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Oxidative decomposition of malonic acid as basis for the action of manganese peroxidase in the absence of hydrogen peroxide

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Abstract Manganese peroxidase (MnP) from the ligninolytic basidiomycetes *Phlebia radiata* and *Nematoloma frowardii* was found to decompose malonate oxidatively in the absence of H_2O_2 in a reaction system consisting of the enzyme, sodium malonate and $MnCl_2$. The enzymatic oxidation resulted in a substantial decrease in malonate concentration and the formation of CO_2, oxalate, glyoxylate and formate. Simultaneously with the decomposition of malonate, Mn(II) was oxidized to Mn(III) leading to high transient concentrations of the latter. MnP action in the absence of H_2O_2 started slowly after a lag period of 3 h. The lag period was considerably shortened after a single addition of Mn(III). Superoxide dismutase and catalase inhibited the enzymatic reaction partly, ascorbate completely. ESR studies demonstrated the formation of a carbon-centered radical during the course of the reaction. We propose that the latter generates peroxides that can be used by MnP to oxidize Mn(II) to Mn(III).

Key words: Manganese peroxidase; Malonic acid; Peroxyl radical; Hydroperoxide; *Phlebia radiata*; *Nematoloma frowardii*

1. Introduction

Manganese peroxidase (MnP) is a ubiquitous enzyme among ligninolytic fungi and has been found both in white-rot and in litter decaying basidiomycetes [1]. It catalyzes the oxidation of Mn(II) to Mn(III), which in turn is capable of oxidizing a variety of organic substances including lignin, humic substances and organopollutants [2–4]. MnP oxidation of Mn(II) and subsequently of secondary substrates requires the presence of dicarboxylic or α-hydroxycarboxylic acids that facilitate the reaction of Mn(II) with the enzyme and stabilize the highly reactive Mn(III) via chelation [5]. It is known that ligninolytic fungi excrete such organic acids, e.g. oxalate, malonate and malate, and that they have an important role in regulation of ligninolytic activities [6–8].

Due to the participation of MnP in the biodegradation of lignin and other high molecular weight substances, which cannot be taken up into the fungal hyphae, the extracellular production of H_2O_2 by ligninolytic fungi seems to be essential to the process. Several extracellular oxidases producing H_2O_2 from various low molecular weight substrates have been described [9–11]. However, there are also efficient lignin-degrading fungi in which H_2O_2-generating oxidases are obviously lacking. Thus, *Ceriporiopsis subvermispora* – which is characterized by its selective decay of lignin in wood – does not excrete H_2O_2-generating oxidase [12]. MnP of this fungus has recently been demonstrated to oxidize glyoxylate and oxalate in the presence of dioxygen to form H_2O_2, which in turn is used for the MnP cycle.

We have recently reported that MnP from *Nematoloma frowardii* and *Phlebia radiata* is capable of depolymerizing and even mineralizing synthetic lignin efficiently in the absence of H_2O_2 in a Na-malonate-buffered reaction system [13]. Here we report that the MnP-catalyzed oxidation of malonate is the basis for its function as peroxidase resulting in the formation of high concentrations of Mn(III). Moreover, evidence is provided that radical species derived from malonate are involved in the MnP cycle.

2. Materials and methods

2.1. Organisms and enzyme preparation

The basidiomycetous fungi *Phlebia radiata* strain 79 (ATCC 64658) and *Nematoloma frowardii* strain b19 (DSM 11239, ATCC 201144) were used for MnP production. *P. radiata* was cultured in a low-nitrogen medium containing 168 μM Mn(II) in a 2-l bioreactor [7] and isozyme MnP2 was isolated by FPLC as reported earlier [14]. Cultivation of *N. frowardii* occurred in a nitrogen-sufficient medium containing 300 μM Mn(II) in 1-l culture flasks. Isozyme MnP2 was partially purified by FPLC on a Mono-Q column (Pharmacia, Uppsala, Sweden) as described previously except that a salt gradient was used instead of a buffer gradient [15].

2.2. Enzymatic reaction mixture

The basic reaction mixture contained 50 mM sodium malonate, 1 mM Mn(II) ($MnCl_2$) and 2 μg/ml MnP (corresponding to an MnP activity of 0.7 U/ml in the presence of H_2O_2); H_2O_2 or an H_2O_2-generating enzyme were not added. In some experiments, the enzyme concentration (0.2–10 μg/ml) or the concentration of Mn(II) (0.1–5 mM) was varied. The enzymatic reaction was carried out in 10-ml reaction tubes (total 2 ml) closed with parafilm on a rotary shaker (180 rpm) at 37°C in the dark for 24 h. Controls were run using the same reaction mixtures and conditions, but omitting MnP, Mn(II) or malonate. Every 3–6 h samples (50 μl) were collected and analyzed by HPLC. Oxidation of 2,2′-azinobis(3-ethylbenzo-thiazolinesulfonate) (ABTS, Boehringer-Mannheim Scandinavia, Espoo, Finland) by the reaction solution was determined after 6 and 9 h. Most experiments were carried out with MnP2 from *P. radiata*, and in addition MnP2 from *N. frowardii* was used to confirm the results.

2.3. Photometric assays

MnP activity was routinely measured in the presence of H_2O_2 by the formation of Mn(III)-malonate complexes at 270 nm [5]. The same assay mixture was used to determine MnP activity in the absence of H_2O_2, but the measuring period was extended to 20–50 min. In some photometric tests, the H_2O_2-free reaction mixtures contained additional components: Mn(III) acetate (Aldrich, Steinheim, Ger-

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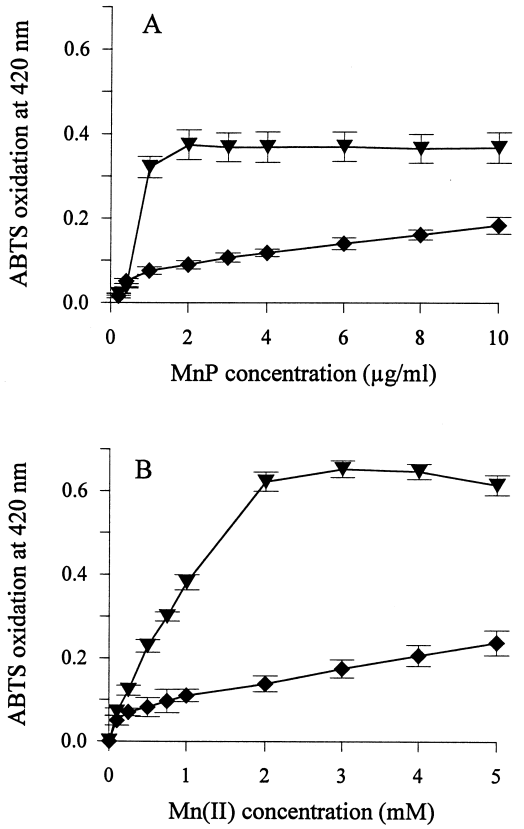

Fig. 1. Influence of MnP (A) and Mn(II) concentration (B) on the oxidation of ABTS by H_2O_2-free reaction mixtures containing malonate (50 mM), $MnCl_2$ (0.1–5 mM) and MnP2 from *P. radiata* (0.2–10 μg/ml). Oxidation of ABTS was measured after 6 (♦) and 9 (▽) h of incubation. The assay solution contained 50 mM malonate (pH 4.5) and 1 mM ABTS; the reaction was started by addition of 20 μl of the reaction mixture and was followed at 420 nm (ca. 30–45 s). Bars on symbols indicate standard deviation of means ($n = 3$).

many), superoxide dismutase (SOD, Sigma, Deisenhofen, Germany), catalase (from *Aspergillus niger*, Sigma) or ascorbate (Sigma). Mn(III) formed in the MnP-Mn(II)-malonate reaction mixture was calculated both directly from the absorption of Mn(III)-malonate complexes ($\varepsilon_{270} = 11.59\ M^{-1}\ cm^{-1}$) [5] and indirectly from the oxidation of ABTS (both methods gave nearly similar results, however the ABTS method was more sensitive). For the latter, 20 μl of the reaction mixture was added to an assay solution containing 50 mM sodium malonate (pH 4.5) and 1 mM ABTS. Oxidation was followed at 420 nm ($\varepsilon_{420} = 36 \times 10^4\ M^{-1}\ cm^{-1}$) over 30–45 s until no further increase in absorbance could be observed [13]. For the calibration of this reaction, different concentrations of freshly prepared Mn(III) acetate in sodium malonate (50 mM) were used. As the result, 1 μM Mn(III) was found to oxidize 0.81 ± 0.03 μM ABTS ($\Delta OD_{420} = 0.0292 \pm 0.0011$; $n = 5$). This ratio was constant in the concentration range of 5–50 μM Mn(III) (ΔOD_{420} 0.145–1.45). All spectrophotometric assays were repeated and confirmed on numerous occasions. The graphic results presented are typical for the particular experiment.

2.4. HPLC analyses

Oxidation of malonate and formation of other organic acids was determined using an HPLC system (HP 1090 Liquid Chromatograph, Hewlett Packard) fitted with an UltraSep ES FS column (250×3 mm, Knauer, Groß-Umstadt, Germany) [4]. Phosphoric acid (10 mM) was used as the solvent at a flow rate of 0.55 ml/min. Routinely, 5 μl of the reaction mixture was injected into the HPLC system and chromatograms were recorded at 210 nm. Authentic standards of malonate (Fluka, Buchs, Switzerland), oxalate (Fluka), glyoxylate (Aldrich), glycolate (Aldrich) and formate (Fluka) were used for calibration and identification of oxidation products formed.

2.5. Spin trapping and ESR analysis

Formation of the nitrosoduren (ND, nitroso-2,3,5,6-tetramethylbenzene; synthesized according to Terabe et al. [16]) spin adduct was used to verify the formation of the carbon-centered acetic acid radical ($COOH\text{-}\dot{C}H_2$) in a phase-transfer reaction system [17]. The reaction mixture contained in a total of 1 ml 50 mM Na malonate (pH 4.5), 0.5 mM $MnCl_2$ and 2 μg MnP. Controls involved reaction in which MnP, malonate or $MnCl_2$ was removed from the complete reaction mixture. The reaction solution was covered with benzene (1 ml) containing ca. 4 μg ND. The biphasic reaction system was flushed with pure oxygen and incubated in gas-tight reaction tubes for 24 h under the conditions described above (see Section 2.2). All spectral recordings were made 5 min following the separation of water and benzene phase. A Bruker spectrometer model ESP 300-E (X-Band) operating at 9.7 GHz with a 100-kHz modulation frequency was used for all recordings.

3. Results

3.1. Oxidation of ABTS by MnP reaction mixtures in the absence of H_2O_2

Reaction mixtures containing only malonate, Mn(II) and MnP showed considerable ABTS oxidizing activity after 6 h of incubation (Fig. 1A). The extent of ABTS oxidation by the reaction solution was dependent on the initial MnP concentration and amounted to 22–256 μM ABTS corresponding to stationary Mn(III) concentrations from 27 to 316 μM. Linearity was observed between ABTS oxidation and MnP concentration in the range from 1 to 10 μg/ml MnP, and ABTS oxidation increased twice when enzyme concentration was increased from 2 to 10 μg/ml. During further incubation, another increase in the 'oxidative strength' of reaction mixtures was observed. Thus after 9 h of incubation, all reaction mixtures containing more than 1 μg/ml MnP oxidized about 515 μM ABTS which corresponds to 635 μM of Mn(III).

Fig. 1B shows the ABTS oxidation by reaction mixtures containing malonate (50 mM) and MnP (1 μg/ml) as well as different Mn(II) concentrations (0.1–5 mM). After 6 h of incubation, ABTS oxidation was the higher the higher the initial Mn(II) concentration. Linearity was observed in the Mn(II) concentration range from 0.5 to 5 mM. Further incubation resulted in an additional increase in ABTS oxidation by the reaction mixtures. Maximum ABTS oxidation (about 900 μM corresponding to 1110 μM Mn(III)) was detected in samples containing 2–5 mM Mn(II).

3.2. Oxidation of malonate

Since the reaction mixtures did not contain H_2O_2, it was investigated whether the oxidation of malonate was the basis for the action of MnP. Fig. 2 shows the time course of malonate disappearance in a reaction mixture containing malonate, Mn(II) and MnP. Within 24 h of incubation, about 60% of malonate (31 mM) was converted. Controls omitting MnP did not decompose any malonate and in the absence of Mn(II), less than 5% of malonate was converted. The decomposition of malonate started after a lag period of 3 h and reached its maximum rate (ca. 1.6 mM/h) after 9 h (Fig. 2). Simultaneously with the disappearance of malonate, the formation of oxalate started and Mn(III) was formed reaching its maximum concentration between 9 and 15 h (ca. 630–670 μM). Traces of oxalate could already be found after 6 h, afterwards substantial amounts of this acid were detectable until the end of the experiment (3–8 mM). A nearly equivalent conversion of malonate to oxalate was observed after 9 h, whereas during the further incubation, considerably more

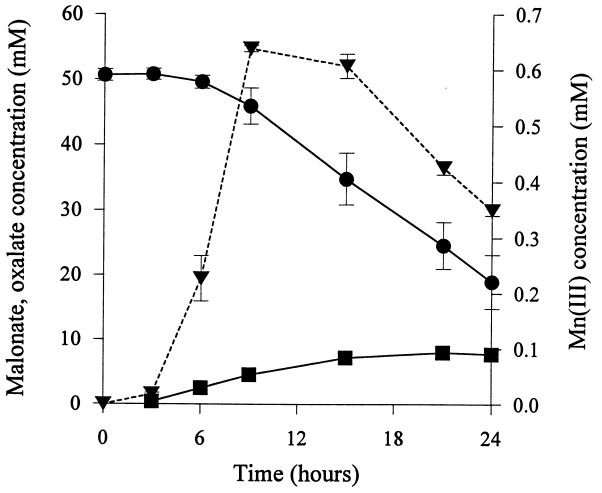

Fig. 2. Decomposition of malonate (●), formation of oxalate (■) and accumulation of Mn(III) (▽) in the absence of H_2O_2 in a reaction mixture containing malonate (50 mM), $MnCl_2$ (1 mM) and MnP (2 μg/ml). Mn(III) concentration was calculated from the oxidation of ABTS. Bars on symbols indicate standard deviation of means ($n = 3$); where absent, bars fall within symbols.

malonate was decomposed than oxalate could be detected, which indicated the preferential further decomposition of oxalate. To prove this assumption, the simultaneous decomposition of malonate (25 mM) and oxalate (25 mM) was followed during an additional experiment. After 4 h of incubation, already 34% of oxalate (8.5 mM) was decomposed while only 3% of malonate (0.75 mM) was converted.

Besides oxalate, glyoxylate and formate were also formed during the oxidation of malonate by MnP (Fig. 3). Maximum concentrations detected were 1.5 mM and 3 mM for glyoxylate and formate, respectively.

3.3. MnP activity in the absence of H_2O_2

The activity of MnP in the absence of H_2O_2 was very low during the first 40 min of incubation and confirmed the lag period found in the previous experiments (Fig. 4). A single addition of small amounts of Mn(III) shortened this lag period considerably indicating that Mn(III) is able to initiate the action of MnP and therefore its own formation. In the presence of Mn(III), the reaction started with a short decrease in absorbance at 270 nm over ca. 60 s demonstrating the consumption of Mn(III) during that initial stage (Fig. 4). Then a rapid increase in absorbance occurred due to the formation of Mn(III)-malonate complexes. The rate of this activity was dependent on the initially added Mn(III) concentration and was the higher the higher the Mn(III) concentration. This result suggests that Mn(III) reacts with malonate to form a product that somehow enables the action of MnP. Since MnP is a peroxidase, only H_2O_2 and/or a hydroperoxide derived from malonate could be this substance.

To prove this assumption, the Mn(III)-stimulated reaction was performed in the presence of ascorbate, SOD and catalase (Fig. 5). Ascorbate (20 μM), which is a scavenger for peroxyl radicals and therefore inhibits the formation of hydroperoxides [20], decreased the MnP activity considerably. After a short increase in absorbance, the reaction stopped and then even a slight decrease in absorbance was observed. Using a higher amount of ascorbate (50 μM) resulted in the complete inhibition of MnP activity. SOD (500 U/ml) and catalase (2000 U/ml) inhibited the oxidation of Mn(II) partially (by about 55% and 50%, respectively) indicating that the formation of Mn(III) is at least in part dependent on superoxide ($O_2^{\bullet-}$) and H_2O_2 formed during the reaction.

3.4. ESR analysis

Difficulties were encountered in experiments designed to trap radicals produced in aqueous reaction mixtures containing MnP, $MnCl_2$ and malonate with ND, because the latter is nearly insoluble in water. To overcome this problem, the reaction solution was covered with a benzenic solution of ND and the biphasic system was incubated under reaction conditions. No ESR signals were observed analyzing the water phase of the reaction system, however, in the benzene phase, a characteristic ESR spectrum was obtained (Fig. 6). The ESR

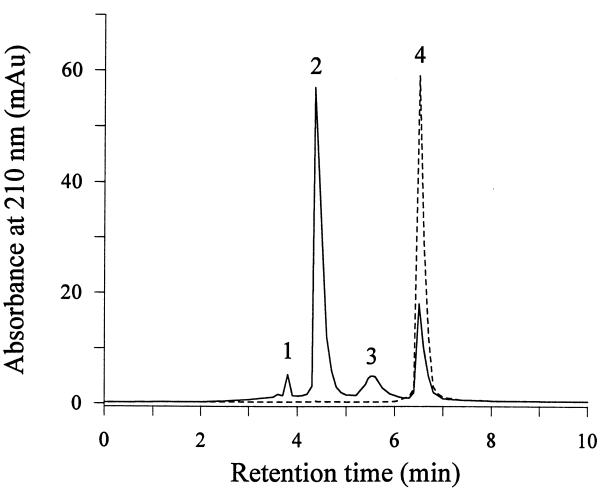

Fig. 3. Decomposition of malonate by MnP in the absence of H_2O_2. Reaction conditions were the same as described in Fig. 2. HPLC elution profiles (recorded after 24 h of incubation) of a control without MnP (dashed line) and a sample containing MnP, Mn(II) and malonate (bold line). 1, glyoxylate; 2, oxalate; 3, formate; 4, malonate.

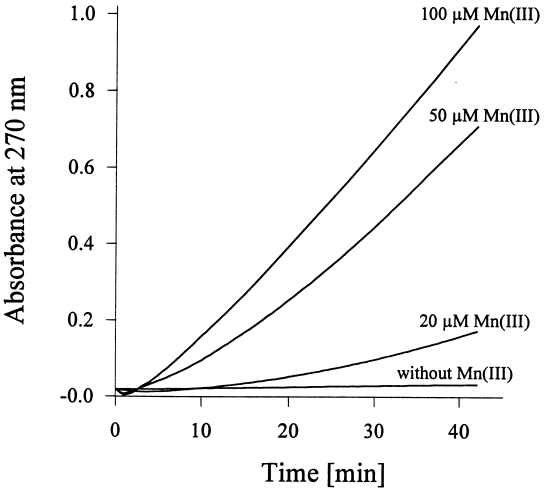

Fig. 4. Oxidation of Mn(II) to Mn(III) by MnP in the absence of H_2O_2. The assay mixture contained: 50 mM malonate (pH 4.5), 1 mM $MnCl_2$ and 2 μg/ml MnP. Mn(III) acetate (20–100 μM) was added to the assay solutions to prove its influence on MnP activity. The curves demonstrate that a single addition of Mn(III) increases the activity of MnP in the absence of H_2O_2 considerably.

spectrum of this ND adduct ($A_N = 13.8$ G; $A_{2H} = 8.3$ G) was only observed when the complete reaction system was used; omitting MnP, Mn(II) or malonate resulted in no detectable ESR signals in the benzene phase (Fig. 6). The characteristic pattern of the ESR spectrum revealed that a R-$CH_2^{\bullet}$ radical was trapped according to Rehorek [17]. It was concluded that Mn(III) cleaves malonate to CO_2 and the acetic acid radical (COOH-$CH_2^{\bullet}$).

4. Discussion

Manganese peroxidase from *P. radiata* and *N. frowardii* decomposed malonic acid in the presence of Mn(II), but in the absence of H_2O_2. The oxidation process resulted in the formation of oxalate as main intermediate product, moreover glyoxalate and formate were formed. The first product in the decomposition of malonate is probably a carbon-centered radical (COOH-$CH_2^{\bullet}$). Simultaneously with the decomposition of malonate high concentrations of Mn(III) were detectable at least over several hours. The main reactions possibly involved in the oxidation of malonate by MnP are formulated as follows:

$$\text{COOH-CH}_2\text{-COOH} + \text{Mn(III)} \rightarrow \text{COOH-CH}_2^{\bullet} + \text{CO}_2 + \text{H}^+ + \text{Mn(II)} \quad (1)$$

$$\text{COOH-CH}_2^{\bullet} + \text{O}_2 \rightarrow \text{COOH-CH}_2\text{OO}^{\bullet} \quad (2)$$

$$\text{COOH-CH}_2\text{OO}^{\bullet} + \text{Mn(II)} + \text{H}^+ \rightarrow \text{COOH-CH}_2\text{OOH} + \text{Mn(III)} \quad (3)$$

$$\text{COOH-CH}_2\text{OOH} + 2\text{Mn(II)} \xrightarrow{\text{MnP}} \text{COOH-CHO} + \text{H}_2\text{O} + 2\text{Mn(III)} \quad (4)$$

$$\text{COOH-CH}_2\text{OO}^{\bullet} + \text{O}_2 \rightarrow \text{COOH-COOH} + \text{O}_2^{\bullet -} + \text{H}^+ \quad (5)$$

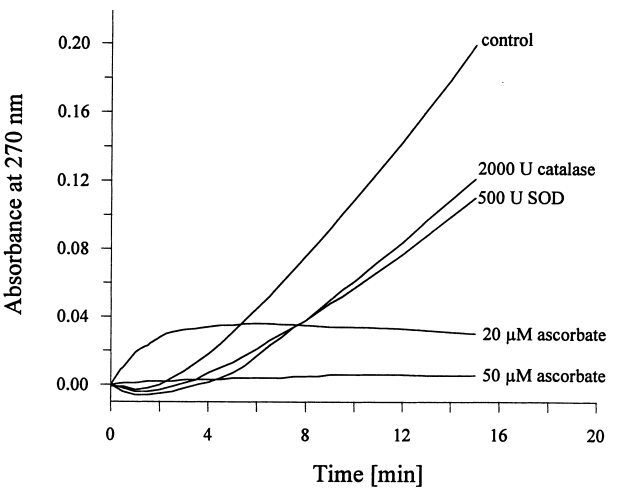

Fig. 5. Influence of catalase, superoxide dismutase (SOD) and ascorbate on the oxidation of Mn(II) by MnP in the absence of H_2O_2. The assay solution contained 50 mM malonate, 1 mM $MnCl_2$, 0.1 mM Mn(III) acetate and 2 μg/ml MnP (=control). Catalase, SOD or ascorbate were added before starting the reaction with MnP.

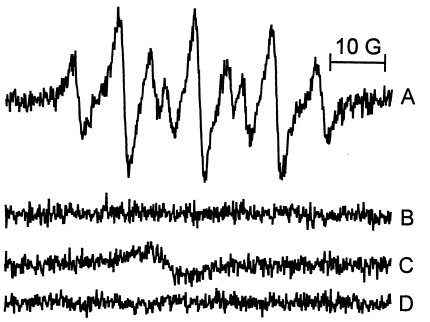

Fig. 6. ESR spectrum of of the ND-CH_2-COOH aduct (acetic acid radical; COOH-$CH_2^{\bullet}$) (A). The reaction mixture (1 ml) contained 50 mM Na malonate (pH 4.5), 1 mM $MnCl_2$ and 2 μg/ml MnP and was covered with 1 ml benzene containing 4 μg ND. Controls did not contain MnP (B), malonate (C) or Mn(II) (D). Recording using the benzene phase was taken 24 h after initiation of the reaction with MnP. Spectrometer settings were: modulation amplitude, 1 G; time constant, 82 ms; scan time 5 min; receiver gain, 8×10^5; and microwave power, 9.7 GHz.

$$\text{COOH-CHO} + \text{Mn(III)} + \tfrac{1}{2}\text{O}_2 \rightarrow \text{HCOOH} + \text{CO}_2^{\bullet -} + \text{Mn(II)} \quad (6)$$

$$\text{COOH-COOH} + \text{Mn(III)} \rightarrow \text{CO}_2 + \text{CO}_2^{\bullet -} + \text{Mn(II)} \quad (7)$$

$$\text{CO}_2^{\bullet -} + \text{O}_2 \rightarrow \text{CO}_2 + \text{O}_2^{\bullet -} \quad (8)$$

$$\text{O}_2^{\bullet -} + \text{Mn(II)} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{Mn(III)} \quad (9)$$

$$\text{H}_2\text{O}_2 + \text{Mn(II)} \xrightarrow{\text{MnP}} \text{H}_2\text{O} + 2\text{Mn(III)} \quad (10)$$

We postulate that a trace amount of Mn(III) formed by autoxidation of Mn(II) initiates the reaction cascade and can be amplified by the action of MnP. This assumption is strongly supported by our finding that a single addition of catalytic concentrations of Mn(III) facilitated the action of MnP in the absence of H_2O_2 considerably. Malonate is decarboxylated by Mn(III) to form the carbon-centered radical of acetic acid (Eq. 1). Mn(III)-catalyzed decarboxylation of organic acids to carbon-centered radicals has also been reported for oxalate [18] and oxaloacetate [19]. Carbon-centered radicals tend to react with dioxygen (O_2) to give the corresponding peroxyl radicals [20]. For the oxidation of malonate by Mn(III), we postulate the formation of the hydroperoxyl acetic acid radical (Eq. 2). Our finding that ascorbate, which is a scavenger of peroxyl radicals [20], inhibited Mn(III) generation and malonate decomposition supports this assumption. The formation of peroxyl radicals from carbon-centered radicals has been well studied in lipid peroxidation [21,22], and was also observed during the oxidation of indole-3-acetic acid by horseradish peroxidase (HRP) [23]. In the next step, the peroxyl radical is transformed to a hydroperoxide (hydroperoxy acetic acid) by an autocatalytic process involving Mn(II). A similar reaction has been reported for the Mn(II)-supported oxidation of malonaldehyde by HRP in the absence of H_2O_2 [24]. The hydroperoxide formed can be used by MnP as co-substrate. As the result, Mn(II) is oxidized to Mn(III) and glyoxylic acid arises (Eq. 4). The reaction of peroxidases using organic hydroperoxides as reductants has been demonstrated for HRP [20]. Catalase cannot decompose hydroperoxides

catalytically [20]. Therefore, a complete inhibition of MnP activity by catalase was not observed in our experiments.

In addition to the conversion to hydroperoxide, the peroxyl radical might also undergo spontaneous reactions to form oxalate and superoxide (Eq. 6). This reaction would be similar to that proposed for the formation of superoxide from the peroxyscatol radical [24] and the α-hydroxybenzyl radical [25]. Oxalate and glyoxylate formed are also substrates for the oxidation by Mn(III) generated by MnP (Eqs. 6 and 7). Their cleavage resulted in the formation of the formate radical as well as of carbon dioxide and formate, respectively. Decomposition of oxalate and glyoxalate by Mn(III) and the formation of the formate radical has already been demonstrated for MnP and lignin peroxidase [4,26]. Moreover, evidence was given that this oxidation process occurs also in the absence of H_2O_2 [12]. The formate radical is known to react with O_2 to produce superoxide plus CO_2 (Eq. 8). Superoxide oxidation of Mn(II) generates Mn(III) and H_2O_2 [27]. The decomposition of superoxide prevents this reaction with Mn(II) and therefore, Mn(III) formation – both spontaneous and MnP-catalyzed (using the H_2O_2 formed; Eq. 10) – is in part inhibited by SOD or catalase, as it was observed in our experiments.

Our results demonstrate that an efficient action of MnP is possible in the absence of H_2O_2, if sufficient amounts of malonate and Mn(II) ions are present. The decomposition of malonate resulting in the formation of radical species and peroxides that can be used by MnP is a partly autocatalytic process. The high reactivity of peroxyl radicals derived from organic acids is probably the basis of many oxidation phenomena observed for MnP (e.g. direct mineralization of aromatic compounds) [2–4,13]. Moreover, current investigation has given evidence that other organic acids – e.g. malate – are also efficiently oxidized by MnP in the absence of H_2O_2.

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