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Oxygen Activation during Oxidation of Methoxyhydroquinones by Laccase from *Pleurotus eryngii*

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Oxygen activation during oxidation of the lignin-derived hydroquinones 2-methoxy-1,4-benzohydroquinone ($MBQH_2$) and 2,6-dimethoxy-1,4-benzohydroquinone ($DBQH_2$) by laccase from *Pleurotus eryngii* was examined. Laccase oxidized $DBQH_2$ more efficiently than it oxidized $MBQH_2$; both the affinity and maximal velocity of oxidation were higher for $DBQH_2$ than for $MBQH_2$. Autoxidation of the semiquinones produced by laccase led to the activation of oxygen, producing superoxide anion radicals ($Q^{\cdot-} + O_2 \leftrightarrow Q + O_2^{\cdot-}$). As this reaction is reversible, its existence was first noted in studies of the effect of systems consuming and producing $O_2^{\cdot-}$ on quinone formation rates. Then, the production of H_2O_2 in laccase reactions, as a consequence of $O_2^{\cdot-}$ dismutation, confirmed that semiquinones autoxidized. The highest H_2O_2 levels were obtained with $DBQH_2$, indicating that $DBQ^{\cdot-}$ autoxidized to a greater extent than did $MBQ^{\cdot-}$. Besides undergoing autoxidation, semiquinones were found to be transformed into quinones via dismutation and laccase oxidation. Two ways of favoring semiquinone autoxidation over dismutation and laccase oxidation were increasing the rate of $O_2^{\cdot-}$ consumption with superoxide dismutase (SOD) and recycling of quinones with diaphorase (a reductase catalyzing the divalent reduction of quinones). These two strategies made the laccase reaction conditions more natural, since $O_2^{\cdot-}$, besides undergoing dismutation, reacts with Mn^{2+}, Fe^{3+}, and aromatic radicals. In addition, quinones are continuously reduced by the mycelium of white-rot fungi. The presence of SOD in laccase reactions increased the extent of autoxidation of 100 μM concentrations of $MBQ^{\cdot-}$ and $DBQ^{\cdot-}$ from 4.5 to 30.6% and from 19.6 to 40.0%, respectively. With diaphorase, the extent of $MBQ^{\cdot-}$ autoxidation rose to 13.8% and that of $DBQ^{\cdot-}$ increased to 39.9%.

The production of extracellular laccase is a common feature of white-rot basidiomycetes (35, 47). These fungi are the only organisms with a demonstrated capacity to both depolymerize and mineralize lignin by an oxidative and nonspecific mechanism (30). Besides laccase, the ligninolytic system of these fungi includes several peroxidases (9, 16, 36, 48), known as lignin peroxidase and manganese peroxidase (MnP), and oxidases that produce the hydrogen peroxide (H_2O_2) needed for peroxidase activities (18, 29). Another enzyme produced by these fungi, which functions in the degradation of not only lignin but also cellulose, is cellobiose dehydrogenase (11). Laccase catalyzes the one-electron oxidation of a wide range of phenolic compounds and aromatic amines (47). For many years, the participation of laccase in lignin degradation was thought to be limited to the oxidation of phenolic lignin units, which comprise only 10 to 20% of the polymer. However, during the present decade, it has been demonstrated that laccase can also oxidize the nonphenolic lignin units in the presence of certain compounds, known as mediators, that include artificial substrates (5, 8) and fungal metabolites (10). Besides their role in extending the kind and number of lignin units that can be oxidized by the action of laccase, natural mediators are important because ligninolytic enzymes have to act indirectly during the early phases of plant cell wall degradation due to size exclusion limitations (13, 14). Other small molecular agents participating in lignin degradation and produced directly or indirectly by ligninolytic enzymes include manganic ion (Mn^{3+}) (24, 27, 50), the cationic radical of the fungal metabolite veratryl (3,4-dimethoxybenzyl) alcohol (26), and activated oxygen species such as the hydroxyl radical ($HO^{\cdot}$) and superoxide anion radical ($O_2^{\cdot-}$) (2, 15, 27). Except for $O_2^{\cdot-}$, all of these compounds are able to oxidize lignin units. However, the $O_2^{\cdot-}$ produced by white-rot fungi (12) can participate in the production of H_2O_2 via both dismutation ($2\,O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2$) and Mn^{2+} oxidation with concomitant production of Mn^{3+} ($O_2^{\cdot-} + Mn^{2+} + 2H^+ \rightarrow H_2O_2 + Mn^{3+}$) (1). It can also be involved in $HO^{\cdot}$ production through the iron-catalyzed Haber-Weiss reaction ($O_2^{\cdot-} + H_2O_2 \rightarrow HO^{\cdot} + HO^- + O_2$) (4). Furthermore, by reacting with phenoxyl radicals produced from lignin model compounds, it can result in oxidative degradation being favored over coupling reactions (15).

Most enzymatic reactions demonstrating $O_2^{\cdot-}$ generation have been carried out with enzymes other than laccase (25, 31, 32, 37, 41). During a comparative study of substrate specificity of the two laccase isoenzymes produced by *Pleurotus eryngii*, we detected, for the first time, $O_2^{\cdot-}$ production in reactions involving benzohydroquinones (38). Although laccase catalyzes the four-electron reduction of O_2 to H_2O, the semiquinones produced in the one-electron oxidation of hydroquinones are able to autoxidize to a certain extent, reducing O_2 to $O_2^{\cdot-}$. For this O_2 activation mechanism to be effective, it is essential that hydroquinones are available during lignin degradation and that semiquinones are converted into quinones mainly via autoxidation. As previously rationalized by Schoemaker et al. (44, 45), white-rot fungi can convert all aromatic rings in the lignin polymer to either ring-opened products or quinones-hydroquinones by a combination of oxidative reactions, involving ligninolytic enzymes and active oxygen species, and reductive reactions, carried out by cell-bound systems. This way, lignin mineralization can be accomplished by as-yet-uncharacterized

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intracellular processes. Another source of quinones is the large amount of white-rot fungi-produced methoxylated and hydroxylated aromatic metabolites (22, 46), which are substrates of the ligninolytic enzymes (23, 34). Quinones are usually reduced to hydroquinones when they are in contact with white-rot fungi mycelium (7, 45). Although it has been postulated that this reaction could lead to the rapid intracellular degradation of quinones (33, 44), a redox cycling process, involving the oxidation of hydroquinones by laccase, was established during the incubation of *P. eryngii* with several quinones (21). Therefore, it is quite likely that hydroquinones will be present and available for oxidation by laccase and ligninolytic peroxidases under natural conditions of lignin degradation by white-rot fungi. On the other hand, there are many factors controlling the extent of semiquinone autoxidation, including the reduction potential of quinones, which is affected by the nature, number, and position of the substituents (6). Thus, during the oxidation of the hydroquinones produced by *P. eryngii* from 1,4-benzoquinone, 2-methyl-1,4-benzoquinone, and duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) by laccase, the level of O_2 activation increased as the number of methyl substituents increased (21). Among these quinones, only 1,4-benzoquinone is a breakdown product of lignin (it is derived from *p*-hydroxyphenyl lignin units and *p*-coumarate residues, which are specially abundant in grasses). However, less than 1% of the 1,4-benzosemiquinone produced by *P. eryngii* laccase isoenzyme I autoxidized (38). For this reason, we planned a new study with hydroquinones derived from guaiacyl and syringyl units of lignin (2-methoxy- and 2,6-dimethoxydroquinone, respectively). The study focused on the extracellular portion of the quinone redox cycling process (activation of oxygen during methoxyhydroquinone oxidation by laccase). Special attention was paid to the possibility of other reactions (besides autoxidation) in which semiquinones are consumed and to factors affecting the extent of the autoxidation reaction.

MATERIALS AND METHODS

Chemicals. H_2O_2 (Perhydrol; 30%) was obtained from Merck. Xanthine (Xn), NAD(P)H, and the chelating resin Chelex 100 were purchased from Sigma. 1,4-Benzohydroquinone (BQH_2), 2,6-dimethoxy-1,4-benzoquinone (DBQ), and 2-methoxy-1,4-benzohydroquinone ($MBQH_2$) were from Aldrich. 2,6-Dimethoxy-1,4-benzohydroquinone ($DBQH_2$) was prepared from DBQ by reduction with sodium borohydride (3), and 2-methoxy-1,4-benzoquinone (MBQ) was produced from $MBQH_2$ by oxidation with silver oxide (23). All other chemicals used were of analytical grade.

In an attempt to reduce the amounts of trace transition metals present in most laboratory chemicals and reagents, the phosphate buffer and the solutions of the compounds used as substrates in enzymatic reactions were treated with the chelating ion-exchange resin Chelex 100, in accordance with the manufacturer's instructions.

Enzymes. Laccase (EC 1.10.3.2) from *P. eryngii* (isoenzyme I) was produced in glucose-ammonium medium and purified by using Sephadex G100 and Mono Q columns as previously described (38). Bovine liver superoxide dismutase (SOD; EC 1.15.1.1) and catalase (EC 1.11.1.6), buttermilk xanthine oxidase (XnO; EC 1.1.3.22), porcine heart diaphorase (EC 1.8.1.4), and rabbit liver cytochrome P450 reductase (EC 1.6.2.4) were obtained from Sigma.

Enzymatic assays. Laccase activity was determined by spectrophotometrically monitoring product formation, using as substrates 500 μM $MBQH_2$ (ε_{360} = 1,252 M^{-1} cm^{-1}) and $DBQH_2$ (ε_{397} = 562 M^{-1} cm^{-1}). Diaphorase and cytochrome P450 reductase were assayed by measuring the oxidation of 200 μM NADH and NADPH, respectively, in the presence of 500 μM MBQ. The extinction coefficient for nucleotides at 340 nm was corrected from 6,220 to 7,120 M^{-1} cm^{-1} because MBQ absorbs light of the same wavelength (ε_{340} = 900 M^{-1} cm^{-1}). All of the above-described reactions were done in 20 mM phosphate buffer, pH 5.0, at room temperature. International units (micromoles per minute) of enzymatic activity were used.

Analytical techniques. H_2O_2 levels were estimated by measuring the production of O_2 with a Clark-type electrode after addition of 100 U of catalase/ml (heat-denatured catalase was used in blanks). The amount of H_2O_2 was calculated taking into consideration the stoichiometry of the catalase reaction (2 H_2O_2:1 O_2). The oxygen electrode was calibrated by the same procedure with known amounts of H_2O_2 from the commercial solution, which were estimated spectrophotometrically (ε_{230} = 81 M^{-1} cm^{-1}).

Quantitative determinations of $MBQH_2$ and $DBQH_2$ were performed by high-performance liquid chromatography (HPLC), using standard calibration curves for each compound. To stabilize the concentration of hydroquinones until HPLC analysis, the pH was lowered to 2 at the end of the reactions. Samples (20 μl) were injected into a Pharmacia HPLC system equipped with a Spherisorb S50DS2 column (Hichrom). The analyses were done at 30°C at a flow rate of 1 ml min^{-1} with methanol–10 mM phosphoric acid (20:80) as the eluent. The UV detector operated at 280 nm.

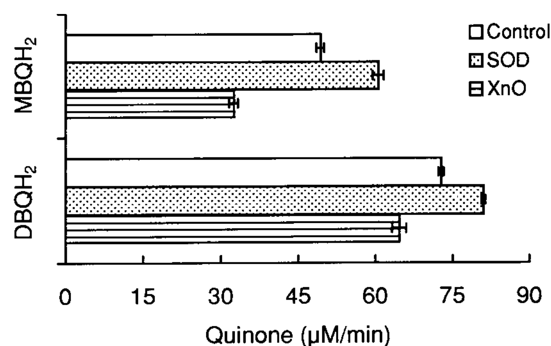

FIG. 1. Effect of SOD and the XnO-Xn system on quinone production rates during oxidation of $MBQH_2$ and $DBQH_2$ by laccase. The reactions were carried out in 20 mM phosphate buffer, pH 5, containing 500 μM hydroquinones (control), 100 U of SOD/ml, 100 mU of XnO/ml, and 250 μM Xn. Heat-denatured enzymes were used in blanks. Means and 95% confidence limits of five replicates are shown.

RESULTS

In our previous study of *P. eryngii* laccase isoenzymes (38), K_m and V_{max} values of laccase I for BQH_2 were found to be 4,600 μM and 21.2 U/mg, respectively. In the present study, for reactions carried out in 20 mM phosphate buffer (pH 5.0), the introduction of methoxyl groups to BQH_2 increased not only the V_{max} (to 200.0 U/mg [for $MBQH_2$] and 667.5 [for $DBQH_2$]), as expected since methoxyl is a benzene ring-activating group that lowers the reduction potential of hydroquinones, as well as the affinity of the enzyme for its substrate (K_m = 190.0 μM [for $MBQH_2$] and 9.9 μM [for $DBQH_2$]). To determine whether the semiquinones produced by laccase were converted into quinones via autoxidation, which is a reversible reaction ($Q^{\cdot-} + O_2 \leftrightarrow Q + O_2^{\cdot-}$), the effect of systems consuming and producing $O_2^{\cdot-}$ (SOD and XnO-Xn, respectively) on quinone production rates was studied. SOD is a useful tool for studying the involvement of $O_2^{\cdot-}$ in autoxidation reactions (39, 40). The presence of SOD (which would shift the semiquinone autoxidation equilibrium to the right) during the oxidation of 500 μM concentrations of $MBQH_2$ and $DBQH_2$ by laccase increased the initial rates of production of MBQ and DBQ by 23 and 11%, respectively (Fig. 1). Increasing the $O_2^{\cdot-}$ content of the reaction mixture by adding XnO and Xn (thereby shifting the semiquinone autoxidation equilibrium to the left) resulted in 34 and 11% decreases in these rates, respectively. In addition to showing the existence of semiquinone autoxidation reactions, these results indicated that $DBQ^{\cdot-}$ autoxidized to a greater extent than did $MBQ^{\cdot-}$ (the effects of the SOD and XnO-Xn systems on the semiquinone autoxidation reaction were lessened when the equilibrium was shifted more to the right). Autoxidation of semiquinones was confirmed by estimating the production of H_2O_2 derived from $O_2^{\cdot-}$. The levels of H_2O_2 (means ± 95% confidence limits) found after oxidation of 500 μM concentrations of $MBQH_2$ and $DBQH_2$ were 4.7 ± 0.2 and 23.9 ± 0.4 μM, respectively. These levels were used to quantify the extent of semiquinone autoxidation, tak-

TABLE 1. Production of H_2O_2 and hydroquinones during reduction of MBQ and DBQ by cytochrome P450 reductase in the absence and presence of laccase[a]

Quinone	Mean concn ± 95% confidence limit (μM)			
	H_2O_2		Hydroquinone	
	−Laccase	+Laccase	−Laccase	+Laccase
MBQ	4.1 ± 0.2	0.6 ± 0.0	13.2 ± 0.9	0.0
DBQ	20.9 ± 1.0	16.2 ± 0.7	8.5 ± 0.7	0.0

[a] H_2O_2 levels were estimated after NADPH was fully oxidized. Reaction mixtures contained 20 mM phosphate buffer (pH 5), 5 mU of cytochrome P450 reductase/ml, 50 μM NADPH, and 20 μM quinones. Laccase experiments (+) were done with 50 mU of enzyme/ml (estimated with 500 μM $MBQH_2$). Samples containing 100 U of catalase/ml were used as blanks.

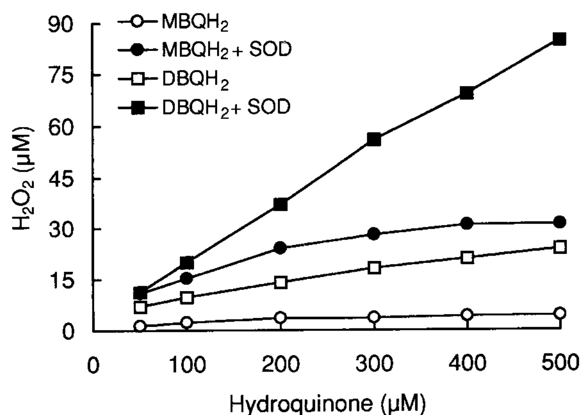

FIG. 2. Effect of SOD on H_2O_2 production during oxidation of $MBQH_2$ and $DBQH_2$ by laccase. H_2O_2 levels were measured after complete oxidation of hydroquinones, which was monitored spectrophotometrically. The compositions of the reaction mixture were as follows: 20 mM phosphate buffer (pH 5), 50 to 500 μM concentrations of hydroquinones, 100 mU of laccase/ml (estimated with 500 μM $MBQH_2$), and 100 U of SOD/ml. Samples containing 100 U of catalase/ml were used as blanks. Means of five replicates are shown (95% confidence limits were less than 5% of the mean).

ing into account the stoichiometry of $O_2^{\cdot -}$ dismutation (2 $O_2^{\cdot -}$:1 H_2O_2) and the amount of semiquinones produced by laccase during the entire reaction (500 μM). First, we demonstrated that the $O_2^{\cdot -}$ produced during semiquinone autoxidation was not reduced to H_2O_2 by the hydroquinones used in this study ($O_2^{\cdot -} + QH_2 \rightarrow H_2O_2 + Q^{\cdot -}$); if it had been, a stoichiometry different from that of $O_2^{\cdot -}$ dismutation would have resulted. Since no quinones were observed after incubating 500 μM concentrations $MBQH_2$ and $DBQH_2$ with the XnO-Xn $O_2^{\cdot -}$-generating system, it was assumed that all H_2O_2 detected after oxidation of methoxyhydroquinones by laccase was produced via $O_2^{\cdot -}$ dismutation. Then, it was estimated that only 2 and 10% of the semiquinones produced by laccase from 500 μM concentrations of $MBQH_2$ and $DBQH_2$, respectively, were autoxidized.

Based on this limited extent of semiquinone autoxidation, it was evident that semiquinones were transformed into quinones by other mechanisms. The likelihood of semiquinone oxidation by laccase and semiquinone dismutation being these mechanisms was investigated. To test the ability of laccase to oxidize methoxysemiquinones, $MBQ^{\cdot -}$ and $DBQ^{\cdot -}$ were produced from their corresponding quinones with cytochrome P450 reductase (this enzyme catalyzes the monovalent reduction of quinones, using NADPH as an electron donor). Then, the level of H_2O_2 derived from semiquinone autoxidation was estimated and the effect of laccase on H_2O_2 levels was evaluated (if laccase were able to oxidize the semiquinones, its presence should decrease H_2O_2 levels by competing with the autoxidation reaction). The level of H_2O_2 was estimated once oxidation of 50 μM NADPH was completed, a process monitored spectrophotometrically at 340 nm. Preliminary experiments revealed that the initial concentration of quinones was a crucial factor for semiquinone autoxidation to proceed, probably due to the reversible nature of this reaction (e.g., no H_2O_2 was generated when 50 μM NADPH and a 400 μM concentration of quinones were used, although NADPH was completely oxidized). The results presented in Table 1 were those obtained with a 20 μM concentration of quinones. The presence of laccase caused 84.4 and 22.7% decreases in the amount of H_2O_2 produced from MBQ and DBQ, respectively, revealing the ability of laccase to oxidize semiquinones. On the other hand, semiquinone dismutation was found to occur in samples lacking laccase in the above-described cytochrome P450 experiment (Table 1). The existence of this reaction was inferred from the presence of $MBQH_2$ and $DBQH_2$ at the end of reactions involving their corresponding quinones. No hydroquinones were found when laccase was present in the reaction mixture.

To favor the conversion of semiquinone to quinones via autoxidation over dismutation and laccase oxidation, two experiments resembling more-natural conditions and involving the removal of semiquinone autoxidation products were carried out. First, SOD was added for a faster consumption of $O_2^{\cdot -}$. Using various concentrations of hydroquinones (50 to 500 μM), it was found that the presence of SOD in the laccase reaction mixture increased H_2O_2 production 6.7- to 8.4-fold (depending on the initial concentration of hydroquinones) in the case of $MBQH_2$ and 1.6- to 3.5-fold in that of $DBQH_2$ (Fig. 2). These results are shown in Table 2 in terms of the extent of semiquinone autoxidation. A negative correlation was found between the initial concentration of hydroquinones, which corresponded with the amount of semiquinones produced by laccase during the entire reaction, and the extent of semiquinone autoxidation. Second, removal of quinones during laccase reactions was achieved by adding diaphorase, a reductase catalyzing the divalent reduction of quinones from NADH oxidation. The final H_2O_2 levels in 1-ml reaction volumes containing, in addition to laccase and diaphorase, 50 nmol of hydroquinones and various amounts of NADH are shown in Fig. 3 (reaction completion was tested by monitoring NADH oxidation at 340 nm). For the correct interpretation of these results, it should be noted that the amount of hydroquinone oxidized (semiquinone produced) by laccase during the reaction was the quantity present at the beginning of the experiment (50 nmol) plus the amount recycled from the NADH oxidation. Therefore, taking into account the stoichiometry of the diaphorase reaction (1 NADH:1 QH_2), it was assumed that 100, 200, and 400 nmol of hydroquinones were oxidized by laccase in samples containing 50, 150, and 350 nmol of NADH, respectively. The complete oxidation of NADH in samples containing an amount larger than that required to reduce the quinone produced by laccase from 50 nmol of hydroquinone demonstrated redox cycling and supported the above assumption. The results shown in Fig. 3 show that H_2O_2 levels were proportional to the amount of $MBQH_2$ and $DBQH_2$ oxidized by laccase. To evaluate the effect of quinone removal on semiquinone autoxidation, these results were compared with those of the control experiment in Fig. 2, in which quinones accumulated in the reaction mixture (Table 2). Quinone recycling by diaphorase increased $MBQ^{\cdot -}$ and $DBQ^{\cdot -}$ autoxidation

TABLE 2. Effect of SOD and diaphorase on extent of semiquinone autoxidation[a]

Amt of semiquinone produced (nmol)	% Autoxidation of:					
	$MBQ^{\cdot -}$			$DBQ^{\cdot -}$		
	Control	With SOD	With diaphorase	Control	With SOD	With diaphorase
50	5.2	43.6		28.0	45.1	
100	4.5	30.6	13.8	19.6	40.0	39.9
200	3.5	24.0	14.7	14.0	37.0	41.7
300	2.3	18.6		12.1	37.2	
400	2.0	15.4	13.1	10.5	34.6	40.5
500	1.7	12.4		9.6	33.9	

[a] The extent of semiquinone autoxidation was calculated from H_2O_2 levels shown in Fig. 2 (control and SOD columns) and Fig. 3 (diaphorase column), taking into account the stoichiometry of the $O_2^{\cdot -}$ dismutation reaction and the amount of semiquinone produced by laccase during the entire reactions.

3.0- to 6.5-fold and 2.0- to 3.9-fold, respectively. In addition, quinone recycling kept the extent of semiquinone autoxidation constant (around 14 and 41% in all samples for $MBQ^{\cdot -}$ and $DBQ^{\cdot -}$, respectively). From these results, it was inferred that quinone accumulation was the factor causing decreased extents of semiquinone autoxidation as the initial concentration of hydroquinones increased in control and SOD experiments. This effect was better shown with $MQH^{\cdot -}$, whose autoxidation reaction equilibrium was less shifted to the right.

DISCUSSION

A diagram of the reactions involved in the conversion of the semiquinones produced by laccase into quinones, including the strategies used to demonstrate their existence, is shown in Fig. 4. This conversion can be carried out by three mechanisms: autoxidation, laccase oxidation, and dismutation. Obviously, the contribution of laccase-mediated hydroquinone oxidation to the production of partially reduced oxygen species will depend on the extent of semiquinone autoxidation. This extent was estimated from H_2O_2 production during laccase reactions after it was demonstrated that other H_2O_2-producing reactions, such as $O_2^{\cdot -}$ reduction by methoxyhydroquinones, did not take place (Fig. 4). The latter reaction, described for certain naphthohydroquinones, leads to a chain reaction in which the $O_2^{\cdot -}$ produced during the autoxidation of semiquinones acts as the propagating species (39). This reaction was evidenced with the hydroquinones produced from 2-methyl-1,4-naphthoquinone (menadione) and duroquinone by *P. eryngii* (21). The buffer and substrate solutions used in the reactions in the present study were treated with the chelating resin Chelex 100 to reduce the levels of trace metal ions, which by reacting with H_2O_2 or $O_2^{\cdot -}$ could lead to H_2O_2 underestimations. As mentioned above, during the incubation of laccase with 100 μM BQH_2, less than 1% of the semiquinone autoxidized (38). The extent of methoxysemiquinone autoxidation was expected to be higher, since electron transfer to O_2 is favored by electron-donating substituents, such as methoxyl groups, which decrease the reduction potential of the semiquinone-quinone couple (6). Besides, $DBQ^{\cdot -}$ autoxidation had been described previously in studies concerning the use of DBQ as an anticancer agent (42). As shown in Table 2, the extent of autoxidation of 100 μM concentrations of $MBQ^{\cdot -}$ and $DBQ^{\cdot -}$ rose to 4.5 and 19.6%, respectively. Despite the considerable increase observed, these results revealed that most of the semiquinone produced by laccase during the oxidation of any hydroquinone derived from lignin units was transformed into quinone through dismutation and laccase oxidation. However, in an in vitro laccase reaction, which is needed for demonstration of oxygen activation, the conditions are far from natural. We have focused our attention on the concentrations of semiquinone autoxidation reaction products because they probably entail the main difference in the extent of the reaction under in vitro and in vivo conditions. On the one hand, the $O_2^{\cdot -}$ produced in vitro disappeared by spontaneous dismutation. Under more-natural conditions, a faster $O_2^{\cdot -}$ consumption is expected because, in addition to undergoing dismutation, it can react with Mn^{2+}, Fe^{3+} (see above), and radicals produced by ligninolytic enzymes (23). Such a faster consumption of $O_2^{\cdot -}$ was simulated by adding SOD to laccase reactions (Fig. 4), and the effects on H_2O_2 levels and the extent of semiquinone autoxidation are quite well illustrated by the results shown in Fig. 2 and Table 2, respectively. In the case of BQH_2, H_2O_2 levels increased from 0.3 μM to 12 and 34 μM in the presence of SOD and Mn^{2+}, respectively (38). On the other hand, whereas quinones accumulate in in vitro reactions, they are redox cycled in the presence of *P. eryngii* mycelium (21). Quinone redox cycling has been simulated in the present study by adding diaphorase to laccase reactions (Fig. 4), and the observed increase in the extent of semiquinone autoxidation (Table 2) show quite well the negative effect of quinone accumulation.

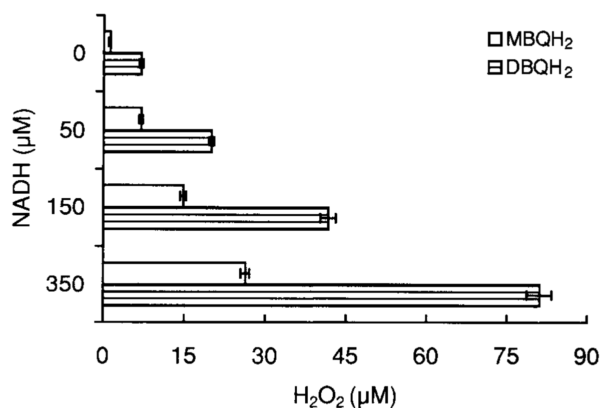

FIG. 3. Production of H_2O_2 during oxidation of $MBQH_2$ and $DBQH_2$ by laccase in the presence of diaphorase and various amounts of NADH. H_2O_2 levels were measured after the oxidation of NADH. The reactions mixtures contained 20 mM phosphate buffer (pH 5), 50 mU of laccase/ml (estimated with 500 μM $MBQH_2$), 50 μM hydroquinones, 150 mU of diaphorase/ml, and 0 to 350 μM NADH. Samples containing 100 U of catalase/ml were used as blanks. Means and 95% confidence limits of five replicates are shown.

Besides semiquinone autoxidation, other mechanisms by which white-rot fungi could produce $O_2^{\cdot -}$ have been reported. First, in reactions involving lignin peroxidase, $O_2^{\cdot -}$ resulted both from the decomposition of a peroxy radical produced

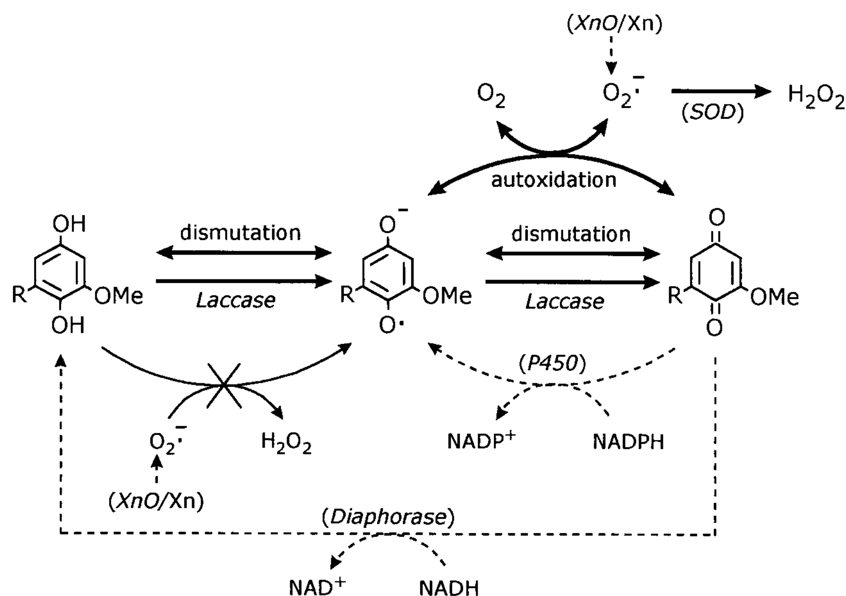

FIG. 4. Scheme of the reactions involved in the conversion of semiquinones into quinones during oxidation of $MBQH_2$ (R=H) and $DBQH_2$ (R=OMe [where Me is a methyl group]) by laccase (bold arrows). The strategies used to demonstrate these reactions are included (dashed arrows and enzymes in brackets).

during oxidation of a lignin model dimer (25) and from autoxidation of formate radicals ($CO_2^{\cdot -}$) produced during the oxidation of oxalate in the presence of veratryl alcohol (41). Second, MnP has also been linked to the production of $O_2^{\cdot -}$, based on the ability of Mn^{3+} to produce $CO_2^{\cdot -}$ from not only oxalate but also glyoxylate (31, 32). Third, cellobiose dehydrogenase has been described to produce $O_2^{\cdot -}$ both directly, via monovalent reduction of oxygen (37), and indirectly, via Fe^{3+} reduction followed by Fe^{2+} autoxidation (51). The interest of researchers in studying the origin of $O_2^{\cdot -}$ is mainly related to its possible participation in Mn^{2+} oxidation (38, 41) and the generation of both H_2O_2 (31, 49) and $HO^{\cdot}$ (41, 51). Since not all white-rot fungi produce the same lignin degradation enzymes, these mechanisms producing $O_2^{\cdot -}$ may have special importance in those fungi lacking MnP activities or extracellular oxidases. As mentioned above, laccase is the most widely distributed ligninolytic enzyme among white-rot fungi, and hydroquinones are intermediates in lignin degradation. These facts, together with the results presented in the present paper, establish the production of $O_2^{\cdot -}$ from semiquinone autoxidation as a firm alternative to any of the above-described mechanisms.

The results shown here also extend our previous findings on oxygen activation by *P. eryngii* through quinone redox cycling (21). This process, which mainly occurs as the monovalent reduction of a quinone to a semiquinone by NAD(P)H-dependent reductases followed by the oxidation of the semiquinone by O_2, has been studied mostly in mammalian systems due to the human-cytotoxic effects of quinones (28). In *P. eryngii*, the process is quite peculiar due to the secretion of quinone reduction products, the participation of ligninolytic enzymes, and the extracellular production of reduced oxygen species. Based on the wide substrate specificity of the reductive and oxidative enzymes involved in the redox cycling of quinones, it is quite likely that the reduction of methoxyquinones by *P. eryngii*, followed by the oxidation of methoxyhydroquinones by laccase, leads to the production of extracellular $O_2^{\cdot -}$ on a constant basis. In addition to quinones acting as carriers of electrons from intracellular NAD(P)H to extracellular O_2, aromatic aldehydes have being described to play the same role in *P. eryngii*. After being reduced to alcohols by intracellular reductases, they participate in the divalent reduction of O_2 in reactions catalyzed by aryl-alcohol oxidase (17, 19). The simultaneous production of $O_2^{\cdot -}$ and H_2O_2 via the redox cycling of the pertinent fungal metabolites or lignin-derived products probably leads to $HO^{\cdot}$ production via the Haber-Weiss reaction, as has already demonstrated during the redox cycling of menadione by *P. eryngii* (20). The latter has been recently used to study the effect of extracellular $HO^{\cdot}$ production on the expression of genes encoding ligninolytic peroxidases (43). Besides being useful tools for fundamental studies, these mechanisms of O_2 activation could be exploited in some biotechnological applications, such as biopulping, and in edible-mushroom cultivation techniques. An enhanced production of reduced O_2 species promoted by redox cycling compounds would favor lignin degradation and competition for the substrate with other microorganisms which are not able to grow under oxidative-stress conditions.

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