ESR Spin-trapping of a Protein-derived Tyrosyl Radical from the Reaction of Cytochrome c with Hydrogen Peroxide*

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The reaction of horse heart cytochrome c with hydrogen peroxide was investigated using the ESR spin-trapping technique and the nitroso spin traps 3,5-dibromo-4-nitroso-benzenesulfonic acid (DBNBS) and 2-methyl-2-nitrosopropane (MNP). The ESR spectra obtained using both spin traps were typical of an immobilized nitroxide and indicated that the adduct was a macromolecule. The intensity of the ESR spectrum corresponding to the DBNBS/cytochrome c radical adduct was greatly enhanced by performing the reaction under anaerobic conditions, which suggested that the spin trap was competing with O_2 for reaction with the radical site(s). Nonspecific proteolysis of either the DBNBS or the MNP adducts revealed isotropic three-line spectra. In addition, a high resolution ESR spectrum for the protease-treated MNP cytochrome c-derived protein radical adduct was obtained. The superhyperfine couplings detected in this spectra were identical to those detected from an authentic MNP/tyrosyl adduct. Carbon-13 labeling of the aromatic ring positions of tyrosine yielded additional hyperfine coupling, demonstrating that the radical site was definitely located on the ring of tyrosine. Mass spectrometry detected as many as four DBNBS/cytochrome c-derived adducts from the reaction of cytochrome c with H_2O_2. Thus, it would appear four radical sites are formed during the reaction, at least one of which is tyrosine.

Oxidative stress in mitochondria has been the focus of much research in the last several decades. Reports of the production of H_2O_2 via the incomplete reduction of O_2 during oxidative phosphorylation have provided the impetus for such studies (1). Under normal physiological conditions, hydrogen peroxide levels are kept relatively low (10^{-9} to 10^{-7} M) through the actions of catalase and glutathione peroxidase (1). However, under certain conditions such as ischemia-reperfusion or inflammation, the production of excessive amounts of hydrogen peroxide does occur (2–3). This production of excess hydrogen peroxide is thought to precede several events that are characteristic of oxidative stress such as lipid peroxidation, glutathione depletion, calcium imbalance, and DNA and protein damage (3–6). Many researchers have promoted the idea that the oxidative damage caused by hydrogen peroxide is conveyed through its reaction with transition metals, which would involve the Haber-Weiss-type reaction sequence (7). Others have reported that the reaction of hydrogen peroxide with heme proteins, such as cytochrome c, produces highly reactive ferryl-heme species that are capable of oxidizing biomolecules and initiating lipid peroxidation (8–12). Radi et al. (9) demonstrated that luminol, ABTS, and 4-aminopyrine oxidation by cytochrome c were all dependent on hydrogen peroxide, but did not involve the hydroxyl radical. In separate studies, it was shown that mitochondrial lipid peroxidation could be initiated by hydrogen peroxide and cytochrome c (12, 13). It was postulated that hydrogen peroxide oxidized cytochrome c to a ferryl-radical, compound I-type species similar to that seen with peroxides and metmyoglobin. The ferryl-radical form of cytochrome c would then catalyze the oxidation of biomolecules. It should be noted, that unlike metmyoglobin, no direct evidence has been obtained for a ferryl or ferryl-protein radical form of cytochrome c. However, because the reactions of both metmyoglobin and cytochrome c with hydrogen peroxide yield oxidizing species that initiate lipid peroxidation and oxidize organic substrates (8–14), we have utilized the ESR spin-trapping technique to determine if protein-centered radicals are formed on cytochrome c following its reaction with hydrogen peroxide.

EXPERIMENTAL PROCEDURES

Materials—Horse heart cytochrome c, sodium phosphate, 4-oxo-TEMPO, DTPA, and horseradish peroxidase were all purchased from Sigma. MNP, potassium cyanide, and sodium dithionite were all obtained from Aldrich and DBNBS was synthesized using the method of Kaur et al. (15). Perdeuterated MNP-d₅ was provided as a gift from Dr. B. Kalyanaraman. Superoxide dismutase, Pronase, and glucose oxidase were purchased from Boehringer Mannheim. The reagents and buffer were prepared using deionized water and were treated with Chelex 100 (Bio-Rad). Prepacked Sephadex G-25 (PD-10) size exclusion cartridges were purchased from Pharmacia (Uppsala, Sweden).

ESR Spin Trapping Experiments—ESR spectra were recorded using a Bruker ECS-106 spectrometer (Billerica, MA) operating at 9.77 GHz with a modulation frequency of 50 kHz and a TM110 cavity. The reactions were initiated by the addition of cytochrome c. For all ESR experiments, the reactions were allowed to proceed for 3 min. The reaction mixture was then loaded onto a Sephadex G-25 size exclusion column and eluted with 0.1 M sodium phosphate buffer (pH 7.4). This was done primarily to remove the DBNBS from the sample and to minimize the contribution of the addition reaction (i.e., one reaction) that DBNBS is known to undergo with tryptophan (16). For the experiment shown in Fig. 5, glucose and glucose oxidase were added to the reaction mixture 3 min before the addition of cytochrome c to produce anaerobic conditions. Prior to the addition of glucose oxidase, the solution containing all reactants except cytochrome c was bubbled with N₂ for 5 min. The

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reactions were all performed in 100 mM sodium phosphate solutions (pH 7.4) that contained 200 \mu M DTPA to inhibit possible trace-transition metal catalysis. The computer simulations were performed using a program that is available through the internet (http://www.niehs.nih/LMB). The details of the program were described in a recent publication (17). The concentration of the DBNBS radical adduct(s) that were detected was estimated by comparing the ESR spectrum from the cytochrome c/H_2O_2 reaction mixture with that of a known concentration of the stable nitroxide 4-oxo-TEMPO (100 \mu M) dissolved in 30% glycerol. The samples were frozen in liquid nitrogen, placed in a TE_20_2 cavity, and the ESR spectra were recorded. The samples were compared at liquid nitrogen temperature so that an immobilized nitroxide could be detected from the 4-oxo-TEMPO. A reasonable comparison of these two radical species was made by first determining the appropriate power and modulation amplitude that gave the greatest signal in each case. The optimum power and modulation amplitude for the DBNBS/cyt c-derived radical adduct was 20 mW and 18 G, respectively. A power of 1 mW and 24 G was found to be optimal for 4-oxo-TEMPO. Comparison of the double integrals from the resulting ESR spectra allowed us to estimate the concentration of the DBNBS/cyt c adduct that was detected from the reaction.

Mass Spectrometry—The mass spectrometric analyses were performed using a Voyager RP (PerSeptive Biosystems, Framingham, MA) time-of-flight dual-stage reflector mass spectrometer. The instrument uses a nitrogen laser at 337 nm to desorb/ionize the samples. The accelerating voltage used was 30 kV and the flight path was 1.3 m. A beam guide wire with an applied voltage was used to refocus dispersed ions back onto the detector. Data from 256 laser pulses (laser energy between 280 and 300 arbitrary units) were averaged on a Tektronix TDS 520A digitizing oscilloscope and then downloaded to a PC. Data analysis was performed using GRAMS 386 software (Galactic Indus-

RESULTS

When cytochrome c was allowed to react with 5 equivalents of hydrogen peroxide in the presence of MNP, a spectrum characteristic of an immobilized nitroxide was detected (Fig. 1A). The MNP/cyt c-derived adduct persisted following size exclusion chromatography, which demonstrated that the radial adduct was a macromolecule (i.e. protein-bound). The adduct was dependent on the presence of MNP (Fig. 1B). In the absence of MNP a broad, featureless, direct ESR spectrum of a free radical with a line width of 10 G was detected. When the MNP/cyt c-derived adduct was submitted to nonspecific proteolysis, an isotropic three-line spectrum was detected with a hyperfine coupling constant of 15.5 G (Fig. 1C). This was similar to that reported by Gunther et al. (18) for the protease digested MNP/metMb adduct.

The fact that only a three-line spectrum was detected following Pronase treatment of the adduct suggested that the radical was located on a tertiary carbon atom (i.e none of the neighboring atoms had nuclear spin). In a previous study, Gunther et al. (18) used a lower modulation amplitude to resolve superhyperfine structure from the DBNBS/metMb adduct. By comparing this superhyperfine structure with that detected from an authentic DBNBS/trp adduct, they concluded that the radical trapped by DBNBS was centered on C-3 of a tryptophan residue in metmyoglobin. We employed the same technique here, except using perdeuterated MNP (i.e. the nine methyl hydrogens were replaced with deuterium). The deuteration of MNP decreases the hyperfine structure from the nine methyl hydrogens which would otherwise broaden the spectrum and prevent the resolution of superhyperfine structure. A three-line spectrum similar to that seen in Fig. 1C was detected when MNP-d_9 was used as the spin trap (a_N = 15.5 G) (data not shown). An 8-gauss scan of the low-field line from this primary triplet using a modulation amplitude of 0.14 gauss revealed superhyperfine structure consisting of five well resolved lines (Fig. 2A). The simulated spectrum in Fig. 2A (dashed line) was obtained using 2 equivalent hydrogens (a_H = 0.66 G (2H)) and 1 non-equivalent hydrogen (a_H = 1.1 G). This was possible that such an adduct might arise from the trapping of a tyrosyl radical at the C-1 position, the sole tertiary carbon in tryptophine. To investigate this possibility, an authentic MNP-d_9/tyr adduct was produced in a separate experiment using horseradish peroxidase and H_2O_2 to oxidize free tryptophine. Once again, a three-line spectrum was observed with a primary nitrogen splitting of 15.6 gauss (data not shown). When an 8-gauss scan was performed using the low-field line from this primary triplet, an identical spectrum to that seen with the MNP-d_9/cyt c derived adduct was detected (Fig. 2B). When all the ring positions of tryptophine were labeled with carbon-13, the three-line spectrum detected with tryptophine (Fig. 3A) was replaced by an eight-line spectrum (Fig. 3B). This demonstrated that the radical site was located on the aromatic ring of tryptophine. The simulation was composed using couplings from one nitrogen (a_N = 15.6 G), an _a-carbon-13 (a_C^13_a = 7.1 G), and 2 equivalent _b-carbon-13 atoms (a_C^13_b = 8.0 G) (Fig. 3B, dashed line).

An immobilized nitroxide was also detected when the reaction was performed in the presence of DBNBS (Fig. 4A). The spectrum was not observed in the absence of hydrogen peroxide (Fig. 4B). Care was taken to ensure that the immobilized radical adduct was not formed by the ene reaction known to occur with DBNBS (16). This reaction involves the addition of the DBNBS nitrosyl function to double bonds and is independent of peroxide. The ene reaction is relatively slow and, thus, the possibility of its interference was eliminated by using short incubation times and a G-25 size exclusion column as described under "Experimental Procedures." Cyanide forms a low-spin
Ferric complex with cytochrome c and inhibits heme-catalyzed reactions (19). Accordingly, the addition of 1M potassium cyanide prior to that of H₂O₂ prevented the formation of the DBNBS adduct (data not shown). There was no effect when 1M potassium cyanide was added after the adduct had already been formed. The addition of DTPA, even at a final concentration of 10 mM, had no effect on the intensity of the ESR spectrum (data not shown). Therefore, the DBNBS adduct was not formed as a result of iron release from cytochrome c. The DBNBS adduct was also detected using ferrous cytochrome c (formed from dithionite reduction followed by size exclusion chromatography). This was not unexpected as the reaction of H₂O₂ with ferrous cytochrome c to form ferric cytochrome c has been reported (20). When DBNBS was incubated alone with the cytochrome c and then reacted with H₂O₂ after passing the sample over a G-25 column, the immobilized nitroxide was not observed (Fig. 4C). Instead, the same spectrum as seen in Fig. 1B was detected. This spectrum was detected again when cytochrome c was allowed to react with hydrogen peroxide in the complete absence of any spin trap (Fig. 4D). When the sample from Fig. 4A was submitted to nonspecific proteolysis, an isotropic ESR spectrum of one major and one or more minor species was detected (Fig. 4E). This spectrum consisted primarily of three lines with a hyperfine coupling constant of 13.6 G, which was similar to that reported for the proteolyzed DBNBS/merMb adduct (18). The intensity of the DBNBS adduct increased dramatically both before and after proteolysis when the reaction was performed in the absence of O₂ (data not shown). This indicated that DBNBS was competing with O₂ for reaction with the radical site(s). In previous studies a protein-derived peroxyl radical has been detected at 77K following the reaction of metmyoglobin with hydrogen peroxide (21). We were unable, however, to detect a peroxyl radical from the cytochrome c/hydrogen peroxide reaction mixture. When the DBNBS/cyt c-derived adduct obtained from the anaerobic re-
action was proteolyzed, a more intense, but otherwise identical spectrum to that seen in Fig. 4E was detected (data not shown).

The concentration of the DBNBS/cyt c-derived adduct that was detected under either aerobic or anaerobic conditions was estimated by comparing frozen samples with the stable nitroxide standard, 4-oxo-TEMPO. The method for quantitation was described under "Experimental Procedures." Using this standard, 4-oxo-TEMPO was detected (data not shown). The ESR spectrum shown in Fig. 4 corresponded to the (M + H)⁺, (M + 2H)²⁺, and (M + 3H)³⁺ molecular ions of cytochrome c. Fig. 5B is an expanded view of the M⁺ ion.

Features from additional radical adducts can be seen in the ESR spectrum shown in Fig. 4E. Thus, it seemed probable that other radical sites were formed during the reaction that were trapped by DBNBS, but not MNP. To confirm that more than one DBNBS adduct was formed, the reaction of cytochrome c with hydrogen peroxide was analyzed using MALDI time-of-flight mass spectrometry (22). This technique uses energy transfer from a UV absorbing matrix to ionize the sample and with hydrogen peroxide, and 30 mM DBNBS. The reaction was allowed to proceed for 20 min, at which time it was put over a G-25 size exclusion column and then allowed to co-crystallize with the MALDI matrix. The method for making the reaction mixture anaerobic was described under "Experimental Procedures."

In the present study, an immobilized nitroxide was detected when either DBNBS or MNP was added to the reaction mixture containing cytochrome c and hydrogen peroxide. This type of protein-derived immobilized nitroxide has been observed from the reaction of other heme proteins such as myoglobin with H₂O₂ (18) and cytochrome P450 with cumene hydroperoxide (23). Nonspecific proteolysis of either the DBNBS or MNP/cyt c-derived adducts definitively indicated that the radical site was located on a tertiary carbon. This was because only a three-line ESR spectrum was observed. If the radical were centered on a carbon that was adjacent to atoms with nuclear spin, additional hyperfine coupling would have been observed. Tyrosine and tryptophan provide two likely possibilities for the formation of a radical adduct that would yield the primary triplet observed in these studies. This is because considerable spin density resides on the C-3 position of the tryptophan radical and on the C-1 ring position of the tyrosyl radical (24, 25). Both of these carbon atoms are tertiary and radical adducts formed from either of them with DBNBS or MNP would yield three-line ESR spectra. In fact, photolysis studies have shown that the MNP adducts obtained from free tryptophan or tyrosine yield three-line ESR spectra (26, 27), although the structure of the tyrosine radical adduct was not characterized until now. The aH values presented here for the DBNBS/cyt c-derived and MNP/cyt c-derived adducts were almost identical to those reported for the corresponding metmyoglobin adducts (18). In addition, the aH value for the MNP/cyt c-derived

**Fig. 5.** MALDI mass spectrum of native cytochrome c. The MALDI mass spectrum was obtained from a sample of ferric cytochrome c (1 mM) that was diluted 1:100 with water before co-crystallization with the MALDI matrix. The acquisition parameters are described under "Experimental Procedures." The mass labels in Fig. 5A correspond to the (M + H)⁺, (M + 2H)²⁺, and (M + 3H)³⁺ molecular ions of cytochrome c. Fig. 5B is an expanded view of the M⁺ ion.

**Fig. 6.** MALDI mass spectra obtained from the reaction of cytochrome with hydrogen peroxide in the presence of DBNBS. A, the initial reaction mixture contained 1 mM cytochrome c, 5 mM hydrogen peroxide, and 30 mM DBNBS. The reaction was allowed to proceed for 20 min, at which time it was put over a G-25 size exclusion column and then allowed to co-crystallize with the MALDI matrix. B, the same as A, except the reaction was performed in the absence of O₂. The method for making the reaction mixture anaerobic was described under "Experimental Procedures."
The coupling to three hydrogens that was detected in the reaction of metmyoglobin with H$_2$O$_2$ using MNP- was centered on a tyrosine residue. We also re-examined the MNP that has similar primary a$^4$ values (26, 27), super-hyperfine structure was needed to determine which MNP adduct was formed from cytochrome c. We were able to resolve this issue by comparing the high resolution ESR spectra obtained from the MNP-d$_5$/cyt c-derived adduct with that of an authentic MNP-d$_5$/trp adduct. The fact that these ESR spectra were identical served as unambiguous proof that the radical trapped by MNP from the reaction of cyt c with H$_2$O$_2$ was centered on a tyrosine residue. We also re-examined the reaction of metmyoglobin with H$_2$O$_2$ using MNP-d$_5$ (26, 27). Gunter et al. (18) recently reported that MNP trapped a tryptophan radical from metmyoglobin. However, we have found that the same high resolution ESR spectrum can be detected from the MNP-d$_5$/metMb adduct (data not shown) as was seen with either the MNP-d$_5$/cyt c-derived or MNP-d$_5$/trp adduct. Thus, MNP trapped a tyrosyl radical, and not a tryptophan radical, from metmyoglobin during its reaction with H$_2$O$_2$. It is possible that the tyrosyl radicals that have been found to cause cross-linking of metmyoglobin (28) were trapped by MNP. Evidence that the radical site was located on the aromatic ring of tyrosine was provided by the fact that an eight-line spectrum replaced the three-line spectrum when tyrosine labeled with carbon-13 in the ring positions was used. The eight-line spectrum was simulated convincingly by incorporating the spin – 1/2 couplings from an $\alpha$ carbon-13 (the one position of the ring) and two equivalent $\beta$ carbon-13 atoms (the two and six positions of the ring). The coupling to three hydrogens that was detected in the MNP-d$_5$ experiment combined with the fact that a significant fraction of the spin density is known to reside on C-1, indicated to us that C-1 was the radical site trapped by MNP. The structure of the radical adduct with the $\alpha$ and $\beta$ carbons labeled is shown in Fig. 3. It should be noted that attempts were made to resolve superhyperfine structure from the MNP-d$_5$/trp adduct (produced by HRP/H$_2$O$_2$, data not shown). However, the resolution of superhyperfine structure from this adduct was poor, and the lines that were seen did not resemble those that were seen from the MNP-d$_5$/cyt c-derived adduct.

The ESR spectrum of the proteolysed DBNBS/cyt c-derived sample revealed an additional adduct (Fig. 4E). Although it was clear from the MNP data that a tyrosyl radical had formed during the reaction, other radical sites may have formed that were trapped by DBNBS but not MNP. We also could not rule out the possibility that DBNBS was trapping a different radical site than MNP. Because of this complexity, we utilized MALDI/MS to detect additional protein-derived adducts, including ESR silent adducts that may be formed if the nitroxide moiety is reduced (i.e. to the hydroxylamine). In the presence of O$_2$ only one DBNBS/cyt c-derived adduct was detected by MALDI/MS. However, when anaerobic conditions were enforced, as many as four DBNBS/cyt c-derived adducts were observed. This indicated that more than one radical site in cytochrome c was formed during the reaction with hydrogen peroxide. The identity of these additional DBNBS adducts remains unknown. It is probable that DBNBS is trapping a radical centered on tryptophan 59, the only tryptophan in cytochrome c. There are also four tyrosines in cytochrome c, so trapping of multiple tyrosine residues is another possibility. If these additional adducts are formed and then rapidly reduced, they would be detectable by MALDI/MS, but not ESR. The fact that these additional adducts were not observed in the presence of O$_2$ may indicate that short-lived peroxyl radicals (which do not form persistent DBNBS radical adducts) were formed more readily than the DBNBS carbon-centered adducts under aerobic conditions. Although we were unable to detect peroxyl radicals with ESR, the fact that both the ESR and MALDI/MS spectra of the DBNBS/cyt c-derived adduct(s) were affected so dramatically by the omission of O$_2$ strongly suggests peroxyl radical formation.

Much work has been performed with metmyoglobin to elucidate the oxidative intermediates formed during the reduction of hydrogen peroxide to water (18, 21, 28–34). The existence of a protein radical in metmyoglobin following the reaction with hydrogen peroxide was demonstrated using ESR as early as 1958 by Gibson et al. (29). However, there seems to be, in general, a void in the literature concerning the oxidative intermediate formed during the reaction of cytochrome c with hydrogen peroxide. To our knowledge this is the first report of tyrosyl radical formation from the reaction of cytochrome c with hydrogen peroxide. An earlier study by Davies et al. (35) reported the formation of an immobilized nitroxide from cytochrome c, but the experiment was done using a Fenton reaction (i.e. ferrous EDTA and hydrogen peroxide), and the adduct was not characterized. The formation of a tyrosyl radical in cytochrome c might be explained in an analogous way to that of metmyoglobin (34). That is, hydrogen peroxide is reduced to water forming a ferryl-porphyrin radical, compound I type species (which, in the case of cytochrome c and metmyoglobin, is very short-lived). Rapid electron transfer from a tyrosine residue to the porphyrin radical could be used to rationalize the absence of spectroscopic evidence for a compound I species. However, there is also a lack of spectroscopic evidence for a ferryl compound II-type species for cytochrome c. It is possible that the ferryl form of cytochrome c also rapidly oxidizes amino acids in the vicinity of the heme or, perhaps, the heme itself. Thus, no spectral intermediates are detected and the Soret absorbance spectrum merely bleaches (36–38). The fact that as many as four DBNBS adducts were detected on one molecule of cytochrome c by MALDI/MS suggested that cytochrome c turned over more than one time under the conditions used here (5 molar equivalents of hydrogen peroxide).

Numerous investigations have demonstrated that, in the presence of hydrogen peroxide, metmyoglobin and cytochrome c, can initiate lipid peroxidation (8, 10–14), and the results presented here could provide a mechanism for cytochrome c-catalyzed lipid peroxidation. Since the cytochrome c-catalyzed lipid peroxidation reported by Radi et al. (10) was not affected by hydroxyl radical scavengers or metal chelators, the Haber-Weiss type reaction was excluded as a possible mechanism. Instead, it was proposed that cytochrome c formed a ferryl radical intermediate that was responsible for catalyzing the initial oxidation. While both cytochrome c and myoglobin catalyze hydrogen peroxide-dependent reactions that are characteristic of a ferryl-heme species, a visible absorption spectrum indicative of a ferryl myoglobin species was obtained decades ago (30), while no such evidence has yet been obtained for cytochrome c. In addition, since the crystal structure of aqueous cytochrome c shows the heme iron to be hexacoordinate, with a methionine ligand occupying the sixth position (39), one might argue that it is not possible for H$_2$O$_2$ to gain access to the heme iron. However, several studies have demonstrated that this ligand is easily displaced by small changes in ionic strength, pH, or the presence of other ligands (19, 40, 41). Thus, it is not unreasonable to presume that H$_2$O$_2$ might displace this ligand. In fact, the heme iron is definitely required as cyanide (a known inhibitor of heme-catalyzed reactions due to its ability to form a ligand in the sixth position) has been shown to
inhibit cytochrome c-catalyzed oxidations (10). In the present study, we also found that cyanide inhibited the formation of the adduct(s).

Considering the data presented here, we suggest that an amino acid radical, formed via the aforementioned mechanism, may be responsible for the previously reported oxidations catalyzed by the cytochrome c/H$_2$O$_2$ system (10–13). From a space-filling model obtained from the crystal structure of cytochrome c, we observed that tryptophan 59 and three tyrosine residues are solvent-exposed. It is also known that an edge of the heme in cytochrome c is solvent-exposed (39). Therefore, a radical formed at any one of these positions may allow the oxidation of molecules that are unable (possibly for steric reasons) to gain access to the heme iron. In the present study, only the tyrosine radical was definitively identified using MNP-d$_2$-catalyzed oxidations (10). In the present study, we also found that cyanide inhibited the formation of the adduct(s).

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