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EPR Spin-Trapping of a Myeloperoxidase Protein Radical

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Incubation of myeloperoxidase (MPO) with H_2O_2 in the presence of the spin trap DBNBS (3,5-dibromo-4-nitrosobenzenesulfonic acid) results in the EPR-detectable formation of a partially immobilized protein radical. The radical was only formed in the presence of both MPO and H_2O_2, indicating that catalytic turnover of the protein is required. The changes in the EPR spectrum of the adduct upon treatment with pronase confirm that the spin trap is bound to a protein residue. These results establish that MPO, like lactoperoxidase [Lardinois, O. M., Medzihradszky, K. F., and Ortiz de Montellano, P. R. (1999) *J. Biol. Chem.* 274, 35441–35448], reacts with H_2O_2 to give a protein radical intermediate. The protein radical may have a catalytic role, may be related to covalent binding of the prosthetic heme group to the protein, or may reflect a process that leads to inactivation of the enzyme.

Key Words: **myeloperoxidase; peroxidases; spin trapping; protein radical.**

Myeloperoxidase (MPO) is a member of the mammalian family of hemoprotein peroxidases that includes lactoperoxidase (LPO), eosinophil peroxidase, and thyroid peroxidase (1, 2). All of these enzymes, with the exception of thyroid peroxidase, are primarily involved in defense of the host against pathogenic infections. Two distinct catalytic activities of the mammalian peroxidases support their biological function: (a) their ability to oxidize halides to reactive hypohalides, and (b) their ability to catalyze the one-electron oxidation of phenols and other readily oxidized substrates to free radical species (3). The first step in both of these catalytic processes is reaction of the ferric peroxidase with H_2O_2 to give a Compound I species in which the iron is oxidized to a ferryl species and either the porphyrin or the protein (P stands for both in Eq. [1]) is oxidized to a radical cation (Eq. [1]):

$$P(Fe^{III}) + H_2O_2 \rightarrow P^{\bullet+}(Fe^{IV}{=}O) + H_2O \quad [1]$$

$$P^{\bullet+}(Fe^{IV}{=}O) + Cl^- + H^+ \rightarrow P(Fe^{III}) + HOCl \quad [2]$$

The Compound I with a porphyrin radical cation is thought to be responsible for the oxidation of halides to hypohalides (Eq. [2]) (4, 5). MPO is unique in that it readily oxidizes chloride ion to hypochlorous acid, but the other mammalian peroxidases oxidize iodide, bromide, and pseudohalides such as thiocyanate (3, 5).

The second catalytic activity of the peroxidases, one-electron oxidation of susceptible substrates such as phenol (PhOH in the equations), can be catalyzed by either Compound I species (Eq. [3]) or by Compound II (Eq. [4]), the intermediate in which the $Fe^{IV}{=}O$ species remains intact but the porphyrin or amino acid radical has been quenched:

$$P^{\bullet+}(Fe^{IV}{=}O) + PhOH \rightarrow P(Fe^{IV}{=}O) + PhO^{\bullet} + H^+ \quad [3]$$

$$P(Fe^{IV}{=}O) + PhOH + H^+ \rightarrow P(Fe^{III}) + PhO^{\bullet} + H_2O \quad [4]$$

It is likely that the Compound I species with a protein radical is formed in all cases by decay of the initially formed porphyrin radical cation. Evidence from the plant and fungal peroxidases indicates that, in some instances, the protein radical is important for substrate oxidation. Thus, the oxidation of cytochrome c by cytochrome c peroxidase appears to be mediated by the protein radical (6), and recent work shows that the oxidation of veratryl alcohol by lignin peroxidase is mediated by a tryptophan radical at the protein surface (7).

One of the primary features that distinguishes the mammalian peroxidases from the plant and fungal enzymes is the fact that the prosthetic heme group in the mammalian enzymes is cross-linked to the protein. In LPO, the heme is bound to the protein through ester

Abbreviations used: LPO, lactoperoxidase; MPO, myeloperoxidase; heme, iron protoporphyrin IX regardless of the oxidation and ligation states; DBNBS, 3,5-dibromo-4-nitroso-benzenesulfonic acid; EPR, electron paramagnetic resonance.

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links between two of the original heme methyl groups and two carboxylic acid sidechains in the active site of the protein (8–10). The two ester links are supplemented in MPO by third bond between one of the heme vinyl groups and a methionine residue (11–14). The nature of the covalent links in eosinophil and thyroid peroxidase has not yet been clearly defined but the links are likely to be similar to those in LPO and MPO. We first demonstrated that the covalent bonds in LPO are formed by an autocatalytic process in which noncovalently bound heme becomes covalently bound in the presence of H_2O_2 (8). Similar processes have been invoked more recently for covalent binding of the heme group in eosinophil and thyroid peroxidases (15, 16). Although the mechanism of the cross-linking reactions has not yet been further defined, it is very possible that a protein radical intermediate formed in an initial catalytic event will play a critical role in the heme cross-linking reactions.

Until recently, only indirect evidence existed for the formation of a protein radical in the mammalian peroxidases. Thus, it had been shown for LPO and MPO that the Compound I with the UV-vis spectrum characteristic of a ferryl/porphyrin radical cation decays with time to a species with the UV-vis spectrum of a species in which the porphyrin radical cation is lost (17–20). No direct evidence was available that the porphyrin radical disappears due to oxidation of the protein to a protein radical. However, we recently used spin-trapping techniques to demonstrate that LPO reacts with H_2O_2 to give a protein radical that is delocalized over several residues (21). We report here spin-trapping studies of the reaction of MPO with H_2O_2 that clearly demonstrate that it also forms a protein radical species. Although we have not been able to determine the amino acid(s) carrying the unpaired electron due to the unavailability of sufficient quantities of MPO, these studies provide the first direct evidence for the existence of an MPO protein radical.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade and were purchased from Sigma (St. Louis, MO) or Boehringer (Mannheim, Germany). Lyophilized MPO from human polymorphonuclear leukocytes (180–220 U/mg protein, A_{425}/A_{280} = 0.72) was purchased from Calbiochem (La Jolla, CA). Lyophilized LPO from bovine milk (80–150 U/mg protein, A_{412}/A_{280} = 0.88–0.95) was from Sigma. The enzyme preparations, shown to be homogeneous by SDS-PAGE, were desalted over prepacked Sephadex G-25 (PD-10) gel filtration cartridges (Amersham Pharmacia) before use. Peroxidase activities were estimated by the ABTS assay as described previously (21) and were in agreement with the supplier's stated activity.

Spin trapping experiments. EPR measurements were performed with an ER/200D EPR spectrometer from Bruker Inc. (Billerica, MA) operating at 9.80 GHz with a TM cavity. X-Band, first derivative absorption spectra were obtained with the following settings: microwave power, 25 mW; center field, 3480 Gauss; time constant, 100 ms; sweep time, 50 s; modulation, 0.32 mT at a frequency of 100 kHz; and total sweep width, 125 Gauss. Spectra were taken at 18–22°C. The magnetic field range and center were estimated by comparing the EPR spectrum from a MPO/H_2O_2 reaction mixture with that of the stable nitroso compound potassium nitrosodisulfonate (PADS). The PADS splitting was taken to be 13.091 Gauss and the center peak to correspond to a g value of 2.0056. The reactions were initiated by adding H_2O_2 to the mixture of MPO and the spin trap in 50 mM potassium phosphate buffer (pH 6.8), containing 200 μM diethylenetriaminepentaacetic acid to inhibit possible catalysis by trace-transition metals.

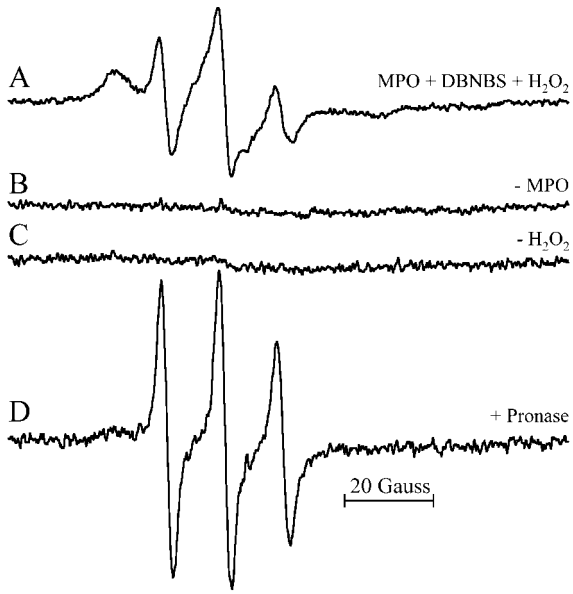

FIG. 1. EPR spectra obtained from the reaction of human MPO with H_2O_2 in the presence of DBNBS: (A) the reaction mixture containing 70 μM MPO, 900 μM H_2O_2, and 18 mM DBNBS; (B) the same as A, except MPO was not added; (C) the same as A, except H_2O_2 was not added; (D) an aliquot of the solution used to obtain spectrum A 5 min after the addition of Pronase (final concentration, 2 mg/ml). The instrumental parameters were as follows: modulation amplitude, 4 G; time constant, 100 ms; receiver gain, 10×10^5; modulation frequency, 100 kHz; microwave frequency, 9.80 GHz; microwave power, 25 mW.

RESULTS

The addition of 12 equivalents of H_2O_2 to 70 μM MPO in the presence of 18 mM DBNBS produced an anisotropic EPR spectrum consistent with the formation of a partially immobilized radical adduct (Fig. 1A). No signals were observed in the absence of the peroxidase (Fig. 1B) or H_2O_2 (Fig. 1C). When the DBNBS/MPO-derived adduct was submitted to nonspecific proteolysis with pronase, an isotropic three-line spectrum was detected with a hyperfine coupling constant of 13.4 G. Data for the corresponding LPO/H_2O_2/DBNBS system studied previously (21) are presented in Fig. 2 for comparison. The top spectrum is characteristic of a highly immobilized nitroxide radical (Fig. 2A). As with MPO, treatment of the sample with pronase yielded an isotropic three-line spectrum typical of rapidly tumbling nitroxide radicals (Fig. 2B) with a hyperfine cou-

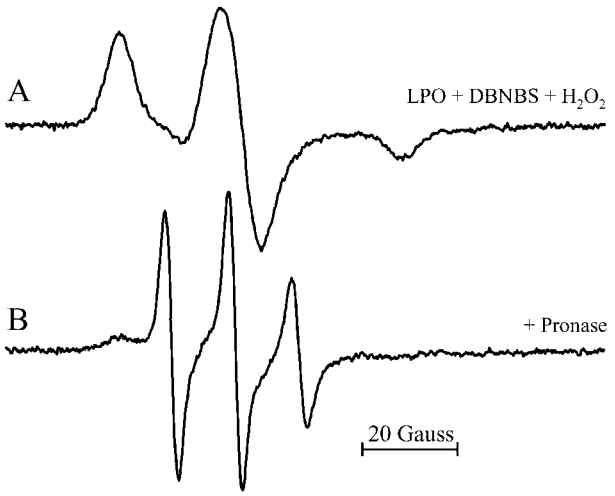

FIG. 2. EPR spectra obtained from the reaction of bovine LPO with H_2O_2 in the presence of DBNBS: (A) the reaction mixture containing 250 μM LPO, 900 μM H_2O_2, and 18 mM DBNBS; (B) an aliquot of the solution used to obtain spectrum A 5 min after the addition of Pronase (final concentration, 2 mg/ml). The instrumental settings were the same as in the legend to Fig. 1.

pling constant of 13.4 G. Note that the spectra in Figs. 1D and 2B and the dominant three-line spectrum in Fig. 1A exhibit the same g values and hyperfine splitting constants. Figure 3 compares the EPR spectrum of the DBNBS/MPO-derived adduct with a simulated spectrum obtained by summing 3 parts of spectrum 2A (~72% of the spin concentration) and 4 parts of spectrum 2B (~28% of the spin concentration). The data suggest that the DBNBS/MPO-derived adduct is a composite of two components; one highly immobilized, giving rise to peaks 1 and 5, and the other mobile, giving rise to peaks 2, 3, and 4. These two components could represent two states of the same protein-derived adduct with slow transitions between the states ($<10^{-6}$ s). Alternatively, they could represent two different DBNBS adducts with different mobilities. Due to the variation between preparations in the magnitude of the more mobile component (not shown) we favor the second alternative. In any case, the highly significant increase in the mobility of the nitroxide probe on addition of pronase unambiguously demonstrates that at least a fraction of the radical adduct is protein-bound.

DISCUSSION

The demonstration that a protein radical is formed in the reaction of MPO with H_2O_2 is consistent with the earlier observation that the Compound I with a porphyrin radical cation decays to a Compound II-like species in which the porphyrin radical cation is no longer present (20). Due to the quantities of MPO required to carry out spin trapping experiments, and the cost of the enzyme, we have not been able to identify the protein residues that are trapped by DBNBS and which therefore, by inference, are oxidized to the free radical state by electron transfer to the Compound I porphyrin radical cation. Nonspecific proteolysis of the DBNBS/MPO-derived adducts yielded an isotropic three-line spectrum, which definitively indicated that the radical site was located on a tertiary carbon. As discussed in more detail by Barr *et al.* (22), tyrosine and tryptophan are the most likely residues for the formation of a radical adduct with the observed triplet EPR signal. There are 15 tyrosine and 10 tryptophan residues in MPO. Analysis of the amino acid accessibility deduced from the MPO X-ray crystal structure (11, 12) showed that only 7 tyrosines (at position 7, 293, 309, 313, 316 and 557) and 4 tryptophans (at position 47, 271, 513 and 514) are located in exposed regions of the protein and are therefore capable of forming DBNBS/Tyr or DBNBS/Trp adducts.

Decay of the Compound I with a porphyrin radical cation to the protein radical yields a form that is inactive in the oxidation of chloride ion, although it retains the ability to oxidize classical peroxidase substrates. The transformation from a porphyrin to a protein radical thus competes with the oxidation of chloride ion to hypochlorous acid. The rate of the reaction of MPO Compound I with chloride ion has been reported as 4.6×10^6 M^{-1} s^{-1} (25°C) and 2.5×10^4 M^{-1} s^{-1} (15°C) (4, 5). Thus, in the presence of physiological concentrations of chloride ion the decay of Compound I to the protein radical would have to occur at a rate comparable to these rates to compete effectively with chloride ion oxidation. The rate of decay of the Compound I porphyrin radical cation to the protein radical is not known. The only available rate is that for a H_2O_2-dependent process that leads to the spectroscopically detectable disappearance of the Compound I porphyrin radical cation ($k = 8.2 \times 10^4$ M^{-1} s^{-1}) (20), but this reaction is a completely different process that is not thought to involve formation of the protein radical. Competition between decay to the protein radical and oxidation of chloride would be significant because formation of the protein radical would inhibit chloride oxidation until the enzyme is returned to the ferric

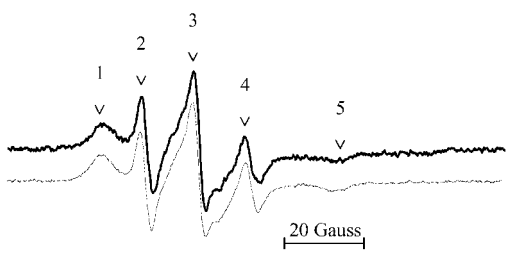

FIG. 3. Spectrum 1A (thick line) compared with synthesized spectra obtained by summing spectra 2A and 2B (thin line). Arrows indicate features corresponding to distinct spectral components (defined in text).

state by electron transfer from a one-electron donor. Indeed, peroxidase substrates such as ascorbic acid and acetaminophen have been shown to enhance the chlorinating activity of MPO, presumably by rescuing MPO from the Compound II state in which it is unable to oxidize chloride ion (23, 24).

The reason for transformation of the MPO Compound I porphyrin radical cation into a protein radical is unclear. Conversion of the Compound I form that oxidizes chloride into a form that does not provides a possible mechanism for restricting the production of a toxic product. Alternatively, it is possible that the peroxidase activity of the enzyme, which is not impaired by this transformation, has an independent function. This function could be oxidation of peroxidase substrates at the protein surface, in analogy to the case of cytochrome c peroxidase (6) and ligninase (7). A third possibility is that the protein radical stems from a vestigial process related to that which is responsible for the original autocatalytic crosslinking of the heme groups to the protein (8). It is also possible, of course, that the protein radical reflects a decay mechanism that has no specific physiological function but simply leads to eventual protein inactivation. Regardless of its specific function, conversion of Compound I from a porphyrin radical to a protein radical form in both LPO and MPO suggests that this transformation is a general one for the mammalian peroxidases.

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REFERENCES

1. Kimura, S., and Ikeda-Saito, M. (1988) Human myeloperoxidase and thyroid peroxidase, two enzymes with separate and distinct physiological functions, are evolutionarily related members of the same gene family. *Proteins* **3,** 113–120.
2. Dunford, H. B. (1999) Heme peroxidase and catalase families and superfamilies: Crystal structures. *In* Heme Peroxidases, pp. 33–37, Wiley-VCH, New York.
3. Dunford, H. B. (1999) Myeloperoxidase and eosinophil peroxidase: Phagocytosis and microbial killing. *In* Heme Peroxidases, pp. 349–385, Wiley-VCH, New York.
4. Marquez, L. A., and Dunford, H. B. (1995) Kinetics of oxidation of tyrosine and dityrosine by myeloperoxidase compounds I and II. Implications for lipoprotein peroxidation studies. *J. Biol. Chem.* **270,** 30434–30440.
5. Furtmüller, P. G., Burner, U., and Obinger, C. (1998) Reaction of myeloperoxidase compound I with chloride, bromide, iodide, and thiocyanate. *Biochemistry* **37,** 17923–17930.
6. Dunford, H. B. (1999) Yeast cytochrome c peroxidase. II. Interaction with cytochrome c. *In* Heme Peroxidases, pp. 252–269, Wiley-VCH, New York.
7. Doyle, W. A., Blodig, W., Veitch, N. C., Piontek, K., and Smith, A. T. (1998) Two substrate interaction sites in lignin peroxidase revealed by site-directed mutagenesis. *Biochemistry* **37,** 15097–15105.
8. DePillis, G. D., Ozaki, S., Kuo, J. M., Maltby, D. A., and Ortiz de Montellano, P. R. (1997) Autocatalytic processing of heme by lactoperoxidase produces the native protein-bound prosthetic group. *J. Biol. Chem.* **272,** 8857–8860.
9. Rae, T. D., and Goff, H. M. (1998) The heme prosthetic group of lactoperoxidase. Structural characteristics of heme l and heme l-peptides. *J. Biol. Chem.* **273,** 27968–27977.
10. Kooter, I. M., Pierik, A. J., Merkx, M., Averill, B. A., Moguilesky, N., Bollen, A., and Wever, R. (1997) Difference Fourier transform infrared evidence for ester bonds linking the heme group in myeloperoxidase, lactoperoxidase, and eosinophil peroxidase. *J. Am. Chem. Soc.* **119,** 11542–11543.
11. Zeng, J., and Fenna, R. E. (1992) X-ray crystal structure of canine myeloperoxidase at 3 Å resolution. *J. Mol. Biol.* **226,** 185–207.
12. Fenna, R., Zeng, J., and Davey, C. (1995) Structure of the green heme in myeloperoxidase. *Arch. Biochem. Biophys.* **316,** 653–656.
13. Taylor, K. L., Strobel, F., Yue, K. T., Ram, P., Pohl, J., Woods, A. S., and Kinkade, J. M. (1995) Isolation and identification of a protoheme IX derivative released during autolytic cleavage of human myeloperoxidase. *Arch. Biochem. Biophys.* **316,** 635–642.
14. Kooter, I. M., Moguilevsky, N., Bollen, A., van der Veen, L. A., Otto, C., Dekker, H. L., and Wever, R. (1999) The sulfonium ion linkage in myeloperoxidase. Direct spectroscopic detection by isotopic labeling and effect of mutation. *J. Biol. Chem.* **38,** 26794–26802.
15. Fayadat, L., Niccoli-Sire, P., Lanet, J., and Franc, J.-L. (1999) Role of heme in intracellular trafficking of thyroperoxidase and involvement of H_2O_2 generated at the apical surface of thyroid cells in autocatalytic covalent heme binding. *J. Biol. Chem.* **274,** 10533–10538.
16. Oxvig, C., Thomsen, A. R., Overgaard, M. T., Sørensen, E. S., Højrupt, P., Bjerrum, M. J., Gleich, G. J., and Sottrup-Jensen, L. (1999) Biochemical evidence for heme linkage through esters with Asp-93 and Glu-241 in human eosinophil peroxidase. The ester with Asp-93 is only partially formed *in vivo. J. Biol. Chem.* **274,** 16953–16958.
17. Kimura, S., and Yamazaki, I. (1979) Comparisons between hog intestinal peroxidase and bovine lactoperoxidase-compound I formation and inhibition by benzhydroxamic acid. *Arch. Biochem. Biophys.* **198,** 580–588.
18. Hu, S., and Kincaid, J. R. (1991) Resonance Raman structural characterization and the mechanism of formation of lactoperoxidase compound III. *J. Am. Chem. Soc.* **113,** 7189–7194.
19. Taurog, A., Dorris, M., and Doerge, D. R. (1994) Evidence for a radical mechanism in peroxidase-catalyzed coupling. I. Steady-state experiments with various peroxidases. *Arch. Biochem. Biophys.* **315,** 82–89.
20. Marquez, L. A., Huang, J. T., and Dunford, H. B. (1994) Spectral and kinetic studies on the formation of myeloperoxidase compounds I and II: Roles of hydrogen peroxide and superoxide. *Biochemistry* **33,** 1447–1454.
21. Lardinois, O. M., Medzihradszky, K. F., and Ortiz de Montellano, P. R. (1999) Spin trapping and protein cross-linking of the lactoperoxidase protein radical. *J. Biol. Chem.* **274,** 35441–35448.
22. Barr, D. P., Gunther, M. R., Deterding, L. J., K. B. Tomer, and Mason R. P. (1996) ESR spin-trapping of a protein-derived tyrosyl radical from the reaction of cytochrome c with hydrogen peroxide. *J. Biol. Chem.* **271,** 15498–15503.
23. Bolscher, B. G. J. M., Zoutberg, G. R., Cuperus, R. A., and Wever, R. (1984) Vitamin C stimulates the chlorinating activity of human myeloperoxidase. *Biochim. Biophys. Acta* **784,** 189–191.
24. Marquez, L. A., and Dunford, H. B. (1993) Interaction of acetaminophen with myeloperoxidase intermediates: Optimum stimulation of enzyme activity. *Arch. Biochem. Biophys.* **305,** 414–420.