RESEARCH COMMUNICATION

Anionic tobacco peroxidase is active at extremely low pH: veratryl alcohol oxidation with a pH optimum of 1.8

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Tobacco peroxidase (36 kDa, pI 3.5) exhibits unique catalytic and spectral properties that are modulated by pH, calcium and magnesium ions. It catalyses the oxidation of veratryl alcohol by hydrogen peroxide over a wide pH range (1.5–5.0) in the presence of these metal ions with a pH optimum of 1.8. This is the only example of a holoperoxidase described so far that is active and comparatively stable at such a low pH. The enhancement of tobacco peroxidase activity by magnesium ions is to our knowledge the first example of a magnesium-induced peroxidase activation. UV/visible spectra of tobacco peroxidase showed that the Soret band shifted and its absorption coefficient increased upon the addition of calcium or magnesium ions and on lowering

the pH. The tobacco peroxidase spectrum at pH 1.85, in the presence of calcium chloride (> 50 mM), is similar to that of lignin peroxidase at pH 6.0, with the Soret band shifting from 403 to 409 nm and the molar absorption coefficient increasing from 108 000 to $148\,000 \pm 2000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (results given $\pm$ S.E.M.; $n = 3$). The data provide evidence for a low-affinity site for bivalent metal ion binding in addition to the two constitutive calcium sites that are present in all plant peroxidases. The presence of a glutamic acid residue (Glu-141) at the entrance to the haem-binding pocket, analogous to Glu-146 in lignin peroxidase and not present in other plant peroxidases, may account for these novel properties.

INTRODUCTION

Haem-containing peroxidases are widespread in Nature and perform important physiological functions that include lignification, suberization and stress/pathogen response [1]. According to current classification, the plant peroxidase superfamily includes prokaryotic enzymes (bacterial catalase-peroxidases, yeast cytochrome *c* peroxidase, plant ascorbate peroxidases), fungal enzymes (lignin and manganese peroxidases and plant-type enzymes) as well as those from plants [2]. The enzymes differ significantly from each other in their primary structures and substrate specificities, although the crystal structure motifs and catalytic centres are very similar [3–6]. Characteristic features of fungal enzymes include their stability at low pH and their ability to catalyse the oxidation of veratryl alcohol by hydrogen peroxide with a pH optimum of 3.0 [7]. The recently reported data on lignin peroxidase-type activity with soybean peroxidase at low pH in the presence of calcium ions [8] caused us to extend our studies on the properties of a newly isolated tobacco peroxidase [9] that we have recently overexpressed in transgenic tobacco and tomato plants. Over-production of the enzyme was associated with increased plant lignification, suggesting that the enzyme was lignin-forming [10]. In this paper, we present data demonstrating for the first time the catalytic activity of a plant peroxidase at extremely low pH, and show that this tobacco peroxidase has a UV/visible spectrum with characteristics resembling those of a fungal lignin peroxidase.

MATERIALS AND METHODS

Hydrogen peroxide, calcium chloride, magnesium chloride and zinc acetate were from BDH Merck, manganese sulphate, veratryl alcohol, veratraldehyde (3,4-dimethoxy-benzaldehyde), 2-hydroxy-3-methoxybenzyl alcohol, 3-hydroxy-4-methoxybenzyl alcohol, 4-hydroxy-3-methoxybenzyl alcohol and 1,4-dimethoxybenzene were purchased from Aldrich. Veratryl alcohol was

vacuum distilled before use. The purity of veratryl alcohol before and after vacuum distillation was judged by reverse-phase HPLC on a column (4.6 × 150 mm; Columbus 5 μ M C18-100-A purchased from Phenomenex) using isocratic elution in methanol/1% acetic acid (40:60 v/v). The major impurity found in both preparations was veratraldehyde ($\approx 0.1\%$).

Horseradish peroxidase isoenzyme C [Reinheitszahl ratio (RZ), A_{412}/A_{278} 2.8] was purchased from Biozyme and used without further purification.

Lignin peroxidase isozyme H2 was isolated from the media of 7-day old, agitated, carbon limited cultures of *Phanerochaete chrysosporium* IMI174727 (BKM-F-1767, A.T.C.C. 24725), and purified as described in [7] using a Pharmacia FPLC Mono-Q column. Identity of the isoenzyme (RZ > 3.6) was confirmed by analysis of the first 19 residues of the N-terminal sequence and comparison with the published gene sequence [11].

Tobacco anionic peroxidase (pI 3.5 [9]) was purified from transgenic tomato fruits that were overexpressing the enzyme [12]. Tomato fruits (1 kg) were homogenized in 0.5 litres of 100 mM Tris/HCl buffer, pH 6.0. The disrupted tissues were removed by centrifugation (4000 g, 20 min), and the resulting homogenate was fractionated with 20–40% satd. ammonium sulphate. The precipitate was dissolved in water and applied to a Sephacryl S-200 column (5.2 × 90 cm) equilibrated with 10 mM Tris/HCl buffer, pH 6.0. Active fractions were collected and applied to a DEAE-cellulose column (5.2 × 5 cm) equilibrated with the same buffer. The adsorbed fractions were eluted with 100 mM Tris/HCl buffer, pH 6.0, and then purified by FPLC gel filtration using a HiLoad 16/60 Superdex 75 column (Pharmacia Biotech) equilibrated with 50 mM Na-phosphate buffer, pH 7.0, containing 150 mM NaCl. Active fractions (RZ 3.0) were collected and used in this study. The enzyme was homogeneous, as judged by SDS/PAGE, with a molecular mass of 36 kDa. The enzyme properties were identical with those of the enzyme obtained after overexpression of the same gene in tobacco [9].

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Soybean peroxidase (pI 4.1, RZ 1.5), was purchased from Enzymol. International Inc., Columbus, OH, U.S.A., and used after further purification using FPLC gel filtration as described above for tobacco peroxidase. The resulting enzyme preparation (RZ 3.0) was homogeneous as judged by SDS/PAGE (molecular mass 37 kDa).

The chemistry of degradation of veratryl alcohol in 100 mM Na-phosphate buffer, pH 1.8, in the presence of 100 mM MnSO_4 was studied by means of HPLC.

Spectra were recorded using a Shimadzu UV-2101PC spectrophotometer. The enzyme activity toward veratryl alcohol was measured using a molar absorption coefficient of $9300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 310 nm for the product veratraldehyde [7]. The reaction was initiated by the addition of hydrogen peroxide if it is not otherwise stated. The steady-state kinetics of veratryl alcohol oxidation by tobacco peroxidase were studied in the range of veratryl alcohol and hydrogen peroxide concentrations of 0.2–4.0 mM and 0.01–0.20 mM respectively, in 100 mM Na-phosphate buffer, pH 1.85, containing 200 mM calcium chloride or 40 mM magnesium chloride. Hydrogen peroxide concentrations were determined using a molar absorption coefficient of $43.6 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 240 nm [13]. Protein concentrations were determined spectrophotometrically as described by Kalb and Bernlohr [14].

RESULTS AND DISCUSSION

Tobacco peroxidase catalysed the oxidation of veratryl alcohol by hydrogen peroxide over a wide pH range (1.5–5.0) in the presence of calcium or magnesium ions according to the spectroscopic and HPLC data. The differential spectra in the course of veratryl alcohol oxidation by tobacco peroxidase showed an increase in absorbance, with the maximum at 310 nm characteristic for veratraldehyde, and thus could be easily distinguished from spectra for the oxidation of analogous alcohols (2-hydroxy-3-methoxybenzyl-, 3-hydroxy-4-methoxybenzyl- and 4-hydroxy-3-methoxybenzyl alcohols exhibiting maxima at 292 nm for example). The redox potential of the tobacco peroxidase at pH 2.7 was sufficiently high to oxidize 1,4-dimethoxybenzene.

The pH optimum for veratryl alcohol oxidation (pH 1.8, Figure 1A) was much lower than those reported previously for lignin peroxidase (pH 3.0) in the absence of calcium cations [7] and soybean peroxidase in the presence of 200 mM calcium chloride (pH 2.4) [8]. Lignin peroxidase H2 was essentially inactive at pH 1.8, and soybean peroxidase showed traces of activity (Figure 1B).

Rapid inactivation of tobacco peroxidase was observed at elevated concentrations of hydrogen peroxide ($> 0.1 \text{ mM}$), especially at non-saturating concentrations of veratryl alcohol ($< 1 \text{ mM}$). A more pronounced inactivation at low veratryl alcohol concentrations suggests that the stabilizing effect of veratryl alcohol reported for lignin peroxidase [7] may also apply to tobacco peroxidase.

The enzymic activity of tobacco peroxidase was strongly dependent on the presence of calcium or magnesium cations (Figure 1A inset). Curvature in the time course of steady-state assays (Figure 1B) provides some evidence for a higher stabilizing effect of calcium ions compared with that of magnesium ions, although the activation effect (expressed as a ratio of the initial slopes of the time-course curves in the absence and presence of metal ions) of magnesium ions was higher than that for calcium ions under the optimal assay conditions (100 μM H_2O_2 and 5–10 mM veratryl alcohol).

The stabilization effect of calcium ions was more evident

Figure 1 Veratryl alcohol oxidation catalysed by tobacco peroxidase at 25 °C

(A) pH-dependence of the reaction rate: (Δ) 100 mM Na-phosphate containing 100 mM CaCl_2 ; ($\blacksquare$) 100 mM Na-acetate containing 100 mM CaCl_2 (to avoid precipitation of calcium phosphate at higher pH); ($\square$) 100 mM Na-phosphate containing 40 mM MgCl_2 . Concentration used: 5 mM veratryl alcohol, 50 μM H_2O_2 , 0.2 μM enzyme. The highest rate observed for 100 mM CaCl_2 (18 $\mu\text{M}/\text{min}$) and 40 mM MgCl_2 (22 $\mu\text{M}/\text{min}$) containing buffers was taken as 100%. Inset, effect of metal concentration $[\text{Me}^{2+}]$ on the reaction rate at pH 1.85 in the presence of 5 mM veratryl alcohol and 50 μM H_2O_2 . The rate in the absence of added metal ions (6 $\mu\text{M}/\text{min}$) was taken as 100%. (B) Curvature in the time course of steady-state oxidation of 10 mM veratryl alcohol by 100 μM H_2O_2 in 100 mM Na-phosphate buffer, pH 1.8: (a, b and c) catalysed by 0.8 μM tobacco peroxidase in the presence of (a) 200 mM CaCl_2 , (b) 40 mM MgCl_2 and (c) in the absence of added metal ions; (d) catalysed by 0.8 μM soybean peroxidase in the presence of 200 mM CaCl_2 ; and (e) catalysed by 0.2 μM lignin peroxidase in the absence of added metal ions.

during the spectral studies at extremely low pH. In the absence of added metal ions, or in the presence of magnesium ions, the tobacco peroxidase Soret band decreased by 50% at pH 1.8 within 15 min, whereas in the presence of calcium ions the enzyme was stable for 1.5 h. To our knowledge this is the only example of a holoperoxidase that is stable at such a low pH. A pH < 3.0 is widely used to isolate plant peroxidase apoenzymes, whereas the pH optimum for veratryl alcohol oxidation catalysed by tobacco peroxidase corresponds approximately to a 100 mM phosphoric acid solution.

Surprisingly, calcium and magnesium ions also stimulated the activity of lignin peroxidase at pH 1.8, although it is well known that 20 mM calcium chloride inhibits lignin peroxidase activity [15], and nothing has been reported on the effect of magnesium ions on lignin peroxidase. In the absence of added metal ions the

Figure 2 Spectral changes induced by calcium addition and pH lowering

(A) The shift in the Soret band and the increase in absorption coefficient upon calcium chloride addition and pH lowering. (a) Tobacco peroxidase ($1 \mu\text{M}$) in 200 mM Na-acetate buffer, pH 5.0; (b) the same buffer containing 100 mM CaCl_2 ; (c) 100 mM Na-phosphate buffer, pH 1.85; (d) the same buffer containing 100 mM CaCl_2 . (B) Comparison of tobacco peroxidase and lignin peroxidase spectra. (a) Spectrum of $6 \mu\text{M}$ tobacco peroxidase in Na-phosphate buffer, pH 1.85, containing 100 mM CaCl_2 ; (b) spectrum of lignin peroxidase H2 in Na-phosphate buffer, pH 6.0, and (c) 1 min after addition to Na-phosphate buffer, pH 1.85, containing 100 mM CaCl_2 .

Figure 3 Double-reciprocal plot for steady-state kinetics of veratryl alcohol oxidation by hydrogen peroxide catalysed by tobacco peroxidase in 100 mM Na-phosphate buffer, pH 1.85, containing 200 mM CaCl_2

Veratryl alcohol (VA) concentrations used, mM: (■) 0.34; (□) 0.85; (◇) 1.7. Inset, the secondary plot of intercepts versus inverse concentration of veratryl alcohol.

enzyme was completely inactive, independent of the order of reagent addition. However, it was possible to record the spectral changes at 310 nm in the presence of calcium or magnesium ions only if the reaction was initiated by addition of the enzyme.

Calcium ions again had a more pronounced stabilizing effect: in the presence of 40 mM magnesium chloride the enzyme was inactivated within 3 min, whereas in the presence of 200 mM calcium chloride it was active for at least 15 min. The initial rates of 10 mM veratryl alcohol oxidation by hydrogen peroxide ($100 \mu\text{M}$) in the presence of lignin peroxidase H2 ($0.2 \mu\text{M}$) were equal to 0.14 mM/min and 0.17 mM/min in the presence of calcium and magnesium ions respectively. This observation illustrates the well-known fact that a functioning enzyme is often better protected against inactivation than a non-functioning enzyme.

Manganese ions (100 mM) caused a time-dependent increase in absorbance at 310 nm at pH < 2 in the absence of hydrogen peroxide and enzyme, due to a non-enzymic reaction involving manganese(II), oxygen and veratryl alcohol to yield veratraldehyde, as judged by HPLC. The magnitude of the activation effect for tobacco peroxidase, including this background reaction, in the presence of $50 \mu\text{M}$ hydrogen peroxide and 5 mM veratryl alcohol, was two times lower than that for magnesium or calcium ions under the same assay conditions. Moreover, in the presence of manganese ions, the enzyme was inactivated within 2–3 min, as judged by the time-course curvature, compared with the 50 min required for inactivation in the presence of calcium ions. No reaction with veratryl alcohol (5 mM) was detected in the presence of zinc cations with or without hydrogen peroxide ($50 \mu\text{M}$) at pH 2.7 in 1% acetic acid solution.

UV/visible spectra of tobacco peroxidase showed that the Soret band shifted and its absorption coefficient increased upon addition of calcium or magnesium ions at pH 5.0 (Figure 2A). This behaviour was not observed with horseradish or soybean peroxidase at that pH. Lowering the pH to 1.85 in the absence of added metal ions, or in the presence of magnesium ions, also induced spectral changes for tobacco peroxidase, but they were complicated by a shift in the Soret band to 400 nm that is associated with the onset of haem bleaching. The addition of calcium at low pH prevented haem bleaching and enhanced the spectral changes for the tobacco peroxidase, which has a spectrum at pH 1.85 in the presence of 50–200 mM of calcium chloride that is very similar to that of lignin peroxidase at pH 6.0 (Figure 2B, curves a and b). The lignin peroxidase spectrum at pH 1.85 indicated significant rapid haem bleaching and subsequent inactivation that was complete within seconds of lowering the pH, both in the presence and the absence of added metal ions (Figure 2B, curve c). Under these conditions, tobacco peroxidase exhibited no decrease in the Soret band and no changes in the enzyme activity toward veratryl alcohol over a period of 1.5 h. However, prolonged incubation (> 10 h) resulted in haem bleaching and corresponding enzyme inactivation.

It is well known that the electronic absorbance spectra of lignin and manganese peroxidases (Soret maximum at 409 nm, $\epsilon = 165000 \text{ M}^{-1} \cdot \text{cm}^{-1}$) differ from those of plant enzymes (Soret maximum at 403 nm, $\epsilon = 102000 \text{ M}^{-1} \cdot \text{cm}^{-1}$). In the case of tobacco peroxidase, addition of calcium ions (optimal 100 mM) and lowering the pH causes the spectrum to change from that of a plant enzyme to more closely resemble that of a lignin peroxidase: the Soret band shifts from 403 to 409 nm and the molar absorption coefficient increases from $108000 \pm 2000 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Figure 3) (results given $\pm$ S.E.M.; $n = 3$). Changes are also seen in the 450–700 nm region of the spectrum. For example, lowering the pH causes the growth of a band at 636 nm similar to that observed for lignin peroxidase (results not shown).

The spectral changes observed in the case of veratryl alcohol oxidation showed Compound I as the main steady-state form of the tobacco peroxidase. The enzyme was inactivated in the

reaction course via a decrease in the Soret band. Compound I conversion back to the native enzyme upon addition of veratryl alcohol was observed only with very low hydrogen peroxide concentrations corresponding to single turnover conditions. The steady-state kinetics in the presence of 40 mM MgCl_2 were consistent with a Ping Pong mechanism at low hydrogen peroxide concentrations (< 0.1 mM). The equation for the initial reaction rate was $[E_0]/v = 1/k_u + 1/k_{\text{H}_2\text{O}_2}[\text{H}_2\text{O}_2] + 1/k_{\text{VA}}[\text{VA}]$, with rate constants of $0.46 \pm 0.05 \text{ s}^{-1}$ (k_u), $(1.2 \pm 0.1) \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($k_{\text{H}_2\text{O}_2}$) and $(1.0 \pm 0.1) \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (k_{VA}) (see the scheme below).

where E, EI and EII are ferric enzyme and Compounds I and II respectively, and VA and VA^{*+} are veratryl alcohol and a radical product of its one-electron oxidation. Under steady-state conditions, $k_{\text{H}_2\text{O}_2} = k_1$, k_{VA} is a function of two rate constants, $k_2 k_3 / (k_2 + k_3)$, and k_u corresponds to a rate-limiting unimolecular step.

The same qualitative picture was obtained using 200 mM CaCl_2 (Figure 3). The corresponding rate constants were equal to $0.25 \pm 0.05 \text{ s}^{-1}$ (k_u), $(5.0 \pm 0.2) \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ ($k_{\text{H}_2\text{O}_2}$) and $(2.8 \pm 0.5) \times 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (k_{VA}) respectively. The specific activity measured under optimal conditions (100 μM hydrogen peroxide, 5 mM veratryl aldehyde, 100 mM calcium chloride, pH 1.85) was $0.30 \pm 0.05 \mu\text{mol/min}$ per mg of enzyme, which is about two orders of magnitude lower than for lignin peroxidase [7] and similar to that of soybean peroxidase [8] when assayed under optimal conditions.

The unimolecular rate-limiting step (k_u) is most likely product dissociation. This requires the existence of a relatively stable complex between one of the enzyme forms and the initial oxidation product of veratryl alcohol. This could be either EII-VA^{*+} (k_u') or E-VA^{*+} (k_u''), or if both complexes have similar dissociation rates, either could be partially rate-limiting. This is consistent with the recently shown existence of such a complex for lignin peroxidase Compound II [16,17].

The ability of tobacco peroxidase at low pH to oxidize substrates with high redox potentials, such as veratryl alcohol, in the presence of calcium and magnesium cations may be of physiological importance. Calcium plays an important role in plants by modulating cell growth and accelerating peroxidase secretion into the cell wall [18]. It is likely that carboxylate groups are involved in calcium binding. If this is so, then the effects of calcium binding and protonation at low pH could be either competitive or synergistic, that is some sites are protonated and others are occupied by a metal cation. The enhancement of tobacco peroxidase activity by magnesium ions is to our knowledge the first example a magnesium-induced peroxidase activation.

The concentrations of calcium chloride used to stabilize soybean peroxidase (200 mM [8]) or to stimulate lignin peroxidase at pH 1.8 (200 mM, this work), or that causing the spectral changes for tobacco peroxidase (100 mM) (Figure 2A), are too high for any specific interaction. However, calcium and magnesium ions do affect the tobacco enzyme activity at concentrations as low as 1 mM (Figure 1A inset). Thus, there must be a low-affinity site for bivalent-metal-ion binding. The existence of

such a centre has been proposed recently for barley peroxidase in order to explain the spectral changes induced by 50 mM calcium ions and the effect on the kinetics of Compound I formation [19]. Thus, there is some evidence for the presence of a low-affinity binding site for bivalent metals in plant peroxidases.

The present study describes for the first time a plant peroxidase able to oxidize high-redox-potential substrates with a pH optimum as low as 1.8. The high stability of the tobacco peroxidase holoenzyme at the extremely low pH compared with other peroxidases could be due to the presence of the negatively charged glutamic acid residue (Glu-141 [10]) at the entrance to the haem-binding pocket. This is in contrast with many other plant peroxidases that contain a phenylalanine residue at this position (Phe-143 for horseradish peroxidase [20] and Phe-143 for peanut peroxidase [6]). However, lignin peroxidase does have a glutamate (Glu-146) in this position that is hydrogen-bonded to His-82 on the distal side of the haem-binding pocket [5]. Protonation of Glu-141 and conformational changes induced by calcium cations with tobacco peroxidase could result in the formation of a new hydrogen-bond, which influences the structure of the enzyme active centre, preventing haem bleaching at extremely low pH. The high catalytic activity of tobacco peroxidase Compound II, compared with the lower activity and high stability of the enzyme Compound I in acidic media (the converse is true for all other native peroxidases studied so far), has also been attributed to the presence of the glutamate residue at the entrance to the haem-binding pocket [9].

The main conclusion of the present work is that tobacco peroxidase exhibits unique catalytic properties and has spectral characteristics that can be modulated by pH, calcium and magnesium ions. These attributes may have physiological importance and commercial potential.

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