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Biochemistry

Glyoxal oxidase of *Phanerochaete chrysosporium*: Its characterization and activation by lignin peroxidase

(hydrogen peroxide/methylglyoxal/white-rot fungus/wood decay)

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ABSTRACT **Glyoxal oxidase (GLOX) is an extracellular H_2O_2-generating enzyme produced by ligninolytic cultures of *Phanerochaete chrysosporium*. The production, purification, and partial characterization of GLOX from agitated cultures are described here. High-oxygen levels are critical for GLOX production as for lignin peroxidase. GLOX purified by anion-exchange chromatography appears homogeneous by NaDodSO$_4$/PAGE (molecular mass = 68 kDa). However, analysis by isoelectric focusing indicates two major bands (pI 4.7 and 4.9) that stain as glycoproteins as well as for H_2O_2-producing activity in the presence of methylglyoxal. Purified GLOX shows a marked stimulation in activity when incubated with Cu^{2+}; full activation takes more than 1 hr with 1 mM $CuSO_4$ at pH 6. The steady-state kinetic parameters for the GLOX oxidation of methylglyoxal, glyceraldehyde, dihydroxyacetone, glycolaldehyde, acetaldehyde, glyoxal, glyoxylic acid, and formaldehyde, were determined by using a lignin peroxidase coupled-assay at pH 4.5. Of these substrates, the best is the extracellular metabolite methylglyoxal with a K_m of 0.64 mM and an apparent rate of catalysis, k_{cat}, of 198 s^{-1} under air-saturated conditions. The K_m for oxygen is greater than the concentration of oxygen possible at ambient pressure—i.e., >1.3 mM at 25°C. Importantly, oxygen-uptake experiments show that purified GLOX is inactive unless coupled to the peroxidase reaction. With this coupled reaction, for each mol of methylglyoxal, veratryl alcohol (a lignin peroxidase substrate), and oxygen consumed, 1 mol each of pyruvate and veratraldehyde is produced. The importance of these results is discussed in relation to the physiology of lignin biodegradation and possible extracellular regulatory mechanisms for the control of oxidase and peroxidase activities.**

Extracellular peroxidases are important components of the ligninolytic system of *Phanerochaete chrysosporium* [for recent reviews, see Buswell and Odier (1), Kirk and Farrell (2), and Tien (3)]. Both lignin peroxidase (4, 5) and manganese peroxidase (6, 7) are secreted by the fungus, and both use H_2O_2 as electron acceptor in their catalytic cycles. Glucose 1-oxidase (8), glucose 2-oxidase (9), fatty-acyl-CoA oxidase (10), and methanol oxidase (11) have been proposed to supply the required H_2O_2. However, these oxidases are intracellular and their effectiveness in supplying extracellular H_2O_2 might be limited by intracellular catalase (12, 13).

Glyoxal oxidase (GLOX) is an extracellular H_2O_2-producing enzyme found in ligninolytic cultures of *P. chrysosporium* (14). The enzyme catalyzes the oxidation of a number of simple aldehydes and α-hydroxy carbonyl compounds; two substrates of the oxidase, glyoxal and methylglyoxal, are also found in the extracellular fluid of ligninolytic cultures grown on defined medium (14). The temporal correlation of GLOX, lignin peroxidase, and oxidase substrate appearances in cultures suggests a close physiological connection between these components. The scale-up and purification of GLOX are reported here, together with a characterization of the enzyme, not only of its physicochemical properties but also its dependence on a peroxidase system for activity. An extracellular regulatory mechanism for control of H_2O_2 production is apparent.

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MATERIALS AND METHODS

Enzyme Production. *P. chrysosporium*, strain BKM-F-1767 (ATCC 24725), was grown at 39°C in rotating (120 rpm) 2-liter flasks containing 750 ml of liquid medium. The standard medium was as described (14) with 0.01 M *trans*-aconitic acid as buffer and 1% glucose as carbon source (14). After 2 days of growth under air, the cultures were flushed daily with 100% O_2 and stoppered. For some experiments, the standard medium was supplemented after 2 days of growth with 0.1% Tween 20 (25% stock solution), 8 mM benzyl alcohol (neat stock solution), and an additional 6-fold concentration of trace metals [100× stock solution (15)]. These additives have been shown to stimulate lignin peroxidase production in agitated cultures of *P. chrysosporium* BKM-F-1767 (T. K. Kirk, M. Tien, S. Croan, T. McDonagh & R. L. Farrell, unpublished proceedings of the Third International Conference on Biotechnology in the Pulp and Paper Industry, Stockholm, Sweden, June 16–19, 1986).

Enzyme Purifications. Seventeen 2-liter flasks of day 5 cultures were harvested and filtered to give 11.9 liters of culture fluid, which were then concentrated by ultrafiltration (Minitan concentrator, Millipore; 10-kDa cutoff membrane) to 910 ml. The concentrate was then treated with 1/3 volume of cold acetone and filtered through glass wool to remove extracellular polysaccharide. The filtrate was dialyzed twice against 4 liters of 5 mM sodium phosphate buffer (pH 7.0) containing 0.5 mM $CuSO_4$ and 0.5 mM EDTA and then was further concentrated to 15 ml with a YM 10 filter (Amicom). The sample was then loaded on a DEAE Bio-Gel A (Bio-Rad) column (1.5 × 20 cm) equilibrated with the same buffer, and 12.5-ml fractions were collected (30 ml/hr) at room temperature. The column was washed with starting buffer for the first 250 ml, and then the column was developed with 240 ml of a linear NaCl gradient (0–0.15 M, 300 ml total) followed by a 1 M NaCl wash. Active fractions were consolidated to give protein, which appeared homogeneous by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (NaDodSO$_4$/PAGE).

GLOX-free lignin peroxidase isozymes were eluted with 1 M NaCl from the DEAE Bio-Gel A column, after the 0–0.15 M NaCl wash, in purifications similar to the one described above. These enzymes were dialyzed against 5 mM Na^+ phosphate (pH 7) for use in the coupled reactions with GLOX at pH 4.5 (see below).

Abbreviation: GLOX, glyoxal oxidase.

Enzyme Assays. Two standard assays for GLOX were used in this study. The first measures the H_2O_2-dependent oxidation of phenol red at pH 6 in a coupled reaction with horseradish peroxidase (14). This assay was used primarily to detect activity of cultures and has the advantage in that the peroxidase is commercially available. The second assay for GLOX was developed because anomalies in enzyme activity (e.g., lags and activations) became apparent as GLOX was purified; therefore, conditions that better approximated that of cultures were used to characterize the interactions of the oxidase/peroxidase systems. The standard reactions contained 20 mM sodium 2,2-dimethylsuccinate (pH 4.5), 5 mM methylglyoxal, 2 mM veratryl alcohol (redistilled under vaccuum), GLOX, and non-rate-limiting amounts of GLOX-free lignin peroxidase. Progress of the reactions was followed at 310 nm for veratraldehyde formation ($\varepsilon_{310} = 9300\ M^{-1}\cdot cm^{-1}$). Lags in activity with purified preparations of GLOX were eliminated with 5 μM H_2O_2. After the fast uncoupled reaction, in which lignin peroxidase consumes the exogenous H_2O_2, the much slower (but extensive) GLOX-dependent reaction was measured.

Two assays for lignin peroxidase were also required. The first assay was used to measure activity in cultures and is the more sensitive assay because of the lower pH conditions. Reactions contained 25 mM sodium tartrate buffer (pH 3.0), 2 mM veratryl alcohol, culture filtrate, and 0.4 mM H_2O_2; activity was followed by the increase in absorbance at 310 nm because of the H_2O_2-dependent oxidation of veratryl alcohol to veratraldehyde (16). The second assay for lignin peroxidase was used to measure activity at the pH of cultures and conditions similar to that of the GLOX assay at pH 4.5 (see above). Reaction mixtures contained 20 mM sodium 2,2-dimethylsuccinate (pH 4.5), 2 mM veratryl alcohol, lignin peroxidase, and 0.4 mM H_2O_2.

Protein Determinations. The method of Bradford (17) was used for protein determinations with bovine serum albumin as standard.

Molecular Weight Determinations. A Superose 12 (Pharmacia) gel filtration column equilibrated with 50 mM sodium 2,2-dimethylsuccinate (pH 6) and a flow rate of 0.5 ml/min was used for molecular mass determination of native GLOX. Standard proteins for calibration were glucose oxidase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and vitamin B_{12} (1.35 kDa) from Sigma. A Pharmacia Phast System was used for molecular weight determination of denatured GLOX by NaDodSO$_4$/PAGE with 10–15% gels and conditions as recommended by the manufacturer. Standard proteins were ovalbumin (45 kDa), bovine albumin (66 kDa), phosphorylase *b* (97.4 kDa), and β-galactosidase (116 kDa) from Sigma.

Isoelectric Focusing. Isoelectric focusing and subsequent activity staining for GLOX were as described (14) with pH 3–6 gels (Serva). Standard proteins were amyloglucosidase (pI 3.55), trypsin inhibitor (pI 4.55), β-lactoglobulin A (pI 5.13), and bovine carbonic anhydrase (pI 5.85) from Sigma.

Glycoprotein Analysis. Purified GLOX (200 ng) was stained for glycoprotein after isoelectric focusing by a method developed by Pharmacia. The gel was treated in a series of solutions as follows: 20% trichloroacetic acid for 5 min at 20°C, distilled water for 5 min at 20°C, 0.7% periodic acid/5% acetic acid for 10 min at 20°C, distilled water for 2 min at 20°C, distilled water for 3 min at 20°C, Schiffs reagent for 10 min at 20°C, 5% sodium metabisulphite/5% acetic acid for 5 min at 50°C, 5% methanol/7.5% acetic acid for 5 min at 50°C, 50% methanol/30% acetic acid for 5 min at 50°C, and 50% methanol/30% acetic acid for 2 min at 50°C.

Stoichiometry. The stoichiometry of the GLOX–lignin peroxidase-coupled reaction at pH 4.5 was determined with methylglyoxal as the oxidase substrate and veratryl alcohol as the peroxidase substrate. A limiting amount of 89 μM methylglyoxal and excess 2 mM veratryl alcohol was used to quantify the pyruvate and veratraldehyde produced. The progress of the reaction and the final concentration of veratraldehyde formed was determined at 310 nm ($\varepsilon_{310} = 9300\ M^{-1}\cdot cm^{-1}$). Pyruvate was measured with lactate dehydrogenase/NADH (Δ 340 nm) after adjusting the pH to 7.0 with sodium phosphate and ultrafiltration through a YM10 membrane (Amicon) to eliminate possible interferences due to peroxidative reactions with NADH. The methylglyoxal concentrations of stock solutions were measured by the formation of *S*-lactoylglutathione in the presence of glyoxalase I (Sigma) and glutathione (Sigma) at 240 nm (18). Stoichiometry for oxygen was determined by comparing the rate of oxygen consumption to the rate of veratraldehyde production under identical reaction conditions (5 mM methylglyoxal/2 mM veratryl alcohol/0.036 unit of GLOX per ml/excess lignin peroxidase at air saturation and 25°C). Oxygen consumption was determined with a YSI model 53 oxygen monitor (Yellow Springs Instruments) fitted with a Gilson single-port 1.5-ml reaction chamber.

Steady-State Kinetics. The GLOX-lignin peroxidase coupled assay (see above) at pH 4.5, 25°C, and 0.26 mM O_2 (air-saturated) was followed at 310 nm to determine the apparent kinetic parameters for GLOX. Initial velocities were determined after full activation of the enzyme by the peroxidase system (i.e., lignin peroxidase, veratryl alcohol, and a catalytic amount of H_2O_2). To determine the K_m for O_2, a YSI model 53 oxygen monitor fitted with a Gilson single-port 1.5-ml reaction chamber was used to measure oxygen consumption; O_2 levels were varied by admixture of O_2-saturated and air-saturated solutions before initiation of the reactions. Lineweaver–Burk double-reciprocal plots were used to determine the apparent kinetic parameters.

RESULTS

Production of GLOX in Agitated Cultures. From earlier studies (14), it was clear that GLOX is a minor protein component in extracellular fluid of nonagitated ligninolytic cultures of *P. chrysosporium*. Consequently, a major obstacle for the purification and characterization of the oxidase was obtaining the enzyme in sufficient quantities. The conditions found to increase GLOX production are similar to those previously found to be successful with lignin peroxidase (15, 19) and were examined here because of the temporal correlations of the activities (14).

Enzyme activities were examined with agitated cultures (see *Materials and Methods*) under three different culture conditions: (*i*) with standard nitrogen-limited medium, (*ii*) the same as condition *i* except with daily flushings of 100% O_2 after 2 days, and (*iii*) the same as condition *ii* except with additions of 0.1% Tween 20/8 mM benzyl alcohol and 6-fold trace metals after 2 days. Results indicate that neither GLOX nor lignin peroxidase activity is found in culture fluids unless the cultures are supplied with high O_2 concentrations (Fig. 1). GLOX production was stimulated by the additions of Tween 20, benzyl alcohol, and extra trace metals, but not to the same extent as seen with lignin peroxidase. Nevertheless, GLOX activity was typically 3–5 times higher than observed in nonagitated cultures (14) and much easier to scale up.

Purification of GLOX. Culture condition *ii* (see above) was used to scale up GLOX production for purification. Fig. 2 shows the elution profiles for protein and enzyme activities from a DEAE Bio-Gel A column. After a 250-ml wash with starting buffer, the column was developed with 240 ml of a linear NaCl gradient (0–0.15 M, 300 ml total) followed by a 1 M NaCl wash. Active fractions eluted at 325–400 ml were consolidated to give protein that appeared to be homogeneous by NaDodSO$_4$/PAGE (Fig. 3), with an activity yield of 38% (Table 1).

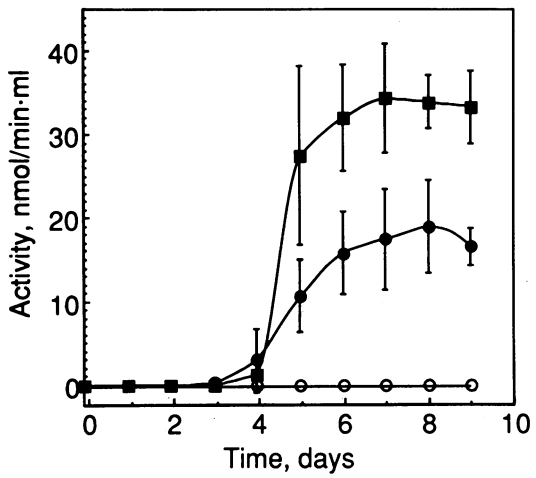

FIG. 1. Expression of GLOX activity in agitated cultures. Three culture conditions were tested: (*i*) with standard nitrogen-limited medium (○), (*ii*) the same as condition *i* except with daily flushings of 100% O_2 after 2 days of growth (●), and (*iii*) the same as condition *ii* except with additions of 0.1% Tween 20, 8 mM benzyl alcohol, and 6-fold trace metals after 2 days of growth (■). Activity plotted was determined with the horseradish peroxidase-coupled assay for four replicate flasks. Similar lignin peroxidase activity profiles were observed. For comparison of GLOX and lignin peroxidase activities assayed at pH 4.5, day 7 cultures had lignin peroxidase activity of 4 nmol/min·ml (32 nmol/min·ml at pH 3.0) and GLOX activity of 16 nmol/min·ml for culture condition *ii*. With culture condition *iii*, lignin peroxidase activity was 75 nmol/min·ml (594 nmol/min·ml at pH 3.0) and GLOX activity was 22 nmol/min·ml.

Molecular Weight. Analysis of crude extracellular proteins and purified GLOX by $NaDodSO_4$/PAGE indicated that the oxidase is a minor protein component of 68 kDa (Fig. 3). The dominant proteins of the crude preparation had molecular masses of ≈42 and ≈47 kDa, in reasonable agreement with the reported molecular masses of lignin peroxidase (16, 20) and manganese peroxidase (6, 7), respectively. Gel permeation chromatography of GLOX indicated a protein of 47 kDa. Increasing the ionic strength (0.15 M NaCl) of the elution buffer did not affect the low apparent molecular mass determination with this procedure. The molecular mass determinations of GLOX under denaturing and native conditions suggest that GLOX is monomeric.

Isoelectric Point and Glycoprotein Analysis. Protein staining of purified GLOX after isoelectric focusing showed two bands corresponding to isoelectric points of 4.7 and 4.9. A third very minor band (pI ≈ 4.5) was also detected at heavier protein loadings and also stained for activity. The proteins of pI 4.7 and 4.9 correspond to those identified previously in stationary cultures (14). Analysis indicated that these are glycoproteins; results for the minor protein were inconclusive.

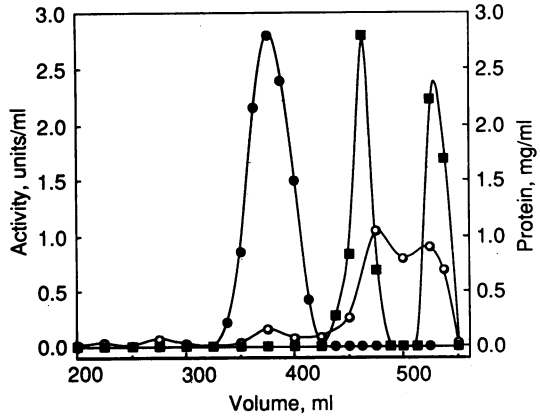

FIG. 2. Enzyme activity profiles with DEAE Bio-Gel A. Activities of the concentrated extracellular protein (see Table 1) were fractionated by ion exchange with a NaCl gradient (see text). GLOX activity (●), lignin peroxidase activity (■), and protein (○) were determined.

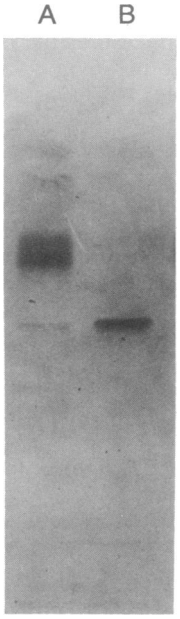

FIG. 3. $NaDodSO_4$/PAGE of purified GLOX and crude extracellular proteins. The acetone-treated (to remove interfering polysaccharide) extracellular protein (lane A; 230 ng) and purified GLOX (lane B; 50 ng), as described in Table 1, were analyzed by $NaDodSO_4$/PAGE. A minor protein of ≈68 kDa in the crude mixture corresponds to the purified GLOX.

Activation by Cu^{2+}. When GLOX was purified without Cu^{2+} in the buffers, there normally was great loss in activity, which could be partially regained by incubation with Cu^{2+}. For example, when a GLOX preparation that was purified by chromatofocusing and gel permeation in a preliminary study was incubated at 25°C with 1 mM Cu^{2+} and 50 mM sodium 2,2-dimethylsuccinate (pH 6), it showed a 5-fold increase in activity in 1 hr with 50% of this activation occurring in 8 min. No activation was observed with the divalent cations Mg^{2+}, Zn^{2+}, Fe^{2+}, or Mn^{2+}.

Stoichiometry. The stoichiometry of the GLOX-lignin peroxidase coupled reaction at pH 4.5 was determined with methylglyoxal as the oxidase substrate and veratryl alcohol as the peroxidase substrate. From 89 nmol of methylglyoxal and excess veratryl alcohol (2 μmol) were produced 85 nmol of pyruvate and 85 nmol of veratraldehyde. Stoichiometry for oxygen was determined by comparing the rate of oxygen consumption with the rate of veratraldehyde production; a 1:1 ratio was determined for O_2/veratraldehyde. These results indicate, within experimental error, that for every 1 mol

Table 1. Purification of GLOX

Purification step	Volume, ml	Units*	Protein, mg	% yield	Units/mg
Culture fluid	11,900	274	13†	100	21
Concentration	910	295	13†	108	23
Acetone treatment	1,550	178	13†	65	14
Concentration	15	130	13	47	10
DEAE Bio-Gel A	75	105	0.8	38	131

*Units are defined as μmol/min.
†Protein concentrations are based on the fourth step because of the low concentrations.

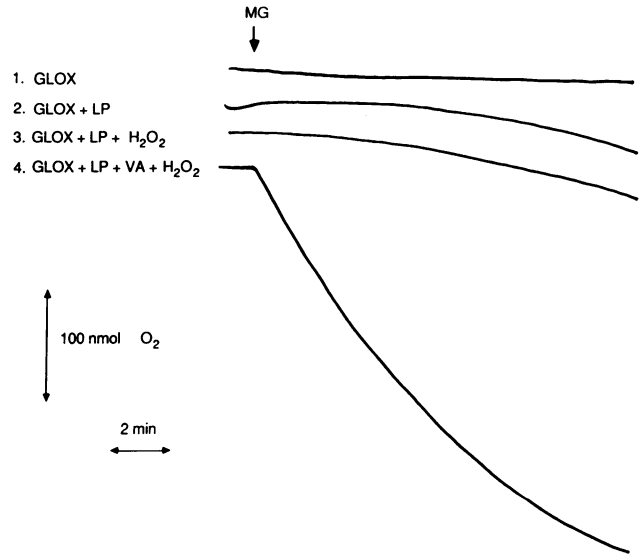

FIG. 4. Activation of GLOX by a peroxidase system. Traces are shown for O_2 consumption by different reaction mixtures containing, where indicated, 60 milliunits of GLOX, 114 milliunits of lignin peroxidase (LP), 1 mM veratryl alcohol (VA), 3.3 μM H_2O_2, and 5 mM methylglyoxal (MG). Reactions were buffered with 20 mM sodium 2,2-dimethylsuccinate at pH 4.5 in a total reaction volume of 1.5 ml.

of methylglyoxal and O_2 consumed, 1 mol of pyruvate and veratraldehyde are produced in this coupled assay.

Activation of GLOX by Lignin Peroxidase. To study the lignin peroxidase-dependent activations of GLOX, an assay for GLOX independent of the activator was required. Therefore, a Clark-type oxygen electrode was used to monitor the rates of oxygen consumption. Overlay traces for O_2 consumption are shown in Fig. 4 for a series of reaction conditions. In each case the reaction was initiated with methylglyoxal. Surprisingly, purified GLOX was inactive as indicated by the lack of O_2 consumption on addition of methylglyoxal. Full activation of GLOX was observed, however, with additions of lignin peroxidase, veratryl alcohol, and a catalytic amount (5 nmol) of H_2O_2. Further control experiments indicated that all of the components were necessary for this activation and that H_2O_2 was required to eliminate a lag in activity.

Steady-State Kinetics. The apparent K_m and k_{cat} values of the various substrates for GLOX were determined by using the lignin peroxidase-coupled reaction at pH 4.5 (Table 2). The second-order rate constants (k_{cat}/K_m) indicate that methylglyoxal is the best of the examined substrates for GLOX. Interestingly, there is little variance in the k_{cat} values, but wide variance in K_m. The rates of oxidation with 5 mM methylglyoxal (pH 4.5; standard assay conditions) and with various concentrations of O_2 indicate that the K_m for O_2 is >1.3 mM at 25°C—i.e., greater than the concentration at 100% O_2 saturation.

Table 2. Steady-state kinetics of GLOX

Substrate	K_m, mM	k_{cat}, s^{-1}	k_{cat}/K_m, $M^{-1}\cdot s^{-1}$	% relative activity
Methylglyoxal	0.64	198	309,600	100
Glyoxylic acid	2.5	96	38,200	12.4
Glycolaldehyde	8.3	208	25,000	8.1
Acetaldehyde	8.3	148	17,800	5.7
Formaldehyde	23	254	11,000	3.6
Glyoxal	11.5	118	10,400	3.3
Dihydroxyacetone	38	188	5,000	1.6
DL-Glyceraldehyde	42	148	3,600	1.1

Apparent kinetic parameters were determined with the lignin peroxidase-coupled assay at pH 4.5, 25°C, and air-saturation. Because specific activities of the GLOX preparations varied slightly, they were normalized to the most active preparation based on their activity with methylglyoxal.

DISCUSSION

Characteristics consistent with the total ligninolytic system of *P. chrysosporium* are (*i*) the production of GLOX in response to high oxygen and (*ii*) the high K_m of the oxidase for O_2 (thus making its activity responsive to O_2 concentrations under physiological conditions). High levels of oxygen not only derepress the ligninolytic system, as measured by the conversion of [^{14}C]lignin to $^{14}CO_2$ (21, 22) but also increase the rate of lignin degradation after the system is established (21). Also, a study with various strains of *P. chrysosporium* indicated that H_2O_2 stimulated lignin oxidation in cultures (23). Therefore, the rate of lignin biodegradation may be a reflection of the rate-limiting activity of oxidative enzymes such as GLOX.

The conditions used to characterize the kinetics of the GLOX-catalyzed oxidations were designed to mimic conditions of ligninolytic cultures. Thus, reactions were coupled to the oxidation of veratryl alcohol with lignin peroxidase in pH 4.5 buffer. [Note: veratryl alcohol, which has a lignin aromatic substituent pattern, is a secondary metabolite synthesized *de novo* from glucose (24, 25), and the pH optimum for ligninolysis is ≈4.5 (22).] Accordingly, GLOX appears to be kinetically competent with respect to lignin peroxidase in cultures—i.e., the activity of GLOX is of the same order of magnitude as that of lignin peroxidase under saturating conditions. Therefore, the rates of reaction in a coupled GLOX/lignin peroxidase system *in vivo* would appear to be controlled by levels of substrates for GLOX in culture and by oxygen concentration.

The second-order rate constants (k_{cat}/K_m) determined for GLOX in this study indicate methylglyoxal, a metabolite that has also been identified in cultures (14), to be the best substrate. Interestingly, the k_{cat} determinations for GLOX with all of the eight substrates tested are considerably higher than that observed for lignin peroxidase (maximum turnover number of 7.8 s^{-1}) with veratryl alcohol (26). Consequently, much less GLOX protein is required to match the catalytic activity of lignin peroxidase at pH 4.5, which might explain why GLOX is a very minor component in cultures compared to the peroxidases.

GLOX is expressed when *P. chrysosporium* is grown on glucose or xylose (data not shown), the major sugar components of lignocellulosics. However, the physiological substrates for GLOX are not these growth-carbon compounds but apparently are intermediary metabolites (14). Thus, the organism produces H_2O_2 without the expense of producing an assortment of extracellular sugar oxidases. Interestingly, proposed products of ligninolysis, such as glycolaldehyde (16), are also substrates for GLOX, suggesting that once the oxidase system is "primed" with substrates derived from carbohydrates, the system can in part be perpetuated by the action of lignin peroxidases on lignin itself. The broad substrate specificity of GLOX for simple aldehydes should allow this versatility.

The activation of GLOX by the lignin peroxidase system (lignin peroxidase plus veratryl alcohol) is another characteristic of the oxidase system that may have considerable physiological significance. A property of the lignin peroxidase of prime concern is the irreversible inactivation by H_2O_2 in the absence of a suitable substrate both *in vitro* (27) and *in vivo* (28). The reversible inactivation of GLOX in the absence of a peroxidase substrate may prevent accumulation of high

H_2O_2 concentrations *in vivo* that otherwise would inactivate lignin peroxidase.

The activation of GLOX by a peroxidase system is not restricted to lignin peroxidase/veratryl alcohol. Purified GLOX that is inactive as determined by O_2 consumption can be reactivated under conditions of the horseradish peroxidase/phenol red assay. Furthermore, purification of GLOX is not a requisite for reversible inactivation of the enzyme; crude enzyme preparations lose activity (determined by O_2 consumption) with dialysis and storage but can be reactivated by including a peroxidase substrate (data not shown). The reversible inactivation of GLOX can not be explained simply as inhibition by H_2O_2 because small additions of H_2O_2 are required to eliminate a lag in the activation, suggesting that it is the turnover of the peroxidase that is required and not simply the removal of H_2O_2. A detailed mechanism for the inactivation and activation of GLOX will require a better understanding of the enzyme's active site.

Activation of glyoxal oxidase by the lignin peroxidase system finds an analogy in the activation of galactose oxidase from the fungus *Dactylium dendroides* by horseradish peroxidase (29–31). Galactose oxidase is of similar molecular weight, has substrates in common (e.g., dihydroxyacetone, glyceraldehyde, and glycolaldehyde), and requires Cu^{2+} for activity. In the case of GLOX, it is not yet known whether Cu^{2+} is a component of the active site of the enzyme or simply an effector. Nevertheless, there are obvious differences between the two oxidases; in contrast to GLOX, galactose oxidase is a highly basic protein and oxidizes galactose (29–31). Whether GLOX has spectral characteristics (32) like galactose oxidase remains to be determined. However, the GLOX–lignin peroxidase interaction described here can be put into a possible physiological context, unlike the activation of galactose oxidase by horseradish peroxidase.

In conclusion, the scale-up production, purification, and partial characterization of GLOX have been accomplished. Properties of GLOX suggests that it is important in the oxidative/peroxidative reactions of ligninolytic cultures. These include not only the extracellular location of the oxidase, the temporal correlation of expression with lignin peroxidase, and the responses to oxygen concentration but also the modulation of the oxidase activity by interaction with the peroxidase system.

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