Spectral and Kinetic Properties of Oxidized Intermediates of *Coprinus cinereus* Peroxidase

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Spectra of native *Coprinus cinereus* peroxidase and its oxidized intermediates, compounds **I**, **II** and **III**, the kinetics of compound **I** formation and guaiacol activity of the enzyme have been studied at 25 °C. Mechanistic aspects of the oxidation of the enzyme and its oxidized intermediates indicate that the enzyme operates via the classic peroxidatic cycle. Isosbestic points in the UV–VIS range between native enzyme–compound **I** are 353, 426, 453 and 547 nm, between native enzyme–compound **II** 343, 414, 465, 530, 629 and 668 nm, between native enzyme–compound **III** 344, 411, 475, 534 and 604 nm and compound **I**–compound **II** 394 and 576 nm. Soret-region maximum molar absorption coefficients [mM^{-1} cm^{-1}] with wavelengths of maximum absorption in brackets are for the native enzyme 109 (405 nm), for compound **I** 63 (402 nm), for compound **II** 92 (419 nm) and for compound **III** 105 (417 nm). The native enzyme exhibited a shift in the Soret absorption maximum wavelength from 393 to 405 nm over the pH range 3.58–6.01. No shift occurred from pH 6.01–10.83. The pH dependence of compound **I** formation showed the presence of an acidic heme-linked group in the enzyme with a pK_a of 4.9 ± 0.1. Hydrogen peroxide reacts with the basic form of the native enzyme, forming compound **I** with a second-order rate constant of $(7.1 \pm 0.1) \times 10^6$ M^{-1} s^{-1}. Guaiacol activity of the enzyme reaches a maximum at pH 8.

Many peroxidases are heme-containing enzymes capable of oxidizing a large variety of reducing substrates (electron donors) at the expense of equivalents from the oxidizing substrate (electron acceptor) which is a peroxide (hydrogen peroxide, a hydroperoxide or a peroxy acid). The central iron ion in the porphyrin ring changes its formal oxidation state during each step of the reaction cycle. The normal or classic peroxidase cycle [eqns. (1)–(3)], applicable for horseradish peroxidase (HRP),[1-4] involves three oxidation states of the enzyme, redox reactions occurring on the iron ion and the porphyrin ring. The three oxidation states are native enzyme (peroxidase), compounds **I** and **II** (cpd. **I** and **II**).

$$\text{Peroxidase} + H_2O_2 \rightarrow \text{Cpd. I} + H_2O \quad (1)$$

$$\text{Cpd. I} + AH_2 \rightarrow \text{Cpd. II} + AH^{\bullet} \quad (2)$$

$$\text{Cpd. II} + AH_2 \rightarrow \text{Peroxidase} + AH^{\bullet} + H_2O \quad (3)$$

The iron ion changes its formal oxidation state during the cycle according to the following sequence: $Fe^{III} \rightarrow Fe^{V} \rightarrow Fe^{IV} \rightarrow Fe^{III}$. The native resting peroxidase (ferric, Fe^{III}) undergoes a two-electron oxidation with formation of cpd. **I** (ferryl, Fe^{IV}), binding one oxygen atom from hydrogen peroxide to the ferryl ion ($Fe^{IV} = O$) with simultaneous release of water. Electronically, the iron ion in cpd. **I** has an oxidation state of IV, the other equivalent being donated from the porphyrin ring whereby a π-cation radical is formed. The native enzyme is regenerated in two distinct one-electron reductions. First, cpd. **II** is formed, by transfer of an electron (in general terms described by transfer of a hydrogen atom) from the reducing substrate to the porphyrin ring, reconstituting the electronic structure of the ring as in the native enzyme. Therefore, the iron ion in cpd. **II** retains its oxidation state of IV and the double-bonded oxygen atom. The proton from the hydrogen atom is captured by a distal base or a water molecule (affected by the base) within the enzyme molecule. Second, cpd. **II** is supplied with a hydrogen atom from another molecule of reducing substrate ($Fe^{IV} \rightarrow Fe^{III}$), a singly bonded hydroxy group now being attached to the iron ion (Fe^{III}–OH). This group is given off as another water molecule, abstracting the proton which the distal base/water molecule received in the first reduction. For the above reaction cycle the reducing substate, AH_2 is a hydrogen donor, which is oxidized into the free radical product, $AH^{\bullet}$. The fate of the latter can be that of dimerization, oligomerization or polymer-

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ization, cooxidation (reaction with AH_2 or $AH^{\bullet}$), scavenging by molecular oxygen (formation of a peroxyl radical, $-OO^{\bullet}$) or reduction of molecular oxygen (formation of superoxide ion, $O_2^{-\bullet}$). Phenolic compounds and aromatic amines are particularly reactive reducing substrates acting as hydrogen donors. For a few substrates, iodide ion as a classical example,[5] the cycle proceeds without formation of cpd. **II**. In this case the reducing substrate undergoes a single two-electron oxidation and the enzyme undergoes the cycle $Fe^{III} \rightarrow Fe^{V} \rightarrow Fe^{III}$.

In addition to cpd. **I** and **II**, the peroxidase can exist in yet two other oxidation states, cpd. **III**, also called oxyperoxidase, and ferroperoxidase (ferrous, Fe^{II}). In cpd. **III** the oxidation state of the iron ion can be regarded as II and III, the electronic structure being that of a resonance hybrid with two contributing structures: $Fe^{II}–O_2 \leftrightarrow Fe^{III}–O_2^{-\bullet}$. Though cpd. **III**, in the case of HRP is not a participant in the normal peroxidatic cycle, it is, however, chemically reactive.[6,7] Inspection of the resonance hybrids reveals two of three general approaches for formation of cpd. **III**. Reduction of native enzyme ($Fe^{III} \rightarrow Fe^{II}$) with agents such as dithionite under aerobic conditions, will result in formation of cpd. **III** when complexation of molecular oxygen and ferrous ion (Fe^{II}) is allowed to take place. Reaction of native enzyme (Fe^{III}) with superoxide ion,[8] $O_2^{-\bullet}$ will likewise give cpd. **III**. Yet another general approach for formation of cpd. **III** can be taken by adding a large excess of hydrogen to the native enzyme. From native, cpd. **III** might be formed if hydrogen peroxide can also act as a reducing substrate: cpd. **I** is reduced to cpd. **II**, and from cpd. **II** two routes are possible. One proceeds by dissociation of OH^- and association of H_2O_2[9] to Fe^{III} followed by oxidation of hydrogen peroxide to the hydrodioxyl (also called perhydroxyl or hydroperoxyl) radical, $HO_2^{\bullet}$, which is in equilibrium with its conjugate base $O_2^{-\bullet}$. The other involves reaction of Fe^{III} with H_2O_2 as a reducing substrate.

Here we report spectral characteristics of oxidized intermediates (CiP-**I**, CiP-**II** and CiP-**III**) of *Coprinus cinereus* peroxidase (CiP). Also, kinetic data are obtained for formation of CiP-**I** using transient-state kinetics, and for guaiacol oxidation under steady-state conditions.

Experimental

Coprinus cinereus (CiP) was provided in pure form, as seen by SDS-PAGE, by Novo Nordisk A/S, Denmark. Before use, samples were dialyzed extensively against Milli Q water. Enzyme concentration, purity and stability was as reported earlier.[10] Enzyme samples had RZ (A_{405}/A_{280}) values in the range 2.45–2.50. In addition to the spectral stability reported earlier, functional stability was also measured by a guaiacol oxidation assay (see below). The activity of the dialyzed enzyme changed less than 10 % within a month when stored at 5 °C. Enzyme stock solution concentration s were determined with errors of 0.1 μM.

Absorption spectra in the UV–VIS range were recorded on a Cary 219 spectrophotometer. In the visible range enzyme concentrations of ca. 6 μM were used, for the UV range enzyme concentrations were ca. 2 μM. CiP-**I** was formed from native CiP at pH 6.96 with a 1.2 molar ratio of hydrogen peroxide to the enzyme. CiP-**II** was formed from native CiP at the same molar ratio of hydrogen peroxide to native enzyme as for CiP-**I**, but experiments were conducted at pH 10.16. CiP-**II** was obtained by the spontaneous conversion of the newly formed CiP-**I** at pH 10.16, and no addition of reducing substrate was required. The reaction to obtain CiP-**III** from native CiP was performed at pH 6.96 using dihydroxyfumaric acid and hydrogen peroxide[8] to generate superoxide ions.

Kinetics of pH dependent CiP-**I** formation was also measured quantitatively by monitoring the change in absorbance at 405 nm under pseudo-first-order conditions with hydrogen peroxide in excess. Kinetic measurements were conducted using a Photal (formerly Union Giken) RA 601 Rapid Reaction Analyzer, interfaced with a digital computer system, Alps Electric Co., Model No. CP 10 A and an Epson VP-550 recorder. One reservoir contained enzyme in buffer, at concentrations of ca. 1 μM after mixing, while the other reservoir contained hydrogen peroxide in buffer. Hydrogen peroxide concentrations were in at least a tenfold excess of the enzyme in order to maintain pseudo-first-order kinetics. Pseudo-first-order rate constants, k_{obs}, were determined by means of a curve-fitting computer analysis of exponential curves. From these curves second-order rate constants, k_{app}, were obtained by taking the slope of plots of k_{obs} versus total hydrogen peroxide concentration. The second-order pH-independent rate constant, k_1, and the acid-dissociation constant of the enzyme, K_1, were determined by means of a non-linear least-squares computer analysis of plots of k_{app} versus pH.

Guaiacol oxidation and determination of steady-state rate constants were measured according to the literature descriptions.[11,12] For pH-dependent activity measurements concentrations of CiP, guaiacol and hydrogen peroxide were adjusted in order to obtain maximum steady-state conditions.

All experiments were performed at (25.0 ± 0.5 °C) and a constant ionic strength (μ) of 0.1 M was maintained by addition of potassium sulfate. All reagents were of the highest grade available. Hydrogen peroxide was from BDH Chemicals and used, in dilute aqueous solution, without further purification. The concentration of this stock solution was determined by oxidation of iodide to iodine.[13] 4-Methylphenol (*p*-cresol) was from Sigma. Solutions of *p*-cresol were prepared immediately before use and the concentration determined spectrophotometrically[14] at 277 nm using a molar absorption coefficient of 1.69 mM^{-1} cm^{-1}. Dihydroxyfumaric acid was from Sigma. Aqueous stock solutions, concentrations determined by weight, were made before use and degassed with nitrogen. 2-Methoxyphenol (guaiacol) was from Sigma; concentrations of aqueous stock solutions were determined from absorption measurements at 274 nm with a molar absorption coefficient[15] of 2.57 mM^{-1} cm^{-1}. The following buffers were used

in 0.01 M concentrations: citric acid–sodium citrate (pH 3.58–6.01), potassium phosphate (pH 6.41–7.57), Tris-hydrochloric acid (pH 8.03–9.09) and sodium carbonate–sodium hydrogen carbonate (pH 9.50–10.83). The pH was measured with a Fisher Microprobe Electrode, a Fisher Digital pH Meter and Fisher standard buffers.

Results

In Figs. 1 and 2, spectra of native CiP, CiP-**I** and CiP-**II** are shown for the Soret and visible regions, respectively. The spectrum of native CiP has the characteristic absorption maxima (405, 504 and 645 nm) reported earlier where the ε_{405} value was determined based upon the pyridine hemochromogen method.[10] For CiP-**I** two peaks are observed at 402 and 658 nm. CiP-**II** shows a peak at 419 nm in addition to the characteristic double peak at 530 and 554 nm. The spectra of CiP-**III** and native CiP are shown in Figs. 3 and 4. Maximum absorption for CiP-**III** occurs at 417, 547, 582 and 649 nm; the double peak at 547 and 582 nm is characteristic for CiP-**III**. From peak and isosbestic absorbances (stable with respect to time) and concentrations of native

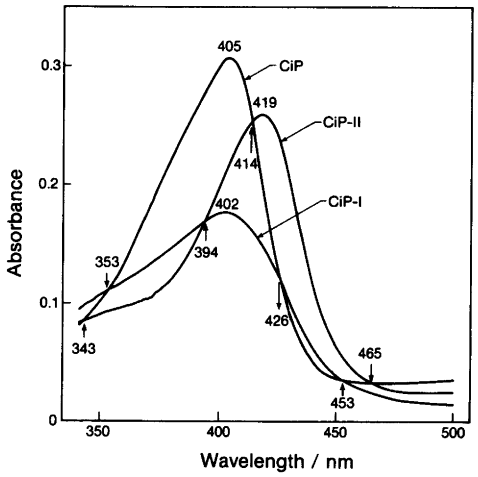

Fig. 1. Spectra of native CiP (2.8 μM, pH 6.96), CiP-**I** (pH 6.96, 1.2 molar ratio of hydrogen peroxide) and CiP-**II** (pH 10.16, 1.2 molar ratio of hydrogen peroxide) in the Soret region.

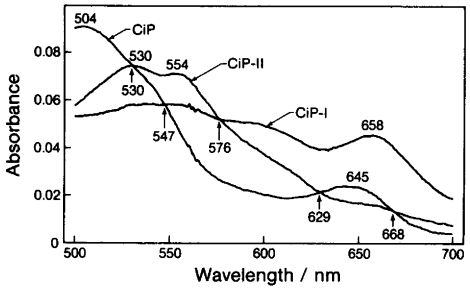

Fig. 2. Spectra of native CiP (7.1 μM, pH 6.96), CiP-**I** (pH 6.96, 1.2 molar ratio of hydrogen peroxide) and CiP-**II** (pH 10.16, 1.2 molar ratio of hydrogen peroxide) in the visible region.

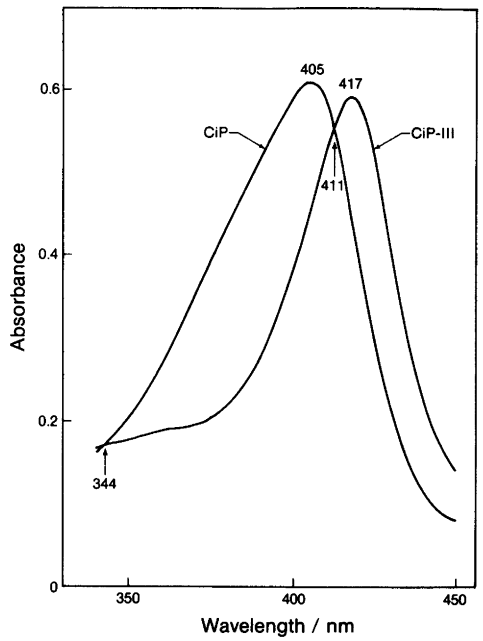

Fig. 3. Spectra of native CiP (5.4 μM, pH 6.96) and CiP-**III** in the Soret region. The spectrum of CiP-**III** was obtained by addition of dihydroxyfumaric acid (hundredfold molar excess) and hydrogen peroxide (1.2 molar ratio).

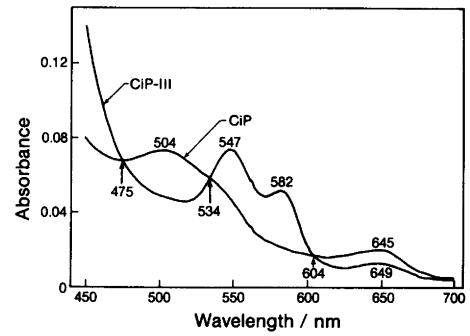

Fig. 4. Spectra of native CiP and CiP-**III** in the visible region. Conditions as in Fig. 3.

enzyme and CiP-**I**, **II** or **III**, maximum and isosbestic molar absorption coefficients were calculated for the four species of CiP. It can be seen that native enzyme has the largest molar absorption coefficient, 109 mM^{-1} cm^{-1} at 405 nm, close to the value for CiP-**III** at 417 nm (105 mM^{-1} cm^{-1}), while the maximum value of CiP-**I** is almost half of that of native enzyme at a slightly lower wavelength (402 versus 405 nm). Table 1 summarizes spectral properties for the four species of CiP.

Formation of CiP-**I** from native CiP was performed by addition of hydrogen peroxide. Various molar ratios were tried. It was found that a 1.2 molar ratio of hydrogen peroxide to CiP gave optimum spectra with respect to maximum absorption and stability with respect to time. Optimum CiP-**I** formation was seen at neutral pH. CiP-**I**

was formed completely within 5 min, as seen by no additional spectral changes, and remained stable for another 4–5 min followed by a slow decay to the native enzyme. Approximately 20 % absorbance was lost within half an hour, but less than 5 % was lost within 10 min.

Formation of CiP-**II** occurred at pH 10.16 with a 1.2 molar ratio of hydrogen peroxide to CiP. Optimum spectra with respect to maximum absorption and stability with respect to time were seen. Experiments were also carried out to form CiP-**II** at pH 10.16 using *p*-cresol as the reducing substrate.[14] The molar ratios of [CiP]:[H_2O_2]:[*p*-cresol] were 1.0:1.2:0.5. However, less stable CiP-**II** spectra were seen than in the presence of hydrogen peroxide only. CiP-**II** was formed completely within 14 min and remained stable for another 5 min. Thereafter a very slow conversion into native enzyme could be seen. The conversion was nearly complete after 90 min.

An optimum CiP-**III** spectrum with respect to absorption and time was obtained using a hundredfold molar ratio of dihydroxyfumaric acid and a 1.2 molar ratio of hydrogen peroxide to CiP. CiP-**III** was formed as a stable species in about 6 min and remained stable, with only a few per cent loss of absorbance, for 15 min. CiP-**III** was also formed at pH 6.96 from superoxide by the xanthine/xanthine oxidase system[16,17] or with a large molar excess of hydrogen peroxide as described in the introduction. These reactions did not give stable spectra of CiP-**III**, however.

Table 1. Wavelengths of absorption peaks, λ, (molar absorption coefficients of peaks, ε), isosbestic points, λ_i, and (molar absorption coefficients for isosbestic points, ε_i) for the four species of CiP. Molar absorption coefficients are given with an error of 1 mM^{-1} cm^{-1}.

Enzyme species	Absorption peaks λ/nm (ε/mM^{-1} cm^{-1})	Isosbestic points λ_i/nm (ε_i/mM^{-1} cm^{-1})	
		CiP	CiP-I
CiP	405 (109) 504 (14) 645 (4)		
CiP-I	402 (63) 658 (6)	353 (39) 426 (43) 453 (13) 547 (8)	
CiP-II	419 (92) 530 (11) 554 (10)	343 (31) 414 (90) 465 (12) 530 (11) 629 (3) 668 (2)	394 (60) 576 (7)
CiP-III	417 (105) 547 (14) 582 (10) 649 (2)	344 (32) 411 (103) 475 (13) 534 (11) 604 (3)	

Table 2. Absorption maxima, λ_1, λ_2 and λ_3 of CiP (3.2 μM) in the UV–VIS range as a function of pH. Buffers used for the various pH values are described in the Experimental section.

pH	λ_1/nm	λ_2/nm	λ_3/nm
3.58	393	506	650
4.66	398	504	650
6.01	405	504	645
6.96	405	504	645
8.03	405	504	645
9.01	405	504	645
10.05	405	504	645
10.83	405	504	645

Table 2 shows the influence of pH on the spectrum of the native enzyme in the UV–VIS range. A shift of λ_{max} from 405 nm to 393 nm occurs when the pH is changed from 6.01 to 3.58. No shift was seen for pH values above 6.01. Also, for absorption peaks in the visible range, smaller significant shifts were seen in the pH range 3.58–6.01. Shifts of the wavelength of the maximum absorption were accompanied by a decrease in absorbance, but it was less than 5 % as compared with the absorbance at λ_{max} for the pH range 6.01–10.83.

Kinetics of CiP-**I** formation was studied using pseudo-first-order conditions. Fig. 5 shows a typical plot of absorbance at 405 nm versus time for reaction of native enzyme with hydrogen peroxide. Pseudo-first-order rate constants, k_{obs} were evaluated from curve-fits of exponential curves. The k_{obs} values were measured as a function of total hydrogen peroxide concentration, [H_2O_2], and were shown to increase linearly with the concentration of hydrogen peroxide, fitting eqn. (4).

$$k_{obs} = k_{app}[H_2O_2] + k_{-app} \quad (4)$$

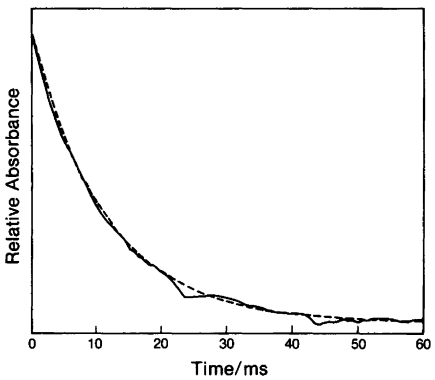

Fig. 5. Time course of the reaction of 1.0 μM CiP with (10.0 ± 0.1) μM hydrogen peroxide in phosphate buffer (pH 6.01, μ = 0.1 M). The dashed line is the computed first-order curve-fit. The ordinate, relative absorbance at 405 nm, is given without values because of the relative behavior of the exponential function.

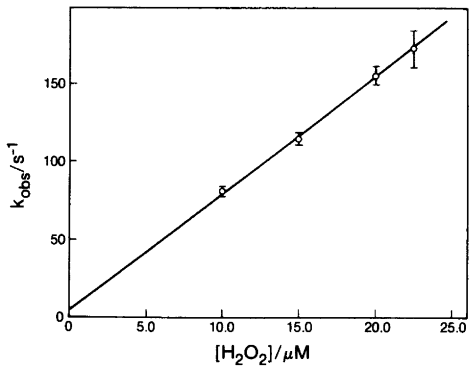

Fig. 6. Pseudo-first-order rate constants of the reaction of hydrogen peroxide with CiP plotted as a function of $[H_2O_2]$ (total hydrogen peroxide concentration during this kinetic experiment). CiP, 1.0 μM in phosphate buffer (pH 8.03, μ = 0.1 M). Error bars show standard deviations calculated from at least seven measurements for each hydrogen peroxide concentration.

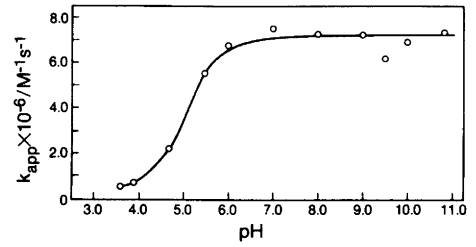

Fig. 7. pH dependence of the apparent second-order association rate constant of hydrogen peroxide reaction. Conditions as in Fig. 6, except that pH was varied from 3.88 to 10.83 and therefore various buffers were used. The error for each point is less than 5 %.

An example, typically for the data obtained from these experiments, is shown in Fig. 6 for pH 8.03. From the slope, the value of the apparent second-order forward rate constant, k_{app} is $(7.4 \pm 0.2) \times 10^6$ M^{-1} s^{-1} and from the intercept, the apparent first-order reverse rate constant, k_{-app} = (5.2 ± 2.7) s^{-1}, or close to zero, to within experimental error. The pH dependence of compound **I** formation appears as that of a titration curve for a simple acid. In Fig. 7 the measured apparent second-order association rate constant, k_{app} is plotted against pH; and inflection point is found around pH 5. If it is assumed that pH dependence is controlled by protonation/deprotonation of a protic group in or close to the active site of the enzyme, the following reaction scheme (Scheme 1) is the simplest one fitting the experimental data obtained.

$$\begin{array}{l} \text{CiP} + \text{H}_2\text{O}_2 \xrightarrow{k_1} \text{CiP-I} \\ \text{H}^+ \downarrow\uparrow K_1 \\ \text{CiP-H}^+ \end{array}$$

Scheme 1.

For this reaction scheme k_1 is the pH-independent second-order association rate constant, and K_1 the acid-dissociation constant of the acid group in the enzyme. The apparent second-order association rate constant can be expressed as eqn. (5). Fitting data by a least-squares analysis

$$k_{app} = k_1/(1 + [H^+]/K_1) \quad (5)$$

to eqn. (5) gave the following results: $k_1 = (7.1 \pm 0.1) \times 10^6$ M^{-1} s^{-1} and $pK_1 = 4.9 \pm 0.1$.

The pH-dependent activity of CiP was assayed using guaiacol as the reducing substrate and hydrogen peroxide as the oxidizing substrate. During the enzymatic oxidation it is assumed that tetraguaiacol is formed[18] as the brownish red reaction product. The course of the reaction was monitored at 470 nm, starting the reaction by addition of hydrogen peroxide. The ratio of guaiacol, hydrogen peroxide and CiP was the same in all experiments. The pH was varied from 3.88 to 10.83. At pH 6.96 concentrations of guaiacol, hydrogen peroxide and CiP were: 0.9 mM, 0.3 mM and 1.2 nM, respectively. For high and low pH values (pH 4.57, 10.16 and 10.83) concentrations of the three reactants were half those given above. All concentrations were measured twice; the reference curvette contained the same concentrations of reactants as the sample cuvette, except for CiP which was excluded. The temperature was 25 °C and concentration of buffer components 0.01 M. One enzyme activity unit, U, is defined as the amount of enzyme producing 1 μmol of tetraguaiacol per minute under the specified conditions. Specific activities were calculated from eqn. (6), where ΔA min^{-1} is the change in absorbance at 470 nm per minute, x the total volume (ml) of the reaction mixture, 26.6 the molar absorption coefficient (mM^{-1}) of tetraguaiacol at 470 nm for a 1 cm light path cuvette, y the volume (ml) of the aqueous enzyme stock solution added to the cuvette, z the concentration (M) of the aqueous enzyme stock solution and 38000 the molar mass (g mol^{-1}) of CiP determined for this batch of enzyme.[10]

$$\text{Specific activity} = (\Delta A \text{ min}^{-1})\, x/26.6\, y\, z\, 38000 \text{ (U mg}^{-1}\text{)} \quad (6)$$

Fig. 8 shows the pH dependence of CiP guaiacol activity. Maximum activity is seen at pH 8, decreasing on both sides of this value. Activities, observed over the entire pH range of the study were low at extreme pH values, but significant and measurable. Linear slopes for at least 1 min reaction time were seen.

For comparison of activities and measured steady-state rate constants with literature values,[19,20] assays were performed according to the literature descriptions.[11,12] Steady-state determination of rate constants for the reaction with hydrogen peroxide and CiP (CiP-**I** formation), pH 6.93 and for the reaction with guaiacol and CiP-**II**, at the same pH gave: $(3.7 \pm 0.3) \times 10^6$ M^{-1} s^{-1} and $(2.4 \pm 0.2) \times 10^5$ M^{-1} s^{-1},

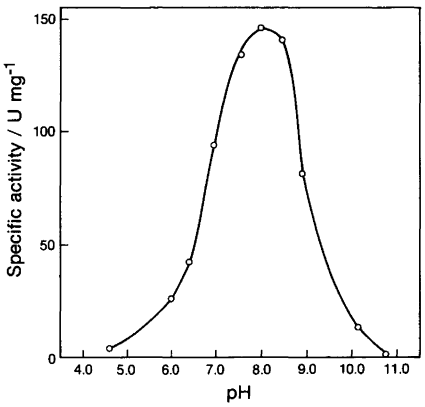

Fig. 8. CiP oxidation of guaiacol as a function of pH. Conditions are described in detail in the Experimental and Results sections. The error for each point is less than 5 %.

respectively. For this enzyme batch a specific activity of (94 ± 2) U mg^{-1} at pH 6.96 was calculated using eqn. (6).

Discussion

Spectra reported in Figs. 1–4 are the ones which display maximum absorption and stability with respect to time. Native CiP is spectroscopically very stable with respect to time: after dilution of the enzyme solution to μM concentrations, the spectra remain unchanged for at least an hour. From Table 2 it is seen that lower pH values cause changes in the Soret maximum absorption band. The absorption peak shifts from 405 to 393 nm at lower pH and ε_{393} is 106 mM^{-1} cm^{-1} compared with ε_{405} 109 mM^{-1} cm^{-1}. Spectral characteristics of CiP and its oxidized intermediates are very similar to those of HRP.[4] Absorption maxima are the same to within 5 nm. Spectra of native CiP, CiP-**I** and CiP-**II** reported earlier[19] and those obtained in this work are similar with respect to maximum absorption and general appearance. Most significant differences are seen for CiP-**II** spectra, which are incomplete in the previous publication owing to the presence of native enzyme, as also noted by the authors.[19] An absorption spectrum of CiP-**III** has not been reported previously. However, CiP-**III** was formed from an undefined molar ratio of hydrogen peroxide at an undefined pH and characterized by electronic absorption and magnetic resonance spectroscopies.[20] Maximum absorption wavelengths were given as 416, 548 and 584 nm, which are to within 2 nm of those obtained in this work.

From the time-dependence of spectra given in Figs. 1–4 and of various spectra for oxidation of *p*-cresol (not shown), it was observed that CiP-**I** was converted into native enzyme at neutral pH. Also a CiP-**II** spectrum, at basic pH, eventually reverted to a spectrum of native enzyme. CiP-**I** and CiP-**II** are also transiently present at pH values where they are not stable, as seen by shoulders, broadening of peaks and decreasing absorption. These observations indicate that CiP uses the normal peroxidatic cycle, when oxidizing substrates such as hydrogen peroxide and reducing substrates such as *p*-cresol are used. The spectrum of CiP-**III** decayed to native CiP with no intermediate shoulders or peak broadenings, indicating absence of detectable amounts of CiP-**I** and/or CiP-**II** during the course of this reaction.

No bleaching (decomposition of enzyme, seen by the disappearance of the Soret absorption) was observed when a hundredfold molar excess of hydrogen peroxide was employed at pH 6.96. The mixture (mostly CiP-**I**) slowly reverted to native enzyme.

To within experimental error, pH-dependent formation (Fig. 7) of CiP-**I** gave the same pK_a value (4.9 ± 0.1) of the enzyme as that obtained for cyanide-complex formation,[10] (5.1 ± 0.2). Alternatively, data can be fitted to a mechanism involving a reaction between acidic enzyme (CiP-H$^+$) and the anion of hydrogen peroxide. Curve-fitting of data to the equation for this mechanism gives $k_1 = 7\times10^{13}$ M^{-1} s^{-1} at pH 7, a value exceeding the diffusion-controlled limit. This mechanism is therefore physically impossible. For HRP[21,22] pK_a values close to 4 have been assigned to cpd. **I** formation depending on the isoenzyme. It has been suggested, based on studies of cpd. **I** formation and known amino acid sequences of the active site of HRP, that the amino acid that most contributes to this pK_a value is a carboxylate and most likely aspartate. Data obtained here support the idea this could also be the case for CiP. But no amino acid sequence of the active site of CiP is yet available. However, comparisons[23] of amino acid sequences of several peroxidases (manganese, lignin, yeast cytochrome c, horseradish and turnip) reveal a conserved aspartate residue hydrogen-bonded to the proximal histidine. The pH-independent second-order association rate constant $[(7.1 \pm 0.1)\times10^6$ M^{-1} s$^{-1}]$ is less than half that of HRP Cl and E5,[22,24,25] $(1.8 \pm 0.4)\times10^7$ M^{-1} s^{-1} but greater than that for HRP A2, $(1.8\times10^6$ M^{-1} s$^{-1})$.[22] Comparison with other plant peroxidases[26] shows that CiP gives a slightly lower value of the rate constant for CiP-**I** formation. On the other hand, the value for CiP (a fungal peroxidase) is slightly higher than values of manganese-dependent and lignin-degrading peroxidases[27,28] from the fungus *Phanerochaete chrysosporium*, $[(2.0 \pm 0.1)\times10^6$ M^{-1} s^{-1} and $(6.5 \pm 0.2) \times 10^5$ M^{-1} s$^{-1}]$, respectively.

Only minor differences in the steady-state-determined rate constants (hydrogen peroxide CiP reaction and guaiacol CiP-**II** reaction) for guaiacol oxidation are seen when the results are compared with those reported earlier.[19] Rate constants obtained here $[(3.7 \pm 0.3)\times10^6$ M^{-1} s^{-1} and $(2.4 \pm 0.2)\times10^5$ M^{-1} s^{-1}, respectively] are close to those measured earlier, $(3.04\times10^6$ M^{-1} s^{-1} and 2.36×10^5 M^{-1} s^{-1}, respectively). The steady-state rate constant of the CiP–hydrogen peroxide reaction, $(3.7 \pm 0.3)\times10^6$ M^{-1} s^{-1} can be compared with to the same value determined from transient-state kinetics, $k_1 = (7.1 \pm 0.1)\times10^6$ M^{-1} s^{-1}. Because the steady-state experiment approximates the rate expression for formation of CiP-**I** (which assumes that the experi-

mental conditions are such that CiP-**I** formation is completely rate-controlling), whereas the transient-state experiment transforms formation of CiP-**I** into an accurate pseudo-first-order reaction, steady-state values might be expected to be lower. The two values differ by nearly a factor 2. Steady-state rate constants for HRP oxidation of guaiacol[19] are similar to those of CiP.

The specific activity for this enzyme batch is (94 ± 2) U mg^{-1} at pH 6.96, and the maximum activity at pH 8 is (146 ± 3) U mg^{-1} (Fig. 8). From Fig. 7 it can be seen that CiP-**I** formation displays no pH dependence in the alkaline pH region. Similarly, from Table 2 it is evident that the native enzyme is spectroscopically stable in the alkaline pH region. Therefore, the decrease in guaiacol oxidation at lower pH values can be explained by protonation of CiP-**I**, while the decrease at higher pH values can be explained by either deprotonation of guaiacol or deprotonation of CiP-**II**, assuming that, (i) the phenol reacts in the unionized form, as seen for HRP and (ii) that the classic peroxidatic cycle prevails, in which the rate-controlling step is the CiP-**II** reaction. Inflection points in Fig. 8 are seen at pH ca. 6.5, corresponding to the pK_a for deprotonation of CiP-**I**, (4.9 ± 0.1), and at pH ca. 9.0, corresponding to 10.2, the pK_a of guaiacol.[29] No data are available for the pH dependence of the CiP-**II** reaction, and therefore no data on the pK_a of deprotonation of CiP-**II** exist, but for HRP[30] a pK_a of 8.7 has been assigned to cpd. **II**; it is the acid form of HRP cpd. **II** that is reactive.

In conclusion, *Coprinus cinereus* peroxidase shows spectral properties similar to those of the classic peroxidase, horseradish peroxidase. The fungal peroxidase can exist as two distinct oxidized intermediates, indicating that it reacts via the normal peroxidatic cycle. As with horseradish peroxidase it also forms a further oxidized form of the enzyme, compound **III**. Kinetically, the enzyme has a lower rate of formation of compound **I** as compared with horseradish peroxidase, but the reactions for these two enzymes are governed by a similar pK_a and the enzymes react in the same basic form. Steady-state rate constants for the *Coprinus cinereus* catalyzed oxidation of guaiacol are also similar to those for horseradish peroxidase.

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