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Lignin peroxidase compound III

Formation, inactivation, and conversion to the native enzyme

Hiroyuki Wariishi and Michael H. Gold

Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, OR 97006-1999, USA

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At pH 3.0 in the absence of a reducing substrate and the presence of only 20 equivalents of H_2O_2, lignin peroxidase (LiP) is readily converted to LiP compound III (LiPIII) ($Fe(III)O_2^{\cdot-}$ complex). LiPIII which is produced via the reaction of LiP compound II (LiPII) with H_2O_2 is not part of the peroxidase catalytic cycle, and is readily and irreversibly inactivated. Veratryl alcohol (VA), a *Phanerochaete chrysosporium* secondary metabolite, protects the enzyme from inactivation via two mechanisms. Acting as a substrate, VA reduces LiPII to regenerate the native enzyme. Secondly, the binding of VA to LiPIII rapidly displaces $O_2^{\cdot-}/HO_2^{\cdot}$, thereby converting LiPIII directly to the native enzyme. VA is not consumed during this displacement reaction. These results help to explain the role of VA in stabilizing the enzyme in the presence of excess H_2O_2.

Lignin peroxidase; Compound III; Superoxide; Lignin degradation; Veratryl alcohol; (*Phanerochaete chrysosporium*)

1. INTRODUCTION

Lignin peroxidase (LiP), a heme-containing glycoprotein, has been purified from the extracellular medium of the white rot basidiomycete *Phanerochaete chrysosporium* [1–3]. The H_2O_2-oxidized states of LiP [4–6] are similar to those of HRP [7]. The enzyme has an unusually low pH optimum (<3.0) [5,6] and catalyzes the H_2O_2-dependent oxidation of a variety of lignin model compounds [1–3,8,9]. At pH 3.0 in the presence of 20 equivalents of H_2O_2 and in the absence of a reducing substrate, lignin peroxidase compound III (LiPIII) is formed readily [6]. In this report, we demonstrate that the formation of LiPIII, an oxidized intermediate not involved in the normal peroxidase catalytic cycle [4,7], can lead to inactivation of the enzyme. We also demonstrate that the conversion of LiPIII back to the native enzyme is dependent on veratryl alcohol (VA).

Correspondence address: M.H. Gold, Department of Chemical and Biological Sciences, Oregon Graduate Center, Beaverton, OR 97006-1999, USA

Abbreviations: HRP, horseradish peroxidase; LiP, lignin peroxidase; TNM, tetranitromethane; VA, veratryl alcohol (3,4-dimethoxybenzyl alcohol)

2. MATERIALS AND METHODS

The major isozyme of LiP was purified from cultures of *P. chrysosporium* as previously described [1,3], and dialyzed against deionized water. The H_2O_2 concentration was determined as described [10]. Tetranitromethane (TNM) was dissolved in ethanol (20 μl), and diluted to 1 mM with water immediately before use [11].

Electronic absorption spectra and TNM reduction kinetics were recorded on a Shimadzu UV-260 spectrophotometer equipped with an SEA-11 stopped-flow apparatus (Hi-Tech Scientific). Rapid scan spectra were recorded with a Photol RA601 reaction analyzer as previously described [6]. LiP-catalyzed VA oxidation products were analyzed by HPLC and GCMS (VG Analytical 7070E) after extraction and derivatization as previously described [1,3]. Experiments were performed in 20 mM sodium succinate, pH 3.0. VA and TNM were obtained from Aldrich.

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3. RESULTS

3.1. *Formation and irreversible inactivation of LiPIII*

Addition of excess H_2O_2 (>20 equivalents) to native LiP at pH 3.0 resulted in its rapid conversion to LiPIII; absorption maxima at 419, 543 and 578 nm (fig.1A) [4,6]. More than 20 equivalents of H_2O_2 was required to form spectrally pure LiPIII. When >30 equivalents of H_2O_2 was used, the formation of LiPIII was followed by the complete disappearance of the Soret and α and β bands within 60 min (fig.1A). The spectrally featureless form of the enzyme was inactive when assayed with VA. During the enzyme inactivation, LiPIII absorption bands were not shifted, suggesting that LiPIII was converted directly to the inactive form.

3.2. *Effect of VA on the conversion of LiPIII to native LiP*

Addition of 40 equivalents of VA to LiPIII resulted in its rapid conversion to native LiP (absorption bands at 408, 498 and 630 nm) [4,6] (fig.1B). When VA was added prior to the addition of H_2O_2, recovery of native LiP was near-quantitative (fig.1C). Fig.2 shows the rapid scan Soret spectrum of the VA-stimulated conversion of LiPIII. The Soret peak shifted from 419 nm to 408 nm over a 0.2-s time span. The isosbestic point at 413 nm between LiPIII and native LiP suggests that LiPIII was converted directly to the native enzyme.

To determine whether VA was oxidized during the conversion of LiPIII to native LiP, 2 ml of LiPIII (20 μM) was prepared using 40 equivalents of H_2O_2; 15 units of catalase (Sigma) were added to the LiPIII preparation to remove excess H_2O_2, assuring a single turnover event. After 5 min, VA (0.2 mM) was added and the conversion of LiPIII was monitored. At the end of the reaction no veratryl aldehyde was detected and the starting VA was still present in the reaction mixture. VA was also added to LiPI (20 μM) prepared as previously described [6]. In this case, oxidation of VA to veratryl aldehyde was confirmed by HPLC and GC retention times and by the mass fragmentation pattern of the product, which were identical to the standard.

TNM is efficiently reduced by superoxide anion/hydroperoxide radical ($pK_a \simeq 4.5$) to yield the trinitromethane anion [12] which has a strong absorbance at 350 nm. Fig.3 shows that TNM was rapidly reduced during the conversion of LiPIII. TNM reduction was dependent on the presence of

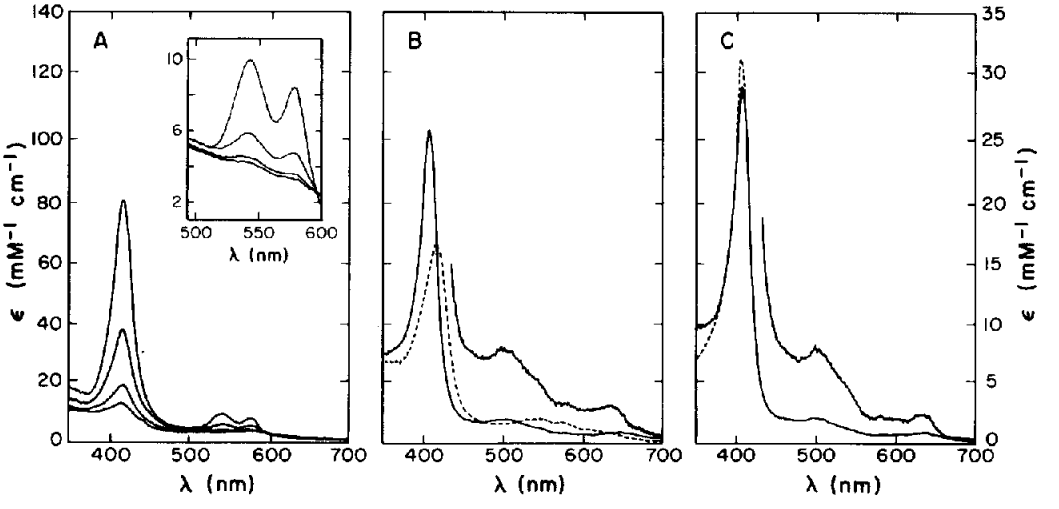

Fig.1. Formation, inactivation and conversion of LiPIII. (A) H_2O_2 (30 equivalents) was added to 1.5 μM LiP (inset 5.0 μM LiP) at pH 3.0. Spectral scans (20 min intervals) monitored the inactivation of LiPIII. (B) LiPIII (– – –) was prepared by adding 20 equivalents of H_2O_2 to 1.5 μM native LiP. VA (40 equivalents) was then added and the conversion to native LiP (——) was observed. (C) 40 equivalents of VA was added to 1.5 μM native LiP (– – –), after which 20 equivalents of H_2O_2 was added. The resulting spectrum (——) is that of native LiP.

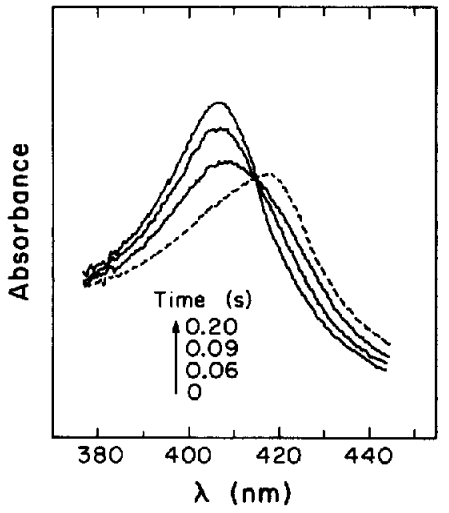

Fig.2. Rapid scan spectra of LiPIII conversion. LiPIII (---) was prepared as in fig.1A after which 40 equivalents of VA was added and rapid scan Soret spectra were taken at the intervals indicated.

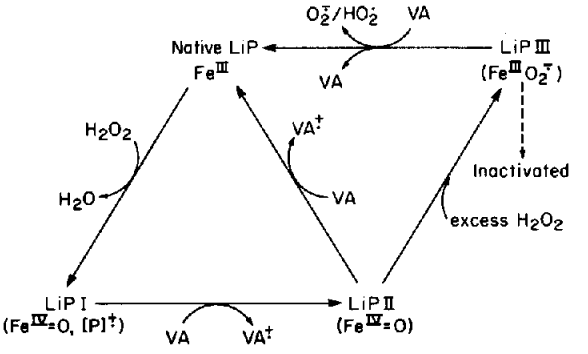

Fig.4. Interrelationships between the four oxidation states of LiP. Reaction path, Native LiP ⟶ LiPI ⟶ LiPII ⟶ Native LiP, indicates the catalytic cycle. $VA^{\cdot+}$ = veratryl alcohol cation radical.

LiPIII and the time course of TNM reduction corresponded closely to that of LiPIII conversion, suggesting that $HO_2^{\cdot}/O_2^{\cdot-}$ was released.

4. DISCUSSION

In the presence of excess H_2O_2 and the absence of a reducing substrate, heme peroxidases are converted from compound II to the compound III state which is either a $[Fe(III)O_2^{\cdot-}]$ complex or $[Fe(II)O_2]$ complex [7,13] (fig.4). Transient state kinetic studies with H_2O_2 in excess have demonstrated the formation of LiPI $(Fe(IV)=O,P^{\cdot+})$ and LiPII $(Fe(IV)=O)$ prior to LiPIII under pseudo-first-order conditions with H_2O_2 in excess [6] (fig.4). LiP is unusual in that only 20–30 equivalents of H_2O_2 are required for LiPIII formation and inactivation (fig.1A). In contrast, ~500 equivalents of H_2O_2 is required to form HRPIII [13] which is also followed by its inactivation ([14]; Wariishi, H., unpublished). Since the formation of LiPIII leads to enzyme inactivation (fig.4), the mechanism of LiPIII conversion

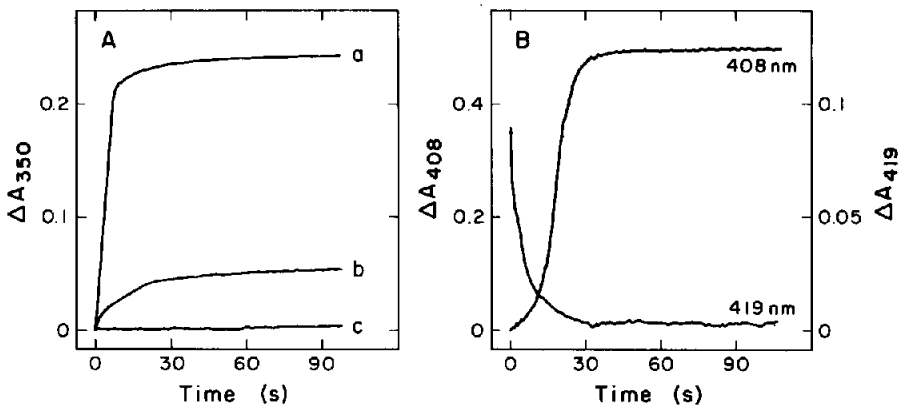

Fig.3. Reduction of TNM during the conversion of LiPIII. LiPIII was prepared by adding 40 equivalents of H_2O_2 to native LiP (5.5 μM). (A) Reaction mixture contained LiPIII, EDTA (0.1 mM), TNM (0.1 mM) in sodium succinate, pH 3.0; reaction was initiated by adding VA (0.23 mM) (a). (b) As in a without TNM. (c) As in a without LiPIII but with H_2O_2. (B) Reaction mixture as in a. Formation of native LiP and decrease of LiPIII were followed at 408 and 419 nm, respectively.

back to the native enzyme was of interest. The reversion of HRPIII to the native enzyme is stimulated by phenolic substrates [15,16]. Preliminary evidence also indicated that VA stimulates the conversion of LiPIII to native LiP [17]. The addition of VA to LiPIII caused the rapid conversion of this oxidized intermediate to native LiP (fig.1B). A single isosbestic point at 413 nm in the Soret region between LiPIII and native LiP indicates that the conversion of LiPIII to native LiP is direct and without intermediates. Ferrous-LiP (absorption maxima 435, 556 nm) [1,4] was not detected during the conversion, suggesting that the LiPIII produced in this study exists as the $[Fe(III)O_2^{\cdot -}]$ complex rather than the $[Fe(II)O_2]$ complex. This was confirmed by measuring the release of $O_2^{\cdot -}/HO_2^{\cdot}$ during the conversion using the $O_2^{\cdot -}$ reduction of TNM as an assay. The release of $O_2^{\cdot -}/HO_2^{\cdot}$ and the total recovery of the added VA indicate that oxidation of the substrate did not occur. These results can best be explained by a displacement mechanism (fig.4) in which the binding of VA to the $Fe(III)O_2^{\cdot -}$ enzyme complex leads to the release of $HO_2^{\cdot}/O_2^{\cdot -}$ and the formation of the native enzyme. Native LiP binds VA at pH 3.0 with an apparent dissociation constant of 4.7 μM (Wariishi, H., unpublished). Superoxide is also released during the conversion of myeloperoxidase compound III [18] to its native state. It is also conceivable that through the release of superoxide which could react with cation radicals, LiPIII may play a role in lignin degradation.

Since VA is a substrate for the enzyme [1–3], its presence also helps ensure that LiPII will be reduced to the native enzyme rather than react with excess H_2O_2 to form LiPIII (figs 1C and 4). VA formation coincides with the onset of ligninolytic activity [8]. Recent work has shown that VA enhances LiP activity in culture by protecting LiP against inactivation by H_2O_2 [19]. We have previously shown that a unique feature of LiP is its ready formation of compound III under relatively low concentrations of H_2O_2 [4,7]. In this study, we have demonstrated that LiPIII is rapidly inactivated. Furthermore, we have demonstrated that VA protects against inactivation by acting both as a substrate which reduces LiPII back to the native enzyme, and by stimulating the conversion of LiPIII to the native enzyme.

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