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Mass Spectral Analysis of Protein-based Radicals Using DBNBS

NONRADICAL ADDUCT FORMATION *VERSUS* SPIN TRAPPING*

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Protein-based radicals generated in the reaction of ferricytochrome *c* (cyt *c*) with H_2O_2 were investigated by electrospray mass spectrometry (ESI-MS) using 3,5-dibromo-4-nitrosobenzenesulfonate (DBNBS). Up to four DBNBS-cyt *c* adducts were observed in the mass spectra. However, by varying the reaction conditions (0–5 molar equivalents of H_2O_2 and substituting cyt *c* with its cyanide adduct which is resistant to peroxidation), noncovalent DBNBS adduct formation was inferred. Nonetheless, optical difference spectra revealed the presence of a small fraction of covalently trapped DBNBS. To probe the nature of the noncovalent DBNBS adducts, the less basic proteins, metmyoglobin (Mb) and α-lactalbumin, were substituted for cyt *c* in the cyt *c*/H_2O_2/DBNBS reaction. A maximum of two DBNBS adducts were observed in the mass spectra of the products of the Mb/H_2O_2/DBNBS reactions, whereas no adducts were detected following α-lactalbumin/H_2O_2/DBNBS incubation, which is consistent with adduct formation via spin trapping only. Titration with DBNBS at pH 2.0 yielded noncovalent DBNBS-cyt *c* adducts and induced folding of acid-denatured cyt *c*, as monitored by ESI-MS and optical spectroscopy, respectively. Thus, the noncovalent DBNBS-cyt *c* mass adducts observed are assigned to ion pair formation occurring between the negatively charged sulfonate group on DBNBS and positively charged surface residues on cyt *c*. The results reveal the pitfalls inherent in using mass spectral data with negatively charged spin traps such as DBNBS to identify sites of radical formation on basic proteins such as cyt *c*.

Reactive oxygen species, such as H_2O_2 and superoxide, are generated by all aerobic cells as by-products of a number of metabolic reactions and in response to various stimuli. Oxidative damage can occur when H_2O_2 reacts with heme proteins, such as ferricytochrome *c* (cyt *c*),[1] to form highly reactive oxyferryl-heme and transient protein-based radical species ($X^{\bullet}$) that are linked to the initiation of lipid peroxidation (1, 2). Detection of $X^{\bullet}$ in biological systems is often difficult because they are short-lived and highly reactive. Spin traps, which are diamagnetic compounds containing a functional group that reacts with $X^{\bullet}$ to form a more stable paramagnetic adduct ($XST^{\bullet}$), are frequently used in electron paramagnetic resonance (EPR) investigations (3). Although EPR signals can provide information about a radical center and its environment, the *specific sites* of radical formation in biomolecules are not identified. Coupling of high performance liquid chromatography (HPLC) and mass spectrometry (MS) has been used to identify spin adducts of various small molecules (4–6). Our research group has extended the use of LC/MS of spin adducts to proteins to overcome the inherent limitations of EPR. We have found that conversion of the spin adduct $XST^{\bullet}$ to a stable diamagnetic mass adduct (XMA) via ascorbate reduction permits the assignment of XMA to a *specific amino acid residue* when spin trapping and peptide mass mapping by on-line LC/MS (ST/LC/MS) are coupled (7–10). In addition to the increased specificity offered by ST/LC/MS, it possesses enhanced sensitivity over EPR, since considerably smaller quantities (picomole *versus* nanomole) of sample can be analyzed (11).

3,5-Dibromo-4-nitrosobenzenesulfonate (DBNBS), sometimes referred to as Perkin's trap, was developed to trap carbon-centered radicals. It is stable to temperature and light, and the introduction of the sulfonate group onto the benzene ring has helped overcome problems encountered in the use of lipophilic nitroso spin traps such as 2-methyl-2-nitrosopropane (MNP) (12). However, it is known that DBNBS adducts can be formed through several nonradical reactions. For example, prolonged incubation of DBNBS with unsaturated fatty acids (13) or with free tryptophan (14) causes chemical modifications through nonradical reactions such as the ene reaction between the nitroso group in DBNBS and the double bond in tryptophan.

Barr and co-workers (11, 15) recently reported the trapping of a protein-based tyrosyl radical by DBNBS in the reaction of cyt *c* with H_2O_2 using EPR. In addition, peaks corresponding to $(DBNBS)_n$-cyt *c* adducts, with $n = 1–4$, were observed by MALDI-MS, suggesting that as many as four protein-based $X^{\bullet}$ species were trapped by DBNBS during the reaction of cyt *c* with 5 molar equivalents of H_2O_2. However, the sites of DBNBS-cyt *c* adduct formation were not identified. The radicals formed in the reaction of cyt *c* with H_2O_2 are of interest, since they could cause mitochondrial membrane damage and play a role in the apoptotic process (16). Using ST/LC/MS, we reexamined the products of the cyt *c*/H_2O_2/DBNBS reaction to determine the extent of protein radical formation. Surprisingly,

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[1] The abbreviations: cyt *c*, ferricytochrome *c*; ACN, acetonitrile; ANS, 8-anilino-1-naphthalenesulfonate; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonate; $DBNBS^{\bullet+}$, oxidized 3,5-dibromo-4-nitrosobenzene sulfonate; DMPO, 5,5-dimethylpyrroline-*N*-oxide; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*″,*N*″-pentaacetic acid; ESI, electrospray ionization; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography; MALDI, matrix-assisted laser desorption ionization; Mb, metmyoglobin; MNP, 2-methyl-2-nitrosopropane; $MNP^{\bullet+}$, oxidized 2-methyl-2-nitrosopropane; MS, mass spectrometry; ST/LC/MS, spin trapping coupled to on-line peptide mass mapping; $X^{\bullet}$, protein-based radical; XMA, diamagnetic mass adduct of $X^{\bullet}$; $XST^{\bullet}$, spin adduct of $X^{\bullet}$.

we detected DBNBS-cyt *c* mass adducts in the absence of H_2O_2, and in the cyanide-ligated protein, even though cyanide binding inhibits heme-mediated peroxidation (11, 17).

Cyt *c*, a highly basic protein (pI ~10), unfolds at low pH, resulting in increased absorption and blue-shifting of the Soret maximum from 408 at pH 7.0 to 394 nm in the acid-denatured protein at pH 2.0 (18). The addition of anions converts the unfolded state of cyt *c* to a conformation resembling a molten globule by reducing the electrostatic repulsion of positive charges on the protein surface via Debye-Hückel screening and ion pairing (18, 19). We provide data here that support the formation of noncovalent adducts under the MS conditions (pH 2.0); in fact, DBNBS was found to stabilize a molten globule state of acid-unfolded cyt *c* at pH 2.0 in a manner similar to that reported for 8-anilino-1-naphthalenesulfonate (ANS) (19). Our results reveal that caution must be used in interpretation of the mass spectra of DBNBS-protein adducts, and the different limitations in EPR and MS approaches to the analysis of spin-trapped species are also highlighted.

EXPERIMENTAL PROCEDURES

Materials—Horse (Type VI), cow and tuna (Type XI) heart cytochromes *c*, horse heart metmyoglobin (Mb), α-lactalbumin from bovine milk, trifluoroacetic acid, and DBNBS were purchased from Sigma and used without further purification. H_2O_2 was purchased from Fisher, while potassium cyanide (KCN) was obtained from BDH Chemicals. Sequencing grade trypsin (Roche Molecular Biochemicals) was used for digestion, and ammonium acetate (JT Baker Chemical Co.) Solutions containing diethylenetriamine-*N*,*N*′,*N*″,*N*″-pentaacetic acid (DTPA, ICN) were prepared using 18-MΩ-cm water obtained from a Barnstead Nanopure system.

Methods—Reactions were carried out by mixing 500 μM protein with 10 mM DBNBS and 0, 1, or 5 molar equivalents of H_2O_2 in 50 mM ammonium acetate solution (pH 7.5) containing 200 μM DTPA. The reactions were terminated after 10 min by injecting the reactants onto a Vydac C_{18} column (4.6 × 300 mm) and separating the protein from salts and low molecular weight reactants by HPLC (HP1090, Hewlett-Packard) using a 10–55% acetonitrile (ACN) gradient in 0.05% trifluoroacetic acid at 1 ml/min over 20 min. The protein peak was collected, lyophilized, suspended in 1:1 methanol/water with 0.5% acetic acid, and infused at a flow rate of 5 μl/min using a syringe pump (Harvard Apparatus) directly into the electrospray ionization (ESI) source of a Finnigan SSQ7000 mass spectrometer (ThermoFinnigan) for molecular weight determination. The protein was further digested with 1:50 (w/w) trypsin at 50 °C for 4 h. The digests were separated for peptide mass mapping on a Vydac microbore C_{18} column (1 × 300 mm) using a 10–55% ACN gradient in 0.05% trifluoroacetic acid at 40 μl/min over 100 min. The spray voltage was set at 4.5 kV, and the capillary temperature was maintained at 210 °C, while the sheath and auxiliary gas pressures were 40 and 15 p.s.i., respectively. Full-scan acquisition was performed in profile mode using scan rates of 320–380 atomic mass units/s.

To probe DBNBS interaction with acid-unfolded cyt *c*, the protein was incubated for 30 min with increasing amounts of DBNBS in 0.05% trifluoroacetic acid (pH 2.0) before carrying out optical or MS measurements. For molecular weight determination, samples were infused into the ESI source of the mass spectrometer by flow injection at 50 μl/min in 75% ACN containing 0.05% trifluoroacetic acid. Optical spectra were recorded on a Beckman DU 650 spectrophotometer between 200 and 600 nm, using a scan rate of 2400 nm/min. Difference spectra were generated using Origin 3.0 software (MicroCal).

RESULTS AND DISCUSSION

Analysis of DBNBS Mass Adduct Formation with Ferricytochrome c in the Presence and Absence of H_2O_2—The deconvolved ESI mass spectrum (Fig. 1*a*) of horse cyt *c* revealed that $(DBNBS)_n$-cyt *c* adducts (n = 1–4) were formed in the reaction of cyt *c* with 5-fold molar excess of H_2O_2, as observed previously by MALDI-MS (11, 15). A similar pattern of $(DBNBS)_n$-cyt *c* adduct formation was observed when cyt *c* was reacted with only 1 molar equivalent of H_2O_2 (Fig. 1*b*) despite the fact that H_2O_2, a two-electron oxidant, can remove a maximum of two electrons to generate two radical sites on cyt *c*. The reaction of horse cyt *c* with 1–5 molar equivalents of H_2O_2 was repeated several times in the presence of DBNBS, and multiple DBNBS adducts were consistently observed. Moreover, in the absence of H_2O_2, up to two DBNBS adducts were detected not only with horse cyt *c*, but also with bovine and tuna cyts *c* (Fig. 1, *c*–*e*). These data suggest that DBNBS interacts with cyt *c* via a non-spin-trapping mechanism.

The cyanide ion serves as a high affinity ligand for the ferric state of cyt *c* by displacing the axial Met^{80} ligand (17, 20). Hence, cyanocyt *c* formation, which inhibits heme-catalyzed reactions and prevents cyt *c* heme degradation by organic hydroperoxides (11, 17), was used to probe direct (*i.e.* non-heme mediated) peroxidation of the polypeptide. Fig. 1*f* reveals that the addition of 3 mM KCN to the horse cyt *c*/H_2O_2/DBNBS reaction results in the same mass spectrum as that observed in the absence of H_2O_2 (Fig. 1, *f versus c*). Therefore, the $(DBNBS)_n$-cyt *c* adducts from the cyanocyt *c*/H_2O_2/DBNBS reaction are unlikely due to peroxidation, supporting the absence of non-heme-mediated oxidation of cyt *c* residues by H_2O_2. Nonetheless, the mass spectra reveal that the $(DBNBS)_n$-cyt *c* adduct intensities increase in the presence of H_2O_2 (Fig. 1, *a* and *b versus c*); hence, spin trapping of X• by DBNBS is also likely occurring, consistent with the EPR data that indicated trapping of a tyrosyl radical in cyt *c* (11).

To further probe the nature of the DBNBS adducts detected by MS, optical spectra of the reaction products obtained for horse cyt *c* in the absence and presence of H_2O_2 were recorded (Fig. 2). The difference spectrum (Fig. 2, *trace 3*) of HPLC-purified products from the cyt *c*/H_2O_2/DBNBS (1:5:20) reaction minus those from the cyt *c*/H_2O_2 (1:5) reaction reveals loss of DBNBS absorption seen at 288 nm (Fig. 2, *trace 2*) and growth of a new absorption band at 300 nm. This is consistent with increased conjugation of the chromophore, where the nitroso group of DBNBS traps a radical on the aromatic ring of a tyrosine residue (11). The possibility that the 300 nm absorption was due to H_2O_2-induced oxidation of DBNBS was also considered, but incubation of DBNBS with 5 molar equivalents of H_2O_2 gave rise to a DBNBS species with an absorption spectrum (Fig. 2, *trace 1*) essentially identical to that of untreated DBNBS (Fig. 2, *trace 2*). Moreover, the cyt *c*/DBNBS (1:10) minus cyt *c* difference spectrum (data not shown) resembles that of DBNBS alone (Fig. 2, *trace 2*). As an additional control, the cyt *c*/H_2O_2 (1:5) minus cyt *c* difference spectrum (Fig. 2, *trace 4*) was generated. A relatively flat base line between 230 and 370 nm with negligible UV absorption was observed, suggesting minimal oxidation of aromatic residues by H_2O_2. Thus, the new band at 300 nm in the cyt *c*/H_2O_2/DBNBS minus cyt *c*/H_2O_2 difference spectrum (Fig. 2, *trace 3*) is not an artifact due to the subtraction procedure, but can be assigned to a protein-based DBNBS spin adduct. Unfortunately, tryptic digestion of the products of the cyt *c*/H_2O_2/DBNBS reaction yielded only native peptides. It has been observed that ascorbate reduction of MNP-Mb spin adducts yields stable XMAs that can be identified by ST/LC/MS (9, 10). However, addition of 5 mM ascorbate to the cyt *c*/H_2O_2/DBNBS reaction did not convert XST• to XMA, since the mass spectrum (data not shown) was the same as that in Fig. 1*a*. This is likely due to either low trapping efficiency of DBNBS toward cyt *c* radicals or instability of the covalently trapped DBNBS-cyt *c* adducts under the digestion conditions. The radical is bonded directly to the nitroxide in the XST• formed with DBNBS, which renders reverse or cleavage reactions of the spin-trapped species favorable (3). Interestingly, Kim and co-workers (21) observed by EPR that the decay of 5,5-dimethylpyrroline-N-oxide (DMPO) adducts of Mb and hemoglobin were accelerated by denaturation (urea or guanidine HCl) and proteolysis of the protein

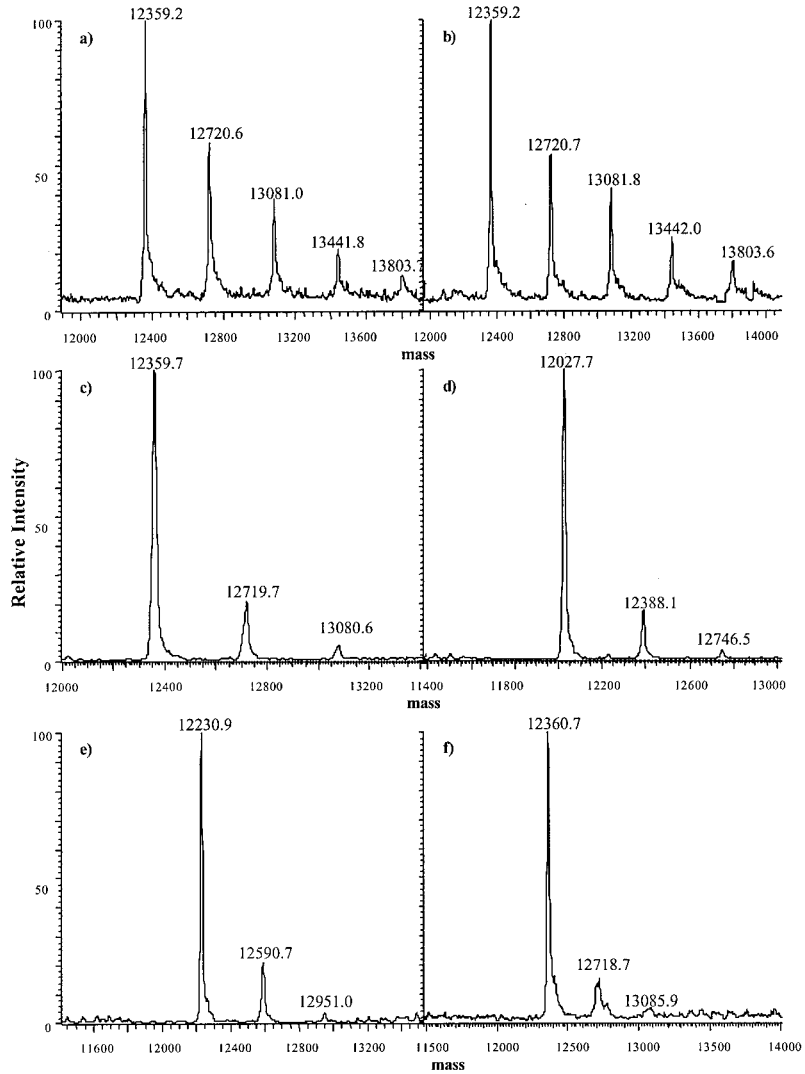

FIG. 1. **Deconvolved ESI mass spectra of the products of a 10-min reaction of 500 μM cyt *c* with H_2O_2 in the presence of 10 mM DBNBS.** *a*, horse cyt *c* with 2.5 mM H_2O_2; *b*, horse cyt *c* with 500 μM H_2O_2; *c*, horse cyt *c* without H_2O_2; *d*, tuna heart cyt *c* without H_2O_2; *e*, cow heart cyt *c* without H_2O_2; *f*, horse cyt *c* with 2.5 mM H_2O_2 and 3 mM KCN (to form cyanocyt *c*). Reactions were carried out in 50 mM ammonium acetate (pH 7.5) containing 200 μM DTPA. The HPLC-purified reaction products (see "Experimental Procedures") were collected, lyophilized, and suspended in 1:1 methanol/water with 0.5% acetic acid. Samples (~1 mg/ml) were directly infused into the ESI source of the mass spectrometer at 5 μl/min for molecular weight determination.

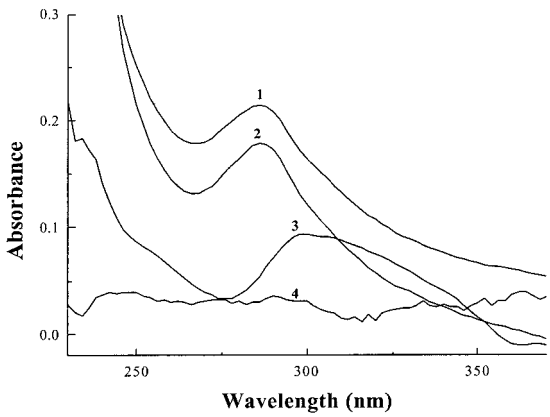

FIG. 2. **Absorption spectra of (*trace 1*) 132 μM DBNBS following a 10-min incubation with 660 μM H_2O_2 and (*trace 2*) untreated 111 μM DBNBS.** Difference optical spectra of the HPLC-purified DBNBS-cyt *c* adduct (~11 μM cyt *c*) minus the products from the 10-min reaction of 11 μM horse cyt *c* with 55 μM H_2O_2 (*trace 3*) and 11 μM horse cyt *c* following a 10-min incubation with 55 μM H_2O_2 minus 11 μM horse cyt *c* (*trace 4*) are shown. All solutions were prepared in 50 mM ammonium acetate (pH 7.5) containing 200 μM DTPA. Spectra were recorded at a scan rate of 2400 nm/min, and difference spectra were generated using Origin 3.0 software.

moiety. The data in Fig. 2 demonstrate, nonetheless, that DBNBS-cyt *c* spin adducts formed in the presence of H_2O_2 can be distinguished from those formed in the absence of H_2O_2 by optical difference spectroscopy.

Many reactions other than spin trapping have been reported for DBNBS. For example, the formation of a nitroxyl free radical was detected by EPR after a 60-min incubation of DBNBS with free tryptophan (14). Chemical modification after 24-h incubation of low density lipoprotein by DBNBS was detected by agarose gel electrophoretic mobility (13). The DBNBS labeling was assigned to the ene reaction between the nitroso group and a double bond, which results in an allylic hydroxylamine (22). However, both reported studies indicated that labeling required prolonged (≥1 h) exposure to DBNBS. We limited cyt *c* exposure to DBNBS to 10 min and immediately separated the protein from the low molecular weight reactants by HPLC.

The possibility of "inverted spin trapping" was also considered, which could occur if the cyt *c*/H_2O_2/DBNBS reaction yielded an oxidized form of the spin trap ($DBNBS^{\cdot+}$). Inverted spin trapping of $DBNBS^{\cdot+}$ by an amino acid residue could lead to a DBNBS-cyt *c* adduct identical to that formed by "normal" spin trapping of $X^{\cdot}$ by DBNBS (reviewed in Ref. 23). Horseradish peroxidase/H_2O_2 ($E°$ $Fe^{IV} = O/Fe^{III} = 0.94$ V) catalyzed the oxidation of DBNBS over a 24-h incubation, as seen by EPR (24). However, $DBNBS^{\cdot+}$ generation was not observed by the peroxidase activity ($E°$ $Fe^{IV} = O/Fe^{III} \sim 1$ V) of Mb/H_2O_2 or cyt *c* oxidase/H_2O_2 (25, 26), the Fenton reaction, Fe^{II}/H_2O_2 ($E°$ $Fe^{III}/Fe^{II} = 0.77$ V) (27), nor in the oxidation of selenite by Ce^{IV} ($E°$ $Ce^{IV}/Ce^{III} = 1.44$ V) (28). Since MNP and DBNBS contain identical redox-active nitroso functional groups, it can be as-

sumed that the $E°$ $DBNBS^{\cdot+}$/DBNBS is comparable with $E°$ $MNP^{\cdot+}$/MNP (2.06 V) (23), which is beyond the reach of the high oxidation states of heme proteins under non-forcing conditions. Hence, "inverted spin trapping" is ruled out in the cyt c/H_2O_2/DBNBS reaction, since (i) the reaction time was only 10 min and (ii) the Fe^{IV} = O center is rapidly converted to Fe^{III} by endogenous electron transfer to heme (data not shown). Also, the reduction potential of the Fe^{III}/Fe^{II} couple in horse cyt c ($E°$ = 0.25 V) is clearly insufficient to oxidize the nitroso group.

DBNBS could also form sulfonamides with the ϵ-amino groups of lysines, which account for ~20% of the residues in cyt c. However, the deconvolved mass spectra (Fig. 1, *a–f*) reveal Δm increments of 360 ± 2 Da for the cyt c adducts, corresponding to ammoniated DBNBS adducts, whereas sulfonamide formation would give rise to Δm increments of 326 Da. Furthermore, peptide mass mapping of the products of the cyt c/H_2O_2/DBNBS reaction yielded exclusively native horse cyt c peptides (data not shown), indicating that no stable derivatives such as sulfonamides were formed. This agrees with the work of Kalyanaraman and co-workers (13) who reported that DBNBS-modified low density lipoprotein was formed by a lysine-independent process.

Analysis of DBNBS Mass Adduct Formation with Metmyoglobin and α-Lactalbumin in the Presence and Absence of H_2O_2—To establish whether or not DBNBS adduct formation with proteins exhibits specificity, Mb and α-lactalbumin were selected for further MS investigations. Mb, a heme-containing protein with a pI of 7.0, is not reported to have a high affinity for anionic compounds (29). Spin trapping of $X^{\cdot}$ generated in the Mb/H_2O_2 reaction has been demonstrated by both EPR and ST/LC/MS using DBNBS and other spin traps, and $X^{\cdot}$ has been assigned primarily to Tyr^{103} (10, 30–33). The difference spectrum (data not shown) of HPLC-purified products from the Mb/H_2O_2/DBNBS (1:5:20) reaction minus those from the Mb/H_2O_2 (1:5) is similar to that seen with cyt c (Fig. 2, *trace 3*), which is indicative of DBNBS-tyrosine spin adduct formation in both proteins. An estimate of ϵ_{302} for the DBNBS-tyrosine spin adduct (42 mM^{-1} cm^{-1}) was obtained by assuming a trapping efficiency of 70% for the tyrosyl radical in Mb, based on the relative intensities of the peaks in the mass spectrum of the Mb/H_2O_2/DBNBS (1:5:20) reaction products (Fig. 3*a*). Using this estimated ϵ_{302}, the yield of DBNBS-cyt c spin adduct formation in the cyt c/H_2O_2/DBNBS (1:5:20) reaction is ~20%, compared with ~10% estimated from EPR measurements (11). Yields of 10–20% are sufficient to identify modified peptides by mass mapping, but tryptic digests of DBNBS-labeled cyt c contained exclusively native peptides, indicating that DBNBS labeling is not stable to peptide mass mapping, as discussed above.

In Fig. 3*a*, the deconvolved mass spectrum of the Mb/H_2O_2/DBNBS (1:5:20) reaction products shows the formation of $(DBNBS)_n$-Mb adducts with n = 1 and 2. DBNBS ene addition to Mb and/or ion pair formation can be ruled out, since only native globin is detected in the deconvolved mass spectrum in the absence of H_2O_2 (Fig. 3*b*). Inverted spin trapping was not reported in the Mb/H_2O_2/DBNBS reaction (26), since the oxyferryl heme of Mb ($E°$ Fe^{IV} = O/Fe^{III} ~ 1 V) cannot oxidize DBNBS. When H_2O_2 was present at 1 molar equivalent (Fig. 3*c*), the relative intensity of the $(DBNBS)_2$-Mb peak was negligible and that of the (DBNBS)-Mb peak decreased by 40% compared with the 1:1 adduct following oxidation with 5 molar equivalents of H_2O_2 (Fig. 3*a*). Likewise, a less intense (DMPO)-Mb peak was observed by ESI-MS upon decreasing the amount of H_2O_2 from 3 to 1 molar equivalents in the Mb/H_2O_2/DMPO reaction (32). It was reported that formation of oxyferryl Mb requires >1 molar equivalent of H_2O_2, since H_2O_2 is consumed

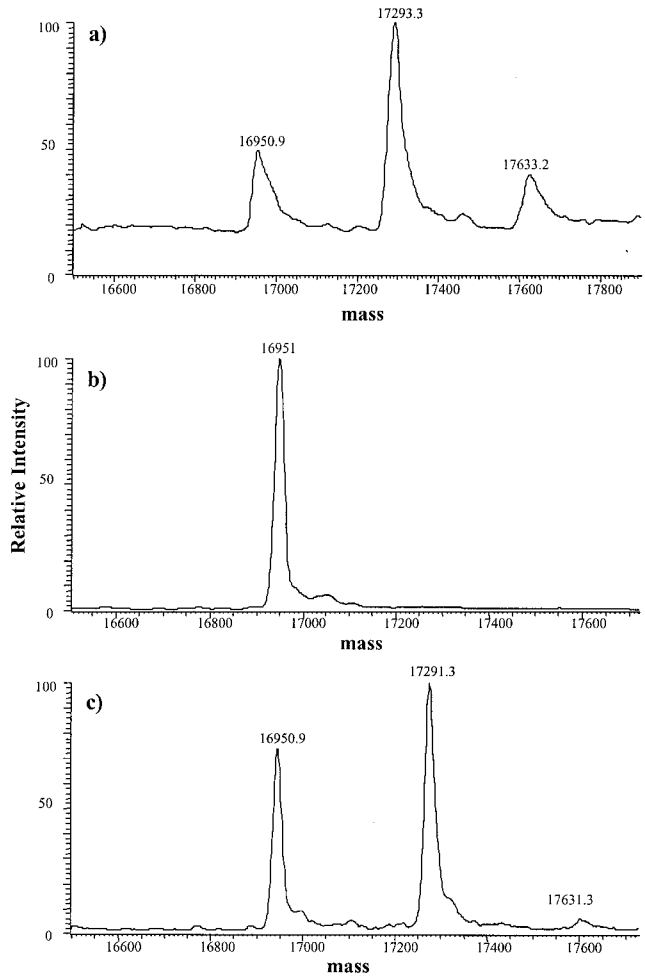

FIG. 3. **Deconvolved ESI mass spectra of the products of a 10-min reaction of 500 μM horse heart metmyoglobin with H_2O_2 in the presence of 10 mM DBNBS: with 2.5 mM H_2O_2 (*a*), without H_2O_2 (*b*), and with 500 μM H_2O_2 (*c*).** Experimental conditions are given in the legend to Fig. 1.

in side reactions at the porphyrin or other locations on the globin (9, 34). Nevertheless, the efficiency of $(XST^{\cdot})_n$-Mb adduct formation depends on the number of oxidizing equivalents present in the Mb/H_2O_2/ST reaction for both DMPO and DBNBS, in contrast to the cyt c/H_2O_2/DBNBS reaction (Fig. 1, *a versus b*).

DBNBS adduct formation with α-lactalbumin was also investigated here, since it is an acidic protein with a pI ~4 and contains basic residues evenly dispersed over its surface. In fact, due to its high negative charge under the spin trapping conditions used (pH 7.5), α-lactalbumin should repel the negatively charged DBNBS. Similarly, Matulis and co-workers (35) observed by fluorescence spectroscopy that very little ANS bound to bovine serum albumin (pI 5.8) at pH > 11. α-Lactalbumin also lacks the heme prosthetic group found in Mb and cyt c; thus there should be no reaction with H_2O_2 to generate $X^{\cdot}$ unless H_2O_2 directly oxidizes the polypeptide. As expected, the deconvolved mass spectra of α-lactalbumin both in the presence (Fig. 4*a*) and absence (Fig. 4*b*) of H_2O_2 revealed no DBNBS adduct formation, since only native α-lactalbumin was detected. This rules out direct peroxidation of the polypeptide, consistent with the results for cyanocyt c (Fig. 2*f*), and it also rules out ion pair formation.

DBNBS Adduct Formation with Horse Cyt c in the Absence of H_2O_2 at pH 2.0—Anion-dependent stabilization of compact structures that resemble the molten globule state have been

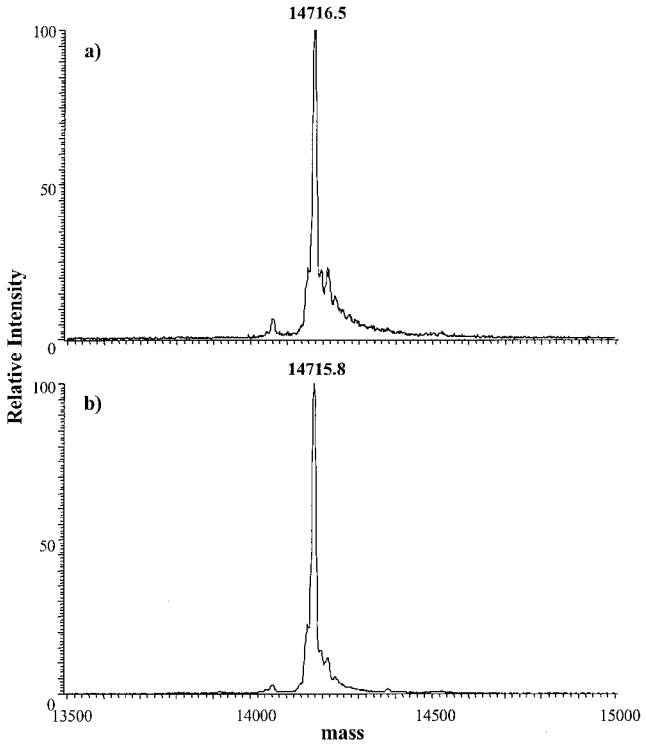

FIG. 4. **Deconvolved ESI mass spectra of the products of a 10-min reaction of 500 μM cow α-lactalbumin with H_2O_2 and 10 mM DBNBS: with 2.5 mM H_2O_2 (*a*) and without H_2O_2 (*b*).** Experimental conditions are given in the legend to Fig. 1.

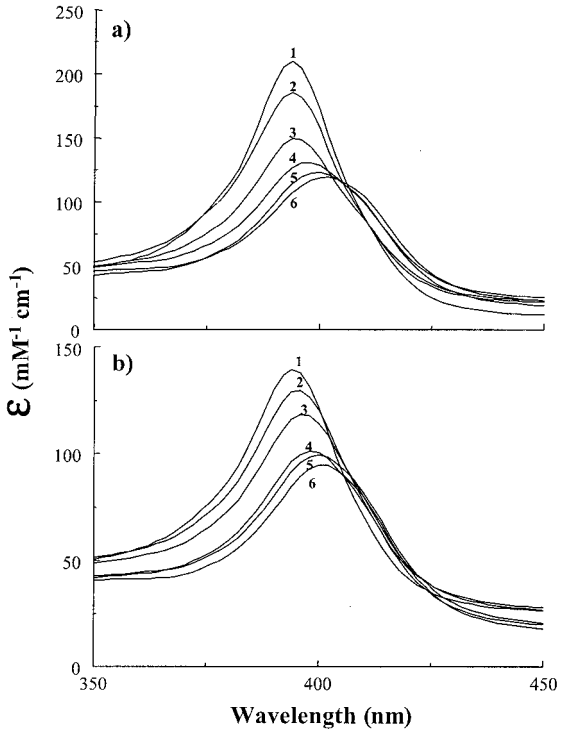

FIG. 5. **Soret absorption spectra of 10 μM non-oxidized (*a*) and oxidized (*b*) horse cyt *c* incubated for 30 min in 0.05% trifluoroacetic acid (pH 2.0) in the presence of 0 (*trace 1*), 1 (*trace 2*), 5 (*trace 3*), 15 (*trace 4*), 20 (*trace 5*), and 25 (*trace 6*) molar equivalents of DBNBS.** Oxidized protein was prepared by treating 100 μM horse cyt *c* with 5 molar equivalents of H_2O_2 for 5 min at pH 7.5. Spectra were recorded at a scan rate of 2400 nm/min.

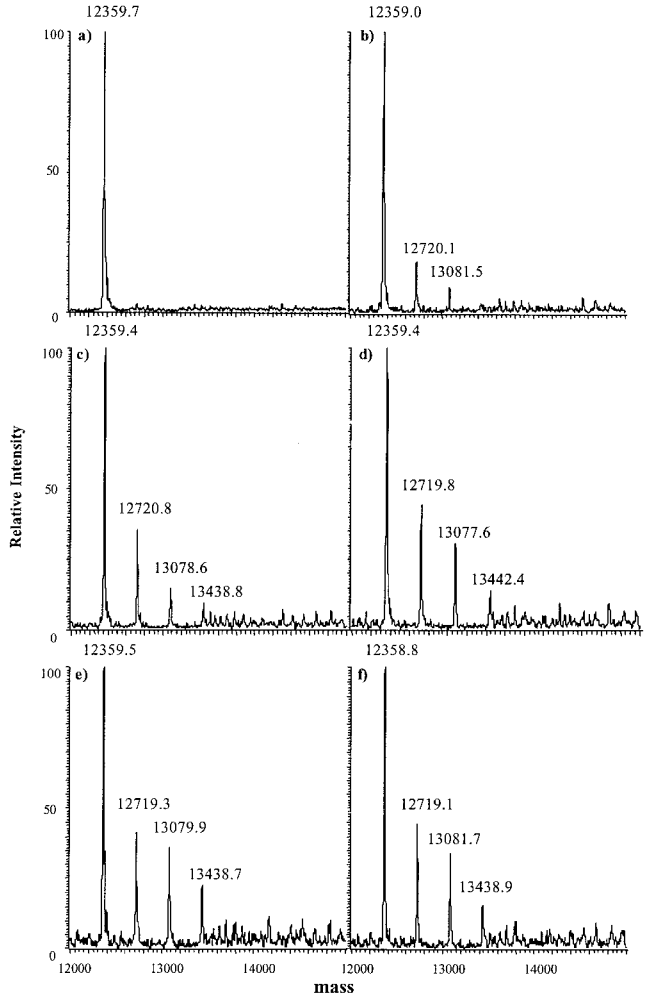

FIG. 6. **Deconvolved ESI mass spectra of 20 μM horse cyt *c* incubated for 30 min in 0.05% trifluoroacetic acid (pH 2.0) in the presence of 0 (*a*), 1 (*b*), 10 (*c*), 15 (*d*), 20 (*e*), and 25 (*f*) molar equivalents of DBNBS.** Samples in 75% ACN containing 0.05% trifluoroacetic acid were infused into the ESI source of the mass spectrometer by flow injection at 50 μl/min.

observed by monitoring the Soret band upon incubation of cyt *c* with ANS or with various strong acids and their neutral salts (18, 19). The molten globule state of cyt *c*, which forms at high salt (500 mM NaCl) and low pH (pH 2.0), is characterized by a red shift from 394 nm (the Soret maximum of acid-denatured cyt *c*) to 400 nm, which is accompanied by band broadening (18). To elucidate the mechanism by which the $(DBNBS)_n$-cyt *c* mass adducts (Fig. 1) are formed, acid-unfolded horse cyt *c* was titrated with DBNBS at pH 2.0, and changes in the Soret and mass spectra were monitored. The Soret maximum red-shifted from 394 to 402 nm following 30-min incubation of cyt *c* with 25 molar excess DBNBS, consistent with molten globule formation (Fig. 5*a*). Similar effects were observed with ANS (19), which promotes the refolding of cyt *c* at low pH by Debye-Hückel screening and ion pair formation (18). The mass spectra in Fig. 1, *a* and b *versus c*, reveal enhanced binding of DBNBS to the H_2O_2-oxidzied cyt *c*, which is also seen in the greater red shifting of the Soret bands in Fig. 5, *b versus a*. Of note, the absorbencies of the acid denatured oxidized cyt *c* (ϵ_{394} = 140 mM^{-1} cm^{-1}) and its DBNBS-stabilized molten globule (ϵ_{402} = 94 mM^{-1} cm^{-1}) are less than those of the unoxidized forms (ϵ_{394} = 209 mM^{-1} cm^{-1}; ϵ_{394} = 120 mM^{-1} cm^{-1}).

DBNBS titration of horse cyt *c* was also monitored by MS (Fig. 6). The $(DBNBS)_n$-cyt *c* (n = 1–4) peaks in the mass spectra of samples with ≥10-fold excess DBNBS recorded with-

out prior HPLC purification are more intense than the corresponding peaks in the HPLC-purified sample (Fig. 6, *c–f versus* Fig. 1*c*). Fig. 6 also reveals that the abundance and stoichiometry of the $(DBNBS)_n$-cyt *c* adducts increased as a function of DBNBS concentration, which is consistent with noncovalent adduct formation. Ali and co-workers (19) observed peaks corresponding to $(ANS)_n$-cyt *c* adducts (n = 1–7) at pH 2.0 by ESI-MS, but no adduct formation was observed with nile red, a neutral hydrophobic dye, revealing the importance of electrostatic interactions.

Precipitation of cyt *c* was observed upon addition of a large excess of DBNBS at pH 7.5, which required limiting the amount of DBNBS added to 500 μM cyt *c* to 10 mM. Ion pair formation between the sulfonate group of DBNBS and basic residues of cyt *c* would reduce the solubility of the protein. Interestingly, cyt *c* is known to possess anionic binding sites; specifically, there are two phosphate binding sites, one near Lys^{87} with a dissociation constant (K_d) of 200 μM and another close to Lys^{25}-His^{26}-Lys^{27} with a K_d of >2 mM (36). However, the DBNBS-cyt *c* adducts must possess K_d values in the low μM range, since phosphate-cyt *c* adducts are not observed by ESI-MS following reversed-phase HPLC purification, whereas DBNBS-cyt *c* adducts are seen in Fig. 1, *c–e*. In fact, a K_d of ~36 μM was estimated from a double-reciprocal plot (ΔAbs^{-1} *versus* $[DBNBS]^{-1}$) of the data in Fig. 5 for both oxidized and nonoxidized horse cyt *c*, similar to the calculated K_d (3–50 μM) for the interaction of ANS to cationic polyamino acids at pH 2.0 (35).

Conclusions—DBNBS complexes with cyt *c* via strong electrostatic interactions at pH 2.0, thereby complicating the analysis of spin trapping by ST/LC/MS. However, noncovalent DBNBS adduct formation clearly shows specificity for cyt *c* (Fig. 1, *c–e versus* Figs. 3*b* and 4*b*), indicating that its formation cannot simply be correlated with the total number of lysine residues, since horse cyt *c* possesses 19, while horse Mb and cow α-lactalbumin have 19 and 12, respectively. Lysine residues are highly conserved in cyts *c* and are clustered predominately around the exposed heme edge, forming anionic binding sites (36). In Mb and α-lactalbumin, the lysine residues are more or less distributed evenly over the entire protein surface. Also, the fact that DBNBS and ANS exhibit comparable efficiencies in inducing cyt *c* folding at low pH, despite the significantly larger hydrophobic moiety of ANS, is consistent with electrostatic interactions being the principal determinant of binding. Ion pair formation between proteins and negatively charged probes such as DBNBS and ANS may be a common occurrence at the low pH values used for ESI-MS analyses in positive ion mode. Therefore, it is essential to carry out the appropriate controls before interpreting MS data involving protein-probe adducts such as the $(DBNBS)_n$-cyt *c* mass adducts observed in the present study. Finally, our results reveal that compared with Tyr^{103} of Mb, the cyt *c* radicals are not very reactive and/or accessible at pH 7.5.

REFERENCES

1. Radi, R., Turrens, J. F., and Freeman, B. A. (1991) *Arch. Biochem. Biophys.* **288,** 118–125
2. Radi, R., Bush, K. M., and Freeman, B. A. (1993) *Arch. Biochem. Biophys.* **300,** 409–415
3. Perkins, M. J. (1980) *Adv. Phys. Org. Chem.* **17,** 1–61
4. Iwahashi, H., Parker, C. E., Mason, R. P., and Tomer, K. B. (1990) *Rapid Commun. Mass. Spectrom.* **4,** 352–354
5. Iwahashi, H., Parker, C. E., Mason, R. P., and Tomer, K. B. (1991) *Biochem. J.* **276,** 447–453
6. Janzen, E. G., Towner, R. A., Krygsman, P. H., Lai, E. K., Poyer, J. L., Brueggemann, G., and McCay, P. B. (1990) *Free Radical. Res. Commun.* **9,** 353–360
7. Filosa, A., and English, A. M. (1999) *Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, June 12–17, Dallas, TX*
8. Tsapraillis, G. (1997) *Probing the Redox-active Residues in Cytochrome Peroxidase.* Ph.D. thesis, Concordia University, Montreal, Canada
9. Fenwick, C. W. (1997) *Study of the Highly Oxidizing Centers in the Metmyoglobin-H_2O_2 Reaction.* Ph.D. thesis, Concordia University, Montreal, Canada
10. Fenwick, C. W., and English, A. M. (1996) *J. Am. Chem. Soc.* **118,** 12236–12237
11. Barr, D. P., Gunther, M. R., Deterding, L. J., and Tomer, K. B. (1996) *J. Biol. Chem.* **271,** 15498–15508
12. Kaur, H., Leung, K. H. W., and Perkins, M. J. (1981) *J. Chem. Soc. Chem. Commun.* 142–143
13. Kalyanaraman, B., Joseph, J., Kondratenko, N., and Parthasarathy, S. (1992) *Biochim. Biophys. Acta* **1126,** 309–313
14. Hiramoto, K., Hasegawa, Y., and Kikugawa, K. (1994) *Free Radic. Res.* **21,** 341–349
15. Deterding, L. J., Barr, D. P., Mason, R. P., and Tomer, K. B. (1998) *J. Biol. Chem.* **273,** 12863–12869
16. Shidoji, Y., Hayashi, K., Komura, S., Ohishi, N., and Yagi, K. (1999) *Biochem. Biophys. Res. Commun.* **264,** 343–347
17. Dyer, C., Schubert, A., Timkovich, R., and Feinberg, B. A. (1979) *Biochim. Biophys. Acta* **579,** 253–268
18. Goto, Y., Takahashi, N., and Fink, A. L. (1990) *Biochemistry* **29,** 3480–3488
19. Ali, V., Prakash, K., Kulkarni, S., Ahmad, A., Madhusudan, K. P., and Bhakuni, V. (1999) *Biochemistry* **38,** 13635–13642
20. Blumenthal, D. C., and Kassner, R. J. (1980) *J. Biol. Chem.* **255,** 5859–5863
21. Kim, Y. M., Jeong, S. H., Yamazaki, I., Piette, L. H., Han, S., and Hong, S. J. (1995) *Free Radic. Res.* **22,** 11–21
22. Mason, R. P., Kalyanaraman, B., Tainer, B. E., and Eling, T. E. (1980) *J. Biol. Chem.* **255,** 5019–5022
23. Eberson, L. (1998) *Adv. Phys. Org. Chem.* **31,** 91–141
24. Nazhat, N. B., Saadalla-Nazhat, R. A., Fairburn, K., Jones, P., Blake, D. R., Nielsen, B. R., Symons, M. C., and Winyard, P. G. (1999) *Biochim. Biophys. Acta* **1427,** 276–286
25. Chen, Y. R., Gunther, M. R., and Mason, R. (1999) *J. Biol. Chem.* **274,** 3308–3314
26. Gunther, M. R., Kelman, D. J., Corbett, J. T., and Mason, R. P. (1995) *J. Biol. Chem.* **270,** 16075–16081
27. Kohno, M., Yamada, M., Mitsuta, K., Mizuta, Y., and Yoshikawa, T. (1991) *Bull. Chem. Soc. Jpn.* **64,** 1447–1453
28. Ozawa, T., and Hanaki, A. (1991) *Bull. Chem. Soc. Jpn.* **64,** 1976–1978
29. Righetti, P. G., and Caravaggio, T. (1976) *J. Chromatogr.* **127,** 1–28
30. Filosa, A., and English, A. M. (2000) *Proceedings of the 48th ASMS Conference on Mass Spectrometry and Allied Topics,* June 11–15, *Long Beach, CA*
31. Witting, P. K., Douglas, D. J., and Mauk, A. G. (2000) *J. Biol. Chem.* **275,** 20391–20398
32. Gunther, M. R., Tschirret-Guth, R. A., Witkowska, H. E., Fann, Y. C., Barr, D. P., Ortiz De Montellano, P. R., and Mason, R. P. (1998) *Biochem. J.* **330,** 1293–1299
33. Catalano, C. E., Choe, Y. S., and Ortiz de Montellano, P. R. (1989) *J. Biol. Chem.* **264,** 10534–10541
34. George, P., and Irvine, D. H. (1952) *Biochem. J.* **52,** 511–517
35. Matulis, D., and Lovrien, R. (1998) *Biophys. J.* **74,** 422–429
36. Moore, G. R., and Pettigrew, G. W. (1987) *Cytochromes c: Biological Aspects*, pp. 83–84, Springer-Verlag, Berlin

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