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Kinetics of Mn^{3+}-Oxalate Formation and Decay in Reactions Catalyzed by Manganese Peroxidase of *Ceriporiopsis subvermispora*

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The kinetics of Mn^{3+}-oxalate formation and decay were investigated in reactions catalyzed by manganese peroxidase (MnP) from the basiomycete *Ceriporiopsis subvermispora* in the absence of externally added hydrogen peroxide. A characteristic lag observed in the formation of this complex was shortened by glyoxylate or catalytic amounts of Mn^{3+} or hydrogen peroxide. MnP titers had a minor effect on this lag and did not influence the decay rate of the complex. In contrast, Mn^{2+} and oxalate drastically affected maximal concentrations of the Mn^{3+}- oxalate complex formed, the decay of which was accelerated at high Mn^{2+} levels. The highest concentration of complex was obtained at pH 4.0, whereas an inverse relationship was found between the pH of the reaction and the decay rate of the complex with MnP present. In the absence of MnP, the best fit for the decay kinetics of the complex gave an order of 3/2 at concentrations in the range of 30-100 μM, with a $k_{obs} = 0.012\ min^{-1}M^{-0.5}$ at pH 4.0. The rate constant increases at lower pH values and decreases at high oxalate concentrations. The physiological relevance of these findings is discaused. © 1998 Academic Press

Key Words: **manganese peroxidase; Mn^{3+}-oxalate; superoxide; hydrogen peroxide; *Ceriporiopsis subvermispora*.**

Manganese peroxidase (MnP)[2] is secreted by white-rot fungi and is thought to play an important role in the degradation of lignin during wood decay. MnP catalyzes the oxidation of Mn^{2+} to Mn^{3+}, which in turn can oxidize phenolic substrates including lignin substructures (1). The kinetics of MnP from *Phanerochaete chrysosporium* has been well characterized (2–4). We have studied the ligninolytic system of another white-rot fungus, *Ceriporiopsis subvermispora*. This fungus selectively decays the lignin of wood and might be useful in softening wood chips prior to mechanical pulping (5). *C. subuermispora* secretes several isoenzymes of MnP and laccase, with isoelectric points that vary with the composition of the growth medium (6, 7). In contrast to *P. chrysosporium*, no lignin peroxidase has been identified from *C. subvermispora*.

We recently published attempts to identify physiological sources of H_2O_2 required for extracellular MnP activity in *C. subvermispora* (8). Efforts failed to find oxidases such as those secreted by other wood-decay fungi. We therefore studied the possibility that hydrogen peroxide might be generated by MnP through the manganese-dependent oxidation of organic acids secreted by the fungus. Indeed, it has been described that MnP from *P. chrysosporium* oxidizes oxalate, producing CO_2 and formate radical (9). The latter reacts with oxygen to give a second molecule of CO_2 and superoxide. Superoxide then is reduced by Mn^{2+} to give hydrogen peroxide and Mn^{3+} (10), both of which further accelerate MnP-catalyzed reactions. Similarly, glyoxylate is oxidized by MnP with the production of formate and formate radical from the organic acid (11). Both oxalate and glyoxylate were found in the extracellular fluid of *C. subvermispora* grown in chemically defined media (8). In cultures containing low nitrogen, a correlation between MnP titers and mineralization of oxalate was observed. *In vitro* assays confirmed the Mn^{2+}-dependent oxidation of [^{14}C] oxalate by MnP and

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[2] Abbreviations used: MnP, manganese peroxidase; SOD, superoxide dismutase.

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that this reaction is stimulated by physiological concentrations of glyoxylate.

It seems probable that the mechanism used to support MnP activity of *C. subvermispora* by oxidation of simple organic acids is also shared by *P. chrysosporium* and other white-rot fungi. Organic acids such as oxalate (2, 3, 12, 13), malonate (3), and glyoxylate (8) are secreted by fungi producing MnP. It is proposed that organic acids also play an important role in MnP-catalyzed reactions by facilitating the release of Mn^{3+} from the active site of the enzyme and stabilizing the metal ion by chelation (2 – 4).

The purpose of this work was to characterize the Mn^{3+}–oxalate complex, a key intermediate in the series of reactions leading to the formation of hydrogen peroxide as a result of oxalate oxidation by MnP. Accordingly, we hereby report the kinetics of Mn^{3+}– oxalate in reactions catalyzed by MnP from *C. subvermispora* in the absence of externally added hydrogen peroxide and test the effects that parameters such as MnP titer, pH, and the concentrations of Mn^{2+} and oxalate might have. The fluctuation of these variables in culture could have a profound effect on the activity of the enzyme. These reactions are followed without the addition of a phenolic substrate so that the primary oxidizing agent generated by MnP, Mn^{3+}–oxalate, could be monitored directly.

MATERIALS AND METHODS

Chemicals. Oxalic acid, hydrogen peroxide, succinic acid, and manganese sulfate were obtained from Merck (Darmstadt, Germany). Glyoxylic acid was purchased from Sigma (St. Louis, MO). Bovine liver catalase and bovine blood superoxide dismutase were obtained from Boehringer Mannheim (Germany). Manganic acetate was from Aldrich (Milwaukee, WI). Stock solutions of Mn^{3+} were prepared fresh each day by dissolving manganic acetate in 96% methanol to a final concentration of 15 mM (14). Hydrogen peroxide concentration was determined by redox titration with potassium permanganate and sulfuric acid as described (15).

Manganese peroxidase production and purification. C. subvermispora strain FP-105752 (Center for Forest Mycology Research of the Forest Product Laboratory, Madison, WI) was used for enzyme production with culture conditions and enzyme purification as previously described (8). MnP was routinely assayed with vanillylacetone as substrate (16) and a unit of activity was defined as the amount of enzyme required to oxidize 1.0 mmol of substrate per minute.

Mn^{3+}–oxalate formation assay. The formation of Mn^{3+}–oxalate complex was continuously measured as an increase in absorbance at 270 nm (4). The amount of Mn^{3+} formed was calculated using an $\varepsilon_{270} = 5600\ M^{-1}cm^{-1}$. This extinction coefficient was determined by adding known amounts of manganic acetate to a solution containing 1.0 mM oxalate, 0.1 mM $MnSO_4$, and 50 mM sodium succinate, pH 4.0. In this system, Mn^{2+} interference was negligible. Mn^{3+}– oxalate complex formed enzymatically was measured in the same solution as above (1 ml) using 0.02 units of MnP instead of manganic acetate. Reactions were initiated by addition of manganese sulfate and they were followed at 30°C for 3 h in a Shimadzu UV160 Spectrophotometer.

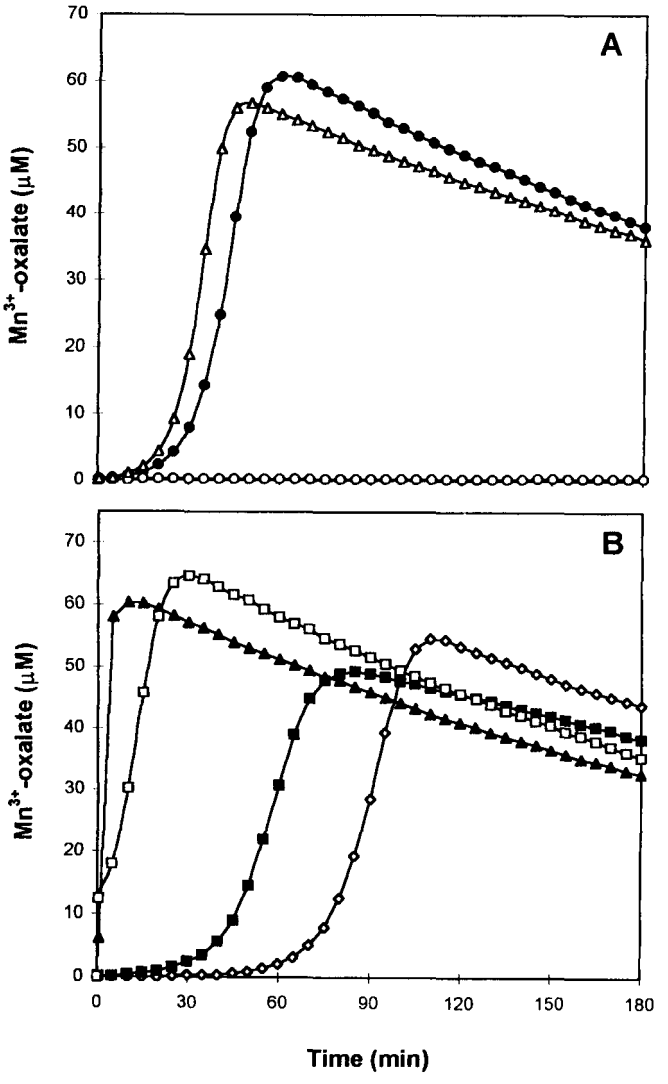

FIG. 1. Mn^{3+}–oxalate kinetics in standard assay with MnP. The formation of the complex in the reaction mixture described under Materials and Methods was monitored at 270 nm (●). Parallel reactions contained, in addition, 0.25 mM glyoxylate (Δ), 5 μM H_2O_2 (▲), 10 μM Mn^{3+} acetate (□), 4 μg SOD (■), 4 μg of catalase (✧), or lacked Mn^{2+} (○).

RESULTS

In our previous studies (8), we determined that the most active mineralization of oxalate in *C. subvermispora* cultures is observed when oxalate and glyoxylate levels are approximately 1 and 0.25 mM, respectively. Accordingly, these concentrations were used for preliminary characterizations of the *in vitro* oxidation of these organic acids (8) and for the kinetic studies of Mn^{3+}–oxalate formation in MnP-catalyzed reactions (Fig. 1). A distinct lag in the formation of Mn^{3+}–oxalate is observed with MnP and oxalate and this lag is shortened by including 0.25 mM glyoxylate (Fig. 1A). As expected, a decrease in the lag is much more pro-

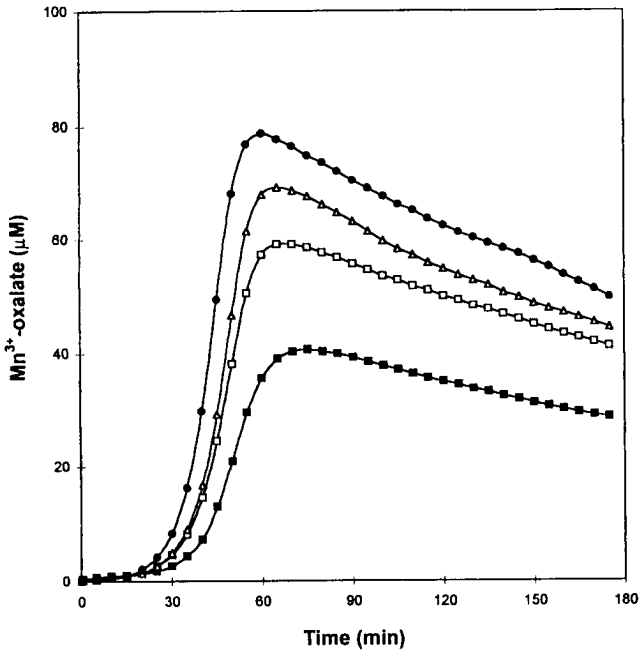

FIG. 2. Effect of MnP titer on Mn^{3+}–oxalate kinetics. The complex was formed in reaction mixtures containing different amounts of enzyme. MnP titers are (units): 0.01 (■), 0.02 (□), 0.04 (Δ), and 0.08 (●).

nounced upon addition of 10 μM Mn^{3+} or 5 μM H_2O_2 (Fig. 1B), the latter being the most effective. In contrast, the lag increases in the presence of superoxide dismutase or catalase (Fig. 1B). This lag in the formation of Mn^{3+}–oxalate, the activating effects of Mn^{3+} and H_2O_2, and the inhibitory effects of SOD and catalase, are all consistent with the oxidation of oxalate as determined by CO_2 evolution (8). A notable characteristic of the kinetic traces in Fig. 1 is that they all peak at approximately 60 μM Mn^{3+}– oxalate and then decay with similar rates.

The titers of MnP were systematically changed to determine the effects on peak concentrations of Mn^{3+}–oxalate formed and the subsequent decay of the complex in reactions containing 1 mM oxalate (Fig. 2). The maximal levels of Mn^{3+}–oxalate were not proportional to MnP titers but instead reached values of decreasing response at higher levels of MnP. Neither was there a direct relationship between Mn^{3+}–oxalate decay rates and MnP titers. A likely contributing factor is the limiting amount of Mn^{2+} in the reaction as the level of Mn^{3+}–oxalate approaches 0.1 mM (the initial concentration of $MnSO_4$). The effect of initial $MnSO_4$ concentrations was determined in the presence of 1 mM oxalate and 0.02 units of MnP (Fig. 3). With 0.04 mM $MnSO_4$, the peak level of Mn^{3+}– oxalate is about half that observed with the standard level of 0.1 mM $MnSO_4$ and the subsequent decay rate is less. With $MnSO_4$ concentrations higher than 0.1 mM, the Mn^{3+}–oxalate peak levels increased but were not proportional to the added $MnSO_4$. The decay of the Mn^{3+}–oxalate, after maximal complex accumulation, was significantly faster at the higher concentrations of $MnSO_4$.

Oxalate concentrations dramatically affected Mn^{3+}–oxalate kinetics: the rate of Mn^{3+}– oxalate formation, the peak levels achieved, and the subsequent decay profiles were distinctive (Fig. 4). At 0.5 mM oxalate, the kinetic trace was similar to that using standard conditions (1 mM oxalate) except that the peak level of Mn^{3+}– oxalate was about half and the lag in its formation was longer. With 2.5 mM initial oxalate, the rate of Mn^{3+}– oxalate formation was slightly faster than with standard reaction conditions and the peak level was significantly higher (about 50%). Furthermore, the subsequent decay of the Mn^{3+}– oxalate appeared to be biphasic with an increased rate of decay after 160 min. At 10 mM oxalate, the rate of Mn^{3+}– oxalate formation was slower, the maximum concentration slightly less, and the subsequent decay rate faster when compared to the standard conditions at 1 mM initial oxalate. At 20 mM oxalate, the asymptotic behavior seemed to be completely abolished with only a very slow increase in absorbance observed over the 180-min time course.

The influence of pH on Mn^{3+}– oxalate kinetics with MnP was also determined (Fig. 5). The highest concentration and sustained level of Mn^{3+}– oxalate was observed at pH 4.0 with standard initial oxalate and $MnSO_4$ concentrations. At pH values above 4.0, the

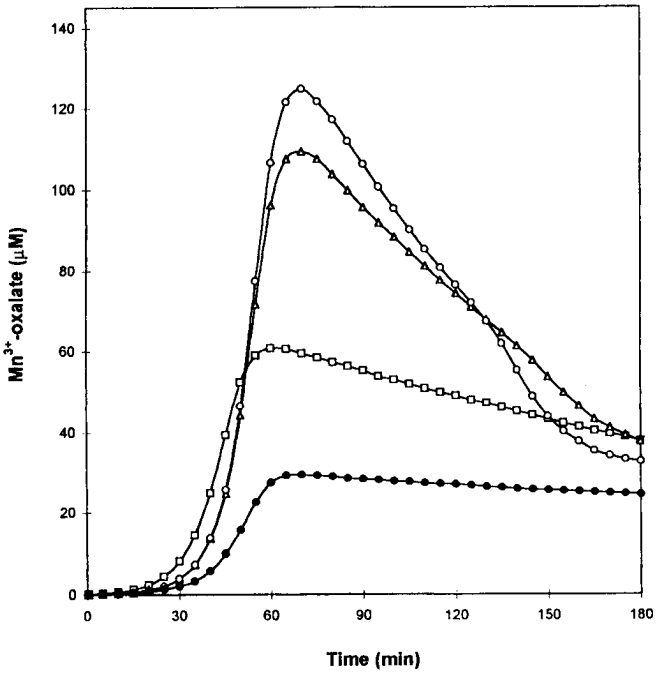

FIG. 3. Effect of initial concentrations of Mn^{2+} on Mn^{3+}–oxalate kinetics with MnP. Concentrations of Mn^{2+} are (mM): 0.04 (●), 0.1 (□), 0.4 (Δ), and 1.0 (○).

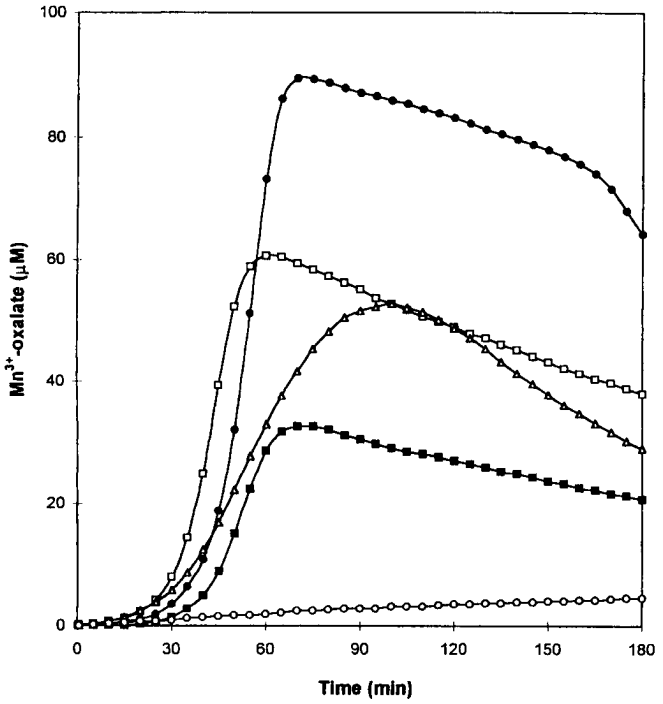

FIG. 4. Effect of initial oxalate concentrations on Mn^{3+}–oxalate kinetics with MnP. Concentrations of oxalate are (mM): 0.5 (■), 1.0 (□), 2.5 (●), 10 (Δ), and 20 (○).

decay rates subsequent to peak complex formation were slower and evidently at pH 6.0 the rate of Mn^{3+}–oxalate formation was so slow that a reflective maximum was not observed during the 3-h time frame. In contrast, decay rates were faster at pH values less than 4.0.

The enzymatic reactions described in Figs. 1–5 show the transient concentrations of the Mn^{3+} complex without direct measure of its rate of formation or decay. To observe the decay kinetics of the complex in the absence of MnP (Fig. 6), the fate of different amounts of chemically formed Mn^{3+}–oxalate was monitored at 270 nm under conditions similar to those employed in MnP-catalyzed reactions (i.e., pH 4.0, 1 mM oxalate, but lacking 0.1 mM $MnSO_4$). Kinetic analysis of the decay reaction was made according to the half-life method described by Levine for reactions with a rate equation of the type $v = k\,[S]^n$, where k is the rate constant and n is the order of the reaction (17). Calculations according to this procedure gave an order of 3/2 in the range of 30–100 μM of complex, with a $k_{obs} = 0.012\ min^{-1}M^{-0.5}$. Addition of MnP at any time during the course of decay restored the complex to the original level (Fig. 7). Thereafter, Mn^{3+}–oxalate decomposed at the rate characteristic of reactions containing the enzyme (see Figs. 1–5).

Similarly, the effects of oxalate and pH on the nonenzymatic decay of the Mn^{3+}–oxalate were determined with chemically generated complex. Oxalate concentration dramatically affected decay rates with the slowest decomposition occurring at high oxalate concentrations (Fig. 8). The pH effect was also significant, with fastest decay at low pH (Fig. 9).

DISCUSSION

We recently proposed a pathway for the production of extracellular hydrogen peroxide in cultures of *C. subvermispora,* which is based on the MnP-catalyzed formation of Mn^{3+}–oxalate complex (8). In the present work we have analyzed the *in vitro* kinetics of this complex under various conditions, because neither fungal culture conditions nor the activities of their enzymes remain constant with time *in vivo.* Parameters that are expected to have considerable effect on MnP activity include the enzyme titers, manganese concentration, chelator identities, chelator concentrations, and pH.

From our earlier studies with cultures of *C. subvermispora,* the highest rates of oxalate oxidation occur when the levels of oxalate and glyoxylate are about 1 and 0.25 mM, respectively. However, these conditions do not remain invariable and the oxalate oxidation rates are not always reflected by the levels of any one parameter, including those of MnP and oxalate. In

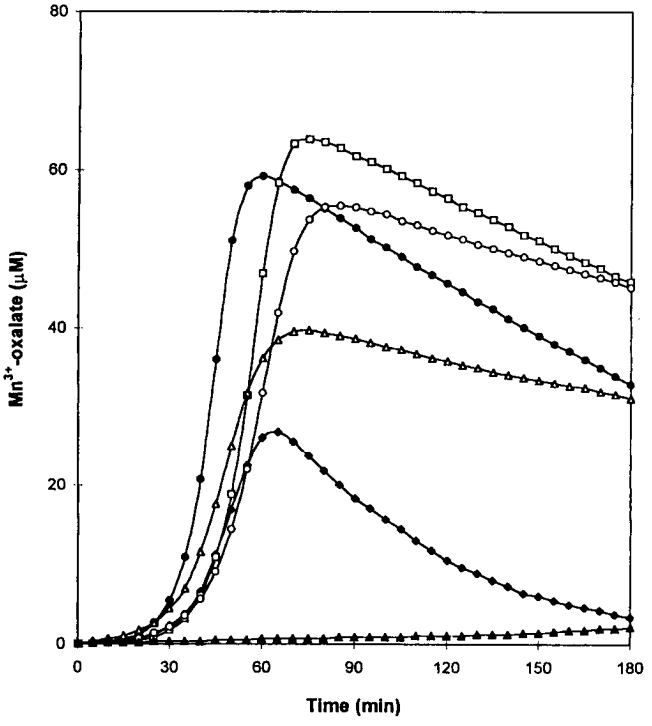

FIG. 5. Effect of pH on Mn^{3+}–oxalate kinetics. The buffer was always 50 mM sodium succinate, adjusted to following pH values: 3.0 (◆), 3.5 (●), 4.0 (□), 4.5 (○), 5.0 (Δ), and 6.0 (▲).

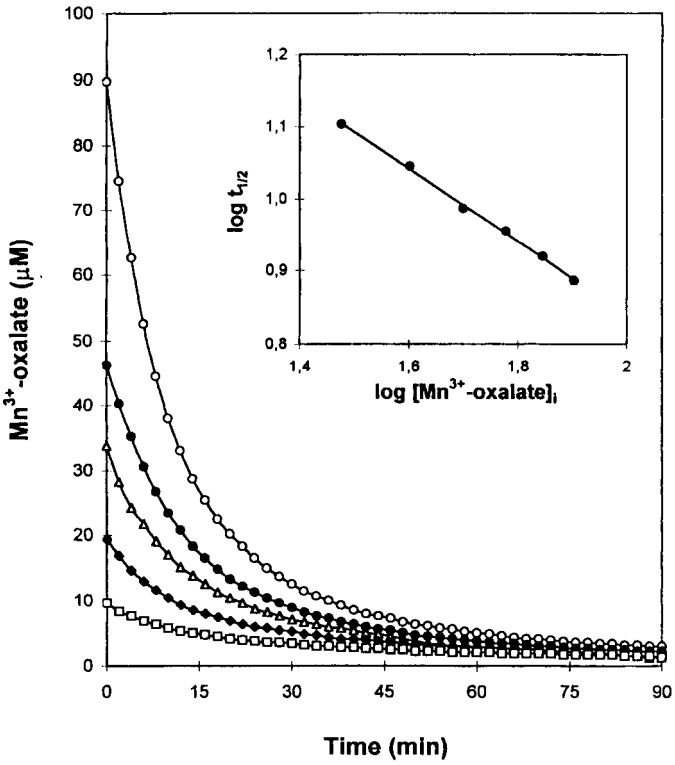

FIG. 6. Decomposition of chemically formed Mn^{3+}–oxalate in the absence of MnP. 1-ml solutions containing 1 mM oxalate in 50 mM sodium succinate, pH 4.0, but lacking $MnSO_4$, were supplemented with the following amounts of manganic acetate (nmol): 90 (○), 47 (●), 34 (Δ), 19 (◆), and 10 (□). Thereafter, the respective decays were recorded at 270 nm. The *inset* illustrates log $t_{1/2}$ as a function of Mn^{3+} concentration during the decomposition of the complex initially present at 90 μM.

these reactions with Mn^{+2} and oxalate, the oxalate is oxidized as the result of Mn^{3+}– oxalate decomposition. Therefore, insights into the factors influencing the oxidation of oxalate by MnP must take into account not only the kinetics of the enzyme but also the reactivity of the Mn^{3+} complex.

The high affinity between manganic ion and oxalate results in the formation of complexes which can be in a Mn^{3+}:oxalate ratio of 1:1, 1:2, or 1:3 (18–20). The specific rate constants of decay of these complexes are 11.8,0.046, and 0.0205 min^{-1}, respectively (18). The three complexes are known to be in rapid equilibrium with one another and their relative fractions in solution can be determined using the respective binding constants (4, 19). In the presence of oxygen, the Mn^{3+}– oxalate complex decomposes according to the following set of reactions:

$$2\ Mn^{3+}\text{–oxalate} \rightarrow 2\ Mn^{2+} + 2\ \dot{C}O_2^- + 2\ CO_2 + 2\ H^+$$

$$2\ \dot{C}O_2^- + 2\ O_2 \rightarrow 2\ \dot{O}_2^- + 2\ CO_2 + 2\ H^+$$

$$2\ \dot{O}_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Calculation of the net reaction is not simple, because besides dismutating, superoxide also contributes to regenerate some Mn^{3+}–oxalate complex through oxidation of Mn^{2+}, with the concomitant formation of additional hydrogen peroxide (10):

$$\dot{O}_2^- + Mn^{2+} + 2\ H^+ \rightarrow Mn^{3+} + H_2O_2$$

The latter reaction may proceed until it reaches an equilibrium, since hydrogen peroxide can in turn reduce Mn^{3+} to Mn^{2+} (14). Also, as oxygen becomes limiting, the formate radical may directly reduce Mn^{3+} to Mn^{2+} (21). Therefore, the complexity of the system may account for the deviation from first order in the decomposition of Mn^{3+}– oxalate, as observed previously by Launer (21), as well as by Anderson and Kochi (22).

The kinetic behavior of Mn^{3+}– oxalate is subjected to additional variables in reactions catalyzed by MnP. Not only does the reactivity of the decomposing Mn^{3+}–oxalate have to be considered, but also the stoichiometry of the Mn^{2+}–oxalate complexes (can be 1:1 or 1:2) and the reactivities of the enzyme intermediates with the complexes and resulting products. Kuan et al. (4) studied the reactions of the free and chelated Mn^{2+} with compounds I and II enzyme intermediates of MnP from *P. chrysosporium*. The optimum pH for turnover

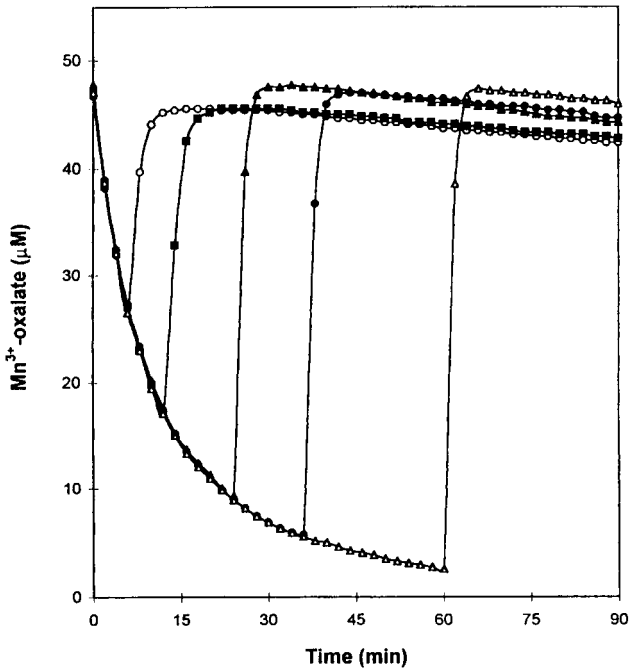

FIG. 7. Effect of MnP addition during decay of chemically formed Mn^{3+}–oxalate complex. MnP (0.04 units) was added to a solutions containing 1 mM oxalate and 47 μM manganic acetate in 50 mM sodium succinate, pH 4.0, at the following time intervals (min): 6 (○), 12 (■), 24 (▲), 36 (●), 60 (Δ).

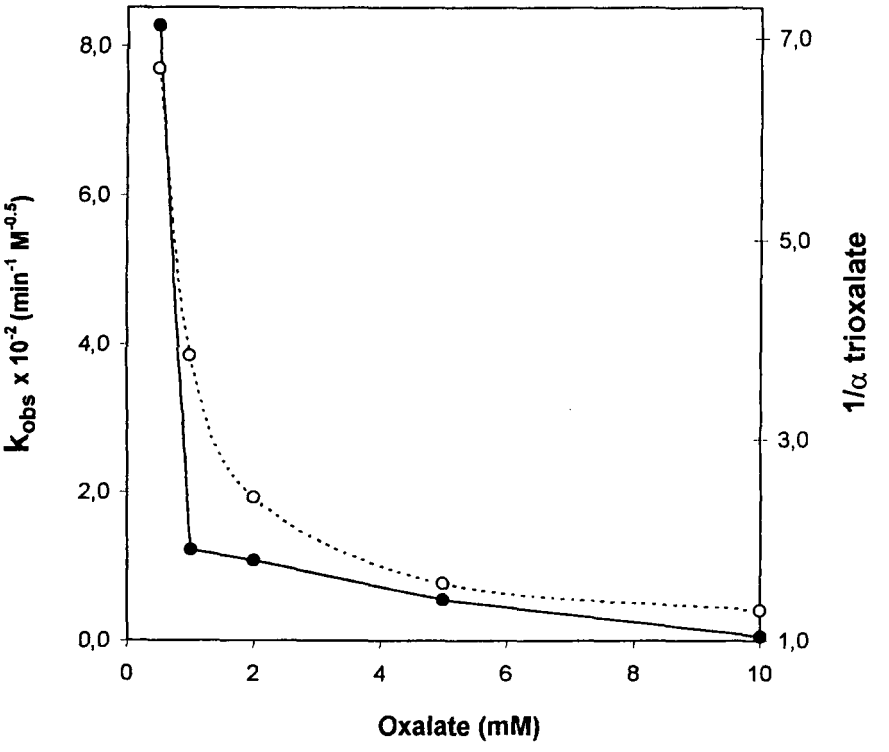

FIG. 8. Effect of oxalate on the decay of chemically formed complex. In these experiments, the complex was formed by adding 50 nmol of Mn^{3+}–acetate to 1 ml solutions containing the indicated concentration of oxalate and 100 mM sodium succinate, pH 4.0, as buffer. For each reaction, the k_{obs} was calculated according to the half-life method described by Levine (17). The dotted line indicates the reciprocal of the fraction (α) of trioxalate Mn^{+3} complex at different concentrations of oxalate (see Discussion).

is pH 4.5 and at this pH the reaction with compound I is too fast to study. Although Kuan *et al.* initially concluded that the 1:1 Mn^{2+}– oxalate complex is the preferred substrate for the oxidation with compound II, it has been recently confirmed that Mn^{2+}– oxalate does not bind to MnP (23). The latter report is in agreement with previous observations by Wariishi *et al.* (3), who had indicated that the free (hexa-aquo) Mn^{2+} is readily oxidized. These authors found no relationship between stimulation of enzyme activity and chelator size, indicating that the substrate is free Mn^{2+} rather than the complex. Kishi *et al.* (2) suggest that the significant stimulation of MnP activity by oxalate is due to its chelation with Mn^{3+}, thereby facilitating the removal of the ion from the enzyme active site. Maximal stimulation of compound II reduction is observed at about 2 mM oxalate (2, 4).

The kinetic traces in Fig. 1 are in good qualitative agreement with our previous experiments (8) where additions of H_2O_2, glyoxylate, or Mn^{3+} were tested for their effects on the lag on CO_2 evolution from oxalate. Interestingly, the levels of peak Mn^{3+}– oxalate concentrations are similar in all cases (approximately 60 μM). At this point of inflection, the rate of Mn^{3+}– oxalate production is equal to the rate of its decomposition. The rate of Mn^{3+}–oxalate decomposition at this concentra-

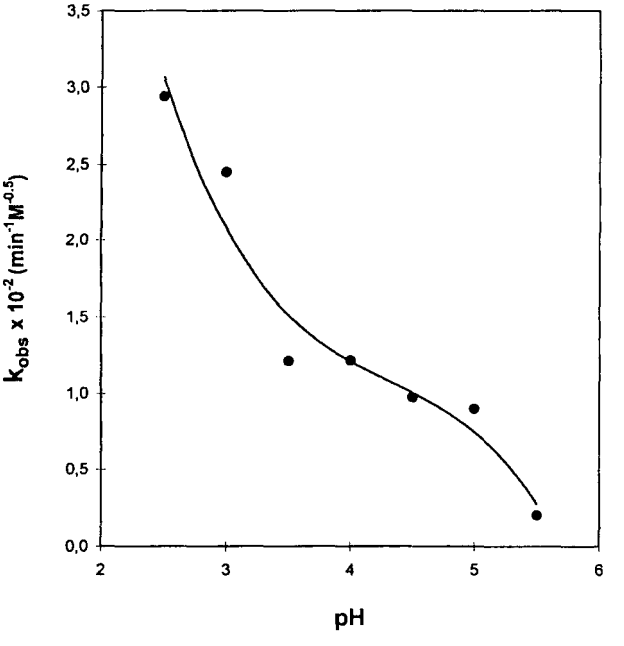

FIG. 9. Effect of pH on the decay of Mn^{3+}–oxalate. The 50 mM sodium succinate buffer was adjusted to the indicated pH values prior to forming the complex. Its decay was thereafter monitored at 270 nm and values of k_{obs} were obtained as indicated in the legend to Fig. 8.

tion can be estimated from the decay traces of Fig. 6 and is approximately 5.6 μM min^{-1} (i.e., $0.012 \times 60^{3/2}$) and therefore it represents an estimate of MnP activity in terms of Mn^{3+}– oxalate produced.

However, the maximal concentration of Mn^{3+}– oxalate observed in reactions is not always proportional to MnP titers (Fig. 2). This nonlinear response could be due, at least in part, to oxygen limitation. Another plausible explanation is that the theoretical limit for Mn^{3+}– oxalate formation is 100 μM, the initial concentration of $MnSO_4$ supplied to the reactions. Therefore the maximal Mn^{3+}– oxalate would be expected to asymptotically approach 100 μM with increasing MnP. How close the Mn^{3+}– oxalate levels approach the theoretical limit will be affected by the K_m of MnP for the depleting levels of Mn^{2+} and by the rate of nonenzymatic decay of the complex, which is faster at higher concentrations. The limiting effect of manganese can be better appreciated in the kinetic traces of Fig. 7, in which addition of MnP restores the level of complex to just about the concentration of Mn^{3+} added initially to the reaction.

Available Mn^{2+} in reactions can have additional effects besides the maximal concentration of Mn^{3+}– oxalate produced (Fig. 3). In reactions where the initial oxalate concentration was 1 mM and the concentration of $MnSO_4$ was varied, the maximal level of Mn^{3+}– oxalate produced was observed with 1 mM $MnSO_4$. Curiously, the rate of complex decomposition at the higher levels of $MnSO_4$ appear exceptionally high after the inflection point. This possibly could be due to the catalase activity of MnP destroying H_2O_2 when unchelated Mn^{2+} is present (24). This would effectively subterfuge the H_2O_2-dependent oxidations of MnP, including the oxidation of Mn^{2+} to Mn^{3+}. A second plausible reason for the rapid decay of Mn^{3+}– oxalate at higher concentrations of Mn^{2+} is that oxygen might be limiting. For example, at 0.4 mM $MnSO_4$, Mn^{3+}–oxalate reaches a maximum near 100 μM. At this concentration, the Mn^{3+}– oxalate complex is expected to decompose at approximately 12 μM min^{-1} ($0.012 \times 100^{3/2}$) and to consume oxygen at approximately half that rate. Given that the concentration of O_2 in aqueous solution is 240 μM (25), it is reasonably assumed that the oxygen is near depletion after about 40 min. The return to Mn^{3+}– oxalate levels around 40 PM might be a reflection of rate-limiting diffusion of oxygen into the solution.

Oxalate concentration also has a dramatic effect on maximal Mn^{3+}– oxalate levels reached in reactions with MnP and 0.1 mM $MnSO_4$ (Fig. 4). Since free Mn^{2+} (hexa-aquo) is the reported substrate for MnP (3, 23), the lack of correlation between oxalate concentration and the peak Mn^{3+}– oxalate levels obtained above 2.5 mM oxalate could be due to an increase in the fraction of the mono and dioxalate complexes of Mn^{2+} (26). On the other hand, an interpretation of the results shown in Fig. 4 must take into consideration the stability of the manganic complexes to spontaneous decay (Fig. 8). Examples to illustrate this argument are the kinetic responses at 1.0 and 2.5 mM oxalate. It would be predicted that if there is a slower decay of the complex at 2.5 mM and if there is equal MnP activity at the two oxalate concentrations, then the maximal Mn^{3+}– oxalate concentration is expected to be higher with 2.5 mM. This is what is observed (Fig. 4). On the other hand, higher concentrations of oxalate favor the formation of di- and trioxalate complexes, which are more stable than the monooxalate complex (18). The relative fraction of each of the three Mn^{+3} complexes with divalent oxalate was calculated (based on initial conditions) and possible correlations with k_{obs} were tested. As it might be predicted by the relative stability of the trioxalate complex, the rate of decay decreases as the concentration of the latter increases. This is confirmed by the correlation observed between the k_{obs} and the reciprocal of the fraction of trioxalate complex (Fig. 8). In these experiments, the concentration of monooxalate complex is negligible. However, this predictive theory based on increased stability of the Mn^{3+}– oxalate at higher oxalate concentrations totally fails at 10 and 20 mM oxalate. Our results suggest that there is a MnP response to higher concentrations of oxalate that is not readily explained by the presteady-state kinetics of MnP with oxalate complexes (4) or by the stability of the complexes. This might also provide insight to why Wariishi *et al.* (3) found no relationship between stimulation of enzyme activity and chelator size when tested at 50 mM.

Maximal levels of complex formation were observed at pH 4.0 with MnP (Fig. 5). This PH value is close to the pK_{a2} of oxalate and is slightly less than the PH optimum for the MnP-catalyzed oxidation of vanillylacetone (27). We have shown that chemically generated complex is more stable when both carboxyls are ionized (Fig. 9), which is consistent with previous results using different oxalate concentrations (18). The increased stability of the complex at higher pH most likely decreases its reactivity with peroxidase substrates. However, we show here that a similar pH optimum is observed even without an aromatic substrate present.

The results presented here support our recently proposed model for extracellular hydrogen peroxide production by *C. subvermispora* that is based on the MnP-dependent oxidation of carboxylic acids secreted by the fungus (8). Indeed, we have confirmed both the formation and the decay of the Mn^{3+}– oxalate complex under conditions mimicking those of the cultures. As mentioned above, the optimum pH for complex formation is in the same range as for MnP activity and for degradation of synthetic lignin (27). The decay of the Mn^{3+}– oxalate at this pH occurs at a rate that appears com-

patible with the maintenance of a sustained level of complex. Glyoxylate stimulates the formation of the complex, providing an explanation for the increased levels of mineralization of ^{14}C-labeled oxalate observed *in vitro* when glyoxylate is present (8). Finally, oxalate was supplied in our experiments at defined initial concentrations which is in contrast to the continuous secretion of this metabolize during wood decay by the fungus. Oxalate stabilizes the complex and extracellular concentrations of this organic acid above critical levels would hinder the steady production of the H_2O_2 required by MnP. This requirement is at least fulfilled in liquid cultures where the concentration of oxalate reaches a maximum of 2.5 mM (8).

ACKNOWLEDGMENTS

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