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Millisecond Radiolytic Modification of Peptides by Synchrotron X-rays Identified by Mass Spectrometry

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Radiolysis of peptide and protein solutions with high-energy X-ray beams induces stable, covalent modifications of amino acid residues that are useful for synchrotron protein footprinting. A series of 5−14 amino acid residue peptides of varied sequences were selected to study their synchrotron radiolysis chemistry. Radiolyzed peptide products were detected within 10 ms of exposure to a white light synchrotron X-ray beam. Mass spectrometry techniques were used to characterize radiolytic modification to amino acids cysteine (Cys), methionine (Met), phenylalanine (Phe), tyrosine (Tyr), tryptophan (Trp), proline (Pro), histidine (His), and leucine (Leu). A reactivity order of Cys, Met ≫ Phe, Tyr, > Trp > Pro > His, Leu was determined under aerobic reaction conditions from MS/MS analysis of the radiolyzed peptide products. Radiolysis of peptides in ^{18}O-labeled water under aerobic conditions revealed that oxygenated radical species from air and water both contribute to the modification of amino acid side chains. Cysteine and methionine side chains reacted with hydroxyl radicals generated from radiolysis of water as well as molecular oxygen. Phenylalanine and tyrosine residues were modified predominantly by hydroxyl radicals, and the source of modification of proline was exclusively through molecular oxygen.

Reactions of oxygenated species with peptides and proteins have been extensively investigated because of important biological effects of protein oxidation in aging, diseases, and radiation damage.[1−9] In addition, mechanisms by which hydroxyl radicals generated either by metal-catalyzed methods[10,11] or through low-flux radiation sources[5] modify peptides and proteins have been characterized. Radiolysis of peptides and proteins by low-flux radiation sources can lead to side chain modification of amino acids, backbone cleavage products, or formation of covalent cross-linked derivatives.[5] Hydroxyl radicals react preferentially with the side chains of aromatic, heterocyclic, or sulfur-containing amino acids.[1,5,7] The rates of these side chain modifications by hydroxyl radicals have been directly measured with pulsed radiolysis and photolysis studies to be $5 \times 10^9-10^{10}$ M^{-1} s^{-1}.[5] In contrast, hydroxyl radical reactions with α-carbon hydrogen atoms (preferentially at alanine and glycine) are slower, occurring at 10^9 M^{-1} s^{-1}.[5,12] It is these latter reactions that lead to backbone cleavage. Thus, it is clear that the side chain modification of aromatic, hetercyclic, or sulfur-containing amino acids occurs faster than backbone cleavage in the presence of hydroxyl radicals produced by ionizing radiation, and our initial investigation is focused on identifying side chain modifications of amino acids by a synchrotron X-ray beam.

The radiolytic chemistry leading to production of hydroxyl radicals from synchrotron X-ray studies[13] as well as radiolysis methods[14] has been discussed in detail. Radiolysis generates hydroxyl radicals according to eq 1. The ionization of water occurs

$$H_2O + \text{ionizing radiation} \rightarrow H_2O^{\bullet+} + e^-_{dry} \xrightarrow{H_2O} H_3O^+ + {}^{\bullet}OH + e^-_{aq} \quad (1)$$

through interactions with high-energy photons or particles. Ionized

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water molecules react with water within a few picoseconds to produce hydroxyl radicals and hydronium ions. When the reaction is performed in the presence of air, superoxide anions and hydroperoxyl radicals are generated from reactions of hydrated electrons with molecular oxygen according to eq 2. An unfocused

$$e^-_{aq} + O_2 \rightarrow O_2^{\bullet -} + H_2O \rightarrow HO_2^{\bullet} + {}^-OH \qquad (2)$$

"white beam" bending magnet beamline at the National Synchrotron Light Source (NSLS) provides $\sim 10^{14}-10^{15}$ photons/s of a continuous spectrum of X-rays ranging from 5 to 30 keV. Under these X-ray beam conditions, hydroxyl radicals are produced within 100 μs at steady-state concentrations near micromolar.[13d]

During synchrotron radiolysis of peptides and proteins, radical species generated on millisecond time scales undergo reactions with amino acid side chains or subsequently cause backbone bond cleavages, in both cases inducing covalent modifications.[15] The extent of reaction with hydroxyl radicals will be affected in part by the accessibility of regions of the protein to solvent.[10,11,16] This can be used to provide detailed information on a protein's higher order structure. Synchrotron radiolysis studies of peptides[17] and proteins[18] at micromolar concentrations have revealed that significant modification of *amino acid side chains* occurs on *millisecond* time scales, and the extent of modification is dependent on the X-ray exposure time as detected by mass spectrometry. All amino acid residues can undergo reactions with hydroxyl radicals produced by ionizing radiation though tyrosine, phenylalanine, tryptophan, histidine, methionine, and cysteine residues are particularly reactive.[1,5−7,19−21]

The present study identifies the time scales and chemistry of modifications of amino acid side chains of peptides using a high-flux, white light synchrotron X-ray beam. This study has enabled the relative reactivity of various amino acids to be measured in a highly solvent accessible environment. Mass spectrometry techniques are especially suited to the characterization of the radiolysis reaction products of peptides at low micromolar concentrations and tandem mass spectrometry is used to identify the sites of amino acid modifications.[22]

These studies establish the feasibility of using the modification of amino acid side chains by oxygenated species as a new method to probe protein structure in solution. The main advantage of synchrotron X-rays is the production of radical species on millisecond time scales. This feature of synchrotron "footprinting" has enabled the rate constants describing the Mg^{2+}-dependent folding of the *Tetrahymena thermophila* ribozyme to be quantitatively determined.[13] Such an approach is now being applied to protein folding kinetics and protein/ligand interactions.

MATERIALS AND METHODS

Peptide samples (70−92% peptide content based on amino acid analysis) were obtained from Sigma Chemicals (St. Louis, MO) and were used without further purification. Solutions of peptides were prepared in deionized water (Barnstead, Dubuque, IA) at a concentration range of 10−270 μM. ^{18}O-Labeled water (87.7 or 97%) was obtained from Isotec Inc. (Miamisburg, OH).

Exposure of the peptides to the X-ray beam was performed at the X-9A beamline of the NSLS at a ring energy of either 2.584 or 2.8 GeV and beam currents ranging from 160 to 250 mA. The X-ray beam is guided in ultrahigh vacuum through a 14-m distance from the bending magnet source to the beam port within the experimental hutch. The current beamline X-9A optics consists of two Be windows (254 and 500 μm) and an Al window (200 μm). Theoretical calculations illustrated that a homogeneous X-ray dose is delivered through the 1-mm path of sample, including losses due to optical components and an air path of 40 cm between the beam port and the sample.[13e]

Time-resolved millisecond X-ray exposures were achieved through the use of an electronic shutter (Vincent Associates, Rochester, NY) equipped with a platinum aperture. A 10-μL portion of peptide solution was dispensed into a 1.5-mL microfuge tube that was held in a holder on its side, and the sample was held by surface tension at the bottom of the tube.[13a] The distance through air between the beam port and the sample in this configuration was 10 cm. The sample tube was aligned with the X-ray beam while the beam dimensions were adjusted to provide full overlap with the 10-μL sample. After X-ray exposure, a 10-μL portion of ethanol was added to the peptide samples to minimize reaction of residual radicals. This manual quench step was completed within 15 s of X-ray exposure. Experiments were also performed by the use of a computer-controlled three-syringe quench-flow apparatus (Kin-Tek, State College, PA)[13d] that enabled the addition of ethanol to the samples 10 ms after X-ray exposure. The distance through air between the beam port and the sample in this set up was 40 cm. Similar radiolysis reaction products of peptides and rates of amino acid side chain modification were observed with all three experimental protocols (data not shown).

After X-ray exposure, the samples were stored at −20 °C. Peptide concentrations were adjusted to 5 μM in 50% methanol

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containing 2% acetic acid for electrospray mass spectrometry analysis. Spectra were collected on a quadrupole ion trap mass spectrometer (Finnigan Corp., San Jose, CA). Peptide solutions were infused at 5 μL/min, and a needle voltage of 4.5 kV was used. Spectra were collected in the profile mode for clear representation of isotope patterns.

Mass Spectrometric Based Reactivity Order of Amino Acids in Peptides. After synchrotron radiolysis, the peptide solutions were analyzed directly by electrospray mass spectrometry without chromatographic separation of the radiolysis products. Mass spectra of radiolyzed solutions typically exhibit ions at $(+16\ \text{u})_n$ intervals above the ions for the unmodified peptides (where $n = 1, 2, 3, ...$). X-ray dose responses (fraction unmodified peptides) were calculated from the ratio of peak intensity for the unmodified peptide relative to the sum of unmodified and modified peptide products.

Tandem mass spectrometry was used to identify the site of amino acid modification. Tandem mass spectra of the peptide mixture after radiolysis contain fragment ions[23] from all modified peptides that exhibit the same mass (i.e., isobaric peptides[24]). When modification is exclusively on one site, fragment ions shift completely with respect to the site of amino acid modification. If the peptide contains two amino acids that are both modified after radiolysis, the precursor ion selected for collisional activation contains two kinds of peptides (in the case of a single modification). Since fragment ions from both modified peptides are generated concurrently, the MS/MS spectrum is composed of fragment ions reflecting modifications of both reactive amino acids. In addition, fragment ions corresponding to the unmodified reactive amino acids are also observed in the MS/MS spectrum of these modified peptides. Fragment ions reflecting modification of one of the reactive amino acids are generated from the peptide with modified amino acid at this site and accompanied with unmodified fragment ions of the other reactive amino acid. The ratio of ion intensities of the modified and unmodified fragment is used to measure relative reactivities at each corresponding amino acid site. The assumption is that hydroxylation or oxygenation of the amino acid side chains does not alter fragmentation pathways or fragment ion intensities.

RESULTS AND DISCUSSION

A series of 5−14 residue peptides with diverse sequences containing the amino acid residues Cys, Met, Phe, Tyr, Trp, Pro, and His were selected for this study. The chemistry of modification for each amino acid side chain type is discussed.

Modification of Phenylalanine Residue. [Glu[1]]fibrinopeptide (EGVNDNEEGFFSAR) contains two phenylalanine residues at positions 10 and 11 that were expected to be highly susceptible to radiolytic modification. Figure 1 shows the electrospray mass spectrum for a 30 μM solution of peptide in water that was exposed to the X-ray beam for 30 ms. The ion for the unmodified peptide is observed at m/z 785.9, and it corresponds to the doubly charged ion $[M + 2H]^{2+}$. The peaks at m/z 793.9, 801.9, and 809.9 suggest

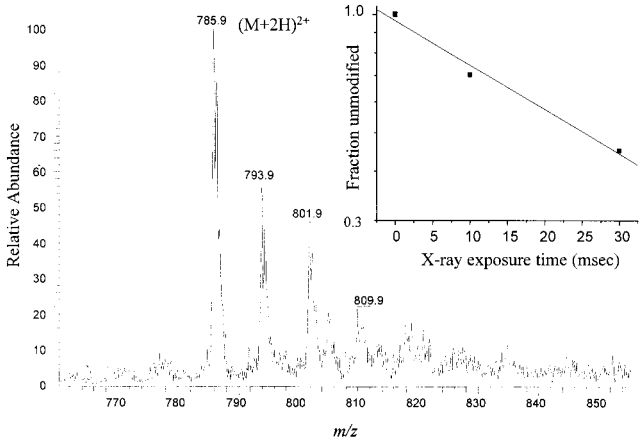

Figure 1. Electrospray mass spectrum for a 30 μM solution of [Glu[1]]-fibrinopeptide (EGVNDNEEGFFSAR) exposed to an X-ray beam (2.8 GeV, 166 mA) for 30 ms. Inset shows a dose response curve ($R = 0.992$) derived from electrospray mass spectral data. "Fraction unmodified" is the ratio of ion intensity of the unmodified peptide to the sum of ion intensity from the modified and unmodified peptide plotted on a semilog scale.

that one to three oxygen atoms are incorporated in this peptide, respectively. Quantitation of the peak intensities of the unmodified and modified peptides shows that 55% of the peptide was modified by the 30-ms exposure to the X-ray beam (Figure 1, inset). The dose response is indicative of a Poisson distribution of modifications.[13]

MS/MS spectra are compared in Figure 2 for ions corresponding to the unmodified peptide and the product of one oxygen addition in order to identify the sites of amino acid modification. MS/MS spectra of this peptide are composed of relatively intense fragment ions of the b and y series that confirm the amino acid sequence from position 3 to 14. The MS/MS spectrum of the modified peptide (Figure 2b) with the m/z 793.7 precursor ion corresponding to $[M + {}^{16}O + 2H]^{2+}$ reveals that fragment ion mass shifts are only observed at the phenylalanine residues.

The exclusivity of phenylalanine modification is clearly observed from the C-terminus ion series, since y_2 and y_3 remain unchanged suggesting the Ser-Ala-Arg portion is unmodified while a mass shift of 16 u is observed for the y_4 ion corresponding to Phe-Ser-Ala-Arg. The y_4 ion of the modified phenylalanine at position 11 is detected at m/z 496. The b_{10} ion corresponding to modification of phenylalanine at position 10 is observed at m/z 1107. Furthermore, modifications of other amino acids in this peptide were not observed. Only unmodified fragments of y_2 and y_3 are detected at a fragment ion signal-to-noise ratio of >10 indicating that the C-terminal arganine, alanine, or serine residues are not modified. Similarly, the N-terminus fragments b_3, b_5, b_8, and b_9 remain unchanged, which reveal that the glutamic acid, glycine, valine, asparagine, or aspartic acid have not been modified.

These spectra also indicate that the modification occurs equally on both phenylalanine residues. The y_4 and b_{10} ions are each divided in two ions differing by 16 u, suggesting partial modifications of both phenylalanine residues, with the extra oxygen residing equally on both phenylalanine residues. The presence of unmodified fragment ions of y_4 at m/z 480 in the MS/MS spectrum of the modified peptide is further evidence that the extra oxygen resides partially on the phenylalanine residue at position

(23) When the peptide bond cleaves at the amide bond and the charge is retained on the N-terminal portion, the fragment is referred to as b-type ion, and charge retention on the C-terminus yields y-type ion: (a) Roepstorff, P.; Fohlmann, J. *Biomed. Mass Spectrom.* **1984**, *11*, 601. (b) Biemann, K. *Biomed. Environ. Mass Spectrom.* **1988**, *16*, 99.

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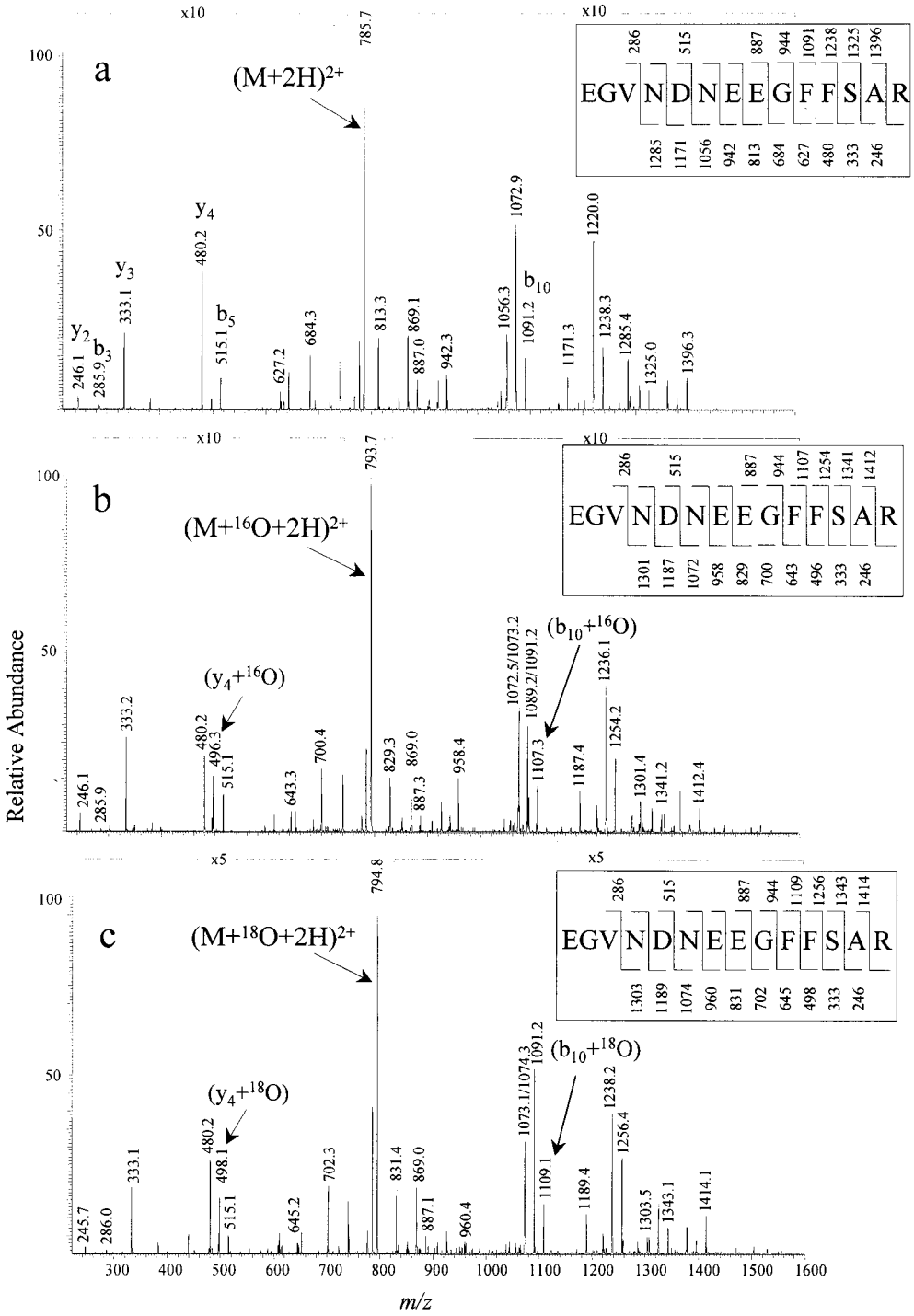


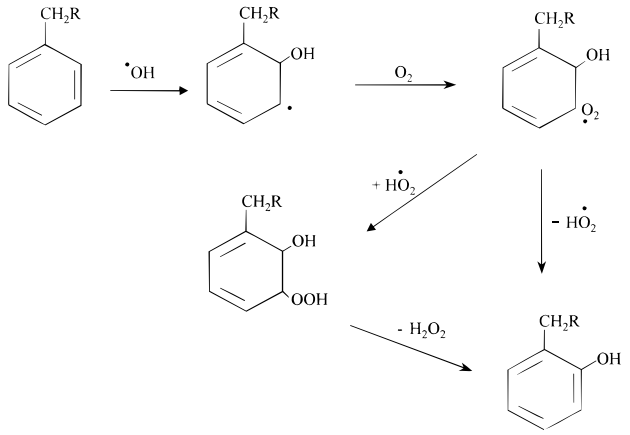
Figure 2. MS/MS spectra for (a) the unmodified [Glu[1]]-fibrinopeptide, (b) modified peptide product from radiolysis in water, and (c) modified peptide product from radiolysis in ^{18}O-labeled water.

10. Similarly, the presence of the unmodified b_{10} ion at m/z 1091 in the MS/MS spectrum of the modified peptide supports that the oxygen resides partially on the phenylalanine residue at position 11.

The MS/MS mass spectrum for the two oxygen product of this peptide (m/z 801.9) revealed that the two oxygens are equally divided between the two phenylalanine residues (data not shown). This precursor ion contained three isobaric peptides, since the two oxygens can be distributed between the phenylalanine residues at positions 10 and 11 or reside exclusively on one or the other. In this case, the y_4 fragment ion was divided into three ions at m/z 480, 496, and 512, representing the unmodified fragment ion, and the ions from addition of one and two oxygens on phenylalanine at position 11. The multiple lines of evidence from singly and doubly modified peptides conclusively demonstrate that the precursor ion of the modified peptide consists of a mixture of two peptides, one with modified phenylalanine at position 10 and the other with modified phenylalanine at position 11.

Mechanism for Modification of the Phenylalanine Residue. Radiolysis of peptide in ^{18}O-labeled water was performed in order to determine the source of the oxygen present in phenyl-

Scheme 1. Mechanism for Hydroxyl Radical Side Chain Modification of Aromatic Unsaturated Residues[5]



alanine residues at positions 10 and 11. Figure 2c shows the MS/MS mass spectrum for the doubly charged ion of the ^{18}O-containing peptide at m/z 794.8. The modified y_4 ion is now observed at m/z 498, and the modified b_{10} ion is observed at m/z 1109. These results are consistent with ^{18}O addition on the Phe-Ser-Ala-Arg fragment ion. The presence of unmodified y_4 and b_{10} ions at m/z 480 and 1091 support that ^{18}O is equally divided between the phenylalanine residues. Although, the unmodified b_{10} ion of m/z 1091 overlaps with $b_{10}-H_2O$ fragment ion of the modified, the presence of the unmodified b_{10} ion is further supported from the m/z 1073 ion that corresponds to $b_{10}-H_2O$ of the unmodified fragment ion. These results also verify that peptide hydroxylation is localized on the phenylalanine residues. The other amino acid side chains were not modified. This MS/MS analysis reveals that ^{18}O has been incorporated in side chains of phenylalanine residues through reactions with hydroxyl radicals generated from radiolysis of the ^{18}O-labeled water.

Multiple hydroxylation of phenylalanine and tyrosine residues has been reported previously in studies of ionizing radiation of peptides.[5] The results of this paper show that synchrotron X-ray radiolysis mechanisms of phenylalanine hydroxylation occur through comparable pathways as depicted in Scheme 1. One mechanism for modification of aromatic unsaturated side chains is through insertion of hydroxyl radicals. The hydroxycyclohexadienyl radicals can react with O_2 to yield peroxy radical intermediates that undergo subsequent reactions involving hydroperoxyl radicals to yield hydrogen peroxide and tyrosine (ortho, meta, and para).[5] Such a mechanism is predominantly consistent with the ^{18}O data of this study. The electrospray mass spectrum of the peptide radiolyzed in ^{18}O-labeled water showed an $^{16}O/^{18}O$ incorporation ratio of approximately one-quarter (data not shown). This ratio of ^{16}O incorporation is higher than expected considering the purity of the ^{18}O-labeled water for this study. The mechanism in Scheme 1 suggests that O_2 can be incorporated in the side chain, affecting the ratio of $^{16}O/^{18}O$ product. Experiments with the use of $^{18}O_2$ gas are required to further explain the role of oxygen in radiolysis reactions of aromatic unsaturated residues.

Modification of the Proline Residue. Proline residues are extensively modified by synchrotron radiolysis. The IgE peptide-III (DSDPR) was selected to characterize reactions of proline in the absence of other competing amino acids. The electrospray

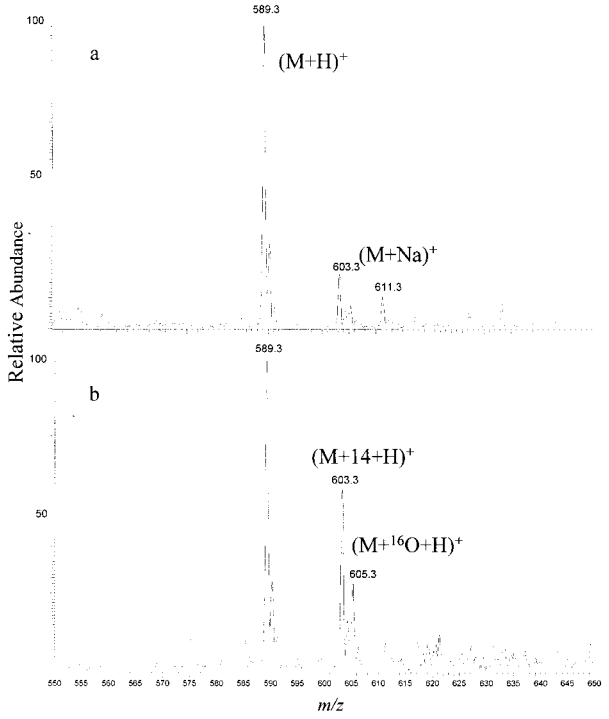


Figure 3. Electrospray mass spectra for a 30 μM solution of IgE peptide (DSDPR) exposed to an X-ray beam (2.8 GeV, 174 mA) for (a) 10 or (b) 30 ms.

mass spectra (Figure 3) from a 30 μM solution of Ig-E peptide after 10- and 30-ms exposure to X-ray beam reveal the radiolyzed products at +14 and +16 u of the unmodified protonated peptide at m/z 589. Approximately 17 and 45% of the peptide were converted to radiolyzed products, respectively. The absence of multiple oxidation products (i.e., +28 or +32) supports that the side chain of proline does not accommodate multiple oxygens. The abundance of the +14 radiolyzed product is approximately twice the +16 product, m/z 603.3 and 605.3, respectively. Both radiolyzed products remained *unchanged* when ^{18}O-labeled water was used as the synchrotron solvent media, indicating that oxygen present in the modification is derived from air in both products (data not shown).

MS/MS studies determined that the site of modification is on the proline residue. A shift of +14 or +16 was detected for the y_2 fragment ion, suggesting modifications of the C-terminal Pro-Arg portion of this peptide. In the absence of ^{18}O-containing products, the mechanism of oxidation for both +14 and +16 products (Scheme 2) is presumed to involve a first-step radical formation through abstraction of hydrogen from the side chain and subsequent reactions with O_2. Loss of water from the hydroperoxyl center produces pyroglutamic acid, while loss of hydroxyl radical and subsequent addition of hydrogen atom result in the formation of hydroxyproline. These products correspond to the +14 and +16 ions observed in Figure 3, respectively. The use of ^{18}O-labeled water generates products in agreement with these mechanisms since the oxidation of proline occurs through the addition of oxygen from air. These products have been reported in metal-catalyzed oxidation of amino acid residues.[1d,25]

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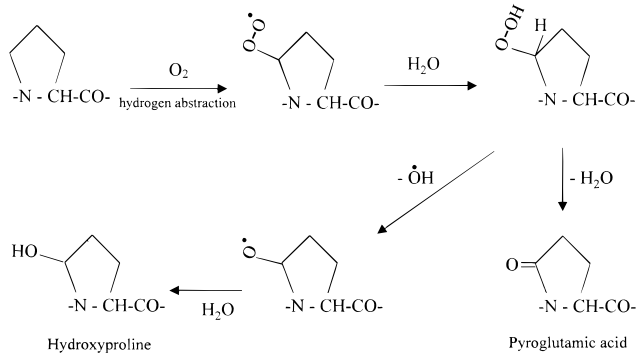
Scheme 2. Mechanism for Side Chain Modification of Proline



Relative Reactivity of Phenylalanine and Proline Residues. To compare the reactivity order of phenylalanine and proline residues, bradykinin-(2−7) (PPGFSP) peptide at a 30 μM concentration was subjected to synchrotron X-ray radiolysis. Electrospray mass spectrometry analysis revealed multiple modifications of this peptide with two series of product ions (data not shown). One series from oxygenation of proline residues generated ions of +14 intervals, and the other series were observed at +16 intervals. The latter series is attributed to side chain hydroxylation of phenylalanine residues and partial oxygenation of proline residues. The reactivity order was determined from the ion abundance of the +14 and +16 radiolyzed products at m/z 615 and 617, respectively. Although this peptide contains three proline residues, the +14 ion abundance was one-third of the +16 ion. These results revealed that the phenylalanine residue is approximately 5 times more susceptible to radiolysis-induced modification than the proline residues in this peptide.

Relative Reactivity of Methionine, Phenylalanine, and Tyrosine Residues. Met-enkephalin-RGL (YGGFMRGL) peptide was selected to compare the reactivity order of methionine to phenylalanine and tyrosine residues. Pulsed radiolysis studies have shown that methionine in peptides reacts with hydroxyl radicals predominantly at the sulfur atom ($k \sim 10^{10}$ M^{-1} s^{-1}) yielding methionine sulfone, $RS(O)CH_3$, and methionine sulfoxide, $RS(O_2)CH_3$, as products.[5] Synchrotron X-ray radiolysis of this peptide was separately performed in both ^{16}O- and ^{18}O-labeled water in order to distinguish mechanisms of radiolysis reactions for these amino acid residues. Tandem mass spectrometry analysis of ^{16}O, ^{18}O, 2(^{16}O), and the (^{16}O + ^{18}O)-containing radiolyzed products revealed that methionine modification occurs first, and its reactivity to oxygen or hydroxyl radical is much greater than phenylalanine and tyrosine. Moreover, methionine selectively reacts with oxygen when oxygen and hydroxyl radicals both compete for reactions with methionine.

MS/MS analysis of the ^{16}O radiolyzed product (not shown) revealed that the y_4 ion, corresponding to the Met-Arg-Gly-Leu portion, was shifted by +16 u and the intensity of unmodified y_4 ion was less than 5% of the modified ion. A shift of +16 u for the y_3 ion was not observed. This indicated that 95% of modification of the Met-enkephalin-RGL peptide was localized to the methionine residue.

The MS/MS spectrum of ^{18}O-containing radiolyzed products (Figure 4b), which corresponds to modifications induced by hydroxyl radicals, shows the ^{18}O modified y_4 ion at m/z 494 with a 60% relative ion abundance. The ion at m/z 476 corresponding to the unmodified y_4 ion is also detected with a 10% relative ion abundance. This shows 85% modification of methionine and that ^{18}O does not reside exclusively on the methionine residue. Fragment ions at m/z 239 and 296, corresponding to modified b_2 and b_3 ions, support partial modification of the tyrosine residue by the ^{18}O-labeled hydroxyl radicals. The reactivity of methionine for hydroxyl radical is greater than that of the phenylalanine or tyrosine residues.

MS/MS mass spectrum of (^{16}O + ^{18}O)-containing radiolyzed product ion detected from radiolysis in ^{18}O-labeled water is shown in Figure 4c. In this analysis, the modified peptide contained one ^{16}O and one ^{18}O. This analysis reveals the selectivity of methionine, phenylalanine, and tyrosine toward the source of radicals. Although methionine modification by ^{18}O-labeled hydroxyl radicals is predominantly observed in Figure 4b, the y_4 fragment ion at m/z 492 signifies that, in this case, methionine is selectively modified by ^{16}O from air (≥90%). This analysis also reveals that phenylalanine and tyrosine are modified exclusively by ^{18}O in relatively equal amounts. Fragment ions of b_2 and b_3 observed at m/z 239 and 296, respectively, reveal ^{18}O modification of the tyrosine residue. The y_5−y_7 fragment ions at m/z 639, 696, and 753 include the +16 modification of the methionine residue. In addition, y_5−y_7 ions that include the ^{18}O modification on the phenylalanine residue are observed at m/z 657, 714, and 771. Since the extra ^{18}O resides on tyrosine and phenylalanine, y_5−y_7 are not fully shifted by +18, and this is further supported by the presence of the unmodified b_2 and b_3 ions at m/z 221 and 278. These results reveal that in a highly solvent accessible environment methionine is more reactive than phenylalanine or tyrosine residues.

Modification of the Cysteine Residue. Since methionine modification by oxidation and hydroxylation was observed, radiolysis of the cysteine-containing fibronectin peptide (RGDC) was investigated. Electrospray mass spectra (Figure 5) for a 30 μM solution of peptide after 10-ms exposure reveal that cysteine residues incorporate up to three oxygens. In this case, incorporation of one oxygen is not observed and total ion current for the modified peptide is greater than the unmodified peptide after only 10 ms of X-ray exposure (35% unmodified peptide remains). Radiolysis of a more concentrated, 90 μM peptide solution also showed the absence of single oxygen incorporation. These results indicate that cysteine residues are very reactive to radiolysis.

Radiolysis of fibronectin peptide in ^{18}O-labeled water revealed that hydroxyl radicals and molecular oxygen both compete in the formation of products. The presence of +32 and +34 ions (Figure 5b), corresponding to the addition of two ^{16}O or one ^{16}O plus one ^{18}O, reveals the incorporation of ^{16}O from air and ^{18}O from hydroxyl radicals generated by synchrotron radiolysis. The mixture of ^{16}O and ^{18}O accounts for the modified peptide incorporating three oxygens as indicated by ions at m/z 498, 500, and 502. Pulsed radiolysis studies of cysteine in air report modifications of cysteine by O_2 and hydroxyl radicals with RSOH and RSO_2H as products.[5] The absence of the singly modified product in synchrotron radiolysis suggests that sulfone may react too rapidly to be detected. Additionally, incorporation of three oxygens or a combination of three oxygens and hydroxyl

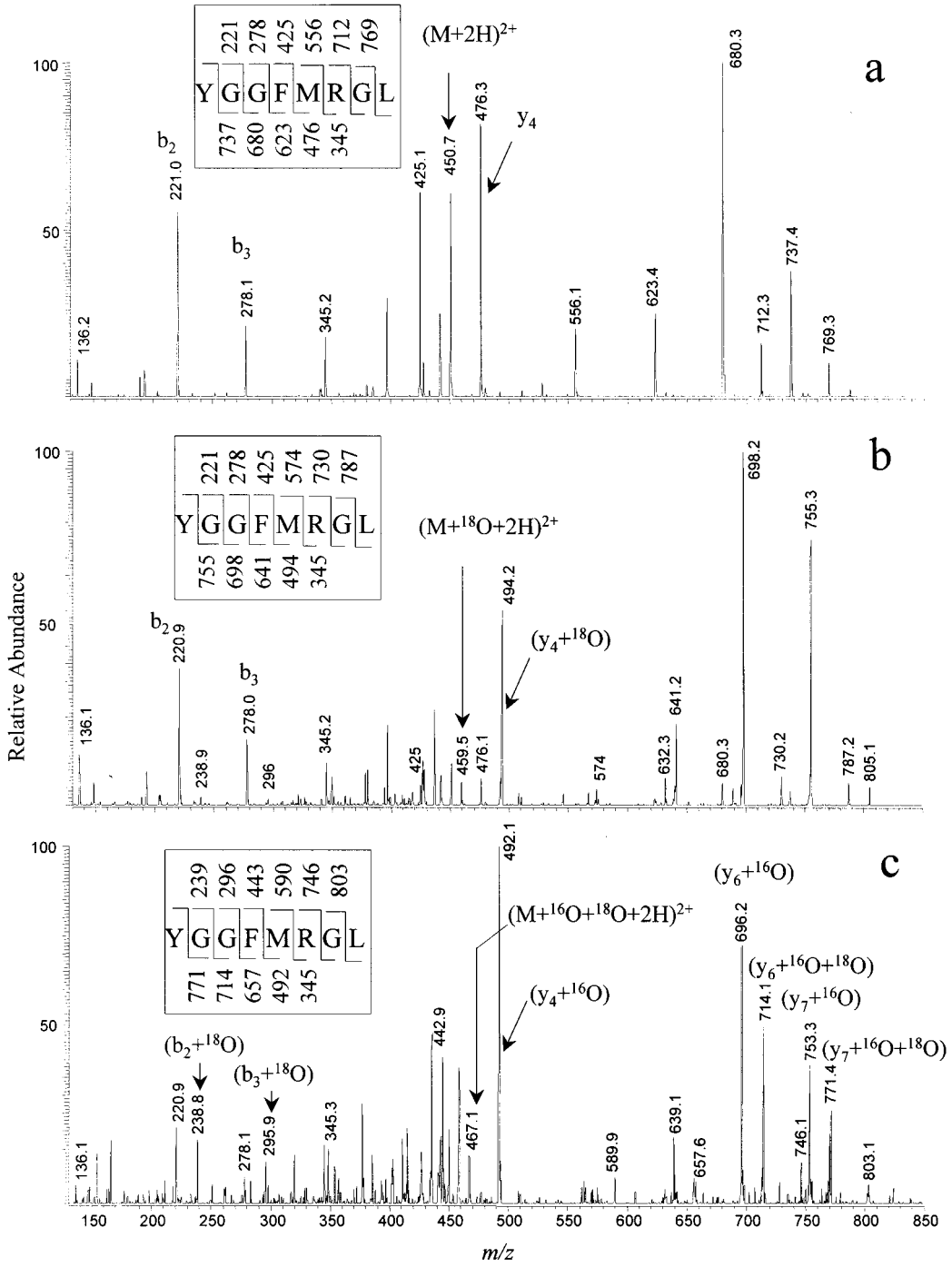


Figure 4. MS/MS spectra for (a) the unmodified Met-enkephalin-Arg-Gly-Leu peptide, (b) singly modified radiolyzed product in O^{18}-labeled water, and (c) doubly modified radiolyzed product in ^{18}O-labeled water.

radicals has not been reported in earlier radiolysis studies of cysteine-containing peptides.[5] The higher flux of radical formation under synchrotron radiolysis could account for the observed differences in products.

MS/MS analysis confirmed that the site of modification is on the cysteine residue (data not shown). Loss of SO_2H_2 was observed as the major fragmentation pathway when the two-oxygen adduct ion of m/z 482 was collisionally activated. The N-terminal b_3 fragment ion corresponding to the Arg-Gly-Asp portion remained unchanged, indicating that the modification was exclusively on the cysteine residue.

Modification of the Tryptophan Residue. U_5−peptide (DL-WQK) was subjected to radiolysis at a 30 μM concentration. Electrospray mass spectrometry analysis revealed radiolyzed products resulting from multiple oxidation after X-ray exposure. MS/MS analysis of the +16 and +32 (not shown) radiolyzed products revealed oxidation of the tryptophan residue when internal fragment ion b_4y_3 at m/z 315 shifted by 16 and 32 u, respectively. This mass shift is also observed for the b_3 and y_3 ions, respectively.

Radiolysis of this peptide in ^{18}O-labeled water was not performed, and the mechanisms of synchrotron radiolysis reactions

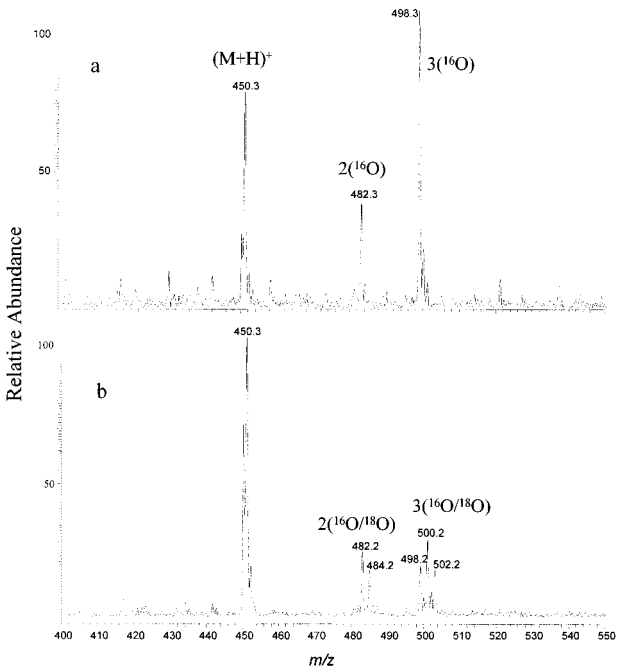


Figure 5. Electrospray mass spectra for a 30 μM solution of fibronectin peptide (RGDC) (a) water and (b) ^{18}O-labeled water, after 10-ms exposure to X-ray beam (2.8 GeV, 177 mA).

of tryptophan residue are presumed to be similar to pulsed radiolysis studies[5] since they are similar to the reactions of other aromatic residues studied. The radiolytic oxidation of the tryptophan residue has been reported to occur predominantly through reactions initiated by hydroxyl radical addition to the unsaturated bonds of the indole moiety or the aromatic ring. A double oxidation product has been proposed through reactions of hydroxyl radicals on the double bond of the indole ring. Reactions of hydroxyl radicals on the aromatic ring proceed through a sequential pathway similar to modifications induced on the phenylalanine residue.[5]

Multiple oxidation of the tryptophan residue has also been reported in the photolysis of gramicidin A,[26] and fluorescence spectroscopy methods have revealed modifications of tryptophan residues of γ-II crystallin[27] after 8−24 h of reaction time. Recently, modifications of tryptophan residues from ^{60}Co radiolysis of α-crystallin have been identified by tandem mass spectrometry.[3b]

The reactivity order of tryptophan was compared to tyrosine and proline residues through radiolysis of the LHRH-peptide (WSYGLRPG). MS/MS analysis of the +16 radiolyzed product of this peptide revealed modification on tyrosine and tryptophan residues corresponding to a shift of +16 for b_2 and y_6 ions. The intensity of the modified y_6 ion was 4 times the unmodified ion, and the intensity of modified b_2 ion was a quarter of the unmodified ion. These results suggest that tyrosine is more reactive than the tryptophan residue. Oxidation of the proline residue was not observed for this peptide.

Reactivity of the Histidine Residue. Pulsed radiolysis[5] and metal-catalyzed oxidation[28] studies report that histidine oxidation gives rise to aspartic acid, asparagine, and 2-oxohistidine products. A recent study on metal-catalyzed oxidation of human relaxin reports 2-oxohistidine and the absence of aspartic acid or asparagine as products based on mass spectrometry analysis of the protein digest.[29] The angiotensin peptide (DRVYIHPFHL) was selected to compare the reactivity of histidine to phenylalanine and tyrosine residues. Mass spectrometry analysis of this peptide after synchrotron radiolysis revealed multiple addition of +16 product ions (data not shown). MS/MS analysis of the +16 product ion revealed that phenylalanine and tyrosine were modified and oxidation of histidine was not observed. The conversion of histidine to aspartic acid or asparagine would cause a mass shift of −23 or −22, respectively, which was not observed by mass spectrometry analysis of angiotensin peptide after X-ray exposure. These results suggest that modification of phenylalanine and tyrosine occur at a faster rate than histidine. Additionally, radiolysis of the RSRHF peptide was performed to further compare the reactivity of histidine to that of the phenylalanine residue. Again, conversion of histidine to aspartic acid or aspargine was not observed in this peptide. MS/MS analysis of the +16 radiolyzed product revealed modification of the phenylalanine residue. These results show that side chain modification of phenylalanine occurs before that of the histidine residue.

To investigate the reactivity of histidine away from other reactive residues, the ASHLGLAR peptide was selected. Exposure of a 30 μM solution to the X-ray beam for 10 ms was sufficient to modify this peptide to a great extent (40% modified products). The electrospray analysis showed radiolytic products with multiple addition of 16 u. MS/MS analysis of the +16 radiolyzed product revealed that both histidine and leucine residues were equally modified. The b_2 and y_2 fragments remained unchanged, indicating that the N-terminal Ala-Ser and C-terminal Ala-Arg were not modified. The presence of b_n and b_n + 16 for b_3, b_4, and b_5 ions revealed that modification was distributed over the histidine and leucine residues, while a complete shift of +16 u for b_6 accounted for total modification and supported that the C-terminal Ala-Ser was not modified. Pulsed radiolysis studies report 3-hydroxy- or 4-hydroxyleucine as major side chain modification products.[5]

CONCLUSIONS

Millisecond exposure of peptide solutions to a white light synchrotron X-ray beam results in extensive modification to some amino acid side chains. Mass spectrometric analysis revealed that a 30-ms X-ray exposure yielded modification of >50% of the phenylalanine residues of fibrinopeptide and 45% of the proline residues in IgE peptide. By comparison, 65% of all cysteine residues were modified with a 10-ms exposure to the X-ray beam. Mass spectral analysis of radiolyzed peptides did not show the presence of cleaved or cross-linked products. A summary of amino acid side chain reactions is presented in Table 1. With the exception of histidine, the most reactive amino acids are the sulfur-containing, aromatic and heterocyclic residues. Multiple side chain modification at these residues is favored over other aliphatic amino acids. However, modification of leucine residues of ASHLGLAR

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Table 1. Synchrotron X-ray Radiolysis Reactions of Peptides

peptide sequence	amino acid side chain reactions
EGVNDNEEGFFSAR	hydroxylation of Phe
DSDPR	oxidation of Pro
PPGFSP	hydroxylation of Phe, oxidation of Pro
YGGFMRGL	oxidation and hydroxylation of Met, hydroxylation of Phe and Tyr
RGDC	oxidation and hydroxylation of Cys
DLWQK	modification of Trp*[a]
WSYGLRPG	hydroxylation of Tyr; modification of Trp*, Pro unmodified
DRVYIHPFHL	hydroxylation of Phe and Tyr; His and Pro unmodified
RSRHF	hydroxylation of Phe; His unmodified
ASHLGLAR	modification of His* and Leu*

[a] Asterisks indicate reactions in ^{18}O-labeled water were not performed to verify the source of oxygen, and mechanisms of modifications are proposed based on earlier studies.[5]

peptide following a 10-ms X-ray exposure shows that synchrotron X-rays induce radiolytic modification of other aliphatic residues. Pulsed radiolysis studies report that these reactions involve formation of carbon-centered radicals through hydrogen abstraction at a rate of $(1-5) \times 10^9$ M^{-1} s^{-1}.[5]

Based upon the peptides selected for this study (Table 1), the order of reactivity determined for synchrotron X-ray exposure is Cys, Met $\gg$ Phe, Tyr > Trp > Pro > His, Leu. The observed side chain reactions occur through pathways similar to those observed following longer exposure to low-flux radiation.[5] Both hydroxyl radicals and molecular oxygen contribute to side chain modifications of amino acids. The use of ^{18}O-labeled water revealed mechanisms of reactions for phenylalanine, proline, cysteine, and methionine residues.

These peptide radiolysis studies using a synchrotron X-ray beam provide a foundation for a new approach to probe protein structures in solution based on side chain modification of amino acids. Synchrotron X-ray induced modification of amino acids has also been identified in proteins.[18] Studies are in progress to determine whether the extent of side chain modification can be correlated with the solvent accessibility calculated from atomic resolution protein structures.[30] Such a relationship has been demonstrated for the methionine residues of human growth hormone subject to oxidative reactions with hydrogen peroxide.[31] These studies suggest that the rates of radiolytic modification of amino acids in proteins are influenced by their solvent accessibility. In principle, the rate of modification of reactive residues can be followed for a protein during a folding transition or a protein/ligand association. The production of stable modification through synchrotron radiolysis experiments makes this method amenable to a wide range of samples as well as solution conditions.

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