

Mass Spectral Analysis of Protein-based Radicals Using DNBBS

NONRADICAL ADDUCT FORMATION *VERSUS* SPIN TRAPPING*

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Protein-based radicals generated in the reaction of ferricytochrome *c* (cyt *c*) with H₂O₂ were investigated by electrospray mass spectrometry (ESI-MS) using 3,5-dibromo-4-nitrosobenzenesulfonate (DNBBS). Up to four DNBBS-cyt *c* adducts were observed in the mass spectra. However, by varying the reaction conditions (0–5 molar equivalents of H₂O₂ and substituting cyt *c* with its cyanide adduct which is resistant to peroxidation), noncovalent DNBBS adduct formation was inferred. Nonetheless, optical difference spectra revealed the presence of a small fraction of covalently trapped DNBBS. To probe the nature of the noncovalent DNBBS adducts, the less basic proteins, metmyoglobin (Mb) and α -lactalbumin, were substituted for cyt *c* in the cyt *c*/H₂O₂/DNBBS reaction. A maximum of two DNBBS adducts were observed in the mass spectra of the products of the Mb/H₂O₂/DNBBS reactions, whereas no adducts were detected following α -lactalbumin/H₂O₂/DNBBS incubation, which is consistent with adduct formation via spin trapping only. Titration with DNBBS at pH 2.0 yielded noncovalent DNBBS-cyt *c* adducts and induced folding of acid-denatured cyt *c*, as monitored by ESI-MS and optical spectroscopy, respectively. Thus, the noncovalent DNBBS-cyt *c* mass adducts observed are assigned to ion pair formation occurring between the negatively charged sulfonate group on DNBBS 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 DNBBS to identify sites of radical formation on basic proteins such as cyt *c*.

Reactive oxygen species, such as H₂O₂ 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 H₂O₂ reacts with heme proteins, such as ferricytochrome *c* (cyt *c*),¹ to form highly reactive oxy-

ferryl-heme and transient protein-based radical species (X[•]) that are linked to the initiation of lipid peroxidation (1, 2). Detection of X[•] 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[•] to form a more stable paramagnetic adduct (XST[•]), 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[•] 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 (DNBBS), 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 DNBBS adducts can be formed through several nonradical reactions. For example, prolonged incubation of DNBBS 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 DNBBS and the double bond in tryptophan.

Barr and co-workers (11, 15) recently reported the trapping of a protein-based tyrosyl radical by DNBBS in the reaction of cyt *c* with H₂O₂ using EPR. In addition, peaks corresponding to (DNBBS)_{*n*}-cyt *c* adducts, with *n* = 1–4, were observed by MALDI-MS, suggesting that as many as four protein-based X[•] species were trapped by DNBBS during the reaction of cyt *c* with 5 molar equivalents of H₂O₂. However, the sites of DNBBS-cyt *c* adduct formation were not identified. The radicals formed in the reaction of cyt *c* with H₂O₂ 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*/H₂O₂/DNBBS reaction to determine the extent of protein radical formation. Surprisingly,

2-methyl-2-nitrosopropane; MS, mass spectrometry; ST/LC/MS, spin trapping coupled to on-line peptide mass mapping; X[•], protein-based radical; XMA, diamagnetic mass adduct of X[•]; XST[•], spin adduct of X[•].

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¹ The abbreviations: cyt *c*, ferricytochrome *c*; ACN, acetonitrile; ANS, 8-anilino-1-naphthalenesulfonate; DNBBS, 3,5-dibromo-4-nitrosobenzenesulfonate; DNBBS^{•+}, 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^{•+}, oxidized

we detected DBNBS-cyt *c* mass adducts in the absence of H₂O₂, 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. H₂O₂ 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',N'*-pentaacetic acid (DTPA, ICN) were prepared using 18-M Ω -cm water obtained from a Barnstead Nanopure system.

Methods—Reactions were carried out by mixing 500 μ M protein with 10 mM DBNBS and 0, 1, or 5 molar equivalents of H₂O₂ in 50 mM ammonium acetate solution (pH 7.5) containing 200 μ M DTPA. The reactions were terminated after 10 min by injecting the reactants onto a Vydac C₁₈ column (4.6 \times 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 μ l/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 C₁₈ column (1 \times 300 mm) using a 10–55% ACN gradient in 0.05% trifluoroacetic acid at 40 μ l/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 μ l/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 H₂O₂—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 H₂O₂, 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 H₂O₂ (Fig. 1*b*) despite the fact that H₂O₂, 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 H₂O₂ was repeated several times in the presence of DBNBS, and multiple DBNBS adducts were consistently observed. Moreover, in the absence of H₂O₂, 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 Met⁸⁰ 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*/H₂O₂/DBNBS reaction results in the same mass spectrum as that observed in the absence of H₂O₂ (Fig. 1, *f* versus *c*). Therefore, the (DBNBS)_{*n*}-cyt *c* adducts from the cyanocyt *c*/H₂O₂/DBNBS reaction are unlikely due to peroxidation, supporting the absence of non-heme-mediated oxidation of cyt *c* residues by H₂O₂. Nonetheless, the mass spectra reveal that the (DBNBS)_{*n*}-cyt *c* adduct intensities increase in the presence of H₂O₂ (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 H₂O₂ were recorded (Fig. 2). The difference spectrum (Fig. 2, *trace 3*) of HPLC-purified products from the cyt *c*/H₂O₂/DBNBS (1:5:20) reaction minus those from the cyt *c*/H₂O₂ (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 H₂O₂-induced oxidation of DBNBS was also considered, but incubation of DBNBS with 5 molar equivalents of H₂O₂ 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*/H₂O₂ (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 H₂O₂. Thus, the new band at 300 nm in the cyt *c*/H₂O₂/DBNBS minus cyt *c*/H₂O₂ 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*/H₂O₂/DBNBS reaction yielded only native peptides. It has been observed that ascorbate reduction of MNP-Mb spin adducts yields stable XMA_s that can be identified by ST/LC/MS (9, 10). However, addition of 5 mM ascorbate to the cyt *c*/H₂O₂/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 $\mu\text{l}/\text{min}$ for molecular weight determination.

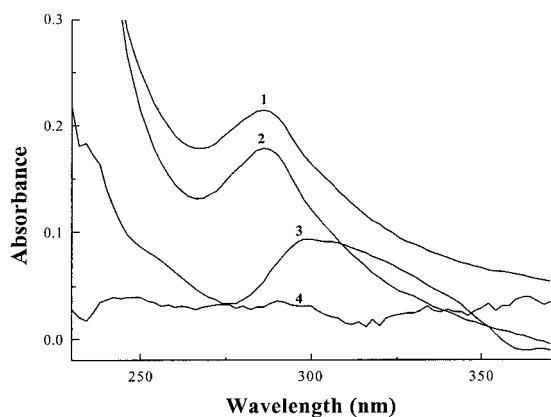
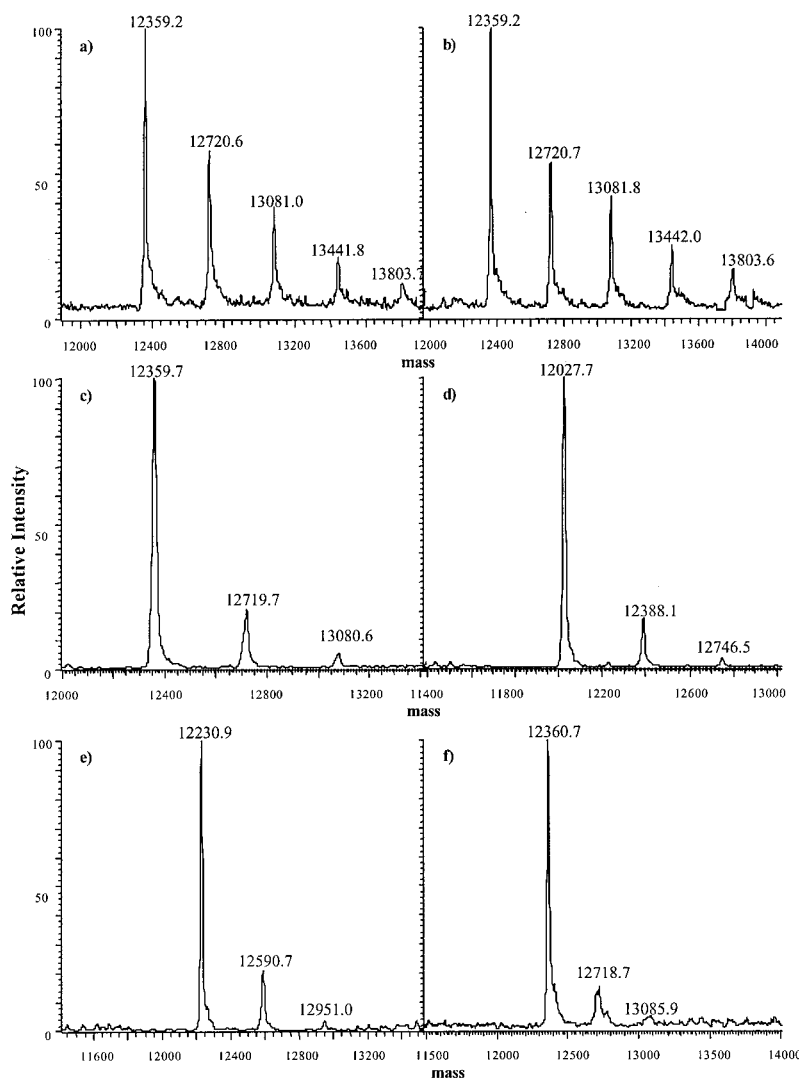


FIG. 2. Absorption spectra of (trace 1) 132 μM DBNBS following a 10-min incubation with 660 μM H_2O_2 and (trace 2) untreated 111 μM DBNBS. Difference optical spectra of the HPLC-purified DBNBS-cyt *c* adduct (~11 μM cyt *c*) minus the products from the 10-min reaction of 11 μM horse cyt *c* with 55 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⁺). Inverted spin trapping of DBNBS⁺ by an amino acid residue could lead to a DBNBS-cyt *c* adduct identical to that formed by "normal" spin trapping of X[•] by DBNBS (reviewed in Ref. 23). Horseradish peroxidase/ H_2O_2 ($E^\circ \text{Fe}^{\text{IV}} = \text{O}/\text{Fe}^{\text{III}} = 0.94$ V) catalyzed the oxidation of DBNBS over a 24-h incubation, as seen by EPR (24). However, DBNBS⁺ generation was not observed by the peroxidase activity ($E^\circ \text{Fe}^{\text{IV}} = \text{O}/\text{Fe}^{\text{III}} \sim 1$ V) of Mb/ H_2O_2 or cyt *c* oxidase/ H_2O_2 (25, 26), the Fenton reaction, $\text{Fe}^{\text{II}}/\text{H}_2\text{O}_2$ ($E^\circ \text{Fe}^{\text{III}}/\text{Fe}^{\text{II}} = 0.77$ V) (27), nor in the oxidation of selenite by Ce^{IV} ($E^\circ \text{Ce}^{\text{IV}}/\text{Ce}^{\text{III}} = 1.44$ V) (28). Since MNP and DBNBS contain identical redox-active nitroso functional groups, it can be as-

sumed that the E° DBNBS⁺/DBNBS is comparable with E° MNP⁺/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*/H₂O₂/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* ($E^\circ = 0.25$ 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*/H₂O₂/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 H₂O₂—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[•] generated in the Mb/H₂O₂ reaction has been demonstrated by both EPR and ST/LC/MS using DBNBS and other spin traps, and X[•] has been assigned primarily to Tyr¹⁰³ (10, 30–33). The difference spectrum (data not shown) of HPLC-purified products from the Mb/H₂O₂/DBNBS (1:5:20) reaction minus those from the Mb/H₂O₂ (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 ($42 \text{ mM}^{-1} \text{ cm}^{-1}$) 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/H₂O₂/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*/H₂O₂/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/H₂O₂/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 H₂O₂ (Fig. 3*b*). Inverted spin trapping was not reported in the Mb/H₂O₂/DBNBS reaction (26), since the oxyferryl heme of Mb ($E^\circ \text{ Fe}^{\text{IV}} = \text{O}/\text{Fe}^{\text{III}} \sim 1$ V) cannot oxidize DBNBS. When H₂O₂ 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 H₂O₂ (Fig. 3*a*). Likewise, a less intense (DMPO)-Mb peak was observed by ESI-MS upon decreasing the amount of H₂O₂ from 3 to 1 molar equivalents in the Mb/H₂O₂/DMPO reaction (32). It was reported that formation of oxyferryl Mb requires >1 molar equivalent of H₂O₂, since H₂O₂ is consumed

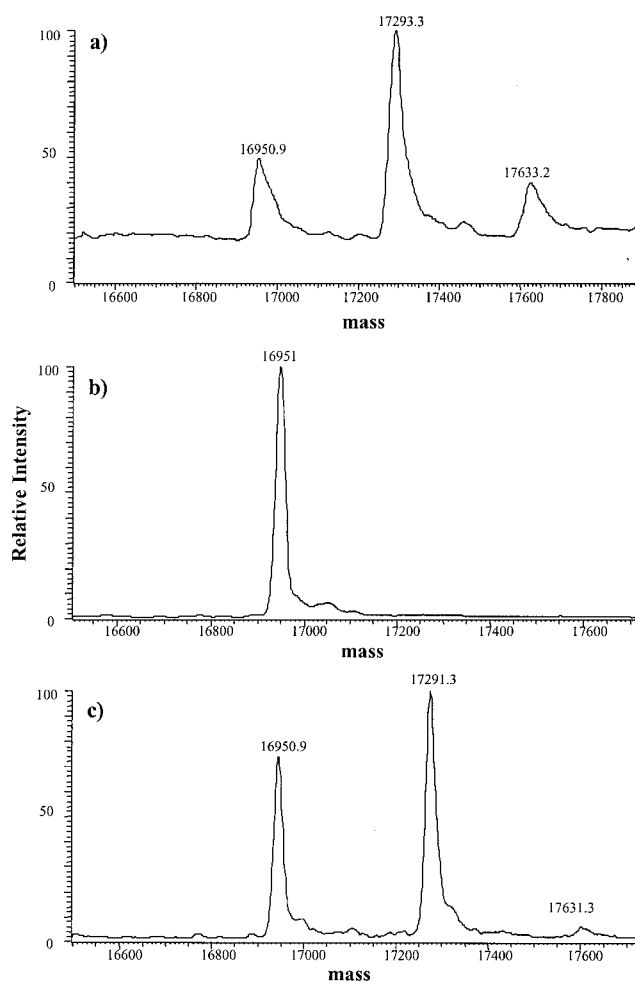


FIG. 3. Deconvolved ESI mass spectra of the products of a 10-min reaction of 500 μM horse heart metmyoglobin with H₂O₂ in the presence of 10 mM DBNBS: with 2.5 mM H₂O₂ (*a*), without H₂O₂ (*b*), and with 500 μM H₂O₂ (*c*). Experimental conditions are given in the legend to Fig. 1.

in side reactions at the porphyrin or other locations on the globin (9, 34). Nevertheless, the efficiency of (XST[•])_{*n*}-Mb adduct formation depends on the number of oxidizing equivalents present in the Mb/H₂O₂/ST reaction for both DMPO and DBNBS, in contrast to the cyt *c*/H₂O₂/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 H₂O₂ to generate X[•] unless H₂O₂ 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 H₂O₂ 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 H₂O₂ at pH 2.0—Anion-dependent stabilization of compact structures that resemble the molten globule state have been

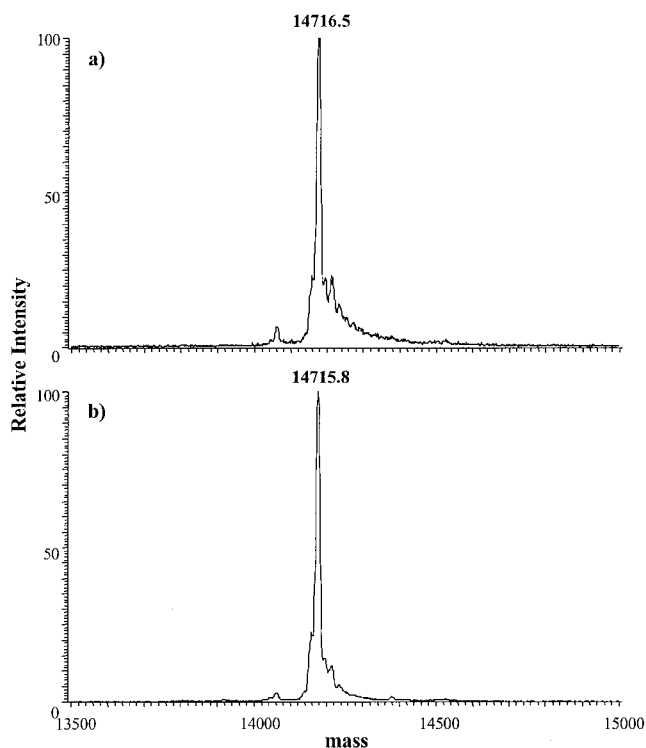


FIG. 4. Deconvolved ESI mass spectra of the products of a 10-min reaction of 500 μM cow α -lactalbumin with H_2O_2 and 10 mM DBNBS: with 2.5 mM H_2O_2 (a) and without H_2O_2 (b). Experimental conditions are given in the legend to Fig. 1.

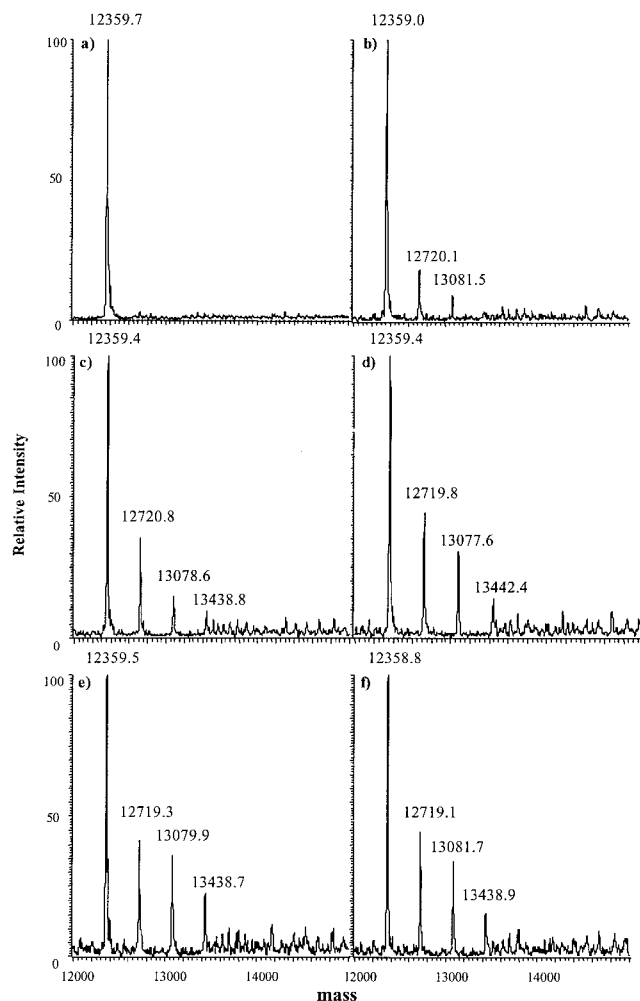


FIG. 6. Deconvolved ESI mass spectra of 20 μM horse cyt *c* incubated for 30 min in 0.05% trifluoroacetic acid (pH 2.0) in the presence of 0 (a), 1 (b), 10 (c), 15 (d), 20 (e), and 25 (f) molar equivalents of DBNBS. Samples in 75% ACN containing 0.05% trifluoroacetic acid were infused into the ESI source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

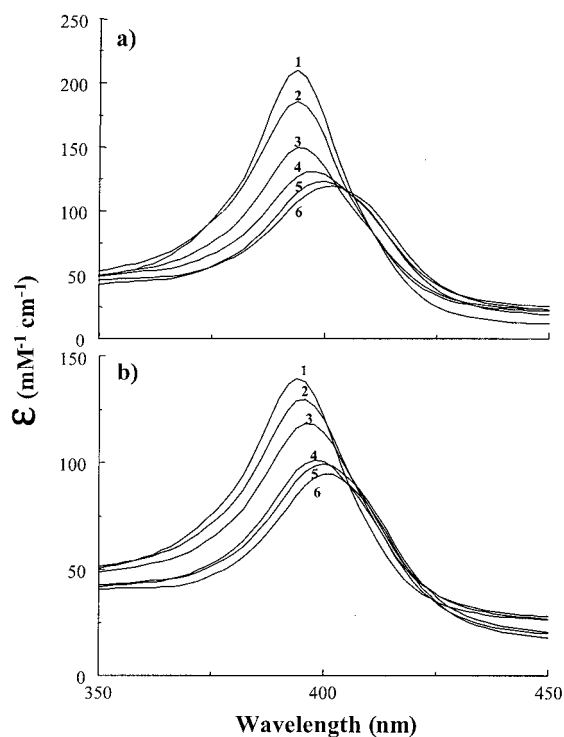


FIG. 5. Soret absorption spectra of 10 μM non-oxidized (a) and oxidized (b) horse cyt *c* incubated for 30 min in 0.05% trifluoroacetic acid (pH 2.0) in the presence of 0 (trace 1), 1 (trace 2), 5 (trace 3), 15 (trace 4), 20 (trace 5), and 25 (trace 6) molar equivalents of DBNBS. Oxidized protein was 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.

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 $(\text{DBNBS})_n$ -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. 5a). 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 -oxidized 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* ($\epsilon_{394} = 140 \text{ mM}^{-1} \text{ cm}^{-1}$) and its DBNBS-stabilized molten globule ($\epsilon_{402} = 94 \text{ mM}^{-1} \text{ cm}^{-1}$) are less than those of the unoxidized forms ($\epsilon_{394} = 209 \text{ mM}^{-1} \text{ cm}^{-1}$, $\epsilon_{394} = 120 \text{ mM}^{-1} \text{ cm}^{-1}$).

DBNBS titration of horse cyt *c* was also monitored by MS (Fig. 6). The $(\text{DBNBS})_n$ -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. 1c). Fig. 6 also reveals that the abundance and stoichiometry of the (DBNBS)_n-cyt *c* adducts increased as a function of DNBNS 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 DNBNS at pH 7.5, which required limiting the amount of DNBNS added to 500 μM cyt *c* to 10 mM. Ion pair formation between the sulfonate group of DNBNS 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 Lys⁸⁷ with a dissociation constant (*K_d*) of 200 μM and another close to Lys²⁵-His²⁶-Lys²⁷ with a *K_d* of >2 mM (36). However, the DNBNS-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 DNBNS-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 [DNBNS]⁻¹) of the data in Fig. 5 for both oxidized and non-oxidized 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—DNBNS 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 DNBNS 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 DNBNS 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 DNBNS 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 Tyr¹⁰³ of Mb, the cyt *c* radicals are not very reactive and/or accessible at pH 7.5.

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