Iodide as the Mediator for the Reductive Reactions of Peroxidases*

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Lignin peroxidase H2 (LiPH2) from the white rot fungus Phanerochaete chrysosporium catalyzed the reduction of cytochrome c, nitro blue tetrazolium, ferric iron, molecular oxygen, and triiodide in a reaction mixture containing LiPH2, H2O2, EDTA, and iodide. Activity followed first order kinetics with respect to EDTA concentration. The reductive activity observed with LiPH2 using iodide as the mediator was comparable to that obtained using a variety of other free radical mediators such as veratryl alcohol, 1,4-dimethoxybenzene, and 1,2,3- and 1,2,4-trimethoxybenzene. EDTA-derived radicals were detected by ESR spin trapping upon incubation of LiPH2 with H2O2, iodide, and EDTA. Reduction activity was also observed using other peroxidases such as lactoperoxidase, horseradish peroxidase, and myeloperoxidase. For the reduction activity of LiPH2, it is proposed that the oxidation of EDTA is mediated by the iodide radical, and the reduction of various electron acceptors is mediated by EDTA radicals. The inhibition of reduction activity at higher concentrations of iodide might be due to the combination of iodide radicals to form I2 which forms a stable triiodide complex by reacting with excess iodide.

Lignin, a complex polymer of phenylpropanoid subunits, is degraded to CO2 by the white rot fungus Phanerochaete chrysosporium under nutrient nitrogen, carbon, or sulfur limitation (1). Degradation of lignin is considered to be a nonspecific and extracellular process (2-4). Some of the important components involved in the degradation of lignin are extracellular peroxidases, VA, and H2O2 (2, 3, 5). These components are secreted by the fungus under lignolytic condition (2).

Many highly chlorinated chemicals are also degraded to CO2 by the fungus, and there is good evidence that the lignin peroxidases are involved in their oxidation (2, 6). However, many of the chemicals would have to be reduced before they can be oxidized. Reductive dechlorination is a fairly common first step in the metabolism of highly chlorinated chemicals, such as 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane. These reductive reactions might be catalyzed by lignin peroxidases, as we have shown (7, 8) that lignin peroxidases were capable of reducing various electron acceptors (molecular oxygen, NBT, cytochrome c, and ferric iron) using veratryl alcohol as the free radical mediator and an organic acid, such as EDTA or oxalate, as a reductant.

Another important requirement would be to prevent the halogenation reactions (9, 10) of lignin peroxidase. Banerjee et al. (11) have reported that EDTA is a strong inhibitor of iodide oxidase activity of horseradish peroxidase. Banerjee (12) proposed that the inhibition of iodide oxidase activity of horseradish peroxidase was due to the binding of EDTA to the enzyme. However, our (13) recent studies have shown that LiPH2 is decarboxylated at the acetic-1-14C in a reaction mixture containing horseradish peroxidase or LiPH2, H2O2, and iodide. Upon further investigation, we have discovered that LiPH2 can catalyze the reduction of various electron acceptors in a reaction mixture containing iodide, H2O2, and EDTA. Activity observed using iodide as the mediator was comparable to that obtained using other free radical mediators, such as VA and a variety of methoxybenzenes. Other peroxidases, such as horseradish peroxidase, lactoperoxidase, and myeloperoxidase, were also found to catalyze the same reactions. These findings are different from previously reported studies (14), where iodide has been used for iodination of organics by peroxidases.

Our results suggest that iodide can function as a free radical mediator for the oxidation of EDTA to EDTA radicals. The latter is a reductant for the reductive activity of peroxidases. The significance of these findings is discussed in relation to the dehalogenation of halogenated organics by the fungus.

EXPERIMENTAL PROCEDURES

Materials

Hydrogen peroxide, cytochrome c (type VI from horse heart), horseradish peroxidase, lactoperoxidase, myeloperoxidase, PBN, Tempol, NBT, and 1,10-phenanthroline were obtained from Sigma. Veratryl alcohol, methoxybenzene, 1,4-dimethoxybenzene, 1,2,3- and 1,2,4-trimethoxybenzene were purchased from Aldrich and purified by distillation. Potassium iodide and EDTA were purchased from Mallinckrodt. Buffers and reagents were prepared with purified water (Barnstead Nanopure II system) and further purified by passing over a Chelex 100 column (Bio-Rad) prior to their use.

Lignin Peroxidase H2 Production and Purification—Culture conditions for the production of LiPH2 from P. chrysosporium and its purification (50 units/mg) were as described previously (15).

Enzyme Assay—The oxidation of iodide to triiodide by LiPH2 was monitored at 353 nm (E = 2.5 × 10^4 M^-1 cm^-1) (10). The reduction of cytochrome c (ΔE(550,nm) = E(P) - E(P)) = 2.1 × 10^4 M^-1 cm^-1) and NBT (E = 1.5 × 10^6 M^-1 cm^-1) were monitored (Shimadzu UV-160 U spectrometer) by measuring the increase in absorbance at 550 and 560 nm, respectively (16). The reduction of ferric ion was determined at 560 nm using the extinction coefficient for the ferrous 1,10-phenanthroline complex of 1.1 × 10^4 M^-1 cm^-1 (17). Oxygen consumption was measured using a Gilson oxy-graph. Reaction conditions are described in the figure legends.

Spin Trapping of EDTA Radicals—EDTA radicals were detected by ESR spectroscopy as its spin adduct with PBN. Reaction mixtures contained 50 mM Chelex-treated sodium phosphate buffer, pH 6.5, 4.7 μM LiPH2, 6 mM EDTA, 250 μM H2O2, 75 mM PBN, and 200 μM iodide. Spectral recording began within 1 min after the initiation of
the reaction with 250 μM H2O2. ESR spectra were recorded at room temperature using a Varian E-109 spectrometer operating at 9.5 GHz with 100 kHz modulation frequency. Hyperfine splitting constants were determined by comparison with the standard Tempol using 17.1 G for αN in water.

RESULTS

Fig. 1 shows the iodide-dependent oxidative and reductive activities of LiPH2 at various concentrations of iodide. For the oxidative activity, I- is oxidized and the product is I2. The reaction components are iodide, H2O2, and LiPH2. For the reductive activity, EDTA and cytochrome c were added to the same reaction mixture resulting in formation of ferrocytochrome c. There is little iodide oxidase activity for iodide concentrations at or below 100 μM. However, cytochrome c reduction occurred at iodide concentrations between 10 and 500 μM with optimal reductive activity at about 50 μM. Furthermore, higher concentrations of iodide inhibited the reductive activity, but not the oxidase activity of LiPH2.

Table I shows the relative rates of reduction of NBT, cytochrome c, ferric iron, and molecular oxygen by LiPH2 using iodide as the mediator in the presence of EDTA and H2O2. With the exception of NBT, the rates of reduction of all electron acceptors were comparable.

Fig. 2 shows the dependence of cytochrome c reduction activity of LiPH2 on EDTA concentration. The reduction activity increased linearly with the increase in concentration of EDTA. Lack of saturation suggests that the binding of EDTA to the enzyme is not responsible for the reduction activity of the enzyme. EDTA was decarboxylated at the

**TABLE I**

| Electron acceptor | Initial rate of reduction (μM/min) |
|-------------------|-----------------------------------|
| Cytochrome c      | 19.5 ± 0.5                        |
| NBT               | 7.1 ± 0.2                         |
| Ferric iron       | 21.2 ± 0.8                        |
| Molecular oxygen  | 20.5 ± 0.9                        |

**Fig. 2.** Dependence of cytochrome c reduction activity on the concentration of EDTA. Reaction conditions were similar to those described in the legend to Fig. 1 except that the concentration of iodide used was 50 μM. Values are expressed as the mean of triplicate samples with standard deviation error bars.

**Fig. 3.** EDTA-dependent reduction of cytochrome c as a function of LiPH2 concentration. Reaction conditions were similar to those described in the legend to Fig. 2 except that the concentration of EDTA used was 3 mM. Values are expressed as the mean of triplicate samples with standard deviation error bars.

Fig. 4 shows the ESR spectrum obtained in a reaction mixture containing LiPH2, iodide, EDTA, and H2O2. A similar ESR spectrum was also observed by using VA or 1,2,3-trimethoxybenzene instead of iodide as previously reported (7) (data not shown). We (7) have previously reported the reduction activity of LiPH2 using VA and a variety of methoxybenzenes as free radical mediators in the presence of EDTA and H2O2. The reduction activity using iodide as the mediator was comparable to that obtained using VA and a variety of methoxybenzenes (Table II). Like LiPH2, horseradish peroxidase, lacto-peroxidase, and myeloperoxidase were also able to catalyze the reduction of cytochrome c using iodide as the mediator (data not shown). Fig. 4A shows the ESR spectrum obtained in a reaction mixture containing LiPH2, iodide, EDTA, and H2O2. A similar ESR spectrum was also observed by using VA or 1,2,3-trimethoxybenzene instead of iodide as previously reported (7) (data not shown). No radicals were detected upon removal of iodide, EDTA, H2O2, or LiPH2. Computer simulation of two major spin adducts from these spectra are shown in Fig. 4B. The hyperfine splitting constants were αN = 15.6 G and αH = 3.63 G for one radical and αN = 16.3 G and αH = 4.07 G for the other. These values are the same as reported earlier by us (7) for EDTA-derived radicals using veratryl alcohol as the mediator. In a separate reaction mixture containing I2 (0.01, 0.1, 1, 10 mM) and EDTA (6 mM), it was found that EDTA was able to reduce I2 (data not shown). However,
2.7 PM LiPH2, 3 mM EDTA, 150 mM H2O2. The concentration of mediator was 1 mM in all cases except for iodide, where it was 50 μM.

TABLE II

| Mediator               | Eₐ* (μM/min) | Rate (μM/min) |
|------------------------|--------------|---------------|
| Iodide                 | 1.2          | 17.1 ± 0.5    |
| Veratryl alcohol       | NA           | 18.5 ± 0.6    |
| Methoxybenzene         | 1.76         | Not detected  |
| 1,2,3-Trimethoxybenzene| 1.42         | 15.2 ± 0.4    |
| 1,4-Dimethoxybenzene   | 1.34         | 13.1 ± 0.8    |
| 1,2,4-Trimethoxybenzene| 1.12         | 11.2 ± 0.6    |

*See Refs. 19 and 20.

** NA, not available.

To determine which enzyme intermediate is involved in the reduction activity of the enzyme, the enzyme was scanned under steady state condition. In a reaction mixture containing 0.1 mM acetate buffer at pH 5.5, 4 μM LiPH2, 50 μM iodide, 3 mM EDTA, 150 μM H2O2, the enzyme appeared to be in compound II form with absorption maxima at 420, 530, and 552 nm (2). When veratryl alcohol (1 mM) was used in the place of iodide, similar absorption maxima were observed.

**DISCUSSION**

The proposed scheme to explain the reductive reactions of peroxidases in a reaction mixture containing H2O2, EDTA, and iodide is shown in Fig. 5. According to this scheme, the iodide radical is formed by LiPH2-catalyzed oxidation. The iodide radical then oxidizes EDTA to yield the EDTA radical, which is the reducing species. There are various lines of evidence to support the scheme proposed. First of all, we showed that the reductive activity of peroxidases using iodide as the mediator was comparable to that obtained using other free radical mediators such as VA (3, 7, 8) or a variety of methoxybenzenes (18) except methoxybenzene. The inability of methoxybenzene to serve as the free radical mediator is due to the fact that it is not a substrate for lignin peroxidases (19). Second, we (7) have shown previously that the cation radical of VA was the oxidant for the oxidation of EDTA to the EDTA radical and the latter was the reductant for the reduction of cytochrome c, NBT, molecular oxygen, and ferric iron. Furthermore, the Eₐ* value of the iodide radical is about 1.21 V (20), a value comparable to that reported for many of the methoxybenzenes which served as a mediator for the reduction activity of LiPH2. The detection of EDTA radicals in the reaction mixture containing LiPH2, H2O2, EDTA, and iodide provides additional evidence for the involvement of iodide radical. The linear relationship between the rate of cytochrome c reduction and the concentration of EDTA also supports the involvement of free radical reactions. As in the case of LiPH2, other peroxidases like horseradish peroxidase, lactoperoxidase, and myeloperoxidase were also found to have reductive activity using iodide as the mediator. EDTA is well known as the reductant for the photolytic reduction of riboflavin. Such reactions are also considered to be free radical based (21).

In previous studies by us (13) and others (9, 10, 14) on the oxidation of iodide by peroxidases, hypoi iodite (I0⁻) or I₂ were proposed to be formed, as against the iodide radical proposed here. The involvement of iodide radical is supported by the fact that enzyme appeared to be in compound II form in the reaction mixture containing LiPH2, iodide, H2O2, and EDTA. Furthermore, the oxidation of EDTA to the EDTA radical is also a 1-electron oxidation process. The inhibition of reductive activity at higher concentrations of iodide might be due to the combination of iodide radicals to form I₃⁻, which forms a stable triiodide complex in the presence of excess iodide. This is the traditional iodide oxidase activity assay for peroxidases. Inhibition might also be due to the reduction of iodine by either EDTA or EDTA radicals.

The significance of our results is that they suggest a possible mechanism for the control of halogenation reactions (9, 10) of lignin peroxidase enzymes of P. chrysosporium. We propose that halogenation reactions of lignin peroxidases might be minimized by suitable reductants. Oxalate might be one of the possible reductants in the case of P. chrysosporium (8) as it is a dicarboxylic acid without a site for halogenation. We speculate that organic acid radicals might even be involved in dehalogenation. In fact, we recently found that CCl₄ was reduced to the trichloromethyl radical via the similar reductive reactions of LiPH2.² Future research is required to identify fungal reductants and to show their involvement in the degradation of halogenated organics by P. chrysosporium. Another important significance of the work presented here is the possibility of production of active oxygen species by

² Shah, M. M., Grover, T. A., and Aust, S. D. (1983) Biochem. Biophys. Res. Commun., in press.
peroxidases using iodide as the mediator and organic acid as the reductant. We (7, 8) have already shown that anion radicals of organic acids are capable of reducing molecular oxygen to superoxide and hydroxyl radicals, and they are known to degrade halogenated organics like 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, chlorophenols, and polychlorinated biphenyls (22-24).

We (25) recently reported that VA, 1,2,3- and 1,2,4-trimethoxybenzene, or 1,4-dimethoxybenzene cation radicals mediated the oxidation of H₂O₂ to produce molecular oxygen by lignin peroxidase. Both molecular oxygen production and reduction activity of LiP might be involved in the degradation of halogenated chemicals by the fungus.

In summary, our results clearly demonstrate that iodide serves as the mediator for the reductive activity of peroxidases. Our results also suggest that the mechanism for the oxidation of iodide by peroxidases could be a 1-electron oxidation process at low concentrations of iodide and in the presence of a reductant such as EDTA. However, future research will be needed to further elucidate the mechanism for oxidation of iodide by peroxidases at various concentrations of iodide, especially in the presence of organic acids.

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