Characterization of Cytochrome c Free Radical Reactions with Peptides by Mass Spectrometry*

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* This work was supported by the National Institutes of Health Office of AIDS Research (for the purchase of the Voyager-RP MALDI mass spectrometer and the Q-Tof hybrid mass spectrometer). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The reactions of horse heart cytochrome c, hydrogen peroxide, and the spin trap 3,5-dibromo-4-nitrosobenzenesulfonic acid with a series of polypeptides were investigated using mass spectrometry. The mass spectra obtained from these reactions revealed that after a free radical has been generated on the heme-containing protein horse heart cytochrome c, it can be transferred to other biomolecules. In addition, the number of free radicals transferred to the target molecule could be determined. Recipient peptides/proteins that contained a tyrosine and/or tryptophan amino acid residue were most susceptible to free radical transfer. Using tandem mass spectrometry, the location of the 3,5-dibromo-4-nitrosobenzenesulfonic acid radical adduct on the nonapeptide RWIIGLNK was unequivocally determined to be at the tryptophan residue. We also demonstrated that the presence of an antioxidant in the reaction mixture not only inhibits free radical formation on horse heart cytochrome c, but also interferes with the transfer of the free radical, once it has been formed on cytochrome c.

A number of toxicities are mediated by mechanisms collectively termed "oxidative stress." Consequently, the study of oxidative stress has been the focus of considerable research in recent years. Oxidative stress mechanisms, reactive oxygen metabolites interact with cellular constituents, including DNA/RNA, proteins, carbohydrates, and unsaturated lipids. In addition, cumulative oxidative damage has been implicated in a number of disease states, e.g. the aging process, cancer, and ischemia-reperfusion. The study of oxidative stress in mitochondria has shown that hydrogen peroxide is produced via the incomplete reduction of oxygen during oxidative phosphorylation (1). Although hydrogen peroxide levels are kept relatively low (10^{-10} to 10^{-7} m) under normal physiological conditions (1), under certain conditions, such as inflammation or ischemia-reperfusion (2, 3), excessive amounts of hydrogen peroxide are produced. These excessive levels are thought to produce toxic effects, such as glutathione depletion, DNA and/or protein damage, and lipid peroxidation (3–6).

Several investigators have postulated mechanisms for oxidative damage, including that oxidative damage caused by hydrogen peroxide is conveyed through its reaction with trace transition metals (7), and that the reaction of hydrogen peroxide with heme proteins, such as cytochrome c, produces highly reactive ferryl-radical species that are then capable of oxidizing biomolecules and initiating lipid peroxidation (8–12). Oxidation of exogenous compounds by cytochrome c has been shown to be dependent on hydrogen peroxide, but did not involve the hydroxyl radical (9). Other reports have shown that mitochondrial lipid peroxidation could be initiated by hydrogen peroxide and cytochrome c (12, 13). It was postulated that hydrogen peroxide oxidized cytochrome c to a ferryl-radical intermediate (i.e. compound I-type species) that was responsible for catalyzing the oxidation of other biomolecules, such as DNA, proteins, and lipids.

This interest in the role of free radicals in the pathogenesis of human disease has led to an increased need for techniques to measure free radicals and their reactions. Detection of free radicals is difficult because they are typically extremely reactive and, consequently, short-lived. Electron spin resonance (ESR) is the only analytical technique currently capable of directly measuring free radicals. One means of increasing the lifetime of a free radical is to react the radical with a trapping agent that will form a reaction product in which a more stable free radical is formed. This procedure is known as spin-trapping. The ESR spectrum of a spin-trapped free radical is typically simpler than that of the free radical but contains correspondingly less information. The combined technique of ESR and spin-trapping is highly selective and sensitive for the detection of free radicals. The spin-trapped radical adducts can build up to a higher steady-state concentration than the primary radical, which makes their detection easier.

While several research groups have successfully applied ESR spin-trapping to the analysis of protein radicals (14–22), the study of oxidative damage to proteins has been less intensively studied than lipid peroxidation due to the large number of different protein targets and the relatively high number of different amino acid residues. Thus, there is a need for techniques with increased specificity and sensitivity that would provide more information about the origin and localization of the protein radical. This additional information should also provide further insight into the mechanisms involved in oxidative damage.

Our research groups have investigated the reaction between horse heart cytochrome c and other proteins and peptides using mass spectrometry (MS).1 MS in combination with proteolysis can be very useful for protein and modified protein determination. In our initial report using ESR and the deuterated spin trap 2-methyl-2-nitroso propane-d_{3}, we suggested that an amino acid radical might be responsible for the oxidation of

1 The abbreviations used are: MS, mass spectrometry; cyt c, cytochrome c; BSTP, resin substrate tetradecapeptide; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonic acid; ESI, electrospray ionization; MALDI, matrix-assisted laser desorption ionization; CID, collision-induced decomposition.
Characterization of Free Radical Transfers Using Mass Spectrometry

FIG. 1. ESI mass spectrum of the reaction mixture of horse heart cytochrome c, hydrogen peroxide, and DBNBS. A = ions of horse heart cytochrome c; B = ions of one DBNBS adduct of horse heart cytochrome c; and C = ions of two DBNBS adducts of horse heart cytochrome c.

EXPERIMENTAL PROCEDURES

Materials—The horse heart cytochrome c (cyt c), porcine renin substrate tetradecapeptide (RSTP) (DRVYIHPFHLLVYS), bradykinin (RP-PGFSPFA), insulin B (oxidized), and RNase A were purchased from Sigma. The synthetic nonapeptide, RWIILGLNK, was synthesized by The Protein Chemistry Laboratory of the University of North Carolina (Chapel Hill, NC). The spin trap, 3,5-dibromo-4-nitrosobenzenesulfonic acid (DBNBS), was synthesized using the method of Kaur et al. (23). Hydrogen peroxide (30%) was obtained from Fisher. Prepacked Sephadex G-25 (PD-10) size exclusion cartridges were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). The concentration devices were obtained from Amicon, Inc. (Beverly, MA). Buffers were prepared using 18-MΩ water (Hydro Service and Supplies, Research Triangle Park, NC).

Reaction Conditions—The cytochrome c free radical reactions were initiated by treating a 500 μM solution of cytochrome c with 5 mM hydrogen peroxide in 50 mM ammonium bicarbonate buffer (pH 7.9). The free radicals generated in this reaction were trapped with 15 mM DBNBS. The reaction was allowed to proceed for 15 min at room temperature, after which excess DBNBS and H2O2 were removed by loading the reaction mixture onto a Sephadex G-25 size exclusion column, and the cytochrome c radical adducts were eluted with 10 mM ammonium bicarbonate buffer.

Transfer of the cytochrome c-derived radicals to other biomolecules was achieved by the addition of 500 μM peptide or protein to the reaction mixture of cyt c and DBNBS with subsequent hydrogen peroxide addition. The RSTP, bradykinin, insulin B (oxidized), and RWIILGLNK radical adducts were separated from the cytochrome c radical adducts by loading the reaction mixture onto a 10,000 molecular weight cutoff concentrator and centrifuging for 30–60 min at 1000–2000 × g.

Electrospray Mass Spectrometry—A Micromass Platform II (Altrincham, UK) single-quadrupole mass spectrometer was used for the acquisition of the electrospray ionization (ESI) mass spectra. The instrument is equipped with an ESI interface and an extended mass range quadrupole (4000 Da). Samples were infused into the mass spectrometer at 5 μl/min using a pressure injection vessel (24).

A Micromass Q-ToF (Altrincham, UK) hybrid mass spectrometer was used for the acquisition of the ESI tandem mass spectrum. The instrument consists of a quadrupole mass filter and an orthogonal acceleration time-of-flight mass spectrometer.

Matrix-assisted Laser Desorption Ionization Mass Spectrometry—The MALDI analyses were performed using a Voyager RP (PerSeptive Biosystems, Framingham, MA) time-of-flight dual-stage reflector mass spectrometer as has been described previously (15).

RESULTS

Trapping of Radicals on Polypeptides Formed by Treatment with Cytochrome c and H2O2—Following the reaction of cytochrome c with hydrogen peroxide in the presence of DBNBS, the positive ion ESI mass spectrum was acquired and is shown in Fig. 1. The mass spectrum reveals molecular ions for the cytochrome c (α series ions) as well as ions that correspond to the addition of one and two DBNBS molecules to cytochrome c (B and C series ions, respectively). None of the ions corresponding to the addition of DBNBS molecules were detected in the absence of either hydrogen peroxide or DBNBS (data not shown). The electrospray data are similar to the MALDI mass spectrum of the same reaction mixture, which revealed the presence of ions corresponding to the addition of up to four DBNBS molecules to cytochrome c (15). The ESR spectrum of this same reaction mixture following treatment with Pronase revealed that one major and at least one minor species were formed (15).

To explore the possibility that an oxidatively induced free radical on cytochrome c can initiate free radical oxidation of other proteins that do not, by themselves, undergo free radical oxidation under identical reaction conditions, the reaction of cytochrome c, H2O2, and DBNBS was repeated in the presence of a peptide or protein. A series of peptides/proteins, including RNase A, insulin B (ox), RSTP, RWIILGLNK, and bradykinin, were subjected to treatment with 5 mM H2O2 in the presence of DBNBS prior to analysis by MALDI/MS. In all cases, the MALDI mass spectra showed that, in the absence of cytochrome c, no free radicals were trapped.

The reactions were then repeated with the addition of an equimolar concentration of cytochrome c. Following the reaction of cyt c, hydrogen peroxide, DBNBS, and RNase A, the mixture was subjected to both ESR spectroscopic and MALDI mass spectrometric analyses. The ESR spectrum of this reaction mixture is identical to the ESR spectrum acquired from the reaction mixture in the absence of RNase A (data not shown). From these ESR data it is not possible, therefore, to ascertain other biomolecules catalyzed by the cytochrome c/H2O2 system (15). In this report, we explore the possibility that an oxidatively induced free radical on horse heart cytochrome c can initiate free radical oxidation of other proteins that do not, by themselves, undergo free radical oxidation under identical reaction conditions.

Electrospray Mass Spectrometry—A Micromass Platform II (Altrincham, UK) single-quadrupole mass spectrometer was used for the acquisition of the electrospray ionization (ESI) mass spectra. The instrument is equipped with an ESI interface and an extended mass range quadrupole (4000 Da). Samples were infused into the mass spectrometer at 5 μl/min using a pressure injection vessel (24).

A Micromass Q-ToF (Altrincham, UK) hybrid mass spectrometer was used for the acquisition of the ESI tandem mass spectrum. The instrument consists of a quadrupole mass filter and an orthogonal acceleration time-of-flight mass spectrometer.
whether or not the cytochrome \( c \) radical could initiate free radical oxidation of the RNase A. Upon MALDI/MS analysis, however, it was determined that two free radicals of RNase A were trapped by the DBNBS (Fig. 2A). The molecular ion of RNase A is observed at \( \sim m/z \) 13,700 with the addition of one and two DBNBS molecules approximately 345 and 690 Da, respectively, higher than the molecular ion. In addition, the molecular ion of cytochrome \( c \) and cytochrome \( c \) with the addition of one and two molecules of DBNBS are observed. As a comparison, the MALDI mass spectrum of the reaction mixture of RNase A, hydrogen peroxide, and DBNBS without the presence of cyt \( c \) is shown in Fig. 2B. The only ion observed is an ion corresponding to the molecular ion of RNase A. No ions corresponding to the DBNBS adducts of RNase A were observed. Similar data were obtained when the peptide insulin B (ox) was added to the cyt \( c \) reaction mixture. Molecular ions for insulin B (ox) as well as ions corresponding to the addition of one and two molecules of DBNBS were observed (data not shown). No DBNBS adduct ions were observed in the mass spectrum of the reaction mixture in the absence of cytochrome \( c \) (data not shown).

In order to try to understand the mechanism involved in the radical peptide reactions, the system was simplified by adding smaller peptides to the reaction mixture. By using smaller peptides, the number of potential free radical sites could be minimized, thus facilitating identification of those sites. In our previous studies involving the cytochrome \( c/H_2O_2 \) system, we discussed the idea that radical formation at any one of the three tyrosine residues or the tryptophan residue on cytochrome \( c \) may allow the oxidation of other biomolecules (15). The synthetic peptide RWIILGLNK (one tryptophan) and RSTP (two tyrosines) were chosen because of the small number of tyrosine and/or tryptophan residues in their amino acid sequences. Fig. 3A shows the MALDI spectrum of the reaction products resulting from the addition of the synthetic peptide RWIILGLNK to the cytochrome \( c/H_2O_2/DBNBS \) reaction. A molecular ion for the peptide is observed at \( \sim m/z \) 1110. In addition, a molecular ion corresponding to one DBNBS adduct of RWIILGLNK is observed at \( m/z \) 1455. The ions of \( m/z \) 1471 and \( m/z \) 1439 correspond to the addition of oxygen and the loss of oxygen, respectively, from the molecular ion of the peptide plus DBNBS. The ion of \( m/z \) 1375 corresponds to the loss of SO\(_3\) from the molecular ion of the DBNBS adduct of RWIILGLNK. The loss of SO\(_3\) is a common mass spectral fragmentation as well as an observed decomposition of DBNBS using ESR (25). The ion labeled as I is an oxidation product of an impurity also observed in the spectrum of the starting material (data not shown).

Fig. 3B shows the ESI mass spectrum resulting from the transfer studies involving RSTP. This spectrum shows the doubly charged molecular ions corresponding to unmodified RSTP, RSTP plus one DBNBS molecule, and RSTP plus two DBNBS molecules. In addition, ions corresponding to salt adducts and addition of oxygen to each of these molecular ion species are observed. Control experiments were performed in which the nonapeptide RWIILGLNK and the RSTP were reacted with hydrogen peroxide and DBNBS in the absence of cytochrome \( c \). The resulting mass spectra from each of these control reactions showed that no DBNBS adducts or oxygen-containing species of either peptide were formed (data not shown). The oxidation species observed in the mass spectra from the cytochrome \( c/H_2O_2/DBNBS/peptide \) reaction mixtures may be due to the reaction of oxygen with the peptide radical (26, 27).

As a control experiment to verify that a tyrosine or tryptophan amino acid is the reactive site for radical transfer on the target peptide, bradykinin was added to the cytochrome \( c \), hydrogen peroxide, and DBNBS reaction mixture. Bradykinin is a small peptide containing nine amino acid residues, none of which are a tyrosine or tryptophan. No peaks corresponding to addition of DBNBS or oxygen to bradykinin were observed in the MALDI spectrum of the reaction (data not shown). Only the molecular ion of bradykinin is observed in the MALDI mass spectrum.

Determination of the Radical Site on Polypeptides—To determine the location of the radical on the target peptides, in-source collision-induced decomposition (CID) spectra were obtained for the adducts of DBNBS with RWIILGLNK and RSTP. By increasing the cone (or skimmer) voltage, peptides often fragment to give structurally informative fragment ions (28). The resultant spectra are the products of fragmentation of all ions in the source, i.e. nonadducted as well as adducted peptides. The source-induced fragmentation spectra of the DBNBS adducts of RWIILGLNK and RSTP show low relative abundance ions that correlate with fragmentation of the DBNBS adduct of the peptide (data not shown). The observed fragment ions correspond to the DBNBS being located on the tryptophan of RWIILGLNK and may correspond to one of the DBNBS adducts being located on tyrosine 4 of RSTP.

To confirm the results of the in-source CID experiments, the tandem mass spectrum of the doubly protonated molecular ion of the DBNBS adduct of RWIILGLNK was acquired (Fig. 4). The sequence-specific ions are labeled on the spectrum. A nearly complete series of both y ions and b ions are observed. The y series ions are C-terminal peptide backbone cleavages and the b series ions are N-terminal backbone cleavages (26, 27). These structurally informative fragment ions allow the unequivocal assignment that the DBNBS is adducted to the tryptophan.

Effect of Antioxidant on Radical Formation—The effect of
the antioxidant vitamin C (ascorbic acid) on protein radical transfer was investigated. The antioxidants fall under two main categories: those that prevent the generation of free radicals and those that intercept any free radicals that are generated (31). Many antioxidants, such as ascorbic acid, can fall into both categories. To investigate the effect of the presence of an antioxidant on the production of the oxidatively derived cytochrome c free radicals, the reaction of cyt c, hydrogen peroxide, DBNBS, and ascorbic acid was repeated in the presence of ascorbic acid. Following the addition of ascorbic acid at the 500 μM, 1 mM, and 5 mM concentration to the reaction mixture, MALDI mass spectra were acquired (Fig. 5). At the 500 μM level, four DBNBS adducts of cytochrome c were detected (Fig. 5A). When the ascorbic acid concentration is increased to 1 mM, the number of DBNBS adducts that can be readily detected was decreased to two (Fig. 5B). At 5 mM ascorbic acid, the relative abundances of the two DBNBS adducts were decreased even further (Fig. 5C).

To determine whether the presence of an antioxidant has an effect on radical formation of target peptides, cytochrome c was first reacted with hydrogen peroxide prior to the addition of an antioxidant. From our previous study, we determined that cytochrome c free radicals could be detected by ESR following H₂O₂ treatment without the presence of a spin trap (15). The ability to obtain the ESR spectrum meant that at least some cytochrome c free radicals are stable for at least 8 min (3-min reaction time plus 5-min ESR acquisition time). Based on this, cytochrome c (500 μM) and hydrogen peroxide (5 mM) were reacted for 5 min. Following this reaction period, a series of
compound(s) were added prior to MALDI mass spectral analysis. First, the spin trap DBNBS was added to verify that the free radicals generated on cytochrome c were still present at 5 min and can be trapped by DBNBS. The MALDI mass spectrum of this reaction showed the addition of one and two DBNBS molecules to cytochrome c (data not shown). Second, RWIILGLNK (500 mM) was added and allowed to react for 2 min prior to the addition of the DBNBS spin trap. MALDI analysis revealed the formation of one radical adduct on RWIILGLNK. A free radical, therefore, was successfully transferred from cytochrome c to the peptide in this reaction (data not shown). Third, RWIILGLNK (500 mM) and varying concentrations of ascorbic acid (500 mM, 1 mM, and 5 mM) were pre-mixed, then added to the cytochrome c/H₂O₂ reaction mixture. Following a 2-min incubation, DBNBS spin trap was added, and the MALDI spectra were acquired. At the 500 mM and 1 mM ascorbic acid levels, RWIILGLNK plus one DBNBS molecule were detected (data not shown). At the 5 mM ascorbic acid level, however, the transfer of free radicals from cytochrome c to the peptide was completely interrupted. No ions corresponding to the addition of the DBNBS spin trap were observed in the MALDI spectrum (data not shown). Only the RWIILGLNK molecular ion was detected.

DISCUSSION

In the present study, multiple DBNBS radical adducts of cytochrome c were identified by ESI/MS following the reaction of cytochrome c with H₂O₂ and DBNBS. From previous ESR studies, protein-derived radical adducts have been observed not only for cytochrome c (15), but other heme-containing proteins such as myoglobin (14) and cytochrome P450 (16). We postulated that a radical formed on a solvent-exposed tyrosine or tryptophan may allow the oxidation of other biomolecules that are unable (possibly for steric reasons) to gain access to the heme iron (15). In a recent report by Østdal et al. (22), free radicals formed in the reaction of metmyoglobin and H₂O₂ in the presence of proteins were investigated using freeze quench and ESR spin-trapping. Although transfer of a protein radical species was detected, the location of the radical species was not determined. In this study, our interest is in determining whether cytochrome c-derived radicals can oxidize and initiate free radical reactions on other proteins, which, by themselves, do not undergo free radical formation, and if radical transfer occurs, to determine the location of the radical on the target peptide.

We initially tested several peptides and proteins to deter-
mine if free radicals could be formed on the biomolecule of interest in the absence of the heme-containing protein, cytochrome c. The compounds were chosen based on the postulate that a tyrosine and/or tryptophan residue was required for radical transfer to occur. It should be noted that no DBNBS/thiyl radical adduct has been reported. Apparently, this radical adduct is unstable, and consequently any cysteine thiyl free radicals formed would most likely not be detected. The substrates were subjected to the identical reaction conditions as for the cytochrome c/hydrogen peroxide/DBNBS reactions. In all cases studies, we were unable to detect a free radical adduct on any of the biomolecules in the absence of cytochrome c.

When the reactions were repeated in the presence of equimolar concentration of the heme-containing protein cytochrome c, radical adduct formation was detected for all substrates that contained a tyrosine or tryptophan amino acid residue (Fig. 3). In the case of the peptide bradykinin, which does not contain a tyrosine or tryptophan residue, no DBNBS adducts could be detected. These data are consistent with our hypothesis that the presence of a tyrosine or tryptophan amino acid residue facilitates the radical transfer reaction. In addition, the fact that cytochrome c is a prerequisite for adduct formation on the target molecule is unequivocal evidence that the radicals are initially formed on cytochrome c and are then transferred to the peptide/protein. The most plausible explanation for these observations is that, initially, oxidation of the heme occurs. The radical may then be transferred intramolecularly to a peripheral tyrosine or tryptophan on the cytochrome c followed by intermolecular transfer to the peptide or protein. Intramolecular electron transfers in peptides have been reported by Bobrowski et al. (32, 33) using pulse radiolysis.

Location of the radical adduct on the peptide may provide insight to a better understanding of the mechanism involved in the transfer of free radicals. Both the in-source collision induced decomposition spectra and the tandem mass spectrum showed structurally informative fragment ions as to the location of the radical adduct on the peptides. For RWIILGLNK, the DBNBS adduct is located on the tryptophan, and for RSTP, it appears that one of the DBNBS adducts is located at tryptophan 4. The in-source CID spectra, however, are complicated, because fragmentations of all ions in the source are observed. Tandem mass spectrometry, on the other hand, allows for selection of a parent ion with the first mass spectrometer to a collision cell with detection of the fragment ions with the second mass spectrometer. In this type of experiment, only ions resulting from fragmentation of the selected parent ion are observed. The tandem mass spectrum of the doubly protonated molecular ion of the DBNBS adduct of RWIILGLNK (Fig. 4) shows a nearly complete series of both C-terminal ions (i.e. y10 to y2) and N-terminal ions (i.e. a1 to b2). The observation of these structurally informative fragment ions allows for definitive identification of the amino acid that has been modified by covalent addition of a DBNBS spin trap molecule. For the peptide RWIILGLNK, the DBNBS radical adduct can thus be unequivocally assigned to the tryptophan residue.

It has been shown that antioxidants, both endogenous and exogenous, are a crucial defense mechanism against free radicals. The mechanisms of action, however, are poorly understood. Antioxidants can be categorized into two types: 1) those that inhibit radical initiation and 2) those that interrupt free radicals. In this study we performed a series of experiments to address the effect of the presence of antioxidants in the reaction mixtures. The trapping of free radicals was monitored by MALDI/MS as varying concentrations of ascorbic acid were added to the cytochrome c/hydrogen peroxide reaction. At the lowest level of ascorbic acid, 500 μM, little if any change in the relative abundance of the radical adducts on cytochrome c was observed (Fig. 5A). With an increase in the ascorbic acid concentration to 1 and 5 mM, the relative abundance of the free radicals detected is greatly reduced (Fig. 5, B and C, respectively). These data conclusively showed that the antioxidant ascorbic acid can be used to inhibit formation of free radicals or promote reduction of cytochrome c free radicals. In contrast to ESR experiments, which cannot, in general, distinguish between ascorbate reduced-trapped radical adduct (ascorbate reduction of a nitrooxide) and radical reduction (34, 35), the mass spectral data show that the relative abundance of the trapped radical is decreased. This indicates that the ascorbic acid can effectively compete for reaction with the radical and/or inhibits formation of the radical species.

To determine the effect of the presence of the ascorbic acid in the reactions containing target peptides, cytochrome c free radicals are formed by treatment with H₂O₂. After a short incubation period, a premixed solution of the target peptide and varying concentrations of ascorbic acid were added. Following an additional 2-min incubation, DBNBS spin trap was added, and the MALDI spectra were acquired. At the 500 μM and 1 mM ascorbic acid levels, RWIILGLNK plus one DBNBS was detected (data not shown). At the 5 mM ascorbic acid level, however, no radical adduct of the peptide RWIILGLNK was observed (data not shown). From these series of experiments, we have shown that the presence of an antioxidant in the reaction mixture prevented the radicals from being transferred to other biomolecules once they were formed on cytochrome c.

In summary, we have shown that mass spectrometry is an extremely useful technique for the analysis of protein-derived radical adducts and for the monitoring of free radical transfers to other biomolecules. Once the free radicals are generated on the heme-containing protein, horse heart cytochrome c they can be transferred to other polypeptides. Our data suggest that, in order for the radical to be successfully transferred, the target peptide/protein must contain either a tyrosine or a tryptophan amino acid residue. In addition, in-source collision-induced decompositions and tandem mass spectrometry provide useful structural information as to the location of the radical adduct on the peptide. The tandem mass spectrum of the molecular ion of the DBNBS adduct of RWIILGLNK produced fragment ions revealing the location of the DBNBS adduct on the tryptophan amino acid. Finally, we have shown that the presence of an antioxidant either inhibits or reduces free radicals on cytochrome c and prevents free radical transfer to other peptides.

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