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Catalysis of Novel Enzymatic Iodide Oxidation by Fungal Laccase

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ABSTRACT

A fungal laccase (*Myceliophthora thermophila*) has been shown to function as an iodide oxidase. Unlike other halides which interact with the type 2 copper site and are inhibitors for the laccase, iodide interacts with the type 1 copper site and serves as a substrate capable of donating an electron to the laccase. Under anaerobic conditions, the interaction between the laccase and iodide results in the reduction of the laccase type 1 copper and the concomitant oxidation of iodide to form iodide. In aerated solutions, the laccase catalyzes the oxidation of iodide to iodine and the concomitant reduction of dioxygen to water. The reaction exhibits typical Michaelis kinetics with a K_m of $0.16 \pm 0.02M$ and a k_{cat} of 2.7 ± 0.2 turnovers per min at the optimal pH (3.4). The catalysis can be enhanced by 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid), which shuttles electrons rapidly between iodide and the laccase. Bilirubin oxidase also demonstrates significant iodide oxidase activity, suggesting that the property could be a common feature for copper-containing oxidases. Possible industrial and medicinal applications for a laccase-based iodine production system are discussed.

Index Entries: Iodide oxidase; laccase; bilirubin oxidase; copper-containing oxidase; fungi; iodine.

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INTRODUCTION

The inhibitory effect of halides has been well-characterized in the past for certain copper-containing oxidases, such as *Rhus* laccase, ceruloplasmin, and tyrosinase (*1–5*). In general, the inhibitory potency of fluoride (F^-), chloride (Cl^-), and bromide (Br^-) is inversely proportional to their radii, possibly due to an access limitation for their bindings to the oxidases; however, iodide (I^-) often defies the order (*6*). For plant and fungal laccases, the interaction with F^- has been used to probe some of their molecular properties, such as redox potential, active copper site, and catalytic mechanism (*7*). Various physical characterizations indicate that the interaction takes place at the type 2 copper site, resulting in an interruption of the electron flow from the donor (bound at the type 1 site) to O_2 (*8*). Similar inhibitory interactions of Cl^- and Br^- with a fungal laccase (*Polyporus versicolor*) have also been reported (*9*). In contrast, the literature contains very little information regarding any interaction between I^- and a laccase.

Our recent work comparing the catalytic properties of various fungal laccases has led to the disclosure that, unlike other halides, I^- can be processed as a substrate by laccase as well as bilirubin oxidase (another multi-copper oxidase [*10,11*]). We report here an investigation on the interaction between I^- and laccase under both anaerobic and steady-state conditions, and discuss potential applications of laccase-based iodine production systems. Part of this work has been disclosed previously in abstract form (*12*).

MATERIALS AND METHODS

Materials

Chemicals used as buffers and substrates were commercial products of at least reagent grade. Sodium fluoride (NaF), sodium chloride (NaCl), sodium bromide (NaBr), and sodium iodide (NaI) of ≥99.99+% purity and NaI of 98% purity (Aldrich, Milwaukee, WI) were used. Ascorbate oxidase (*Cucurbita*), ceruloplasmin (porcine), bilirubin oxidase (*Myrothecium verrucaria*), and tyrosinase (mushroom) were purchased from Sigma (St. Louis, MO). The recombinant *Myceliophthora thermophila* laccase (MtL; MW[SDS-PAGE]: ~70 kDa, A_{280}/A_{600}: 35, Cu/subunit: 4), expressed in an *Aspergillus oryzae* strain, was purified chromatographically on Q-Sepharose and Mono-Q (*13,23*). Spectroscopic assays were conducted either on a spectrophotometer (UV160U; Shimadzu, Kyoto, Japan) with quartz cuvet (1-cm path length) or on a plate reader (Thermomax; Molecular Devices, Menlo Park, CA) with 96-well plate (tissue-culture; Costar, Cambridge, MA). Britton & Robinson (B & R) buffers were made by mixing a stock solution of boric acid/acetic acid/phosphoric acid (0.1/0.1/0.1*M*) with 0.5*M* NaOH to desired pH. All experiments were carried out at 20°C.

Enzymatic Assay

Typical buffered solutions contained 0.2–20 μM MtL and 10–200 mM NaI. Triiodide (I_3^-) formation was spectrally monitored at 353 nm in quartz cuvet with an extinction coefficient of 26.0 + 2.2 [I^-] $mM^{-1}cm^{-1}$ (*14*). The oxidation of 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) ws monitored at either 405 nm (in 96-well plate) or 418 nm (in quartz cuvet), with an extinction coefficient of 36 $mM^{-1}cm^{-1}$ at 418 nm (*15*). The K_m and k_{cat} constants were obtained from initial rate (v), substrate concentration (S), and enzyme concentration (E) with nonlinear regression fitting to $v = k_{cat}*E*S/(K_m + S)$ with the Prizm program (GraphPad, San Diego, CA).

Halide inhibition analyses for NaF, NaCl, and NaBr were performed with 50 nM MtL and 1 mM ABTS in B & R buffer, pH 4.1. The parameter I_{50}, the inhibitor concentration that resulted in 50% remaining laccase activity, was used to compare the inhibition potency of the halides.

Anaerobic Reduction of MtL by I^-

After repetitive evacuation at ≤ 2 torr and argon (99.999%) flushing at 4°C, MtL solutions (30–60 μM) were mixed anaerobically with NaI (5–20 mM) (stored at a side-arm compartment of the cuvet apparatus) at 20°C and spectral changes were monitored at 600 nm in quartz cuvet. The MtL reduction was shown by the disappearance of the absorbance band near 600 nm, corresponding to the reduction of the type 1 copper (II) to copper (I).

RESULTS

Anaerobic Reduction of MtL by I^-

Under anaerobic conditions, the addition of NaI to MtL resulted in a decrease of the absorption near 600 nm and a reduction of the type I copper (II) in MtL (Fig. 1). The disappearance of the absorption band near 600 nm accompanied by the appearance of an absorption band near 350 nm that was congruent with the characteristics of I_3^- (*14*). Apparently, an electron exchange took place between I^- and MtL, resulting in the formation of the reduced MtL and I_3^-.

I^- Oxidation Catalyzed by MtL

Under aerobic conditions, MtL reduced by I^- could be re-oxidized by O_2 and, by doing so, catalyze the coupled oxidation of I^- by O_2. When NaI and MtL were mixed in aerated solutions, a characteristic yellow or violet color (depending on the concentration of the product) developed, whose spectrum indicated the formation of I_3^- (Fig. 2A). Under our conditions, the nonenzymatic I^- oxidation was negligible (data not shown).

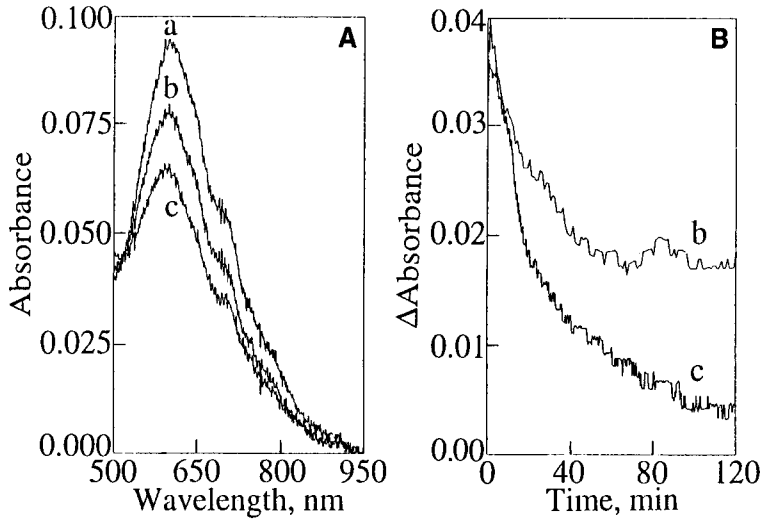

Fig. 1. The anaerobic reduction by MtL by NaI. (**A**) MtL (31 μM) in 8 mM MES, pH 5.3, was anaerobically mixed at 20°C with (a) 0, (b) 5, and (c) 19 mM NaI. The spectra was recorded after the spectral change at 600 nm became stationary. (**B**) The time profiles of the reduction with (b) 5 and (c) 19 mM NaI. The initial rate of (b) and (c) was estimated as 0.21 or 0.71 μM min^{-1}, respectively.

The rate of the catalyzed I^- oxidation was proportional to MtL concentration over the range tested (Fig. 2B). In contrast, only ≤ 5% of the catalytic activity was observed when MtL was heat-inactivated (data not shown). As tested by its ABTS oxidase activity, no inactivation of MtL by I^- at concentration levels up to 0.1M was observed under our conditions.

The MtL-catalyzed I^- oxidation exhibited a typical Michaelis-Menten pattern (data not shown). The catalysis was optimal at pH 3.4 and minimal above pH 6.0. Figure 3 shows the pH dependence of the apparent K_m and k_{cat}. At the optimal pH (3.4), a K_m of 0.16 ± 0.02M and a k_{cat} of 2.7 ± 0.2 turnovers per min (tpm) were observed.

The correlation slope in Fig. 2B yielded a rate of 1.2 tpm with 0.4M NaI in B & R buffer, pH 4.1. Applying the K_m of 0.7M and k_{cat} of 5.2 tpm observed at the same pH, a rate of 1.9 tpm could be calculated. In Fig. 1B, the initial spectral changes of the anaerobic reduction of 31 μM MtL by 5 or 19 mM NaI yielded a rate of 0.21 or 0.71 μM min^{-1} in 8 mM MES, pH 5.3, respectively, while based on the K_m of 3.9M and k_{cat} of 4.3 tpm observed in B & R buffer, pH 5.2, a rate of 0.17 or 0.65 μM min^{-1} could be calculated, respectively. These results suggest that the reduction of the type 1 Cu (II) in MtL by I^- is the rate-limiting step in MtL-catalyzed I^- oxidation by O_2.

When a less pure NaI sample (98%) was used instead of the 99.99+ % sample, ≤ 5% difference in the rate of MtL-catalyzed I^- oxidation was observed. When the MtL sample prepared in 10 mM Tris, pH 7.6, was

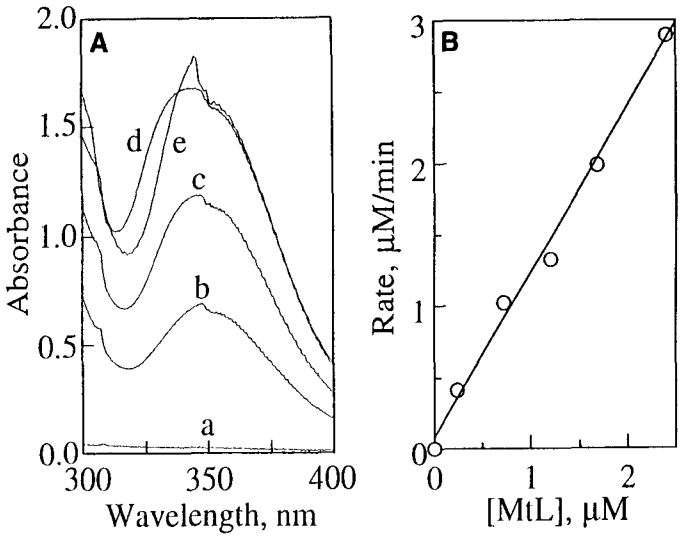

Fig. 2. I^- oxidation catalyzed by MtL. (**A**) The formation of NaI_3 from NaI and O_2 under the catalysis of MtL. The reaction was carried out in 0.1*M* potassium phosphate, pH 2.75, with 0.3 μ*M* MtL and 0.1*M* NaI. The spectra was recorded at (a) 0, (b) 1.3, (c) 3.3, and (d) 6.7 min. The spectrum (e) corresponded to 0.1*M* NaI and 0.060 m*M* I_2 mixed in the buffer with 0.01% ethanol present (come from I_2 stock solution). (**B**) The dependence of the initial I^- oxidation rate on the concentration of MtL. The measurement was carried out with 0.4*M* NaI in B&R buffer, pH 4.1. The correlation line yielded a slope that corresponded to a rate constant of 1.2 tpm.

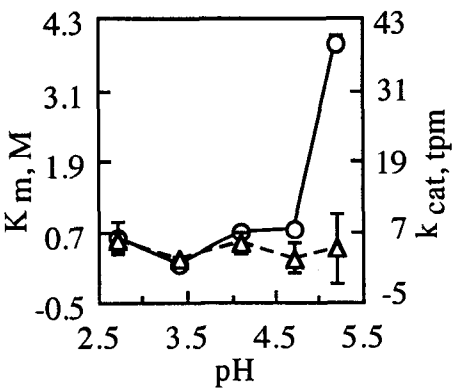

Fig. 3. The pH dependence of K_m (○) or k_{cat} (△) for the MtL-catalyzed I^- oxidation. The experimental details for determining these parameters are given in the Materials and Methods section.

compared to the MtL sample prepared in 18 m*M* citrate, 0.15*M* NaCl, pH 7.0, ≤10% difference in the rate of MtL-catalyzed I^- oxidation was observed. This indicated that other components in the assay solution did not contribute significantly to the catalysis (except for providing buffer condition).

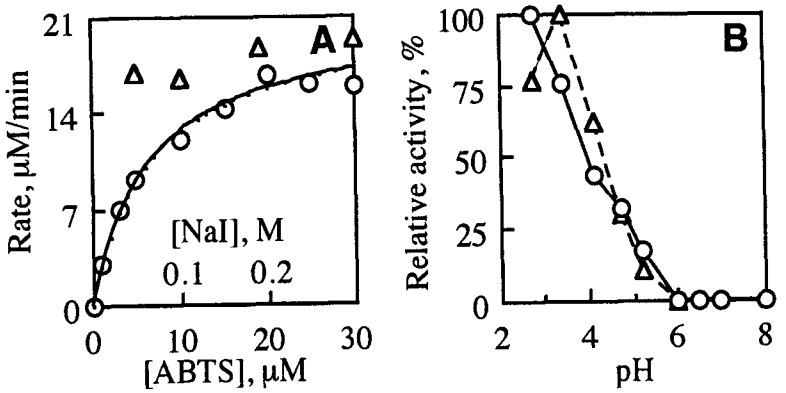

Fig. 4. The enhancement of the MtL-catalyzed I^- oxidation by ABTS. (**A**) The dependence of the initial I^- oxidation rate on ABTS concentration (○) with 0.1*M* NaI; or on NaI concentration (△) with 10 μ*M* ABTS in B&R buffer, pH 4.1, with 24 n*M* MtL. The curve represents the Michaelis-Menten equation fitting result with a K_m of 6.4 ± 0.8 μ*M* and a k_{cat} of 860 ± 40 tpm. (**B**) The pH profiles of the relative catalytic activity. The concentrations were: (○) 0.1*M* NaI, 2.4 n*M* MtL, 10 μ*M* ABTS; (△) 0.1*M* NaI, 14 μ*M* MtL, no ABTS. The optimal I^- oxidation rate was taken as 100%.

Enhancement of the Catalysis by an Active Redox Mediator

ABTS was readily oxidized by MtL and was able to significantly enhance the catalysis of MtL in I^- oxidation. When 20 μ*M* ABTS and 2 n*M* MtL were mixed, the oxidation of ABTS to ABTS cation radical was finished in 5 min at pH 4.1, characterized by the formation of three intense absorbance bands over 600–900 nm (*15*). However, in the presence of 40 m*M* NaI, no detectable ABTS oxidation was observed for 4 h, while I_3^- steadily accumulated with a rate that was significantly faster than the rate observed in a solution in which ABTS was excluded. As shown in Fig. 4A, the presence of ABTS made the initial I^- oxidation rate depend on ABTS concentration (in typical Michaelis-Menten manner), but not on I^- concentration. The apparent K_m of 6.4 ± 0.8 μ*M* and k_{cat} of 860 ± 40 tpm observed for the rate-dependence of the I^- oxidation on the concentration of ABTS are close to the K_m of 5.6 ± 0.5 μ*M* and k_{cat} of 1460 ± 31 tpm observed for the oxidation of ABTS itself catalyzed by MtL. As shown in Fig. 4B, the presence of ABTS also shifted the optimal pH of the I^- oxidation and resulted in a pH profile similar to that for the MtL-catalyzed oxidation of ABTS itself (data not shown).

ABTS cation radical was kinetically competent to oxidize I^-. The reaction between 20 μ*M* cation radical (prepared by pre-incubation of 20 μ*M* ABTS and 2 n*M* MtL for 5 min) and 40 m*M* NaI at pH 4.1 was completed within 15 s, as demonstrated by the disappearance of the absorbance bands over 600–900 nm. However, no reaction between ABTS and NaI or I_2 was detected during 1 h under the same conditions.

Inhibition of MtL by Halides

Addition of NaF, NaCl, or NaBr could cause fast inhibition of MtL. At saturating ABTS concentration (1 m*M*) and pH 4.1, an I_{50} of 0.05, 600, and 1600 m*M* was obtained for NaF, NaCl, and NaBr, respectively. The inhibition was of mixed-type nature and was reversible, since a dialysis or dilution that reduced the halide concentration well below its I_{50} could lead to the recovery of the laccase activity. When ABTS was replaced by I^- as the substrate for MtL, the same I_{50} of 600 m*M* was observed for Cl^- inhibition.

I^- Oxidation Catalyzed by Other Copper-Containing Oxidases

Similar to MtL, bilirubin oxidase was capable of catalyzing the I^- oxidation at significant rate. At pH 4.1, it possesses a K_m of 0.15 ± 0.03*M* and a k_{cat} of 1.7 ± 0.1 tpm. The rate of the catalyzed I^- oxidation was proportional to the concentration of bilirubin oxidase over the range of 0.5–5 μM studied. At pH 2.7, 5.2, 6.0, 7.0, 8.2, and ≥9.0, the activity was 95, 91, 80, 59, 32, and ≤6% of that at pH 4.1, respectively.

Bilirubin oxidase effectively catalyzed ABTS oxidation with rates proportional to its concentration over the range of 1–10 n*M* tested. The optimal activity was found at pH 4.1 with a K_m of 0.12 ± 0.01 m*M* and a k_{cat} of 1310 ± 40 tpm. Addition of ABTS could enhance the oxidase-catalyzed I^- oxidation. The presence of only 10 μM ABTS boosted the rate of the I^- oxidation catalyzed by bilirubin oxidase 50-fold. At the optimal pH (4.1), the fitting of the initial I^- oxidation rate and ABTS concentration to Michaelis-Menten equation resulted in a K_m of 0.18 ± 0.02 m*M* and a k_{cat} of 600 ± 40 tpm.

At pH 5 to 6, ascorbate oxidase and ceruloplasmin at concentrations up to 1.5 μM showed low I^--oxidase activity in the presence of 0.1*M* NaI (rate ≤0.1 tpm). Tyrosinase had a K_m of 0.1*M* and a k_{cat} of 0.1 tpm at pH 2.7 for I^- oxidation with a rate proportional to tyrosinase concentration over the range of 2–20 μM studied. The activity at pH 4.1, 5.2, 6.0, and ≥7.0 was 58, 33, 10, and ≤5% of that at pH 2.7, respectively.

At pH 5 and concentrations up to 1.5 μM, ascorbate oxidase showed no ABTS oxidase activity. Tyrosinase catalyzed the oxidation of ABTS with rates proportional to its concentration over the range of 0.2–2 μM tested. It had an optimal activity at pH 2.7 with a K_m of 0.18 ± 0.01 m*M* and a k_{cat} of 3.1 ± 0.1 tpm. Ceruloplasmin also catalyzed ABTS oxidation with rates proportional to its concentration over the range of 0.1 to 1 μM tested. It had an optimal activity at pH 4.1 with a K_m of 0.11 ± 0.04 m*M* and a k_{cat} of 9.3 ± 1.3 tpm. For these enzymes, the enhancement of the catalyzed I^- oxidation by the addition of ABTS in the solutions was moderate (rates ≤ 10 tpm).

DISCUSSION

As shown before, F^-, Cl^-, and Br^- inhibit fungal laccase activity with a potency in the reverse order of their ionic radius (*9*). The inhibition is interpreted as being realized by the halide binding to the type 2 copper and affected by the defined size of the access channel in a laccase (*8*). Our study demonstrates that unlike other halides, I^- serves as a substrate for MtL (similar I^- oxidase activity has also been observed with *Polyporus pinsitus* and *Scytalidium thermophilium* laccases [*16*]). Unlike the reaction between free Cu (II) (aq) and I^- that produces insoluble CuI, the redox reaction between I^- and MtL does not generate any type 1 Cu (I) -I complex, a product which would inactivate MtL and transform its spectroscopic characteristics. After reducing the type 1 Cu (II) in MtL, I^- is oxidized to $I\cdot$ and I_2 (or I_3^-, in the presence of excess I^-) is steadily formed. The pseudo-first-order rate constants obtained from the anaerobic reduction of the type 1 copper in MtL by I^- and the MtL-catalyzed I^- oxidation in aerated solution are similar, indicating that the electron-exchange between I^- and MtL takes place at the type 1 copper site and is the rate-limiting step in the catalysis.

Although the catalysis of MtL has a typical Michaelis-Menten kinetics pattern, I^- possesses a K_m near 0.1*M*. Since no kinetic difference is observed in regard to the purity of NaI samples (98% or 99.99+%), the buffered solution sources (B & R, potassium phosphate, or MES), and the MtL samples (prepared in 10 m*M* Tris, pH 7.5, or in 18 m*M* citrate, 0.15*M* NaCl, pH 7), the catalysis is attributed to a direct interaction between I^- and MtL, not from a mediation of some redox-active impurities in the solution similar to the mediation of ABTS. Probably, an electron is transferred from I^- to the type 1 copper, which in turn transfers the electron to the trinuclear cluster (comprised by the type 2 and type 3 coppers) where O_2 is reduced to H_2O (*1–5*). However, whether the electron exchange between I^- and laccase's type 1 copper is proceeded in one single step or involves transient yet distinct intermediates remains unclear. Future experiments, such as stopped-flow kinetic analysis and rapid-freeze EPR, would be useful for better understanding of the unusual interaction between I^- and laccase. Nevertheless, the reaction described in this report further expands the scope of chemicals that can serve as substrate for laccase and other copper-containing oxidases.

ABTS cation radical readily oxidizes I^- in solution, while ABTS does not react with either I^- or I_2 at significant rate. As a substrate for MtL, ABTS has a 10^3-fold smaller K_m and a 10^3-fold larger k_{cat} in comparison with I^-. Exploiting these properties, ABTS is able to quickly shuttle electrons between I^- and MtL, and by doing so significantly enhance MtL-catalyzed I^- oxidation. When both ABTS and I^- are present, the bulk ABTS remains unoxidized as long as free I^- is still available to be oxidized. Similar K_m, k_{cat}, and pH profiles govern the dependence of the overall oxidation rate on ABTS concentration in the presence or absence of I^-. These

analyses suggest that, for the ABTS-enhanced catalysis, the rate-limiting step is the oxidation of ABTS.

I_2/I^- (I_3^-/I^-) couple has a redox potential of 0.536 *V* vs NHE (*17*). Simple copper complex Cu (II)-$(NH_3)_4$ has a redox potential of −0.1 *V* (*18*) and simple blue copper proteins (with one type 1 copper, such as azurin) usually have redox potentials in the range of 0.18–0.37 *V* (*1,2*). From a thermodynamic point of view, straight I^- oxidation by simple Cu (II) complexes, as well as simple blue copper proteins, should be quite difficult. For multi-copper oxidases, whose redox potentials probably range from 0.4–0.5 *V* (such as that for ceruloplasmin and *Rhus* laccase) to 0.7–0.8 *V* (such as that for *P. versicolor* laccase [*1,2*]), an electron transfer with I^- should be thermodynamically feasible. However, among the multi-copper oxidases tested, only laccase and bilirubin oxidase have significant activity in oxidizing I^-, while ascorbate oxidase and ceruloplasmin are almost inactive with or without the mediation of ABTS. This indicates that in addition to redox potential, kinetic factors and protein-I^- interaction can also play important roles in the reaction. Tyrosinase, which lacks type 1 copper site but has an enzymatically-active type 3 copper pair (*3,19*), also has low activity in catalyzing I^- oxidation. The low affinity of I^- for these oxidases (including MtL) might be related to structural factors at their substrate channels, which are probably better-suited for conventional substrates, such as ascorbate, phenols, and anilines. The poor performance of ABTS with ascorbate oxidase, ceruloplasmin, and tyrosinase is expected, since ABTS itself is a poor substrate for them. However, this does not exclude the possibility that other substances which are better substrates for these enzymes could mediate the catalysis more significantly. Future experiments with different redox mediators would help to establish whether the I^- oxidase activity is a general property of copper-containing oxidases and/or simple copper complexes (such as Cu-EDTA and Cu-histidines).

In the literature, only peroxidases have been reported to be capable of catalyzing I^- oxidation (*20*). Our results establish that laccase, bilirubin oxidase, and probably other copper-containing oxidases as well, are also capable of catalyzing the reaction. Although the finding might have little relevance to the physiological roles of these oxidases, the catalysis described in this article might be used in various industrial and medicinal applications that involve enzymatic transformation of I^- and production of I_2. For example, I_2 might be produced from brine and other natural sources at industrial scale by a laccase-base system in which the currently used Cl_2 gas and other undesirable chemicals could be replaced by environment-friendly enzymes. The high tolerance of MtL towards Cl^- inhibition ($I_{50} \sim 0.6M$) could make the enzyme more attractive (Cl^- has an $I_{50} \sim 0.01M$ for *P. versicolor* laccase [*9*]). Various industrial, medical, domestic, and personal-care sterilizations might also use a laccase-I^- binary system in which the disinfectant I_2 could be generated *in situ* by mixing active enzyme and I^- salt (a form of iodine much safer than I_2 in terms of storage, transport,

and handling). In general, a laccase-based I_2 generation system could hold advantage over a peroxidase-based system (*21,22*), because the former uses O_2 (air), rather than peroxide or peracid used by the latter, as the electron acceptor. The relatively low reactivity of laccase toward I^-, probably resulting from the structural factors imposed by the enzyme, could be overcome by choosing a suitable mediator, such as ABTS. In principle, the choice of mediator can not only enhance the catalysis, but also fine-tune the reaction in terms of optimizing pH and temperature range, turnover rate, and so on, based on the specificity of the mediator.

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REFERENCES

1. Reinhammar, B. and Malmstrom, B. G. (1981), in *Metal Ions in Biology: Copper Proteins,* vol. 3, Spiro, T. G., ed., Wiley, New York, pp. 109–149.
2. Farver, O. and Pecht, I. (1984), in *Copper Proteins and Copper Enzymes,* vol. 1, Lontie, R., ed., CRC Press, Boca Raton, pp. 183–214.
3. Mayer, A. and Harel, E. (1978), *Phytochem.* **18,** 193–215.
4. Mayer, A. (1987), *Phytochem.* **26,** 11–20.
5. Yaropolov, A. I., Skorobogat'ko, O. V., Vartanov, S. S., and Varfolomeyev, S. D. (1994), *Appl. Biochem. Biotech.* **49,** 257–280.
6. Curzon, G. and Speyer, B. E. (1967), *Biochem. J.* **105,** 243–250.
7. Koudelka, G. B. and Ettinger, M. J. (1988), *J. Biol. Chem.* **263,** 3698–3705.
8. Winkler, M. E., Spira, D. J., LuBien, C. D., Thamann, T. J., and Solomon, E. I. (1982), *Biochem. Biophys. Res. Comm.* **107,** 727–734.
9. Naki, A. and Varfolomeev, S. D. (1981), *Biokhimiya* **46,** 1694–1702.
10. Murao, S. and Tanaka, N. (1981), *Agric. Biol. Chem.* **45,** 2383–2384.
11. Hiromi, K., Tamaguchi, Y., Sugiura, Y., Iwamoto, H., and Hirose, J. (1992), *Biosci. Biotech. Biochem.* **56,** 1349–1350.
12. Xu, F. (1995), *FASEB J.* **9,** Abs498.
13. Berka, B. M., Thompson, S. A., Brown, S. H., Golightly, E. J., Brown, K. M., and Xu, F. (1994), abstr. Western Regional American Chemical Society Meeting and Pacific Conference, October 19–22, Sacramento, CA. A full report is in preparation.
14. Ramette, R. W. and Sandford, R. W. (1965), *J. Am. Chem. Soc.* **87,** 5001–5005.
15. Childs, R. E. and Bardsley, W. G. (1975), *Biochem. J.* **145,** 93–103.
16. Xu, F. Unpublished data.
17. Vanysek, P. (1993), in *Handbook of Chemistry and Physics,* Lide, D. R., ed., CRC Press, Boca Raton, pp. 8–19.
18. Bard, A. J., Parsons, R., and Jordan, J. (1985), *Standard Potentials in Aqueous Solution,* Marcel Dekker, New York, p. 292.
19. Sanchez-Ferrer, A., Rodriguez-Lopoz, J. N., Garcia-Canovas, F., and Garcia-Carmona, F. (1995), *Biochim. Biophys. Acta* **1247,** 1–11.
20. Thomas, J. A. and Hager, L. P. (1969), *Biochem. Biophys. Res. Comm.* **35,** 444–450.
21. Neidleman, S. L. and Geigert, J. (1981), U.S. Patent 4,282,324.
22. Kessler, J. H. (1991), U.S. Patent 4,996,146.
23. Xu, F., Shin, W., Brown, S. H., Wahleithner, J.A., Sundaram, U., and Solomon, E. I. (1996), *Biochim. Biophys. Acta* **1292,** 303–311.