Effect of pH and Oxalate on Hydroquinone-Derived Hydroxyl Radical Formation during Brown Rot Wood Degradation

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The redox cycle of 2,5-dimethoxybenzoquinone (2,5-DMBQ) is proposed as a source of reducing equivalent for the regeneration of Fe$^{2+}$ and H$_2$O$_2$ in brown rot fungal decay of wood. Oxalate has also been proposed to be the physiological iron reductant. We characterized the effect of pH and oxalate on the 2,5-DMBQ-driven Fenton chemistry and on Fe$^{2+}$ reduction and oxidation. Hydroxyl radical formation was assessed by lipid peroxidation. We found that hydroquinone (2,5-DMHQ) is very stable in the absence of iron at pH 2 to 4, the pH of degraded wood. 2,5-DMHQ readily reduces Fe$^{2+}$ at a rate constant of $4.5 \times 10^7$ M$^{-1}$s$^{-1}$ at pH 4.0. Fe$^{2+}$ is also very stable at a low pH. H$_2$O$_2$ generation results from the autoxidation of the semiquinone radical and was observed only when 2,5-DMHQ was incubated with Fe$^{2+}$. Consistent with this conclusion, lipid peroxidation occurred only in incubation mixtures containing both 2,5-DMHQ and Fe$^{2+}$. Catalase and hydroxyl radical scavengers were effective inhibitors of lipid peroxidation, whereas superoxide dismutase caused no inhibition. At a low concentration of oxalate (50 μM), ferric ion reduction and lipid peroxidation are enhanced. Thus, the enhancement of both ferric ion reduction and lipid peroxidation may be due to oxalate increasing the solubility of the ferric ion. Increasing the oxalate concentration such that the oxalate/ferric ion ratio favored formation of the 2:1 and 3:1 complexes resulted in inhibition of iron reduction and lipid peroxidation. Our results confirm that hydroxyl radical formation occurs via the 2,5-DMBQ redox cycle.

A prerequisite to gaining access to the cellulose and hemicellulose components of woody biomass is the circumvention of the lignin barrier. Filamentous fungi, the predominant degraders of wood, have evolved at least two mechanisms to circumvent this barrier. White rot fungi circumvent the lignin barrier by degrading it with extracellular peroxidases (14, 47), with eventual degradation to the level of CO$_2$ (28). In contrast, brown rot fungi cannot degrade the lignin component to CO$_2$. However, these fungi can access the cellulose components with minimal modification of the lignin. These modifications include demethylation of aryl methoxy groups and ring hydroxylation (for a more extensive review, see reference 29).

Due to the limited size of the wood pores and the nonspecific nature of wood degradation, Cowling and Brown (12) suggested that low-molecular-weight oxidants are the initial agents in wood decay. Koenigs (33) showed that a number of wood-decomposing fungi produce H$_2$O$_2$ and noted the similarities between wood treated with the hydroxyl radical and with brown rot fungi (34). Illman et al. (23) subsequently detected the hydroxyl radical in incubations with the brown rot fungus *Poria placenta* by use of electron spin resonance and spin trapping agents. Further supporting the involvement of the hydroxyl radical is the formation of 3-hydroxy derivatives (the expected products from a hydroxyl radical attack) of phthalic hydrazide in incubations with brown rot fungi (5).

The most likely nonphotochemical source of the hydroxyl radical is Fenton’s reagent, defined by the following chemistry: Fe$^{2+}$ + H$_2$O$_2$ → Fe$^{3+}$ + *OH + OH*$. The key reagents are iron, molecular oxygen, and a reducing agent (35), two of which, iron and O$_2$, are readily available. Three different reducing agents for the iron have been suggested for brown rot fungi. One is an enzyme, cellobiose dehydrogenase (22), and the other two are chemicals, oxalate (42) and 2,5-dimethoxyhydroquinone (2,5-DHQ) (26, 38). Although Hyde and Wood (22) demonstrated that cellobiose dehydrogenase can reduce iron, this enzyme has not been found in all brown rot fungi. Schmidt et al. (42) proposed that oxalate serves as a chelator and as a reducing agent for iron-dependent hydroxyl radical formation. More recently, three groups have proposed that 2,5-dimethoxybenzoquinone (2,5-DMBQ) and its hydroquinone, discovered in 1955 by Bu’Lock (8) and again isolated in 1976 by Nakajima et al. (37), serve as the extracellular reducing agents (26, 38). Kerem et al. (26) demonstrated the involvement of this quinone, as well as 4,5-dimethoxy-1,2-benzoquinone, in the extracellular cleavage of polyethylene glycol. The quinone undergoes cyclic oxidation-reduction reactions, serving as a shuttle for electrons from intracellular donors to extracellular acceptors. Although a similar mechanism has been proposed for white rot fungi (4, 17, 18) for hydroxyl radical formation, product analysis suggests that hydroxyl radical oxidation is relatively minor in comparison to peroxidase oxidation (30, 32).

The role of oxalate, ubiquitously found in brown rot fungi, as a chelating agent, and the role of pH, which is altered by the fungus, are not clear. Our objective in this study was to use a lipid peroxidation system to characterize 2,5-DMBQ-dependent hydroxyl radical formation and to determine the effect of oxalate and pH on 2,5-DMBQ-dependent production of the hydroxyl radical. Our results indicate that 2,5-DMBQ plays a key role in hydroxyl radical formation, that oxalate acts as a
sequestering agent, and that pH plays a central role in these reactions.

MATERIALS AND METHODS

Chemicals. 2,5-DMHQ was purchased from TCI America (New York, N.Y.). Linolenic acid, oxalic acid, ferrozine, Lubrol (polyoxyethylene-9-lauryl ether), p-aminobenzoic acid, protocatechuic acid, deferoxamine mesylate, and superoxide dismutase from bovine erythrocytes were purchased from Sigma Chemical Company (St. Louis, Mo.). 2,2-Dimethyl succinic acid (DMS) was purchased from Aldrich Chemical Company (Milwaukee, Wis.). Catalase from Aspergillus niger was purchased from Calbiochem (La Jolla, Calif.).

2,5-DMHQ was chemically reduced by the procedure of Kerem et al. (26). The hydroquinone (2,5-DMHQ) crystals were stored desiccated under an argon atmosphere and were dissolved in argon-purged acetonitrile prior to dilution in solvents.

Organism. Liquid cultures of Gloeophyllum trabeum (Mad 617-R) were prepared with homogenized mycelia. The inoculum was prepared by initial growth in static 250-ml Fernbach flasks containing 50 ml of YM medium (46) at 30°C for 10 days. This mycelial preparation was collected by decanting the YM medium and washing the mat with 500 ml of distilled water, and then the preparation was decanted. The washed mycelium was added to 50 ml of the high-carbon, low-nitrogen liquid medium described by Kerem et al. (26) in a 2-liter Fernbach flask and grown statically at 30°C for 10 days. The mycelium collected from the Fernbach flasks was homogenized and used to inoculate 1 liter of the same medium. Static liquid cultures were grown in 125-ml Fernbach flasks at 30°C with 5 ml of medium containing 1% glucose, 4 mg of NH₄NO₃/liter, BIII trace elements (46), and 1.5 g of DMS (pH 4.5)/liter as described by Kerem et al. (26). Cultures were flushed with water-saturated O₂ on days 3, 6, and 9 of growth.

Oxygen consumption. Oxygen consumption was measured with a YSI model 5300 (Intech Laboratories, Plymouth Meeting, Pa.) oxygen electrode. The concentration of dissolved O₂ used in our calculation was assumed to be 230 µM. Reaction mixtures contained 100 µM FeSO₄ in different buffers with different pH values: 25 mM Tris-Cl for pH 7 to 9 and 25 mM DMS for pH 2.5 to 6. Incubations were performed at 28°C.

Lipid peroxidation. Oxidation of linolenic acid was used to assess hydroxyl radical formation. Malondialdehyde, an oxidation product of linolenic acid, was measured with thiobarbituric acid (7). An extinction coefficient of 1.56 × 10⁵ M⁻¹ cm⁻¹ at 535 nm was used (7). Incubation mixtures, unless otherwise stated, contained 0.25 mg of linolenic acid/ml, 50 µM 2,5-DMHQ, and 100 µM FeCl₃ in 25 mM DMS buffer (pH 4). Reaction rates were obtained by removing 0.5-ml aliquots which were then added to 1 ml of the thiobarbituric acid reagent (7) containing 25 µl of a solution of 2% butylated hydroxytoluene in ethanol. In incubation mixtures where the effect of hydroxyl radical scavengers was determined, 0.2% (wt/vol) Lubrol was added. In experiments where catalase and superoxide dismutase were added, the DMS-buffered incubation mixtures were adjusted to pH 5 with 10 M sodium hydroxide, the concentration of FeCl₃ was reduced to 50 µM, and Lubrol was at 0.06%. These concentrations were utilized to minimize the inhibiting effect of the detergent on the enzymes.

Reduction of iron. Experiments where Fe³⁺ was reduced by 2,5-DMHQ were performed in 25 mM DMS, pH 4.0, and monitored by the formation of the ferrozine-Fe²⁺ complex. At various times, aliquots were removed and 1/10 vol. of 10 mM ferrozone was added (10). An extinction coefficient of 27.9 M⁻¹ cm⁻¹ at 562 nm was used.

Rapid kinetic measurements of iron reduction. Reduction of Fe³⁺ was also measured by rapid kinetic techniques. The stopped-flow apparatus used was purchased from KinTek Instruments (State College, Pa.) and contained a 2.6-cm light path. The rates were determined by averaging kinetic traces from three shots. The reduction of Fe³⁺ was determined by direct reduction of the ferrozine-Fe²⁺ complex. Typical experiments contained 3 mM ferrozone-15 µM FeCl₃ in one syringe and various concentrations of 2,5-DMHQ in the second syringe. Ferrous ion formation was monitored at 562 nm (10). All reactions were performed at 28°C, and the mixtures were buffered with 25 mM DMS, pH 4.0.

Effect of oxalate on the hydroxylation of p-hydroxybenzoic acid. Static liquid cultures (5 ml in 125-ml Fernbach flasks) were grown for 9 days. Gently, 0.5 ml of 10 M p-hydroxybenzoate (in water) was added to the cultures, yielding a final concentration of 0.91 mM. In some of these incubation mixtures, oxalate was also included in the liquid media at final concentrations of 100, 200, 400, and 800 µM. After an 8-h incubation, the extracellular medium was separated from the mycelial biomass by filtration and then analyzed for protocatechuic acid by high-performance liquid chromatography (HPLC) with a Supelcosil LC-18 column (Supelco, Bellefonte, Pa.). Protocatechuic acid was monitored at 254 nm.

The product was identified and quantitated based on a comparison of the retention time to known standards. The column chromatography was operated at 1 ml/min and eluted with a linear gradient of 0 to 30% methanol in 10 mM phosphoric acid.

Calculation of concentration of iron complexes. The relative contribution of each iron form to the total iron concentration is given by equation 1, where [Fe], [Fe(C)], ..., [Fe(C)] are the concentrations of free iron ions, a 1:1 iron-chelator complex, and a 1:1 iron-chelator complex, respectively.

Total Fe = [Fe] + [Fe(C)] + [Fe(C)] + ... + [Fe(C)]

This relationship can be rewritten with binding constants, as in equation 2, where Kᵣ, Kᵣ, ..., Kᵣ are the binding constants for the first, second, and nth molecule of chelator to the iron ion.

Total Fe = [Fe] + Kᵣ[Fe][C] + Kᵣ[Fe][C] + ... + Kᵣ[Fe][C]

The fractions of iron ions as free ions and as the iron complex are calculated with equations 3 to 6, where αFe, αFe(C), and αFe(C) designate the fractions of iron ions in the form of free iron ions, a 1:1 iron-chelator complex, and a 1:1 iron-chelator complex, respectively.

αFe = 1/(1 + Kᵣ[Fe][C] + Kᵣ[Fe][C] + ... + Kᵣ[Fe][C])

αFe(C) = Kᵣ[Fe][C](1 + Kᵣ[Fe][C] + Kᵣ[Fe][C] + ... + Kᵣ[Fe][C])

αFe(C) = Kᵣ[Fe][C][1 + Kᵣ[Fe][C] + Kᵣ[Fe][C] + ... + Kᵣ[Fe][C]]

αFe(C) = Kᵣ[Fe][C][1 + Kᵣ[Fe][C] + Kᵣ[Fe][C] + ... + Kᵣ[Fe][C]]

Binding constants Kᵣ of 2.5 × 10⁶ M⁻¹, Kᵣ of 6.3 × 10⁶ M⁻¹, and Kᵣ of 1 × 10⁸ M⁻¹ were used for the Fe³⁺ complexes with oxalate (36, 44). A Kᵣ of 5.01 × 10⁴ M⁻¹, a Kᵣ of 7.07 × 10⁵ M⁻¹, and a Kᵣ of 1 × 10⁴ M⁻¹ were used for the Fe²⁺ complexes with oxalate (36, 44).

RESULTS

Reduction of iron by 2,5-DMHQ. Fenton’s reagent is composed of the ferrous ion and H₂O₂. Both can be formed from Fe³⁺ and O₂ in the presence of a reducing agent. 2,5-DMHQ has been shown to be the reducing agent formed by brown rot fungi (27, 38). To determine the rate constant for the reduction of iron, we monitored the formation of the ferrous ion-ferrozine complex. However, the rate is too high to monitor by conventional techniques. Thus, we monitored iron reduction in a stopped-flow apparatus with one syringe containing various concentrations of 2,5-DMHQ and another syringe containing the ferric iron-ferrozine complex. Reduction of the ferric complex to the ferrous complex results in an increase in absorbance at 562 nm. The rate of reduction is dependent on the concentration of 2,5-DMHQ (Fig. 1). The second-order rate constant is 4.5 × 10⁷ M⁻¹ s⁻¹.

Autodissociation of the ferrous ion and 2,5-DMHQ. Solutions of ferrous ions alone can form H₂O₂ (6). This autodissociation is a second-order process with respect to the ferrous ion concentration (19) and can be measured by the rate of oxygen consumption. At a low pH, this reaction is slow as the ferrous ion does not readily autodissociate (Fig. 2). Thus, at the acidic pH values observed for fungal growth, the ferrous ion is relatively stable and very little of the reduced oxygen species is formed by this mechanism.

At low pH, ferric ions are readily reduced by 2,5-DMHQ (Fig. 1), but molecular oxygen is not (Fig. 2). In the absence of iron, the rate of 2,5-DMHQ autodissociation increases gradually from pH 3 to 6. In the presence of iron, the rate of 2,5-DMHQ-dependent oxygen consumption is much higher. The rate of oxygen consumption increased linearly with the increases in 2,5-DMHQ concentration and ferric iron concentration (Fig.
3). These results indicate that the predominant route for \( \text{O}_2 \) reduction by 2,5-DMHQ is through an iron-dependent mechanism rather than a direct reaction of 2,5-DMHQ with \( \text{O}_2 \). The formation of \( \text{H}_2\text{O}_2 \) during this autoxidation process was demonstrated by an increase in \( \text{O}_2 \) concentration following the addition of catalase (data not shown).

### 2,5-DMHQ-dependent hydroxyl radical formation

A lipid peroxidation system containing linoleic acid, which forms malondialdehyde and is readily detected using thiobarbituric acid (7), was used to assess hydroxyl radical formation. Malondialdehyde was formed readily in 2,5-DMHQ-containing reaction mixtures (Table 1). In the absence of 2,5-DMHQ or iron, little or no peroxidation was detected. The dependence on iron is consistent with the increased rates of oxygen consumption resulting from the addition of iron. The rate of lipid peroxidation increased with increased pH up to pH 4 (Fig. 4). Above pH 4, the rate of peroxidation decreased, possibly due to the higher rate of ferrous autoxidation.

#### Inhibition of 2,5-DMHQ-dependent lipid peroxidation

The addition of the hydroxyl radical scavengers mannitol and ethanol did not inhibit lipid peroxidation (Table 1). However, when the micelles were dispersed with the detergent Lubrol, mannitol and ethanol could inhibit this one-phase system. This result is consistent with previous observations of hydroxyl radical-dependent oxidation of phospholipid liposomes in which hydroxyl radical scavengers inhibited the reaction only when detergent was added (48). The addition of EDTA or deferoxamine inhibited malondialdehyde formation (Table 1), but the addition of superoxide dismutase did not (Table 1). The addition of catalase resulted in significant, but not complete, inhibition (Table 1). Neither boiled catalase nor boiled superoxide dismutase had a significant effect.

#### Effect of oxalate on lipid peroxidation and reactions with iron

At concentrations up to 50 \( \mu \text{M} \), oxalate stimulates lipid peroxidation (Fig. 5), but further increases in oxalate concentration decreased the rate of peroxidation. The inhibition of 2,5-DMHQ-dependent lipid peroxidation was concentration dependent (Fig. 5). The addition of catalase resulted in significant, but not complete, inhibition (Table 1). Neither boiled catalase nor boiled superoxide dismutase had a significant effect.

### Table 1. DMHQ-dependent lipid peroxidation

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Malondialdehyde produced (nmol/min/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete reaction mixture</td>
<td>1.20 ± 0.02</td>
</tr>
<tr>
<td>Minus 2,5-DMHQ</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Minus ( \text{Fe}^{3+} )</td>
<td>0.01 ± 0.00</td>
</tr>
<tr>
<td>Complete reaction mixture plus hydroxyl radical scavenger</td>
<td></td>
</tr>
<tr>
<td>Mannitol (100 ( \mu \text{M} ))</td>
<td>1.11 ± 0.10</td>
</tr>
<tr>
<td>Ethanol (100 ( \mu \text{M} ))</td>
<td>1.50 ± 0.40</td>
</tr>
<tr>
<td>Lubrol (0.2%, wt/vol)</td>
<td>1.02 ± 0.20</td>
</tr>
<tr>
<td>Lubrol (0.2%, wt/vol), mannitol (100 ( \mu \text{M} ))</td>
<td>0.46 ± 0.05</td>
</tr>
<tr>
<td>Lubrol (0.2%, wt/vol), ethanol (100 ( \mu \text{M} ))</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td>Complete reaction mixture plus chelator or enzyme</td>
<td></td>
</tr>
<tr>
<td>Deferoxamine mesylate (0.2 mM)</td>
<td>0.16 ± 0.05</td>
</tr>
<tr>
<td>EDTA (1 mM)</td>
<td>0.13 ± 0.05</td>
</tr>
<tr>
<td>Catalase (1 ( \text{U} ))</td>
<td>0.91 ± 0.08</td>
</tr>
<tr>
<td>Boiled catalase</td>
<td>1.32 ± 0.08</td>
</tr>
<tr>
<td>Superoxide dismutase (1 ( \text{U} ))</td>
<td>1.26 ± 0.08</td>
</tr>
<tr>
<td>Boiled superoxide dismutase</td>
<td>1.34 ± 0.04</td>
</tr>
</tbody>
</table>

*Results are the means and standard deviations of results from three incubations.
tration decrease the rate of peroxidation. Due to the possible photochemical reactions of oxalate and iron (15, 20, 22), experiments were also performed in the absence of light, with identical results (data not shown).

Under anaerobic conditions and at low concentrations, oxalate stimulates iron reduction by 2,5-DMHQ, but at concentrations above 50 \( \mu M \), the reduction of iron gradually decreases (Fig. 6). Thus, at a high oxalate concentration, the oxalate appears to sequester the iron and to prevent its reaction with 2,5-DMHQ. Again, these experiments were repeated in the dark, with identical results (data not shown).

**Hydroxylation of \( p \)-hydroxybenzoic acid and effect of oxalate.** The effect of oxalate on hydroxyl radical formation in cultures was measured by hydroxylation of \( p \)-hydroxybenzoic acid to form protocatechuic acid (41). Increasing the concentration of oxalate added to high-carbon, low-nitrogen cultures resulted in decreased protocatechuic acid formation (Fig. 7).

**DISCUSSION**

The production of extracellular hydroxyl radicals enables brown rot fungi to oxidize a large number of seemingly unrelated chemicals, such as dimethyl sulfoxide (21), phthalic hydrazide (5), lignin (25), and cellulose (11, 31). The substrate of the hydroxyl radical is hypothesized to be cellulose and hemicellulose. Cleavage of these polymers into small, diffusible fragments allows the fungus to circumvent the lignin barrier and the crystalline structure of cellulose, which are formidable problems for large, bulky enzymes. The formation of a non-photochemically generated hydroxyl radical requires a metal (typically ferric ions), molecular oxygen, and a reducing agent. In biological systems (wood), free iron and molecular oxygen are readily available. Thus, for brown rot fungi, secretion of a reducing agent can result in extracellular hydroxyl radical formation. The delivery of extracellular electrons by 2,5-DMBOQ and 2,5-DMHQ and the subsequent reactions with the ferric ion and molecular oxygen may be summarized as follows (26, 38):

\[
\begin{align*}
2,5-\text{DMBQ} + 2e^- + 2H^+ &\rightarrow 2,5-\text{DMHQ} \\
2,5-\text{DMHQ} + \text{Fe}^{3+} &\rightarrow 2,5-\text{DMHQ}^- + \text{Fe}^{2+} + \text{H}^+ \\
2,5-\text{DMHQ}^- + \text{O}_2 &\rightarrow 2,5-\text{DMBOQ} + \text{O}_2^- \\
2\text{O}_2^- + 2\text{H}^+ &\rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 &\rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH} 
\end{align*}
\]

In reaction 7, 2,5-DMBOQ is reduced by a mycelial reductase to...
yield the hydroquinone 2,5-DHQ (24). At a low pH, secreted 2,5-DHQ is stable for autoxidation but is a good reductant for the ferric ion. Reduction by one electron yields the ferrous ion and the semiquinone radical (reaction 8). The semiquinone radical is further oxidized to the quinone by molecular oxygen to yield superoxide (reaction 9). The predominant source of H$_2$O$_2$ is probably the dismutation of superoxide rather than the autoxidation of the ferrous ion (reaction 10). At a low pH, the oxidation of Fe$^{3+}$ is relatively slow. Thus, the components of Fenton's reagent (reaction 11) are formed with the ferric ion, 2,5-DHQ, and molecular oxygen. In the present study, we (i) determined the rate constant for reaction 8, (ii) determined the effect of pH on the sequence of reactions 8 to 11, and (iii) determined the effect of oxalate on reactions 8 to 11. Reactions 7 to 11 are also consistent with our data on inhibition with superoxide dismutase, catalase, and hydroxyl radical scavengers. Although we obtained only 24% inhibition with 1 U of catalase/ml, this level of inhibition is comparable to or better than that previously observed. For example, in a study by Chen and Schopfer (9), 26 U of catalase/ml caused only 3% inhibition of hydroxyl radical-dependent oxidation of RNA.

The pH is important for 2,5-DMBQ-driven Fenton chemistry since protons (or hydroxide ions) are reactants or products in all five reactions. The pH also affects the chelation (and, thus, the reactivity) of iron by organic acids such as oxalate (22). Fungi often lower the pH of the extracellular medium through secretion of organic acids and thus establish a pH gradient around the mycelium (22). By lowering the pH of the medium, 2,5-DHQ and ferrous ions (Fig. 2) are effectively stabilized and do not autoxidize. Yet, in this pH range, 2,5-DHQ can reduce ferric ions, and the 2,5-DHQ semiquinone can reduce molecular oxygen to form superoxide. As the pH increases toward neutrality, both 2,5-DHQ and ferrous ions are destabilized and more readily autoxidize. These properties could explain the pH profile observed for 2,5-DHQ- dependent lipid peroxidation (Fig. 4), where the highest rates occur at pH 4. As pH increases up to pH 4, the rate of iron-dependent oxidation of 2,5-DHQ increases (Fig. 2), resulting in increased Fe$^{2+}$ and H$_2$O$_2$ concentrations and increasing the rate of hydroxyl radical formation. Above pH 4, the enhanced rate of 2,5-DHQ autoxidation decreases the steady-state level of Fe$^{2+}$, thereby reducing the rate of hydroxyl radical formation.

The pH also impacts the speciation of organic acids with ferric ions. The organic acid oxalate is produced by most, if not all, wood-degrading fungi (43). Hyde and Wood (22) calculated that in a solution of 10 mM oxalate, increasing the pH from 1.5 to 3.5 changes a 50:50 mixture of 2:1 and 3:1 oxalate-Fe$^{3+}$ complexes to 100% of the 3:1 complex. Complexation of Fe$^{3+}$ with oxalate is also affected by changes in oxalate concentration. A concentration range of 100 (2) to 500 (27) μM oxalate has been reported for G. trabeum. Within this range, the dominant ferric ion species is a mixture of the 2:1 and 3:1 complexes (Fig. 8). Whether Fe$^{3+}$ is complexed by two oxalates or three oxalates greatly affects the reactivity of the iron. For example, the reduction potential is 771 mV for free Fe$^{3+}$, 468 mV for the 1:1 complex, 181 mV for the 2:1 complex, and -120 mV for the 3:1 complex (22). Thus, changes in reduction potential caused by speciation changes may preclude certain reductants from reducing the Fe$^{3+}$ (22).

To study the effect of oxalate on 2,5-DMBQ-dependent reactions, we maintained the pH at 4, the approximate pH of fungal cultures, and varied the oxalate concentration within the physiologically reported range. At physiological concentrations, oxalate inhibits iron reduction, hydroxyl radical formation (lipid peroxidation), and hydroxylation of p-hydroxybenzoic acid. Our inhibition studies of iron reduction and lipid peroxidation were performed with 100 μM Fe$^{3+}$. This Fe$^{3+}$ level is much higher than that found in wood; however, this concentration facilitated our in vitro studies. When concentrations of oxalate are less than 100 μM, Fe$^{3+}$ reduction and lipid peroxidation are both enhanced and a mixture of 2:1 and 3:1 complexes is expected (Fig. 8). However, in our experiments, the 1:1 oxalate-iron complex probably dominates because of the high iron concentration used. Thus, enhanced lipid peroxidation and ferric ion reduction may result from oxalate increasing the solubility of ferric ions. Inhibition of 2,5-DHQ-dependent reactions occurs at oxalate concentrations above 100 μM (where the 2:1 or 3:1 complexes are favored under our experimentally high iron concentrations). The reduction potential for 2,5-DMBQ is -590 mV (3), so this inhibition cannot be explained by unfavorable reduction potentials. We cannot explain the inhibition of Fe$^{3+}$ reduction by oxalate. Oxalate may prevent the formation of the ferrozine-Fe$^{3+}$ complex, thereby preventing its detection, but the addition of increasing concentrations of oxalate to ferrozine-Fe$^{3+}$ did not decrease the absorbance (data not shown).

The inhibition of lipid peroxidation by oxalate may also result from the inhibition of iron reduction or the radical scavenging activity of oxalate. The one-electron oxidation of oxalate yields the formate radical. This radical reduces molecular oxygen to form superoxide at a rate limited by diffusion (1) (Fig. 9).

This concentration dependence of Fenton chemistry on oxalate was also reported by Tanaka et al. (45). They studied the change in viscosity of cellulose following oxidation by Fenton’s reagent. A similar effect was also found by Schmidt et al. (42) and Shimada et al. (43). The addition of increasing concentrations of oxalate to fungal cultures also inhibited hydroxyl radical-dependent hydroxylation of p-hydroxybenzoic acid.

In conclusion, our results indicate that at low concentra-
tions, oxalate facilitates hydroxyl radical formation, but at higher concentrations, oxalate inhibits hydroxyl radical formation by brown rot fungi. The ability of oxalate to inhibit hydroxyl radical formation can be attributed to the sequestration of iron by oxalate, which is greatly affected by both the pH and the oxalate concentration (22). Because our experiments were also performed in the dark with similar results, none of the effects observed are due to photochemistry (15, 20). Due to the sensitivity of oxalate-dependent reactions to oxalate concentration and to pH, and in light of the physiological variation in pH (from pH 2 to 6.7) (2, 13) and in oxalate concentration (2, 27), it is not possible to determine an exact role for oxalate in hydroxyl radical formation. Although oxalate is reported as a reductant of Fe$^{3+}$ for brown rot fungi (42), this role has since been questioned due to the photochemical dependence of this process (15, 20). The ability of oxalate to sequester ferric ions may protect brown rot fungi from hydroxyl radicals. Further support for this hypothesis is that white rot fungi, which do not utilize the hydroxyl radical as the major oxidant (30, 32), also produce oxalate (39, 40, 42, 49). Despite reports on how the hydroxyl radical may be formed in white rot fungi (4, 17, 18), it is unlikely that oxalate would have a role in its formation. The chemical signatures of wood affected by white rot fungi and brown rot fungi are different (30). If oxalate has a common role in both fungi, then the hypothesis of Green et al. (16), that oxalate’s role in wood decay is to chelate calcium, resulting in a weakening of the wood structure and increasing the pore size, should be more critically evaluated.

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REFERENCES


FIG. 9. Oxalate yields the formate radical which reduces molecular oxygen to form superoxide.