of peptides were then directly infused into the ESI source with oxygen or increasing concentrations of ozone.

When the corona discharge field strength is powered off and only oxygen is introduced into the ionization chamber, no oxidation of angiotensin I (DRVYIHPFHL, ACTH 1-14 (SYSMEHFRWKGVPV), glu-Fib (EGVNDNEEGFFSAR), collagen binding fragment CB2 (CQDSERTRFY), substrate P (RPKPQQFGLML-NH₂), amyloid β 10-20 (YEVHHQKLVF), and melittin (GIGAVLKEFTLGALISW1KRRQGQ-NH₂) is detected for angiotensin I, CBF, Amyloid β, ACTH 1-14, and glu-Fib (Figures 2 and S1). This is in agreement with previous work that demonstrated oxygen must be coupled with higher electrospray potentials to promote extensive oxidation of peptide and protein ions.34,35 On the A2ZS-6GLab ozone generator utilized for this work, field strengths below 30% are insufficient to generate a corona discharge, therefore, when these peptides are subjected to ozonation, no oxidation occurs until settings rise above 30% (Figure 2). Once above 30%, systematically increasing the ozone concentration promotes steady increasing oxidation of substance P, ACTH 1-14, and melittin. The extent of oxidation begins to plateau at field strengths above 60% (Figure 2). To ensure the greatest sensitivity, a field strength of 80% will be utilized for all following experiments.

Even at these highest ozone concentrations, no oxidation is detected for angiotensin I, CBF, Amyloid β, and glu-Fib (Figures 2 and S1). In contrast, when substance P, melittin, and ACTH 1-14, are directly infused at these high ozone levels, each display a unique oxidation pattern (Figure 1). Substance P undergoes a single oxidation event with a maximum efficiency of 15 ± 2% (Figures 1 A). ACTH 1-14 and
melittin undergo much more significant oxidation and acquire up to three oxygen atoms with 52 ± 5% and 57 ± 3% efficiency, respectively (Figures 1 and 2). The oxidation patterns demonstrated by ACTH 1-14 and melittin are distinctive, with the doubly and triply oxidized melittin and singly and triply oxidized ACTH 1-14 being the most abundant species for each (Figure 1 B and C).

To identify the basis of these oxidation patterns, we subjected each peptide to tandem mass spectrometry (MS/MS). Collisional activation of the singly oxidized substance P enabled assignment of the oxidation event to the Met residue (Figure S2). This spectrum also demonstrates the characteristic loss of methanesulfenic acid (64 Da) a further indicator of Met oxidation (Figure S2).36 When the doubly and triply oxidized species of melittin are sequenced, all oxidation events are assigned to the Trp residue (Figure S3). The observed labeling profile here resembles that found when Trp was exposed to ozone in a FIDI source.32 They found that ozonation of Trp largely resulted in the formation of a doubly and triply oxidized species indicating the formation of N-formylkynurenine (NFKyn) and hydroxy-N-formylkynurenine (HFKyn), respectively.32

ACTH 1-14 contains both Met and Trp residues and MS/MS of the singly oxidized species assigns the oxidation event predominately to the methionine residue but the existence of oxidized y6, y7, y8, y9, and y10 product ions indicates that singly oxidized tryptophan was generated as a minor product (Figure S4). When the triply oxidized species is examined, two oxygens are directly assignable to the Trp residue, and the final oxygen is again primarily located on Met4 (Figure S4). The presence of an unoxidized y11 in both the singly and triply oxidized MS/MS spectra provides evidence for a minor Ser3 oxidation product (Figure S4). This is somewhat surprising as no oxidation was observed on serine residues in glu-Fib, CBF, and melittin. Covalent labels can be sensitive to microenvironments, undergo gas-phase molecular rearrangement, or be lost as a neutral and this may be an example of any one of these processes.37-41 With the exception of this serine residue, these results indicate that the selectivity displayed in the FIDI source is retained in this accelerated ozonation process.32

To examine the dependence of this labeling technology on proton concentration, we examined a mixture of ACTH 1-14 and substance P in the absence of formic acid and in negative-ion mode. When ionized from a solution of 50/50 methanol/water the oxidation patterns of ACTH 1-14 and substance P cations closely resembles that generated from solutions containing formic acid (Figure S5). In contrast, anions generated from the same solutions do demonstrate significant alterations in oxidation profiles (Figure S6). In negative-ion mode, the total oxidation level of ACTH 1-14 is increased. This is primarily driven by the increased abundance of the doubly oxidized species (Figure S6 A). Alterations of substance P oxidation patterns are more subtle with the only change being the generation of a low abundance doubly oxidized species (Figure S6 B). While additional work is needed to examine the basis of these polarity-driven labeling patterns, the retention of labeling selectivity in positive-ion mode in the absence of formic acid, enables the application of this labeling technique to proteins ionized from native conditions.

Ozonation of Proteins

The selective labeling of Trp and Met residues observed in peptides is particularly intriguing as these residues are buried in many natively folded proteins and their labeling may be indicative of protein conformational integrity. To examine this potential, we dissolved cytochrome C in 100 mM ammonium acetate and 49.5/49.5/1 water/methanol/formic acid, directly infused them into the ESI source, and subjected each to ozonation. Cytochrome C has one Trp
and two Met residues. Met80 is the most exposed (33 Å²) and the only Met/Trp residue not indicated as buried by GETAREA (Table S1 and Figure 3). If the selectivity displayed in peptides persists, we anticipate only minor labeling of a natively folded protein and an increase in oxidation as the protein is denatured and buried Trp and Met residues become exposed.

When cytochrome C is ionized from ammonium acetate, the 8 and 7+ charge states are predominately generated (Figure S7). Conversely, ionization from 49.5/49.5/1 water/methanol/formic acid generates a charge envelope spanning from the 7 to the 17+ charge state (Figure S8). Cytochrome C undergoes significant structural perturbations when subjected to decreased pH and organic solvents42,43 and these differences in charge envelopes are likely reflective of the differential electro spray mechanisms of properly folded globular proteins and unfolded chains. Globular proteins are hypothesized to be subject to the charge residue model (CRM) and will be sequestered in the center of the droplet. Unfolded protein chains are subject to the chain ejection model (CEM) and will spend increased time near the liquid/air interface.25 Any conformational selective labeling observed in this work may reflect both solvent accessibility and these differential ESI mechanisms. While this technique offers an exciting opportunity to gather unique insights into the mechanisms driving the electrospray ionization of large biomolecules, that will be explored in future work. To minimize the influence of ionization mechanisms, this work will focus on a single charge state for each protein.

The 8+ charge state is highly abundant in both spectra and was recently proposed to be subject to CRM at both neutral and acidic pH.44 Therefore, the ozonation pattern of that charge state following ionization from both solutions was compared. When exposed to ozone, a single low-yield oxidation event is observed with the natively folded protein (Figure 4 A). This is dramatically different than the much higher yield and five oxidation events observed from the denatured protein (Figures 4 B).45 While top-down sequencing is necessary to assign the modification to a specific residue, four additional oxidation events is the expected result of increased Trp59 and Met65 accessibility.

While examining total oxidation levels enables facile identification of loss of structure, The differential labeling behavior of methionine and tryptophan residues enables the identification of what residue is exposed. To explore this, we utilized carbonic anhydrase which has seven Trp and three Met residues. The methionine residues are completely buried according to GETAREA (Table S2). The Trp residues are also largely buried, with Trp243 (33 Å²) being the only residue with greater than 20 Å² of accessible surface area. If this or the other Trp residues are accessible, the distinctive labeling pattern of Trp will be facilely identifiable.

When this protein is dissolved in 100 mM ammonium acetate in water or 49.5/49.5/1 water/methanol/formic acid and subjected to online ozonation, we again see dramatically different behavior between the two solution conditions. Analysis of the native spectrum is complicated by salt adducts but a low abundance, triply oxidized species is observable (Figure 5) and represents the oxidation of a single Trp residue. The denaturing conditions, however, are sufficient to completely disrupt the protein conformation and displace the catalytic zinc (Figure S9). Ozonation of this species generates a complex series of overlapping products, reflecting numerous exposed Trp and Met residues (Figure S9). Reduction of the methanol and formic acid content to 25 and 0.5%, respectively restores the catalytic zinc and reduces the generated oxidized species to an addition of three and six oxygens, representing the oxidation of tryptophan residues (Figure 5).
observed peptide ozonation occurs within the droplet or at the air/liquid interface.32

Figure 5. Mass spectra of native carbonic anhydrase prior to (top) and following ozonation (Middle). Bottom is mass spectrum of carbonic anhydrase following ionization from 74.5/25/0.5 water/methanol/formic acid.

To examine if this technology is sensitive to more subtle alterations of protein higher-order structure, we again examined cytochrome C. Cytochrome C is more robust to increased concentrations of methanol at pH values above 4.5.42,49 We dissolved cytochrome C into a series of ammonium acetate solutions with progressively increasing methanol content. When these solutions are ozonated, only minor variation in the oxidation pattern is observable at concentrations up to 50% methanol (Figures S11, 6A and B). Above 50%, there is a dramatic increase in the level of oxidation present (Figures S11, 6A and B). This is predominately driven by the formation of a triply oxidized species (Figures 6A and S11). The labeling patterns displayed at even the highest methanol concentrations are significantly more subdued than that observed at 50% methanol and 1% formic acid (Figures 4B, 6A, and S11). Protein signal dropped precipitously at methanol concentrations above 80% due to poor protein solubility. These results are in agreement with previous experiments that demonstrated minimal alterations of cytochrome C conformation at methanol concentrations up to 50% and pH values above 4.5.49,50

While the total oxidation content enables rapid assessment of protein structure, the unique labeling patterns of Met and Trp enable more subtle analyses. Based on the labeling patterns demonstrated in positive-ion mode, the singly and triply labeled species are principally indicators of Met and Trp accessibility, respectively. If the relative abundance of these peaks is plotted individually, more resolved information can be attained (Figure 6C). The singly oxidized species steadily increases in abundance as methanol concentration increases (Figure 6C). This suggests that one of the methionine residues is exhibiting gradually increased solvent accessibility even at “low” methanol concentrations. The triply oxidized species demonstrates no significant oxidation until 60% methanol, where it undergoes a rapid 15% increase in labeling (Figures 6C and S11). Trp59 is buried near the heme, in the core of the protein (Figure 3). This striking increase in the abundance of the triply oxidized species likely represents the destabilization of the hydrophobic core and displacement of Trp59 from the heme. This correlates with previously acquired solution-phase data that demonstrated a sharp increase in Trp59 fluorescence in 60% methanol.50 The origin of the doubly oxidized species is difficult to ascertain as it could be derived from the direct double oxidation of a Tryptophan or oxidation of the second Met residue. This species also displays an increase in labeling at 60% methanol indicating a reliance on the disruption of the hydrophobic core. To confidently assign the precise location of this oxidation event, future work coupling this labeling technique with an instrument platform capable of robust top-down sequencing is needed. While other modes of denaturation still have to be examined, these results demonstrate that online ozone oxidation is sensitive to protein denaturation similarly to other chemical labeling strategies.17,51–53

Figure 6. A) Mass spectra of ozonated cytochrome C after ionization from a solution with the indicated water/methanol ratio, B) Fraction of cytochrome C oxidized after ozonation and ionization from indicated methanol percent, and C) Abundance of specified oxidized cytochrome C species as a function of methanol content. B and C are baseline corrected. (n=3)

Conclusions

We demonstrate that addition of ozone into an electrospray ionization source promotes the oxidation of methionine and tryptophan residues in peptides and proteins at typical ESI voltages. We further demonstrate that this technique can be utilized to oxidize cytochrome c and carbonic anhydrase in a conformationally selective manner. This is particularly exciting as the most accepted model for the ionization of globular proteins, CRM, is thought to occur faster than protein folding. The unique labeling patterns of tryptophan and methionine residues enable assignment of some oxidation events to specific amino acids in cytochrome C. This technique is a promising alternative to other label-based mass spectrometry workflows due to its applicability to all ESI-based mass spectrometers, ability to rapidly assess protein conformation at sub-protein folding timescales, without the need for complex microfluidic setups, or proteolysis.
ASSOCIATED CONTENT

Supporting Information
A schematic of the instrument source, CID tandem mass spectra of oxidized peptides, mass spectra of ACTH-14 and substance P, tables reporting Met and Trp solvent accessibility in cytochrome C and carbonic anhydrase, mass spectra of proteins prior to and following ozonation, and mobility spectra of carbonic anhydrase can be found in the supporting information. The Supporting Information is available free of charge on the ACS Publications website.

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REFERENCES


