Scavenging with TEMPO• To Identify Peptide- and Protein-Based Radicals by Mass Spectrometry: Advantages of Spin Scavenging over Spin Trapping

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Abstract: The detection and characterization of radicals in biomolecules are challenging due to their high reactivity and low concentration. Mass spectrometry (MS) provides a tool for the unambiguous identification of protein-based radicals by exploiting their reactivity with suitable reagents. To date, protein-radical detection by MS has been modeled after electron paramagnetic resonance experiments, in which diamagnetic spin traps, such as 3,5-dibromo-4-nitrosobenzene sulfonic acid, convert unstable radicals to more stable spin adducts. Since MS detects mass changes, and not unpaired spins, conversion of radicals to stable diamagnetic adducts is more desirable. The use of 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO•) in the MS identification of protein-based radicals was explored here to establish whether scavenging via radical combination would give rise to TEMPO adducts that were stable to MS analysis. The horseradish peroxidase/H_2O_2 reaction was used to generate radicals in derivatives of tyrosine, tryptophan, and phenylalanine as models of protein-based radicals. TEMPO• was added as a radical scavenger, and the products were analyzed by electrospray ionization (ESI) MS. Dramatically higher mass-adduct yields were obtained using radical scavenging vs radical trapping, which greatly enhanced the sensitivity of radical detection. The efficiency of TEMPO• in protein radical scavenging was examined in horse heart myoglobin and cytochrome *c* peroxidase (CCP) from *Saccharomyces cerevisiae*. On H_2O_2 binding to their ferric hemes, two oxidizing equivalents are transferred to the proteins as an $Fe^{IV}=O$ species and a polypeptide-based radical. In addition, CCP has been shown to reduce up to 10 equiv of H_2O_2 using endogenous donors, thereby generating as many as 20 radicals on its polypeptide. Following myoglobin and CCP incubation with a 10-fold molar excess of H_2O_2 and TEMPO•, matrix-assisted laser desorption ionization (MALDI) time-of-flight analysis of the tryptic peptides derived from the proteins revealed 1 and 9 TEMPO adducts of myoglobin and CCP, respectively. Given the high scavenging efficiency of TEMPO• and the stability of TEMPO-labeled peptides in ESI and MALDI sources, scavenging by stable nitroxide radicals coupled with MS analysis should provide sensitive and powerful technology for the characterization of protein-based radicals.

Introduction

Protein radicals can lead to cross-linking, backbone cleavage, and formation of peroxy radicals and protein peroxides. In some cases, these radical reactions are physiologically relevant, but in many other cases, protein radicals promote pathological or toxicological processes. Despite their importance, the factors and mechanisms that control the formation, localization, delocalization, and propagation of protein radicals remain obscure. Their direct observation is often disadvantaged by their low concentration (10^{-7} M) and high rates of self-reaction (10^7-10^9 M^{-1} s^{-1}).[1] Radical detection has been assisted by the use of diamagnetic spin traps containing a nitroso or nitrone function that is reactive with primary radicals to form more stable radical adducts.[2-4] To date, electron paramagnetic resonance (EPR) spectroscopy has been the main tool used in protein-based radical detection.[5] In an EPR spin-trapping experiment, the spin trap (ST) is added to a radical-generating reaction, and detection of the spin adduct (R−ST•) is considered evidence that R• is an intermediate in the reaction.[3] However, this is not always the case. For instance, in an attempt to provide evidence for OH• radical production in the Fenton reaction, Lai et al.[6] assigned EPR signals to the MNP−OH• spin adduct, but it was later shown that an identical spectrum was obtained by enzymatic or chemical reduction of the diamagnetic ST, 2-methyl-2-nitrosopropane (MNP).[7] "Inverted spin-trapping" represents another complication in the use of STs. Here, an enzyme or other component oxidizes the ST, which then reacts with an

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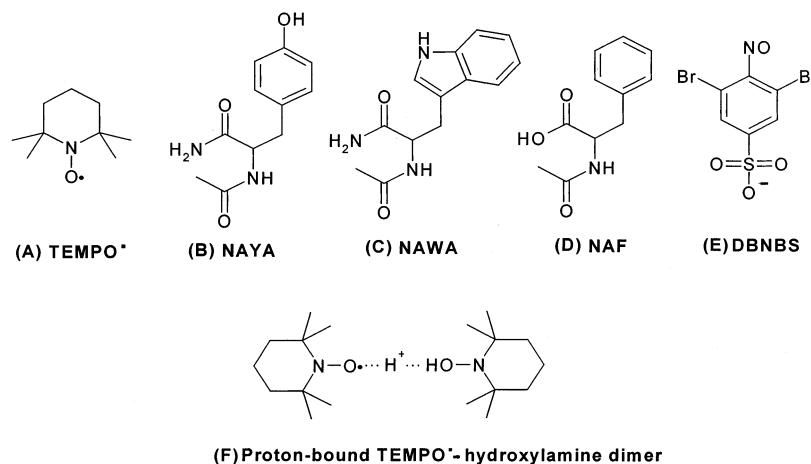
Figure 1. Structures of the reagents and the proton-bound TEMPO[•]−hydroxylamine dimer: TEMPO[•] (2,2,6,6-tetramethylpiperidinyl-1-oxy); NAYA (*N*-acetyl-L-tyrosinamide); NAWA (*N*-acetyl-L-tryptophanamide); NAF (*N*-acetyl-L-phenylalanine); DBNBS (3,5-dibromo-4-nitrosobenzenesulfonate).

amino acid residue, producing the same product as the direct reaction of the ST with a protein radical. It has also been shown that ene addition of 3,5-dibromo-4-nitrosobenzene sulfonic acid (DBNBS) to peptides containing tryptophan (W) gives rise to EPR signals upon oxidation of the resultant hydroxylamine.[8,9] Such processes obviously lead to incorrect assignments of R[•].[10]

In addition to the chemical problems associated with spin trapping, difficulties in EPR spectral interpretation[5,11] often prevent the unambiguous identification of radical sites in proteins. For instance, Mason et al. misassigned W14 and W7 as sites of radical formation in H_2O_2-oxidized myoglobin (Mb) due to ambiguity in the EPR signals.[12] Radicals in cytochrome *c* peroxidase (CCP) also eluded identification, and it was only after considerable investigation that satisfactory simulations provided conclusive evidence that the unusual behavior of the W191 radical in CCP can be attributed to its coupling with the Fe^{IV} center.[13]

To complement and overcome the limitations of EPR approaches for the analysis of protein-based radicals, spin trapping was combined in our laboratory with mass spectrometric detection of the labeled species. Specifically, spin trapping was coupled with peptide mass mapping and sequencing using high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS) (spin trapping coupled with HPLC and ESI-MS (ST/LC/MS)) to localize the actual protein residues labeled by the ST.[10,11] ST/LC/MS has greatly increased the specificity and sensitivity of radical identification and has been used by a number of groups to identify sites of protein-based radicals.[10,11,14−16] For example, ST/LC/MS has been successful in unambiguously identifying the amino acid radicals formed in H_2O_2-oxidized Mb, lacto-

peroxidase, and CCP.[11,14,16,17] Nonetheless, the technology does suffer from the drawbacks of spin trapping mentioned above, and also the relatively low reactivity of STs with proteins requires the use of 100- to 1000-fold excess ST, which complicates the MS analysis. A recent demonstration of this was the assignment of noncovalent mass adducts of cytochrome *c* and DBNBS to spin adducts.[10]

Stable nitroxide radicals, such as 2,2,6,6-tetramethylpiperidinyl-1-oxy (TEMPO[•], Figure 1A), have been known for some time to scavenge carbon-centered radicals in vivo and in vitro.[18−23] TEMPO[•] and its derivatives have also been used extensively as scavengers to control polymerization or polymer degradation in living polymerization reactions.[23−26] Since a spin adduct is not required for MS detection, nitroxide radicals should be effective tools for the identification of peptide and protein-based radicals using MS. The reported rate constants for scavenging ($k_S = 5 \times 10^7$ to 2×10^9 M^{-1} s^{-1})[1,27] or combination of a carbon-centered radical with TEMPO[•] are faster than those for trapping ($k_T = 10^6−10^7$ M^{-1} s^{-1})[2] or spin-adduct formation with a diamagnetic ST.

$$R^{\bullet} + TEMPO^{\bullet} \underset{\rightleftharpoons}{\overset{k_S}{}} R-TEMPO \qquad (1)$$

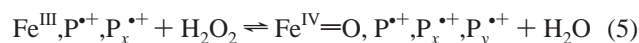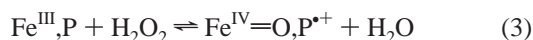$$R^{\bullet} + ST \underset{\rightleftharpoons}{\overset{k_T}{}} R-ST^{\bullet} \qquad (2)$$

In this study, the use of TEMPO[•] in scavenging peptide and protein-based radicals and characterization of the TEMPO adducts by mass spectrometry (MS) were explored. Specifically,

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horseradish peroxidase (HRP) was used to catalyze the one-electron oxidation by H_2O_2 of the aromatic amino acid derivatives, *N*-acetyl-L-tryosinamide (NAYA) (Figure 1B), *N*-acetyl-L-tryptophanamide (NAWA) (Figure 1C), and *N*-acetyl-L-phenylalanine (NAF) (Figure 1D). These derivatives were selected as peptide models since tyrosine (Y) and tryptophan (W) are frequently oxidized amino acids in biology whereas phenylalanine (F) is rarely oxidized.[28] The adducts formed by spin-combination reactions (eq 1) of the one-electron oxidized derivatives ($R^\bullet$) were analyzed by ESI-MS. For all three amino acid derivatives, formation of a stable R-TEMPO adduct competed favorably with self-dimerization ($R-R$ formation). Also, scavenging by nitroxide radicals provided a clear kinetic advantage over spin trapping, which greatly enhanced the sensitivity of MS detection of the mass adducts.

Horse heart Mb and yeast CCP were used as protein model systems. CCP efficiently catalyzes the oxidation of ferrocytochrome *c* by H_2O_2 in yeast mitochondria. In the absence of donor substrates, CCP reduces up to 10 molar equiv of H_2O_2 using endogenous donors on its polypeptide, including Y and W residues, via a heme-mediated process (eqs 3−5).[29] The first molecule of H_2O_2 reacts with ferric CCP (Fe^{III},P) to form compound I ($Fe^{IV}=O,P^{\bullet+}$, where the oxygen atom is assigned an oxidation number of $-II$). Intramolecular electron transfer to the $Fe^{IV}=O$ heme leads to the formation of a new protein-based radical, $P_x^{\bullet+}$ (or $P_x^\bullet + H^+$),[13] and reaction of a second H_2O_2 molecule with the newly formed Fe^{III} regenerates $Fe^{IV}=O$ and an additional protein radical $P_y^{\bullet+}$.

$$Fe^{III},P + H_2O_2 \rightleftharpoons Fe^{IV}=O,P^{\bullet+} + H_2O \qquad (3)$$

$$Fe^{IV}=O,P^{\bullet+} + 2H^+ \rightleftharpoons Fe^{III},P^{\bullet+},P_x^{\bullet+} + H_2O \qquad (4)$$

$$Fe^{III},P^{\bullet+},P_x^{\bullet+} + H_2O_2 \rightleftharpoons Fe^{IV}=O, P^{\bullet+},P_x^{\bullet+},P_y^{\bullet+} + H_2O \quad (5)$$

Up to 10 molar equiv of H_2O_2 can be consumed in reactions 3−5 with no heme damage and no O_2 evolution, eliminating H_2O_2 disproportionation as a mechanism of H_2O_2 consumption.[17] The protein-based radicals formed in CCP have received much attention since $P^{\bullet+}$ in eq 3 represents a cation radical on W191, which was the first catalytically active tryptophanyl radical identified in an enzyme.[13]

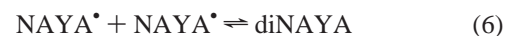Like heme peroxidases, Mb reacts with H_2O_2 to form an oxyferryl ($Fe^{IV}=O$) heme and unstable protein radical species.[30] It has been established by several groups using EPR, ST/LC/MS, and site-directed mutagenesis that Y103, which is in van der Waals contact with the heme and solvent exposed, is the primary site of radical formation in horse heart Mb.[10,11,30−33] Thus, Mb is a well-characterized model to use in the development of protein-based radical detection methods.

The low-molecular-weight R-TEMPO adducts examined here were stable to electron spray ionization mass spectrometry (ESI-

MS) analysis. Furthermore, the TEMPO-labeled proteins were stable to tryptic digestion and peptide analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF-MS). Thus, MS analysis of diamagnetic R-TEMPO adducts should provide an effective and sensitive method for the identification of radicals accessible to scavengers in biomolecules. Such capability should aid greatly in understanding the mechanisms of radical translocation, which, in turn, will assist in comprehending the role of surface-exposed protein radicals in cellular redox signaling[34] and oxidative stress.[28] The present results add to the extensive reports on biological applications of nitroxides in radical scavenging in the mitochondrial respiratory chain,[35] in cytochromes P450,[36] and in preventing Haber−Weiss reactions.[37] Scavenging by nitroxides has also been implicated in protecting against oxidative DNA scission in *Escherichia coli*, membrane damage, loss of contractility in beating cardiomyocytes, the deleterious effects of cardiac reperfusion after ischemia, and lipid peroxidation in hepatic microsomes.[18,21,22,38,39]

Results

Products of Free-Radical Generation in the Aromatic Amino Acid Derivatives. On the basis of the oxidation of free Y by HRP/H_2O_2,[40] the expected radical combination reactions following HRP-catalyzed NAYA (Figure 1B) oxidation by H_2O_2 in the presence of TEMPO$^\bullet$ are

$$NAYA^\bullet + NAYA^\bullet \rightleftharpoons diNAYA \qquad (6)$$

$$NAYA^\bullet + TEMPO^\bullet \rightleftharpoons NAYA-TEMPO \qquad (7)$$

The mass spectral analysis of the NAYA reaction mixture (36 nM HRP, 150 μM H_2O_2, 500 μM NAYA, 500 μM TEMPO$^\bullet$) revealed the presence of two product ions (Figure 2A). Peaks at *m/z* 378.1 and 443.3 are assigned to the MH$^+$ ions of the NAYA−TEMPO adduct (eq 7) and diNAYA (eq 6), respectively, while the peak at *m/z* 222.8 matches that predicted for MH$^+$ of unreacted NAYA. Peaks at *m/z* 156, 157, and 158 due to unreacted TEMPO$^\bullet$,[41] which undergoes unusual ion chemistry in the ESI source (see below), were observed with high relative abundance in all spectra recorded but are omitted for clarity. When 10-fold excess TEMPO$^\bullet$ over NAYA was added to the radical-generating reaction (36 nM HRP, 150 μM H_2O_2, 500 μM NAYA, 5 mM TEMPO$^\bullet$), the NAYA−TEMPO peak (*m/z* 378.1) is the most abundant product ion, while diNAYA (*m/z* 443.3) is undetectable, and the peak containing unreacted NAYA (*m/z* 222.8) is weak (Figure 2B). When the same experiments were performed using 1:2 and 1:5 NAYA/TEMPO ratios, no diNAYA formation was detected by MS in either case (data not shown). The peak at *m/z* 314.1 in Figure

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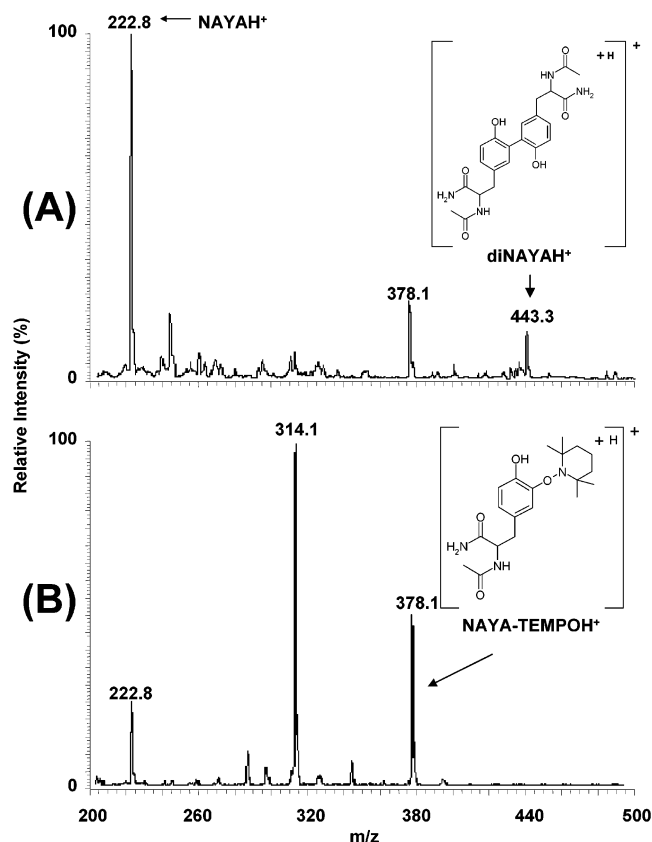
Figure 2. Product ESI mass spectrum of the reaction of 36 nM HRP, 150 μM H_2O_2, and 500 μM NAYA with (A) 500 μM and (B) 5 mM TEMPO•. The reaction was carried out for 5 min in 500 μM NaPi (pH 7.4) with 100 μM DTPA, diluted 10-fold in 50% acetonitrile/0.05% TFA, and directly infused at a rate of 3 μL/min using a syringe pump into the ESI source of the mass spectrometer. ESI conditions were 4.0 kV spray voltage and 180 °C capillary temperature. Insets show proposed structures of the radical combination products at m/z 443.3 and 378.1.[15]

Figure 3. Product ESI mass spectrum of the reaction of 36 nM HRP, 150 μM H_2O_2, and 500 μM NAF with 500 μM TEMPO• (A) between m/z 200 and 500 and (B) between m/z 360 and 420**.** The spectrum in B was expanded 50-fold. Insets show proposed structures of the radical-combination products at m/z 413.0 and 363.0.[60] The experimental conditions are given in the legend to Figure 2.

2B is assigned to a noncovalent proton-linked TEMPO•−hydroxylamine dimer (Figure 1F) since nitroxides tend not to dimerize covalently.[2] The intensity of the dimer peak, which was not seen at <1 mM TEMPO•, is dependent on concentration and indirectly related to capillary temperature and spray voltage (data not shown), consistent with noncovalent dimer formation.

Since TEMPO• catalyzes, via formation of TEMPO+, oxidation of alcohols in the Anelli reaction,[42−44] we investigated the possibility of "inverted spin scavenging". This would involve formation of the NAYA−TEMPO adduct from direct reaction between NAYA and TEMPO+, generated by HRP/H_2O_2 oxidation of TEMPO•, rather than via spin scavenging. Specifically, 5 mM TEMPO• was oxidized by HOCl, and the preformed TEMPO+ incubated with 500 μM NAYA for 10 min. The ESI mass spectrum of the products revealed no NAYA−TEMPO adduct formation (data not shown), indicating that inverted spin scavenging does not occur under the present experimental conditions (500 μM NaPi, pH 7.4).

To further characterize the efficacy of TEMPO• as a radical probe, oxidation of two other amino acid derivatives, NAWA and NAF (Figure 1C,D), was examined. Free W and F have
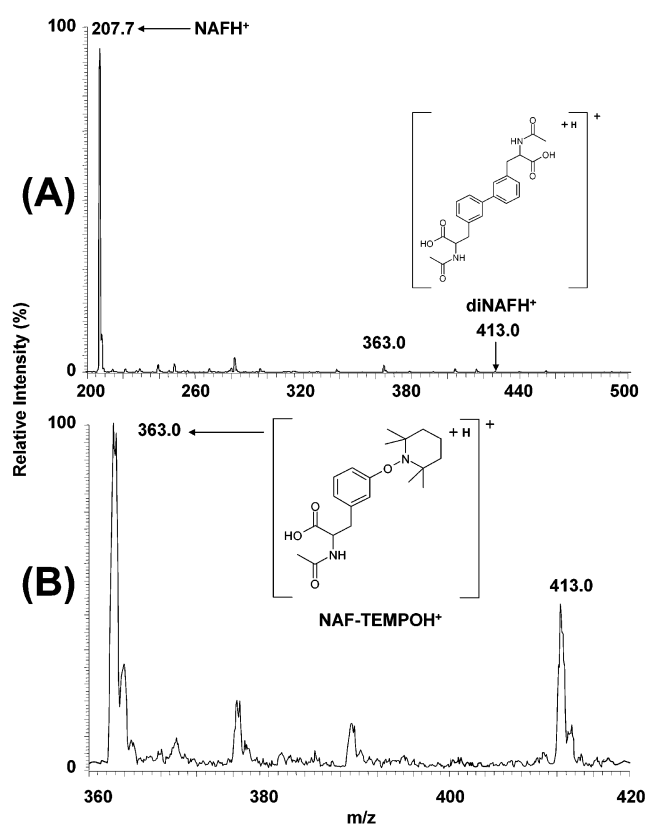
also been used as donor substrates for HRP/H_2O_2, but no reaction products were identified to date.[45] Figure 3 shows the mass spectrum of the reaction products from the 1:1 NAF/TEMPO• reaction (36 nM HRP, 150 μM H_2O_2, 500 μM NAF, 500 μM TEMPO•). The low abundance of the MH+ ions of NAF−TEMPO (m/z 363.0) and diNAF (m/z 413.0) vs that of unreacted NAF (m/z 207.7) clearly demonstrates that this amino acid derivative is a poor donor substrate for HRP/H_2O_2. Nonetheless, the major product ion is NAF−TEMPO (Figure 3B), and this becomes the sole product detected by MS when NAF/TEMPO• is ≥2 (data not shown). These results mirror those found for NAYA and reveal that scavenging of NAF• by TEMPO• is faster than NAF• dimerization. Similar results were obtained in a series of NAWA experiments, and Figure 4 shows the mass spectra of the products from 1:1 (A) and 1:10 (B) NAWA/TEMPO• reactions (36 nM HRP, 150 μM H_2O_2, 500 μM NAWA, and 500 μM and 5 mM TEMPO•).

The high efficiency of spin scavenging with TEMPO• is recognized when adduct yields are compared to those obtained in spin-trapping experiments. For example, in a competition reaction for NAYA• containing equimolar NAYA and TEMPO• and 100-fold excess of the ST, DBNBS (Figure 1E), both NAYA−TEMPO (peak T, m/z 377.9) and NAYA−DBNBS (peak D, m/z 581.3) adducts are detected with similar intensities (Figure 5). DBNBSNa+ and DBNBSH+ ions are observed at

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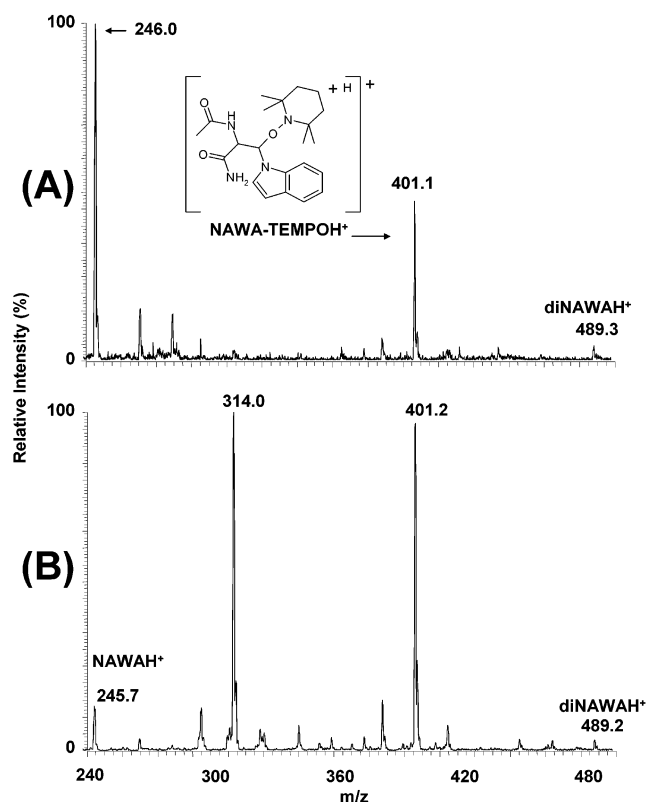
Figure 4. Product ESI mass spectrum of the reaction of 36 nM HRP, 150 μM H$_2$O$_2$, and 500 μM NAWA with (A) 500 μM TEMPO$^\bullet$ and (B) 5 mM TEMPO$^\bullet$. The inset shows the proposed structure of the radical-combination product at m/z 401.1.[60] The experimental conditions are given in the legend to Figure 2.
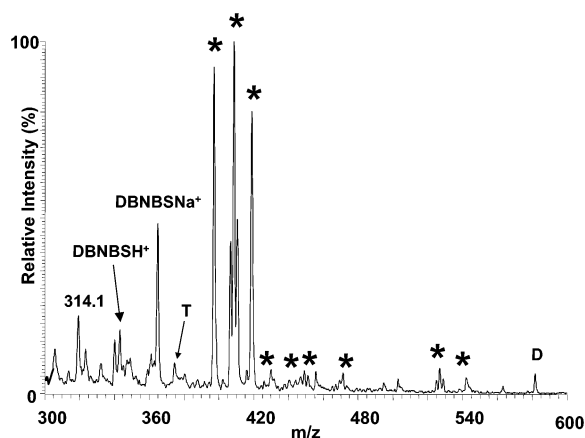

Figure 5. Product ESI mass spectrum of the reaction of 36 nM HRP, 150 μM H$_2$O$_2$, 500 μM NAYA with 50 mM DBNBS, and 500 μM TEMPO$^\bullet$. The NAYA−TEMPOH$^+$ (peak T, m/z 377.9) and NAYA−DBNBSH$^+$ (peak D, m/z 581.3) adducts are labeled, as are the DBNBSH$^+$ and DBNBSNa$^+$ ions at m/z 343.9 and 365.9, respectively. Unidentified peaks present in the spectrum of DBNBS only are labeled with an asterisk (∗). The experimental conditions are given in the legend to Figure 2.

m/z 365.9 and 343.9, respectively, as well as a number of unidentified peaks (marked with an asterisk in Figure 5) that are also present in the ESI mass spectrum of DBNBS alone. These DBNBS-derived species cause ion suppression that significantly decreases the sensitivity of NAYA-adduct detection. A NAWA−DBNBS adduct, which may have been formed by ene addition of DBNBS to W,[8,9] was observed in radical-generating reactions containing 100-fold excess DBNBS (36

nM HRP, 150 μM H$_2$O$_2$, 500 μM NAWA, 5 mM DBNBS), but no NAF−DBNBS adduct was detected in the corresponding NAF reaction (data not shown).

TEMPO$^\bullet$ Adducts Formed in H$_2$O$_2$-Oxidized Mb. The reaction of Mb with H$_2$O$_2$ produces both an Fe^{IV}=O heme and a globin-centered radical (eq 3).[32] The MALDI-ToF mass fingerprint of the tryptic digest of untreated Mb contained 14 peaks, 12 of which were identified as Mb peptides (including two isobaric peptides, Table 1) by searching the NCBI database (www.ncbi.nlm.nih.gov) using the Mascot search engine (Matrix Science),[46] and provided 97% sequence coverage. The presence of 5 mM TEMPO$^\bullet$ during the 10 min reaction of 500 μM Mb and 5 mM H$_2$O$_2$ at pH 5.0 or 7.4, followed by quenching with catalase, tryptic digestion, and MALDI-ToF analysis of the digest, resulted in the identification of TEMPO-labeled tryptic peptide T$_{16}$ which contains Y103 (Table 1). No labeled peptides were observed when the Mb/H$_2$O$_2$/TEMPO$^\bullet$ reaction was repeated at pH 3.0 and 10.0 (data not shown).

Interestingly, no evidence for TEMPO-adducts of undigested, intact Mb was obtained by either MALDI-ToF or LC/ESI-MS analysis at pH 7.4, but a low-intensity TEMPO-adduct peak was observed at 17 107 Da (10%) in the deconvolved ESI mass spectra of the protein from the pH 5.0 reaction (Figure 6A). In contrast, the Mb−DBNBS spin adduct (17 293 Da) was found[10] to dominate the ESI mass spectrum when DBNBS was added as a ST. The Mb−TEMPO adduct was observed only under very soft ionization conditions, and increasing the spray voltage and capillary temperature revealed that cleavage of the Mb−TEMPO bond was quite facile. However, differences in sample preparation, such as lyophilization of the heme-free protein at 25 °C vs direct infusion of the reaction mixture or changing the amount of TFA added, did not affect the relative intensity of the Mb−TEMPO adduct observed in the mass spectrum. These results suggest that Mb−TEMPO is less stable than T$_{16}$-TEMPO due to steric hindrance in the intact protein. The observation of an Mb−TEMPO adduct at pH 5.0 in the ESI mass spectrum (Figure 6A) is consistent with this hypothesis since opening of the heme pocket occurs in Mb at low pH.[11,47]

TEMPO$^\bullet$ Adducts Formed in H$_2$O$_2$-Oxidized CCP. The ability of CCP to turnover 20 equiv of H$_2$O$_2$ using endogenous donors[29] makes it an ideal protein to use in studies of TEMPO-labeling of protein-based radicals. MALDI-ToF analysis of the tryptic digests of untreated CCP revealed 29 peaks, 21 of which were identified as CCP peptides (including three pairs of isobaric peptides, Table 2) by searching the NCBI database with Mascot,[46] and provided 88% sequence coverage (Figure 7). The presence of 5 mM TEMPO$^\bullet$ during a 10 min reaction of 500 μM CCP with 5 mM H$_2$O$_2$ resulted in the detection by MALDI-ToF-MS of eight TEMPO-labeled tryptic peptides (including singly and doubly labeled T$_{27+28+29+30}$, Table 2), but no labeled peptides in the absence of H$_2$O$_2$. Figure 8 compares the MALDI-ToF mass spectra of tryptic peptides in the m/z range 2500−2900 from the control and H$_2$O$_2$ reactions. The mass increases of MH$^+$ ions of peptides T$_{27+28+29+30}$ (m/z 2585.8, Δm 151.8 Da) and T$_{7+8}$ (m/z 2680.8, Δm 153.7 Da) reveals that these peptides are singly TEMPO-labeled since an increase of 155.2 Da is expected per label. Peaks attributed to sodium adducts

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Table 1. Tryptic Peptides from the MALDI-ToF Mass Fingerprints of Myoglobin[a]

peptide[b]	residues[c]	M_r(obs)[d]	M_r(calcd)[e]	sequence[f,g]
T_{18}	134−139	748.2	747.9	ALELFR
T_{6+7}	48−56	1084.4	1086.3	HLKTEAEMK
T_{5+6+7}	46−56	1362.2	1361.6	FKHLKTEAEMK
T_{8+9}	57−71	1507.6	1506.8[h]	KHGTVVLTALGGILK
T_{9+10}	58−72	1507.6	1506.8[h]	HGTVVLTALGGILKK
T_2	17−31	1607.4	1606.8	VEADIAGHGQEVLIR
$T_{18+19+20}$	134−147	1649.6	1651.9	ALELFRNDIAAKYK
T_{16}	103−118	1885.9	1885.2	*Y*LEFISDAIIHVLHSK
T_{16} + TEMPO		**2041.3**	**2040.4**	
T_{3+4+5}	32−47	1938.1	1937.2	LFTGHPETLEKFDKFK
$T_{12+13+14}$	79−98	2248.8	2247.5	KGHHEAELKPLAQSHATKHK
$T_{18+19+20+21}$	134−153	2283.6	2283.6	ALELFRNDIAAKYKELGFQG
T_{1+2}	1−31	3405.4	3404.8	GLSDGEWQQVLNVWGKVEADIAGHGQEVLIR
$T_{16+17+18}$	103−139	4100.3	4099.7	YLEFISDAIIHVLHSKHPGDFGADAQGAMTKALELFR

[a] Tryptic peptides were prepared and analyzed as given in the Experimental Section. [b] Peptides are assigned T_x in order of the expected site of cleavage (C-terminal to K and R residues) and listed based on increasing mass. The peptide found to be TEMPO labeled at pH 7.4 is presented in bold font, and the mass of the labeled species is given in the T_{16} + TEMPO row. [c] Position of peptide in the protein's amino acid sequence. [d] Observed average mass (M_r) of the peptides in Da. The mass accuracy of the MALDI-ToF mass spectrometer is ±0.14% using human keratin and trypsin autodigestion peaks as internal standards. [e] Calculated average M_r based on peptide sequence and a mass increase of 155.2 per TEMPO label. [f] Amino acid sequence presented in one-letter code; peptides were identified by searching the NCBI database (www.ncbi.nlm.nih.gov) using the Mascot search engine.[46] [g] Y and W residues, which are considered possible sites of TEMPO labeling, are in italic. [h] These peptides are isobaric given the mass accuracy of the instrument (±0.14%).
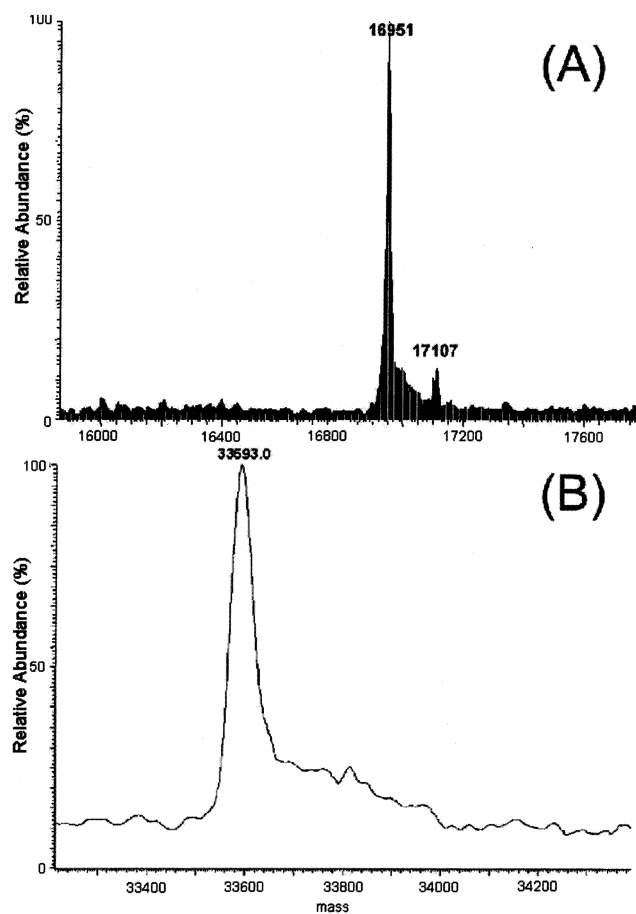

Figure 6. Deconvolved ESI mass spectra of the intact proteins from the 10 min reactions of 500 μM heme protein with 5 mM H_2O_2 in the presence of 5 mM TEMPO in 500 μM NaPi containing 100 μM DTPA: (A) Mb from a reaction carried out at pH 5.0; (B) CCP from a reaction carried out at pH 7.4. The HPLC-purified protein products were collected, lyophilized, resuspended in 50% acetonitrile/0.05% TFA to a concentration of ~1 μg/mL, and directly infused at a rate of 3 μL/min using a syringe pump into the ESI source of the mass spectrometer. The ESI conditions are given in the legend to Figure 2.

(+23 Da), which are commonly observed in MALDI-ToF spectra,[48] are denoted with an asterisk in Figure 8.

Three of the singly TEMPO-labeled peptides contain a sole W or Y residue (W126 in $T_{14+15+16}$, Y153 in T_{18+19}, and Y251 in $T_{28+29+30}$), which are the likely sites of radical scavenging. The other peptides contain multiple W or Y residues (Y36, Y39, and Y42 in T_6; Y244 and Y251 in $T_{27+28+29+30}$ and $T_{27+28+29+30+31}$; Y187, W191, Y203, and W211 in T_{23}). In doubly TEMPO-labeled $T_{27+28+29+30}$, both Y244 and Y251 are assumed to be labeled, as is Y251 in singly TEMPO-labeled $T_{28+29+30}$. The other doubly TEMPO-labeled peptide, T_{23}, contains W191, the site of radical formation in compound I,[13] in addition to Y187, Y203, and W211. Sequencing of the labeled peptides is currently underway to unambiguously determine the oxidized residues. The errors in M_r(obs) for some of the TEMPO-labeled CCP peptides (Table 2) fall outside the range of mass accuracy (±0.14%) obtained using keratin and trypsin autodigestion peaks as internal standards (Figure 7). Postsource cleavage of the TEMPO label could contribute to the slightly increased deviation of M_r(obs) from M_r(calc) for the labeled peptides.

Although extensive TEMPO-labeling of CCP peptides was detected by MALDI-ToF analysis, as with Mb, labeling of the undigested protein was not detected by MS. For example, when 500 μM CCP was reacted with 5 mM H_2O_2/5 mM TEMPO• for 10 min at pH values of 3.0, 5.0, 7.4, or 10.0, and analyzed by LC/ESI-MS or MALDI-ToF-MS, only native protein peaks (M_r ~ 33 593 Da) but no CCP−(TEMPO)$_n$ peaks were observed in the mass spectra (Figure 6B).

Discussion

Scavenging of Radicals Generated in the Aromatic Amino Acid Derivatives. TEMPO• is a π-radical molecule where the unpaired electron occupies a π* orbital between the oxygen and nitrogen atoms. The result of this electron delocalization is a relatively stable structure that provides a unique means by which to scavenge radicals. The effective N−O bond order is 1.5, since there are also filled σ- and π-bonding orbitals between these atoms.[2]

The generation of dityrosine in proteins in vivo is a normal physiological process in specialized cells such as those involved
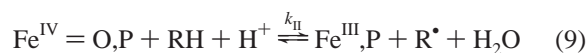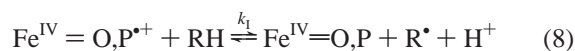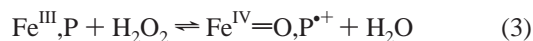
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Table 2. Tryptic Peptides from the MALDI-ToF Mass Fingerprints of Cytochrome *c* Peroxidase[a]

peptide[b]	residues[c]	M_r(obs)[d]	M_r(calcd)[e]	sequence[g,h]
T$_{33}$	279–287	1022.4	1021.2	DAPSPFIFK
T$_1$	3–12	1083.5	1082.3	TLVHVASVEK
T$_{32}$	269–278	1132.3	1131.3	LLENGITFPK
T$_{21}$	167–179	1296.0	1295.6	EVVALMGAHALGK
T$_{18+19}$	150–160	1360.4	1359.5	DAG*Y*VRTFFQR
T$_{18+19}$ + TEMPO		**1519.2**	**1514.7**	
T$_{31+32}$	265–278	1607.3	1606.9	AFEKLLENGITFPK
T$_{3+4}$	15–29	1789.1	1789.0	SYEDFQKVYNAIALK
T$_{28+29+30}$	250–264	1880.1	1882.0	E*Y*ANDQDKFFKDFSK
T$_{28+29+30}$ + TEMPO		**2037.2**	**2037.2**	
T$_6$	32–48	2015.9	2017.1	EDDE*Y*DN*Y*IG*Y*GPVLVR
T$_6$ + TEMPO		**2169.5**	**2172.3**	
T$_{3+4+5}$	15–31	2057.2	2058.4[h]	SYEDFQKVYNAIALKLR
T$_{17+18}$	131–149	2057.2	2056.1[h]	VDTPEDTTPDNGRLPDADK
T$_{32+33}$	269–287	2133.4	2134.5	LLENGITFPKDAPSPFIFK
T$_{5+6}$	30–48	2284.8	2286.5[h]	LREDDEYDNYIGYGPVLVR
T$_{14+15+16}$	124–143	2284.8	2285.5[h]	IP*W*RCGRVDTPEDTTPDNGR
T$_{14+15+16}$ + TEMPO		**2440.7**	**2440.7**	
T$_{20+21+22}$	161–183	2518.3	2519.0	LNMNDREVVALMGAHALGKTHLK
T$_{27+28+29+30}$	244–264	2584.8	2585.9	*Y*LSIVKE*Y*ANDQDKFFKDFSK
T$_{27+28+29+30}$ + TEMPO		**2736.6**	**2741.1**	
T$_{27+28+29+30}$ + 2TEMPO		**2895.1**	**2896.3**	
T$_{7+8}$	49–72	2679.8	2679.9	LA*W*HISGT*W*DKHDNTGGS*Y*GGT*Y*R
T$_{7+8}$ + TEMPO		**2833.5**	**2835.1**	
T$_{32+33+34}$	269–294	2907.2	2905.3[h]	LLENGITFPKDAPSPFIFKTLEEQGL
T$_{9+10+11+12}$	73–97	2907.2	2906.3[h]	FKKEFNDPSNAGLQNGFKFLEPIHK
T$_{7+8+9}$	49–74	2954.6	2955.2	LA*W*HISGT*W*DKHDNTGGSYGGTYRFK
T$_{27+28+29+30+31}$	244–268	3063.3	3061.4	*Y*LSIVKE*Y*ANDQDKFFKDFSKAFEK
T$_{27+28+29+30+31}$ + TEMPO		**3222.1**	**3216.6**	
T$_{23}$	184–212	3362.6	3363.6	NSG*Y*EGP*W*GAANNVFTNEF*Y*LNLLNED*W*K
T$_{23}$ + 2TEMPO		**3674.7**	**3675.0**	
T$_{3+4+5+6}$	15–48	4053.3	4049.6	SYEDFQKVYNAIALKLREDDEYDNYIGYGPVLVR
T$_{12+13+14}$	91–127	4201.7	4198.9	FLEPIHKEFPWISSGDLFSLGGVTAVQEMQGPKIPWR

[a] Tryptic peptides were prepared and analyzed as given in the Experimental Section. [b] Peptides are assigned T$_x$ in order of the expected site of cleavage (C-terminal to K and R residues) and listed based on increasing mass. Peptides found to be TEMPO labeled at pH 7.4 are presented in bold font, and the masses of the labeled species are given in the T$_x$ + TEMPO rows. [c] Position of peptide in the protein's amino acid sequence. [d] Observed average mass (M_r) of the peptides in Da. The mass accuracy of the MALDI-ToF mass spectrometer is ±0.14% using human keratin and trypsin autodigestion peaks as internal standards. [e] Calculated average M_r based on peptide sequence and a mass increase of 155.2 per TEMPO label. [f] Amino acid sequence presented in one-letter code; peptides were identified by searching the NCBI database (www.ncbi.nlm.nih.gov) using the Mascot search engine.[46] [g] Y and W residues, which are considered possible sites of TEMPO labeling, are in italic. [h] These peptides are isobaric given the mass accuracy of the instrument (±0.14%).

in the biosynthesis of thyroxin and melanin.[49] Dityrosine is also a marker for assessing oxidative damage or exposure of proteins to harmful environmental agents such as UV radiation[50] in vitro and in vivo.[40,51,52] HRP (Fe^{III},P) catalyzes the oxidation by H$_2$O$_2$ of free Y ($E°' = 0.93$ V)[53] via the mechanism shown in eqs 3, 8, and 9 with k_I and k_{II} values of 5.0×10^4 and 1.1×10^3 M^{-1} s^{-1}, respectively[54,55]

$$\text{Fe}^{III}\text{,P} + \text{H}_2\text{O}_2 \rightleftharpoons \text{Fe}^{IV}{=}\text{O,P}^{\bullet+} + \text{H}_2\text{O} \qquad (3)$$

$$\text{Fe}^{IV}{=}\text{O,P}^{\bullet+} + \text{RH} \overset{k_I}{\rightleftharpoons} \text{Fe}^{IV}{=}\text{O,P} + \text{R}^{\bullet} + \text{H}^{+} \qquad (8)$$

$$\text{Fe}^{IV}{=}\text{O,P} + \text{RH} + \text{H}^{+} \overset{k_{II}}{\rightleftharpoons} \text{Fe}^{III}\text{,P} + \text{R}^{\bullet} + \text{H}_2\text{O} \qquad (9)$$

The resultant tyrosyl radical (R$^{\bullet}$) rapidly dimerizes to dityrosine,[50] which provides a common method of dityrosine synthesis in vitro.[56] Mass spectral analysis of the HRP/H$_2$O$_2$/

NAYA (500 μM)/TEMPO$^{\bullet}$ (500 μM) products revealed the presence of a NAYA–TEMPO peak and a less intense diNAYA peak (Figure 2A). These results were encouraging since they demonstrated that the NAYA–TEMPO adduct is stable under MS conditions and that NAYA–TEMPO formation (eq 7) competes with NAYA$^{\bullet}$ dimerization (eq 6) in solutions containing equimolar NAYA and TEMPO$^{\bullet}$. Furthermore, the data in Figure 5 demonstrate that spin scavenging is much more efficient than spin trapping, and the large excess of ST required to trap the NAYA$^{\bullet}$ radical complicates the mass spectrum.

Dityrosine formation and the reaction of TEMPO$^{\bullet}$ with carbon-centered radicals both have rate constants in the range of 10^9 M^{-1} s^{-1}.[1,40,57] Thus, dimerization should not compete with scavenging by 10-fold excess TEMPO$^{\bullet}$ and no diNAYA peak was detected under such conditions (Figure 2B). Similar results were observed with NAWA ($E°' \sim 1.1$) and NAF ($E°' \sim 1.4$)[28] as substrates (Figures 3 and 4), demonstrating the ability of TEMPO$^{\bullet}$ to scavenge these less easily formed amino acid radicals and to compete with their dimerization. Interestingly, the homodimerization of both W and F has been shown to stabilize proteins against proteolytic cleavage in vitro,[58,59] and

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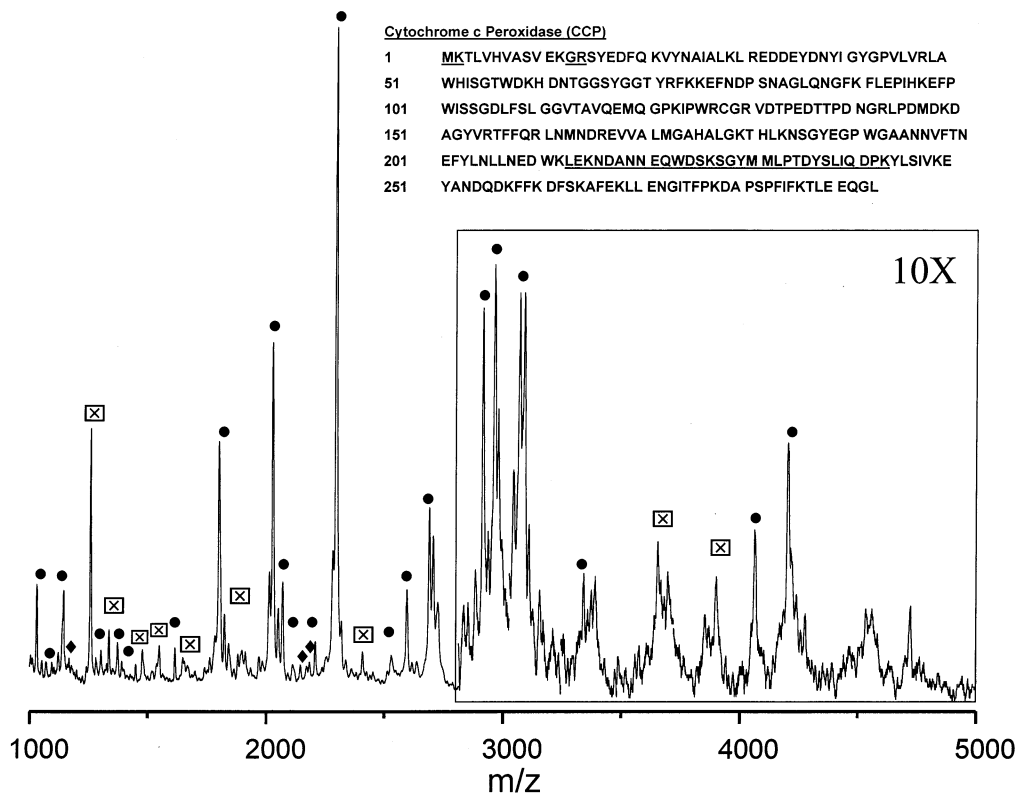
Figure 7. MALDI-ToF mass fingerprint of the tryptic peptides of untreated CCP: (solid circle) identified CCP tryptic peptides, which are listed in Table 2; (solid diamond) trypsin autodigestion peptides; (× inside box) human keratin peaks. The CCP sequence coverage was 88%, and the missing residues are underlined (insert). Tryptic peptides were prepared and analyzed as described in the Experimental Section. The MALDI-Tof mass spectrometer was operated in the linear, positive-ion, and delay modes with an accelerating voltage of $+15$ kV. Baseline correction and noise removal were performed using the default parameters (peak width 32, flexibility 0.5, degree 0.1, resolution, $R \sim 500$) of the Data Explorer software on the instrument.
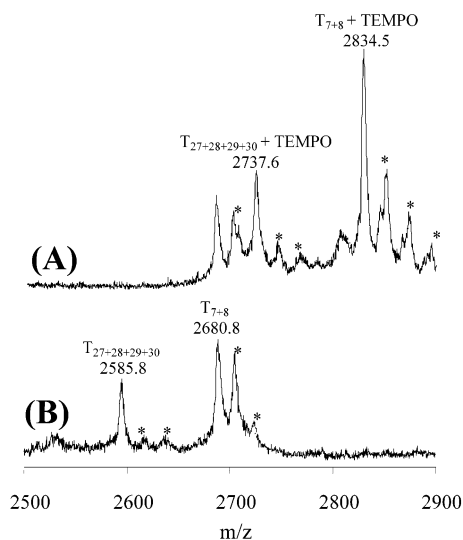

Figure 8. MALDI-ToF mass fingerprint in the region of tryptic peptides $T_{27+28+29+30}$ and T_{7+8} of (A) untreated CCP and (B) CCP oxidized by H_2O_2 in the presence of TEMPO•. Tryptic peptides were prepared and analyzed as described in the Experimental Section and MS conditions are given in the legend to Figure 7. Na^+ adducts ($+23$ Da) are labeled with an asterisk (∗). The doubly TEMPO-labeled MH^+ ion of $T_{27+28+29+30}$ is not observed in this particular spectrum.

W dimers stabilize antiparallel β-structures in model peptides.[60] A soluble fluorescent compound isolated from certain scorpi-

ons[61] was recently shown to be a ditryptophan derivative, revealing the biological relevance of W dimerization.

Protein-Based Radical Scavenging. Much work has focused on the location of polypeptide-based radicals in H_2O_2-oxidized Mb.[10,11,30−33] TEMPO labeling of T_{16} was detected here by MALDI-ToF-MS (Table 1). This is attributed to scavenging of a radical localized on Y103 at pH 5.0 and 7.4 (Table 1), since we and several other groups have confirmed Y103-labeling by ST/LC/MS, site-directed mutagenesis, and protein cross-linking experiments.[10,11,30−33]

With 7 W and 14 Y, CCP contains an unusually high number of redox-active amino acid residues for a 294-residue protein. These numbers are especially notable when compared with other peroxidases of similar size, such as HRP (one W and five Y), ascorbate peroxidase (two W and seven Y), lignin peroxidase (three W and no Y), or manganese peroxidase (one W and no Y). For many years, CCP was one of a few enzymes known to utilize a W (W191) radical during catalytic turnover.[13,62−64] A function of CCP is believed to be the removal of H_2O_2 using electrons obtained (via ferrocytochrome c) from the electron-transport chain.[65−68] It was recently reported that CCP is also involved in conveying an oxidative-stress signal to the transcrip-

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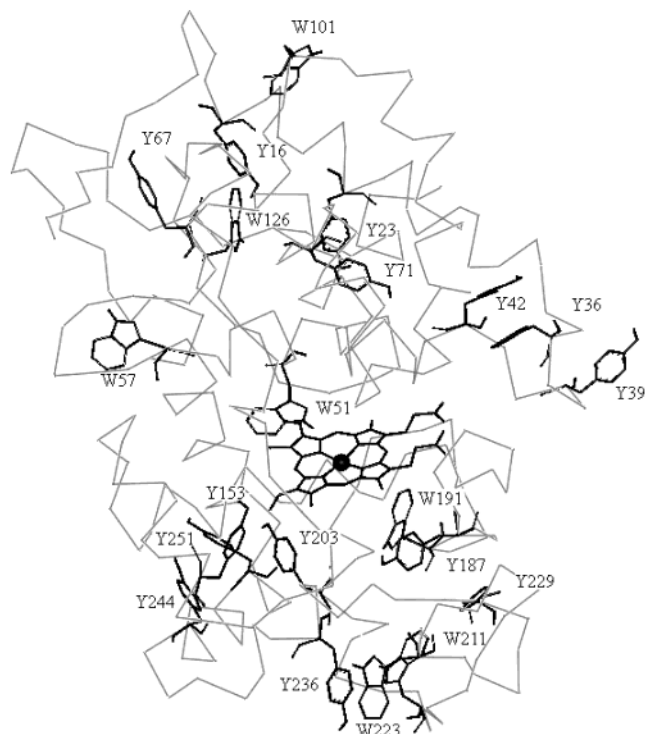
Figure 9. The C_α backbone of CCP showing the location of the 7 tryptophan (W) and 14 tyrosine (Y) residues relative to the heme. Finzel, B. C.; Poulos, T. L.; Kraut, J. *J. Biol. Chem.* **1984**, *259*, 13027–13036.

tion factor, Pos9p (or Skn7p), in *Saccharomyces cerevisiae*.[69] Since the W191F mutant, which has no ferrocytochrome *c* oxidizing activity, is also able to activate Pos9p-dependent transcription,[69] the signaling function of CCP is independent of electron flux in the electron-transport chain. Therefore, identification of the sites of heme-mediated H_2O_2-induced radical formation in CCP (eqs 4 and 5) is of interest, since such radicals are likely involved in oxidative-stress signaling in yeast mitochondria.[69,70]

Previously we reported that the presence of the ST, MNP (11 mM), during the reaction between 20 μM CCP and 200 μM H_2O_2 resulted in the detection by LC/ESI-MS of triply MNP-labeled CCP. Peptide mass mapping and sequencing by CID of the MNP-labeled peptides revealed the sites of adduct formation to be Y236, Y153, and peptide T_6 containing Y36, Y39, and Y42 (Figure 9).[17] Similar results were obtained by Zhang et al. who found MNP adducts on Y39 and Y153.[16] Although only a 10-fold molar excess of TEMPO• over CCP was used here vs the 55- or 90-fold molar excess of MNP used in previous studies,[16,17] nine TEMPO-labeled peptides were found by MALDI-ToF-MS analysis of the CCP tryptic peptides (Table 2). These results highlight the much greater efficiency of spin scavenging compared to spin trapping as anticipated from the NAYA results (Figure 5).

Interestingly, Pfister et al. found that sequentially mutating W191, W51, Y187, Y229, Y236, Y36, Y39, and Y42 to F

residues resulted in higher stability of compound I, revealing the importance of these residues as endogenous donors.[71] Such results reinforce the importance of the role played by Y and W residues in CCP compound I stability and possibly also in oxidative-stress signaling.[71] With the exception of T_{26} (residues 227–243 including Y229 and Y236), which was not detected (Figure 7, inset), TEMPO-labeled peptides containing the residues mutated were found here in the mass fingerprint (Table 2), as well as seven additional peptides with W or Y residues that were not mutated by Pfister et al.[71] Intramolecular electron transfer to the CCP heme most likely involves radical hopping, forming transients until a radical is trapped in a relatively stable chemical environment or reaches the protein surface. Work is underway in our lab to determine the sites of radical formation when CCP and several of its W and Y mutants are reacted with 1–10 molar equiv of H_2O_2.

Radical Scavenging vs Electron Donation by TEMPO•. In our previous ST/LC/MS studies on protein-based radicals, the STs used exhibit reduction potentials ($E° \sim 2$ V)[72] more positive than those of the $Fe^{IV}=O$ centers ($E°' < 1.2$ V)[10,11,14] generated on reaction of heme proteins with H_2O_2 (eq 3). In contrast, oxidation of TEMPO• to the nitrosium ion, TEMPO$^+$ ($E° \sim 0.64$ V),[43] by HRP/H_2O_2 is thermodynamically favorable. To investigate if this occurs, the Soret band of 1 μM HRP was monitored following addition of 1 mM H_2O_2 with and without 2.5 mM TEMPO•. H_2O_2 consumption in the absence of TEMPO• gave rise to a time-dependent Soret shift from 403 to 370 nm, indicative of heme destruction and HRP inactivation,[73–75] while in the presence of TEMPO•, the Soret band remained unaltered and all the H_2O_2 was consumed (data not shown). The observation of a more abundant TEMPO$^+$ ion in the product mass spectrum of 36 nM HRP, 150 μM H_2O_2, and 500 μM TEMPO• in the absence vs presence of NAYA (data not shown) confirms that TEMPO• acts as a donor substrate to HRP.

Heme destruction was also observed during 1 μM HRP turnover in a solution of 1 mM H_2O_2 and 2.5 mM NAYA (data not shown). Although phenolic compounds are excellent donor substrates for HRP,[68] studies have revealed that dityrosine is a competitive inhibitor of Y oxidation by HRP/H_2O_2.[76] Thus, diNAYA binding to the enzyme likely prevents access of NAYA to the HRP heme, which is then destroyed by reaction with excess H_2O_2. Heme destruction is eliminated when TEMPO• is present because it acts as a NAYA• scavenger, thereby preventing diNAYA product inhibition. Nonetheless, NAYA is the preferred substrate as evidenced by the large amount of NAYA– TEMPO detected in the mass spectra (Figures 2 and 4) presumably because HRP has a well-defined binding pocket for aromatic donors.[77] Electron donation by TEMPO• is not expected to be kinetically competitive with rapid radical

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scavenging since the buried hemes in peroxidases are reduced with rate constants of $<10^7$ M^{-1} s^{-1}.[78]

In positive-ion mode, ESI sources typically produce protonated molecular ions (MH$^+$).[79] The ESI mass spectra of solutions containing only TEMPO$^•$ exhibit peaks at m/z 156, 157, and 158 that are assigned to TEMPO$^+$, TEMPOH$^{•+}$, and TEMPOH$_2^+$ ions, respectively. The relative abundance of the three species depends on the experimental conditions, and a systematic analysis of this unusual ion chemistry is currently under investigation in our laboratory.

Stability of the R−TEMPO Bond. Evidence of TEMPO-labeling was observed at pH 5.0 (but not at pH values of 3.0, 7.4, and 10.0) in the deconvolved ESI mass spectra of intact Mb (Figure 6A). The Mb−TEMPO adduct disappeared from the mass spectrum when the capillary temperature and spray voltage were increased. Similar results were obtained whether the Mb/H$_2$O$_2$/TEMPO$^•$ reaction products were directly infused into the ESI source or HPLC-purified and lyophilized prior to infusion. No TEMPO-labeling of intact Mb was observed in the MALDI-ToF spectra. These results suggest that the T$_{16}$−TEMPO bond is weaker when the protein is in its folded conformation and that the label is lost under the ionization conditions used to record the ESI and MALDI-ToF spectra. However, when Mb was subjected to tryptic digestion before MS analysis, TEMPO-labeled T$_{16}$ was observed. Similar results were obtained for CCP, where no TEMPO labeling of the intact protein was detected (Figure 6B), but nine labeled peptides were discovered when the enzyme was protolytically cleaved prior to MS analysis (Table 2). Since the NAYA−, NAWA−, and NAF−TEMPO adducts are stable in both the ESI and MALDI sources, steric constraints must destabilize the TEMPO label in a folded or partially folded protein. Given the low bond strength reported for similar carboxylamine (C−ON) bonds in alkoxyamine polymers (100−130 kJ/mol),[80,81] and the inherent instability of the C−ON bond to thermal homolysis,[82] it is not surprising that TEMPO-labeled proteins are less stable to MS than their ST-labeled counterparts, which possess a stronger C−C bond (345 kJ/mol).[83] Relief of steric overcrowding on protein digestion allows the C−ON bond to withstand the MS analysis, as seen clearly for the TEMPO-labeled aromatic amino acid derivatives. In fact, it should be possible to exploit the thermal lability of the C−ON bond to confirm radical scavenging by establishing threshold temperatures for homolysis of peptide−TEMPO and protein−TEMPO adducts. Such investigations are underway, and the results may demonstrate the intriguing possibility of generating polypeptide-based radicals reversibly as in nitroxide-mediated living polymerization.[26] Radical capping may increase the stability of peroxidases, thereby expanding their utility in industrial applications, which currently include food production, fabric dying, and paper-pulp manufacture.[84]

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Conclusions

The use of TEMPO$^•$ as a reagent for the MS detection of biologically relevant carbon-centered free radicals has been shown to be highly effective in two very different radical-generating environments in vitro. In both the HRP-catalyzed peroxidation of aromatic amino acid derivatives (NAYA, NAWA, and NAF) and direct heme-mediated protein peroxidation, TEMPO$^•$ significantly outperforms diamagnetic STs such as MNP and DBNBS in adduct yield. Also, the formation of a diamagnetic TEMPO adduct rather than a spin adduct greatly diminishes the probability of radical side reactions typically associated with STs. Spin scavenging is also expected to be more selective over spin trapping given the high reactivity of stable nitroxide radicals with carbon-centered radicals. Furthermore, the well-documented homolysis of the carboxylamine bond at elevated temperatures can be exploited to confirm spin scavenging. The technology described here should help elucidate mechanisms of translocation of protein radicals and their roles in redox- and oxidative-stress-signaling pathways.

Experimental Section

Materials. Wild-type recombinant cytochrome *c* peroxidase (CCP) was prepared as described previously.[62] Horseradish peroxidase isozyme C (HRP, Grade I) was purchased from Roche. Horse heart myoglobin, bovine catalase, *N*-acetyl-L-tyrosinamide, *N*-acetyl-L-tryptophanamide, *N*-acetyl-L-phenylalanine, 2,2,6,6-tetramethylpiperidinyl-1-oxy, sodium 3,5-dibromo-4-nitrosobenzenesulfonate, and trifluoroacetic acid were from Sigma and used without further purification. HPLC grade acetonitrile was purchased from EM Industries, and H$_2$O$_2$ from Fisher. Sodium phosphate (ICN) buffers were prepared using distilled water (specific resistance 18 MΩ) from a Millipore Simplicity 185 system. All buffers contained 100 μM DTPA (Fisher) to prevent trace-metal redox chemistry.

Radical Generation in the Aromatic Amino Acid Derivatives. A 500 μM portion of NAYA, NAWA, or NAF, 150 μM H$_2$O$_2$, and 1 or 10 molar equiv of TEMPO$^•$ (500 μM or 5 mM) were added to 500 μM NaPi buffer (pH 7.4) containing 100 μM DTPA. The reaction was initiated by addition of 36 nM HRP and allowed stand at room temperature for 5−10 min, flash frozen, and stored at −80 °C for MS analysis. The samples were diluted 10-fold in 50% acetonitrile/0.05% TFA and directly infused using a syringe pump (Harvard Apparatus) into the ESI source of a ThermoFinnigan SSQ 7000 single quadrapole mass spectrometer at a flow rate of 3 μL/min. Ultrapure N$_2$ (Praxair) was used as a nebulizing gas. Typical MS operating conditions were as follows: needle voltage, 4.0 kV; spray current, 2.3 mA; capillary temperature, 180 °C; electron multiplier, 1200 V. The mass range was scanned from 100 to 1000 amu at 3 s/scan in positive-ion mode. Data analysis was performed using Xcalibur software (ThermoFinnigan).

Heme Protein-Based Radical Generation Reactions. A 5 mM portion of H$_2$O$_2$ was added to a mixture of 500 μM CCP (or Mb) and TEMPO$^•$ (500 μM or 5 mM) and allowed to react for 10 min at room temperature in 500 μM NaPi buffer (pH 7.4) containing 100 μM DTPA. Although the reduction of H$_2$O$_2$ by CCP should be completed in ∼1 min,[85] 10 nM catalase was added after 10 min to ensure that all H$_2$O$_2$ was removed. The product mixture was directly infused into the ESI source of the mass spectrometer at a flow rate of 3 μL/min or flash frozen and dried at 25 °C in a Speed Vac (Savant) for 1 h. For further ESI-MS analysis of the intact protein, the lyophilizate was diluted in 50% acetonitrile/0.05% TFA. The MS operating conditions were the same as those used for the amino acid derivatives except the mass range was scanned from 500 to 2000 amu.

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Prior to tryptic digestion, heme-free CCP and Mb were formed by diluting the lyophilizate in 20 μL 50% acetonitrile/0.05% TFA and removing the heme on a reversed-phase C18 4.6 × 250-mm HPLC column (Zorbax) with a 10−50% acetonitrile gradient in 0.05% TFA at 1 mL/min over 20 min. The HPLC purifications were performed using an Agilent HPLC (model 1090) equipped with a control module, binary pump, manual injector, and diode-array UV−vis detector. The heme-free proteins were lyophilized and resuspended in 100 mM NaPi buffer (pH 7.4). Sequencing-grade trypsin (1 μg/μL, Roche) was added at a sample-to-trypsin ratio of 10:1 (w/w), and digestion was carried out at 37 °C for 4−6 h. Digestion was quenched by freezing in liquid N$_2$, and the recovered peptides were dried in the Speed Vac, resuspended in 10 μL of 5% methanol (Fisher)/0.1% TFA, and desalted on C$_{18}$ Zip Tips (Millipore). The desalted peptides were collected from the Zip Tips in 1.5 μL of 60% acetonitrile/0.1% TFA and mixed with 1.5 μL of MALDI matrix solution prepared by combining 10 volumes of acetone saturated with α-cyano-4-hydroxycinnamic acid (Sigma), 1 volume of acetone with 20 mg/mL nitrocellulose (Sigma), 4 volumes of acetone (Fisher), and 5 volumes of 2-propanol (Fisher). Samples (1.5−2.5 μL) were spotted on the MALDI target plate (Applied Biosystems), and analyzed using a Voyager DE Linear MALDI-ToF mass spectrometer (Applied Biosystems) with a resolution of ∼500. Mass spectra were acquired as the sum of the ion signals generated by irradiation of the target with 128 laser pulses and subjected to two-point internal calibration using the trypsin autolysis peptides with M_r 804.9 and 2163.3. This resulted in a mass accuracy of ±0.14% based on the masses obtained for the peptides derived from trypsin auto-digestion and human keratin present in the samples as impurities (see Figure 7). Average peptide masses were assigned and used in the database search. Peptides were selected in the mass range of 1000−4200 Da.

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