The First Evidence Indicating Formation of Superoxide by Manganese-Dependent Peroxidase (MnP) in the Presence of Excess H_2O_2

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(Received December 13. 1999; CL-991049)

Production of O_2^- by manganese peroxidase (MnP) in the absence of free radical intermediates was analyzed by ESR spin trapping experiments with 4-POBN and DMPO. MnPs from *Pleurotus ostreatus* and *Bjerkandera adusta* produced O_2^- after addition of an excess of H_2O_2 independently of the presence of organic acid chelator for Mn(II) and Mn(III). This is the first report demonstrating direct production of O_2^- by MnP.

A heme-containing Mn(II)-oxidizing enzyme, manganese peroxidase (MnP) is implicated to be involved in lignin biodegradation by white-rot fungi. In the catalytic cycle of MnP, ferric enzyme is oxidized by H_2O_2 by two electrons to generate a ferryl-π-porphyrin cation radical, compound I, followed by a one-electron reduction to compound II and then back to ferric enzyme.[1,2] Thus, catalytic cycle of MnP is similar to conventional peroxidases, except that Mn(II) is the obligatory electron donor for reduction of the one-electron deficient enzyme to its resting state. MnP is known to produce O_2^- *via* free radical intermediates from substrates. In the free radical reactions, compounds such as oxalate,[3] glyoxalate,[4] malonate[5] and NADH[6] were oxidized by MnP to yield free radicals, which in turn reduce O_2 to form O_2^-. However, there has not been any report demonstrating direct production of O_2^- by MnP. Rate of O_2^- reduction by Mn(II) [7] was estimated to be about 6×10^6 $M^{-1}S^{-1}$ (M = mol dm^{-3}). Due to the high rate of Mn(II) oxidation by O_2^-, O_2^- generated by MnP is readily consumed by Mn(II)[8] unless an excess amount of O_2^- is produced. This makes detection of O_2^- difficult in the enzymatic reactions containing Mn(II). By using a highly reactive scavenger of O_2^- under minimal concentration of Mn(II), we found that MnP generated O_2^- with an excess of H_2O_2.

MnP was purified from liquid cultures of *P. ostreatus* and *B. adusta*.[9,10] The purified MnP from *P. ostreatus* was allowed to react with 0.5 mM H_2O_2 in the presence of 50 μM Mn(II) and spin trapping agent, 0.1 M 4-POBN in sodium tartrate buffer.[11] The ESR spectra demonstrated that a POBN spin adduct with hfc, A(N), 1.42 mT, A(Hβ) 0.17 mT was produced at 1 min (Figure 1). Hfc of the sextet signals observed was identical to those of O_2^- adduct of 4-POBN.[12] Addition of 80U SOD suppressed the formation of the spin adduct. The same 4-POBN spin adduct was observed when the reactions were carried out in sodium acetate buffer (Figure 2) or in aqueous solutions that did not contain any buffer salts. The production of O_2^- was not observed when any of the reactants were omitted from the complete reaction system (Data not shown). In the MnP reactions shown in Figures 1 and 2, the spin adduct appeared within 1min but increased signals were not observed after 1 min although the MnP maintained Mn(II)-oxidizing activity even after 1 min.[13] This indicates that oxidation of 4-POBN by MnP is not involved in the O_2^- formation. There was

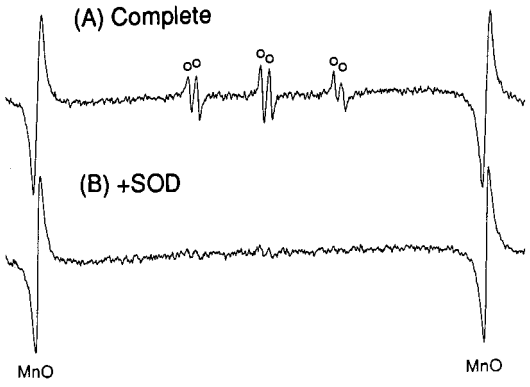

Figure 1 ESR spectra of POBN spin adducts formed by the reaction of *P. ostreatus* MnP in sodium tartrate buffer.

The complete reaction system (A) (200μl) contained 400mU MnP from *P. ostreatus*, 12.5mM sodium tartrate buffer (pH 5.0), 50μM $MnSO_4$, 0.5mM H_2O_2 and 100mM 4-POBN. The reaction system (B) contained 80U of SOD. The spectra were recorded at 1 min.

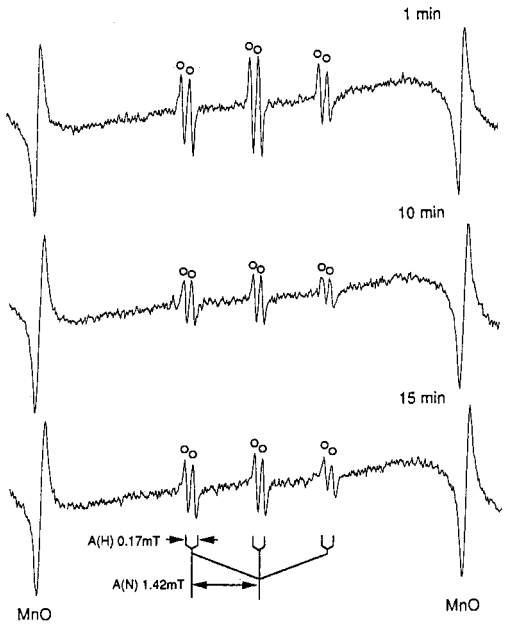

Figure 2 ESR spectra of 4-POBN spin adducts formed by the reaction of *P. ostreatus* MnP with H_2O_2 in sodium acetate buffer.

The reaction system (200 μl) contained 400 mU MnP from *P. ostreatus*, 12.5 mM sodium acetate buffer (pH 5.0), 50 μM $MnSO_4$, 0.5 mM H_2O_2 and 100 mM 4-POBN. The reaction was initiated by addition of H_2O_2.

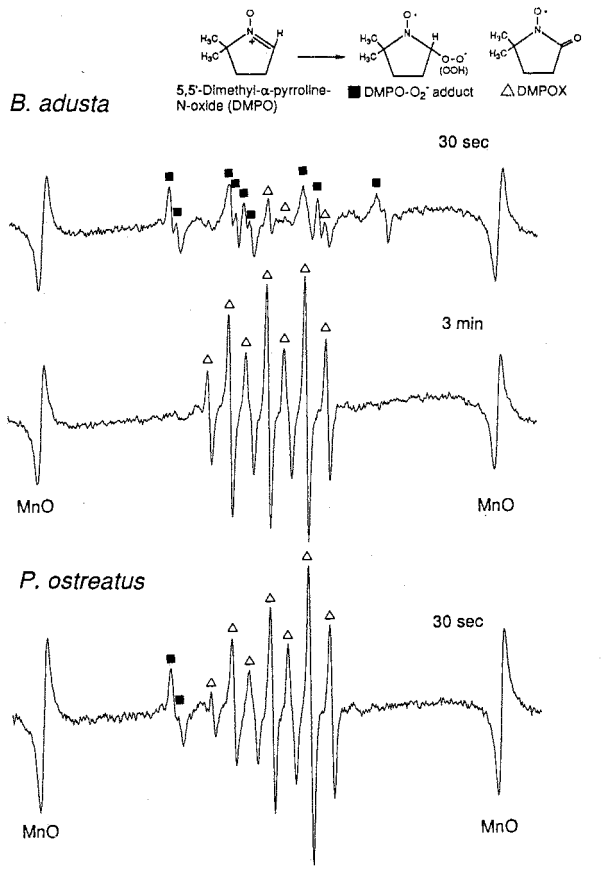

Figure 3 ESR spectra of DMPO spin adducts formed by the reaction of MnPs with H_2O_2 in sodium acetate buffer.

The reaction system (200 μl) contained 400 mU MnPs, 12.5 mM sodium acetate buffer (pH 5.0), 50 μM $MnSO_4$, 0.5 mM H_2O_2 and 90 mM DMPO. The reaction was initiated by addition of H_2O_2.

no spin adduct produced when 50 μM Mn(III)-tartrate[14] was mixed with 0.1M POBN as reported by Shi.[15] Addition of H_2O_2 to the Mn(III)-tatrate solution did not change the ESR spectra.

To confirm the direct generation of O_2^- by MnP, spin trapping experiments were carried out with DMPO (Figure 3). When the MnPs were reacted with H_2O_2/Mn(II) in sodium acetate buffer in the presence of DMPO, formation of DMPO-OOH (A(N) 1.43mT, A(Hβ) 1.15mT, A(Hγ) 0.13mT) was observed at 30 sec. At three minutes after addition of H_2O_2, DMPO was oxidized by enzymatically-generated Mn(III) to yield a DMPOX (A(N) 0.72 mT, A(H_2) 0.41 mT). In the reaction with MnP from *P. ostreatus*, signals from DMPO-OOH were also detected in the spectrum recorded at 30 sec. Thus, direct formation of O_2^- by MnP was demonstrated with two spin trapping agents, 4-POBN and DMPO.

Khindaria reported that reduction of ferric ion was observed in reaction mixtures containing MnP, Mn(II), oxalate, H_2O_2 and ferric chloride.[3] They reported reduction of Fe(III) by O_2^- is dependent on formate radical from oxalate. As they reported, Fe(III) reduction did not proceed in the absence of oxalate when the MnPs were mixed with Mn(II), Fe(III), 1,10-phenanthroline and H_2O_2 (Data not shown). This is consistent with our observation that formation of O_2^- was not involved in the consecutive catalytic cycle. No decrease in O_2 concentration below the starting level was observed in the MnP reactions (Data not shown). These results suggest that O_2^- formation by MnP is not related to reduction of molecular oxygen but to decomposition of Fe(III)-superoxo complex, compound III formed by the excess of H_2O_2. In contrast to release of O_2^- from compound III of lignin peroxidase,[16] the O_2^- formation by MnP required Mn(II). This suggests that reactivation of compound III to a resting state proceeds not only by O_2 generation with Mn(III)[17] but also through dissociation of O_2^- with manganous or manganic ions, as reported by stimulation of O_2^- dissociation from myeloperoxydase compound III by adrenaline.[18] The detection of O_2^- in MnP reactions raise a new issue of how the O_2^- affects on Mn(II)-oxidizing processes of MnPs.

References and Notes

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9 *P. ostreatus* (ATCC 66376) was cultivated in a glucose-yeast extract medium (GY) stationery at 28°C and purified with DEAE-Sepharose CL-6B and MONO-Q columns to obtain a single protein with pI 3.7 (RZ value: 3.6, N-terminal sequence: VTCATGQT-TANE, 1.0 U= 8.85 x 10^{-11} mol) as described before [H. Kamitusji, et al., *Wood Res.*, **86**, 41 (1999)]. Crude MnP from *B. adusta* was collected from 14 day-cultures grown on a glucose-peptone medium [Y. Kimura, et al., *Appl. Microbiol. Biotechnol.*, **32**, 436 (1990)] and purified to a single protein (pI 3.4, RZ value: 3.9, 1.0 U= 4.97 x 10^{-11} mol) with DEAE-Sepharose CL-6B and MONO-Q columns. MnP activity was measured with 0.2 mM 2,6-DMP, 0.5 mM $MnSO_4$, 0.1 mM H_2O_2, in 250 mM sodium tartrate buffer (pH 5.0). One unit (U) of enzyme activity is defined as the amount of enzyme that oxidizes 1 μmol of 2,6-DMP in one minute.
10 To avoid interference of a trace amount of transition metals and buffer salts from ion-exchange chromatography, low molecular mass compounds were removed from the enzyme solutions by successive washing with Milli-Q water in an ultrafiltration concentrator before use.
11 DMPO and 4-POBN were purchased from Sigma Chemical (USA) and Labotech (Japan), respectively. ESR spectral recordings were made in a flat cell (200μl) with JEOL FR-30 X-band ESR spectrometer operating at room temperature with a modulation width 0.079mT, time constant 0.10 sec, scanning time 2 min and microwave power 4mW.
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