# The Effect of Histidine Oxidation on the Dissociation Patterns of Peptide Ions

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Oxidative modifications to amino acid side chains can change the dissociation pathways of peptide ions, although these variations are most commonly observed when cysteine and methionine residues are oxidized. In this work we describe the very noticeable effect that oxidation of histidine residues can have on the dissociation patterns of peptide ions containing this residue. A common product ion spectral feature of doubly charged tryptic peptides is enhanced cleavage at the C-terminal side of histidine residues. This preferential cleavage arises as a result of the unique acid/base character of the imidazole side chain that initiates cleavage of a proximal peptide bond for ions in which the number of protons does not exceed the number of basic residues. We demonstrate here that this enhanced cleavage is eliminated when histidine is oxidized to 2-oxo-histidine because the proton affinity and nucleophilicity of the imidazole side chain are lowered. Furthermore, we find that oxidation of histidine to 2-oxo-histidine can cause the misassignment of oxidized residues when more than one oxidized isomer is simultaneously subjected to tandem mass spectrometry (MS/MS). These spectral misinterpretations can usually be avoided by using multiple stages of MS/MS ($MS^n$) or by specially optimized liquid chromatographic separation conditions. When these approaches are not accessible or do not work, N-terminal derivatization with sulfobenzoic acid avoids the problem of mistakenly assigning oxidized residues. (J Am Soc Mass Spectrom 2007, 18, 553–562) © 2007 American Society for Mass Spectrometry

Mass spectrometry (MS) is a powerful method for identifying amino acid modifications to peptides and proteins. Such identifications are important in studies of protein post-translational modifications and in techniques that use covalent labeling to study protein structure. An emerging set of methods in the latter category are techniques that rely on oxidative modifications as indicators of protein structure. These methods use radicals (such as ·OH) to modify solvent-exposed [1–10] or metal-bound amino acids [11–18]. Tandem MS (MS/MS), typically in conjunction with proteolytic digestion, is then used to identify oxidatively modified residues and information about protein structure is then derived. A modified amino acid is determined by finding product ions whose *m/z* ratios are shifted from expected values.

Although oxidative modifications often do not change peptide ion dissociation patterns, there are several examples in which they do. Oxidative modifications to cysteine and methionine residues have very noticeable effects on peptide ion dissociation patterns. For example, oxidation of cysteine to cysteic acid in some cases can lead to very selective peptide dissociation and in other cases to more efficient overall peptide dissociation [19–22]. The strong acid character of cysteic acid enhances peptide bond dissociation at its C-terminal side and can allow mobilization of an additional proton that initiates cleavages more efficiently at other peptide bonds as well. Oxidation of cysteine to cysteine sulfinic acid also leads to selective dissociation on the C-terminal side of this residue when the peptide charge state does not exceed the number of arginine residues in the peptide [23, 24]. Methionine oxidation to methionine sulfoxide can also have a dramatic effect on peptide ion dissociation patterns [25–29]. When the number of protons on the peptide does not exceed the number of basic residues, product ion spectra of peptides containing methionine sulfoxide are dominated by a neutral loss of methane sulfenic acid ($CH_3SOH$) [29]. Indeed, in many cases no other sequence information is present, highlighting the effect that this oxidative modification can have.

Whereas cysteine and methionine residues are readily oxidized, other amino acids such as those with aromatic side chains are also susceptible to oxidation [30], and oxidation of some of these residues might affect peptide ion dissociation patterns. During our work with oxidized peptides, we have observed that oxidation of histidine to 2-oxo-histidine can change peptide dissociation patterns in very noticeable ways. Understanding the effect of this oxidative modification

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to histidine on dissociation patterns is important for correctly interpreting tandem mass spectra of peptide ions containing this residue. In general, histidine oxidation is not only important for methods that rely on oxidative modifications as indicators of protein structure, but more broadly speaking oxidative modifications to this residue are commonly found in proteins from cells that have undergone oxidative stress [31–33]. Indeed, oxidation of histidine to 2-oxo-histidine in proteins has been suggested as a good marker of cellular oxidative stress [34]. Thus, any studies that use MS to understand protein modifications associated with oxidative stress are likely to analyze peptides and proteins with 2-oxo-histidine. In this work we describe the effect that this oxidative modification to histidine can have on the dissociation patterns of peptide ions, attempt to understand its cause, and suggest a means of avoiding spectral misinterpretations that are possible when this modification is present in peptides.

## Experimental

### *Materials*

Hydrogen peroxide (30%), formic acid, tris(hydroxymethyl)-aminomethane (Tris), and tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were obtained from EM Science (Gladstone, NJ). Dithiothreitol (DTT), sodium ascorbate, ascorbic acid, copper (II) sulfate, 3-morpholinopropanesulfonic acid (MOPS), ammonium acetate, triethylamine, and tetrahydrofuran (THF) were purchased from Sigma–Aldrich (St. Louis, MO). Acetic acid and HPLC-grade methanol were obtained from Fisher Scientific (Fair Lawn, NJ). Chymotrypsin was obtained from Roche Diagnostics (Indianapolis, IN) and trypsin was obtained from Promega (Madison, WI). Distilled, deionized water was generated with a Millipore (Burlington, MA) Simplicity 185 water purification system.

The peptides angiotensin I (DRVYIHPFHL) and angiotensin II (DRVYIHPF) were obtained from Sigma. The prion peptide Ac-PHGGGWGQ-$NH_2$ was a gift from Prof. Colin Burns of East Carolina University. The peptides VSGFHPSDIEVDLL and VNHVTLSQPK are proteolytic fragments of the protein β-2-microglobulin (β2m), which was obtained from Research Diagnostics (Flanders, NJ). To digest β-2-microglobulin (β2m), a 200 μL solution of the protein (10 μM) buffered at pH 7.4 with MOPS was mixed with 100 μM DTT, 5 μg of chymotrypsin, 5 μg of trypsin, and incubated overnight at 37 °C. The reaction was terminated by changing the pH of the solution to about 2 by the addition of acetic acid. The peptide NVMGHNW is a proteolytic fragment of azurin from *Pseudomonas aeruginosa*, which was obtained from Sigma. A 500 μL solution of azurin (30 μM) at pH 7.4 was digested overnight with 5 μg of trypsin, 5 μg of chymotrypsin, and 10 mM DTT at 37 °C. The reaction was terminated by lowering the pH to about 2 with acetic acid. The peptide HYGKHHQTY is a proteolytic fragment of Fe-superoxide dismutase (Fe-SOD), obtained from Sigma. Fe-SOD (30 μM) was digested in the same manner as azurin, but no DTT was added.

### *Peptide Oxidation*

All the peptides and proteins were oxidized using metal-catalyzed oxidation (MCO) reactions as described previously [14, 15, 18]. β2m, azurin, and Fe-SOD were oxidized at protein concentrations of 20–60 μM in solutions that were buffered with Tris/Tris-HCl or MOPS at 25–100 mM in open microcentrifuge tubes. Total sample volumes were <200 μL. For β2m, an equimolar concentration of copper (II) sulfate was added, whereas azurin and Fe-SOD natively bind Cu and Fe, respectively, so no metal was added. As we described previously [14, 15, 18], each of these proteins is selectively oxidized at the amino acids that bind these metals. Detailed MCO reaction conditions for β2m [15], azurin [14], and Fe-SOD [18] can be found in our previous work. Angiotensin I, angiotensin II, and the prion peptide were oxidized at concentrations between 100 and 500 μM. After adding an equimolar amount of copper (II) sulfate, the MCO reactions were initiated by the addition of ascorbate (10 mM), whereas atmospheric $O_2$ acted as the oxidant. Reactions were stopped by the addition of 1% (by volume) of glacial acetic acid.

### *Peptide Derivatization*

N-terminal derivatization of the peptides was performed using 2-sulfobenzoic acid anhydride. This anhydride was prepared at a concentration of 0.1 M in dry THF just before use. The peptide solution was diluted with triethylamine to a final concentration of 50 mM and an equal volume of the 2-sulfobenzoic acid anhydride solution was added so its final concentration was 50 mM. The mixture was vortexed for 2 min and excess solvent was evaporated under a stream of $N_2$ gas to dryness. The sample was redissolved in water and the peptide was purified using $C_{18}$ zip-tips.

### *Instrumentation*

All mass spectral analyses were performed on a Bruker (Billerica, MA) Esquire LC quadrupole ion trap mass spectrometer. Typically, the needle voltage was kept at 3–4 kV; the capillary temperature was set to 250 °C; 10–60 V was applied to skimmer 1; and the capillary offset voltage was set between 20 and 60 V. For direct injection experiments the sample was delivered at 1 μL/min using a syringe pump. HPLC-MS analyses of the peptides were conducted using an HP1100 (Agilent, Wilmington DE) system with a Zorbax $C_{18}$ column (4.6 × 150 mm; Agilent). The LC effluent was split in a 1:4 ratio with the smaller outlet being fed into the electrospray ionization (ESI) source of the quadrupole ion trap mass spectrometer. For separation of the oxidized an-