Spectroscopic studies of the type 2 and type 3 copper centres in the mercury derivative of laccase

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INTRODUCTION

Laccase is one of the blue copper oxidases, which catalyse the four-electron reduction of O₂ to water [1-3]. Laccase depends upon four copper ions distributed among three different binding sites for its function, and each copper ion appears to have an important role in the catalytic mechanism [4-6]. However, the detailed investigation of specific chromophores is complicated by overlap among the respective spectroscopic signals. For example, the visible-absorption bands of the type 1, or blue, copper centre are so intense that it is hard to identify the visible transitions of the type 2 and the (binuclear) type 3 sites. A start in unravelling the visible-absorption spectrum was made by Graziani et al., who found decreased absorbance in the region of 740 nm in a type-2-depleted (T2D) form of the protein, and they originally assigned the absorption to the type 2 chromophore [7]. Later, LuBien et al. discovered that the absorbance re-appeared when the protein was treated with peroxide, and they suggested that the decrease in absorbance at 740 nm was due to reduction of the type 3 site instead [8]. Reinhammar & Oda, on the other hand, have reported that there is no significant change in the absorbance in this region after removal of the type 2 copper [9]; however, the type 3 copper centres apparently remain oxidized in their preparation. Overlap is also a problem in interpreting the e.p.r. spectrum, even though only the type 1 and type 2 copper centres exhibit signals in the resting enzyme. Laccase reportedly undergoes temperature-dependent [10] and pH-dependent [4,5] structural transitions, which may directly involve the type 2 site; however, changes in the e.p.r. spectrum of the type 2 copper are difficult to resolve because of interference from the type 1 signal.

Partly in order to overcome problems of spectral overlap, we have prepared a mixed-metal derivative of Rhus laccase (referred to below as the MDL) that contains Hg(II) in one of the copper-binding sites [11]. With the aid of a synchrotron source we have measured the Hg e.x.a.f.s. and confirmed that Hg(II) selectively replaces the type 1 copper [12]. In a previous study we obtained structural information about the type 2 copper when we observed superhyperfine structure from three equatorial nitrogen donors in the S-band e.p.r. signal of the mercury derivative [13]. Further studies, detailed below, establish that the oxidized type 3 site exhibits a weak absorbance at 740 nm and that the e.p.r. signal of the type 2 copper reflects the pH-dependent structural transition previously invoked to explain kinetic data [4].

EXPERIMENTAL

Materials

An acetone-dried powder of the latex from the Chinese natural lacquer tree (Rhus vernicifera) was obtained from Saito and Co., Osaka, Japan. The ligand 1,9-diethyl-3,6,9-triaza-undecane (Et₂dien) was purchased from Pfaltz and Bauer, Waterbury, CT, U.S.A. All other materials were reagent grade.

Methods

Laccase and stellacyanin were extracted and purified by the method of Reinhammar [14] with minor modifications. The final A₃₉₀/A₄₄₄ absorption ratio of purified laccase was 16 ± 1:1. The MDL was prepared as before [11] with two improvements in the procedure: (1) the apoprotein was dialysed into 0.1 M-imidazole/acetic acid buffer, pH 6.25, before the addition of Hg(II) and Cu(I); (2) and, as opposed to an atomic absorption standard solution, a stock solution of mercuric acetate in 0.025 M-Tris/HCl buffer, pH 7.8, was used as the source of mercury. The MDL analysed for 3.0 ± 0.1 copper atoms and 1.0 ± 0.1 mercury atom per molecule of protein. Integration of the e.p.r. spectrum showed there

Abbreviations used: MDL, mercury derivative of laccase; T2D, type-2-depleted; Et₂dien, 1,9-diethyl-3,6,9-triaza-undecane.

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was 1.0±0.1 e.p.r.-active copper atom per molecule of protein.

T2D laccase was prepared by a modification of the method published by Graziani et al. [7]. In our procedure all dialyses were carried out in a hollow-fibre device at 5 °C. The first step was dialysis against 2 mM-K₄Fe(CN)₆ in 0.05 M-sodium acetate buffer, pH 5.2. The enzyme solution was then dialysed for 1 h against the same buffer solution except that 2 mM-dimethylglyoxime was also present. This was followed by dialysis against 2 mM-K₄Fe(CN)₆ and 1 mM-EDTA in 0.05 M-sodium acetate buffer, pH 5.2. Finally, the EDTA and ferrocyanide were removed by exhaustive dialysis against 0.1 M-sodium phosphate buffer, pH 6.0. The T2D derivative analysed for 3.0±0.1 copper atoms per molecule of protein.

Buffer solutions were prepared from deionized water and passed through Chelex-100 resin (Bio-Rad Laboratories, Richmond, CA, U.S.A.), previously equilibrated with the same buffer, in order to remove trace metal ion contaminants. During analysis, buffers were deoxygenated by bubbling with N₂ gas that had passed through a solution containing V²⁺ ions over zinc amalgam. For the pH-dependent e.p.r. experiments involving the mercury derivative, a universal buffer was employed that was made from acetic acid, boric acid, sodium phosphate and NaOH.

The protein concentration was determined spectrophotometrically by the biuret method [15], calibrated with native laccase on the assumption that freshly isolated laccase binds four copper ions. Copper was determined with biquinoline in an acetic acid medium [16], and mercury was also determined spectrophotometrically with dithizone [17]. Spin concentrations were obtained from double integrations of e.p.r. spectra by means of a calibration curve obtained with stellacyanin standards.

Solid Et₄dien,3HNO₃ was prepared from the amine by addition of conc. HNO₃, and precipitation was induced by the addition of ethanol and diethyl ether. The salt was recrystallized from ethanol. An aqueous solution of the salt was standardized by NaOH titration. The complex Cu(Et₄dien)²⁺ was generated in solution by mixing solutions of Cu(NO₃)₂ and Et₄dien.HNO₃ (1:2). Solutions of the complex were buffered with Mops, Caps (3-cyclohexylamino-1-propanesulphonic acid) or Mes buffers where the ionic strength was maintained at 0.1 with KNO₃.

Instrumentation

The u.v.-visible-absorption spectra were run on a Perkin–Elmer Lambda 4C spectrophotometer. The e.p.r. data were obtained on a Varian E-109 spectrometer with a Varian E-935 data system. The pH was measured with a Radiometer model PHM 64 pH-meter at room temperature (21 °C).

RESULTS

Absorption data

Since the absorption band at 330 nm is a shoulder on the u.v. absorbance of the aromatic side chains, Δε₃₃₀ values were obtained by placing a sample of reduced laccase in the reference beam. The reduced enzyme was obtained by anaerobic addition of a slight excess of ascorbate to native laccase. When exposed to 5 mol of H₂O₂/mol of copper, the A₃₃₀ and A₇₄₀ values increased for the MDL and T2D laccase as well as for native laccase. Before oxidation with peroxide, Δε₃₃₀ values of 1440±100 M⁻¹·cm⁻¹ and 870±100 M⁻¹·cm⁻¹ were obtained for the MDL and T2D laccase respectively. In the case of native laccase the results indicated that about 25% of the type 3 copper sites in the resting enzyme remained in the reduced state, in reasonable agreement with the results obtained by Solomon and co-workers [18,19]. Fig. 1 and Table 1 indicate that Δε₃₃₀ = 2800±100 M⁻¹·cm⁻¹ for the native protein after treatment with peroxide, and virtually the same Δε₃₃₀ was observed for peroxide-treated MDL. In the case of T2D laccase a slightly lower Δε₃₃₀ was observed, even after addition of a large excess of peroxide, but the 614 was the

Fig. 1. Difference spectra of laccase derivatives

Difference absorption of the oxidized forms of native laccase (----), T2D laccase (-----) and MDL (-----) versus reduced laccase at room temperature in 0.1 M-phosphate buffer, pH 6.0. The absorbance at 425 nm is due to a pigment that could not be removed from laccase; it is present in all samples.

Table 1. Absorption data for laccase derivatives at room temperature in pH 6 buffer

<table>
<thead>
<tr>
<th>Laccase derivative</th>
<th>λ₃₃₀ (nm)</th>
<th>Δε (M⁻¹·cm⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>330</td>
<td>614 (5200)</td>
<td>Present work</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>614 (5400)</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>614 (5700)</td>
<td>[9]</td>
</tr>
<tr>
<td>MDL</td>
<td>330</td>
<td>740 (380*)</td>
<td>Present work</td>
</tr>
<tr>
<td>T2D</td>
<td>330</td>
<td>614 (5200)</td>
<td>Present work</td>
</tr>
<tr>
<td>Oxidized T2D</td>
<td>330</td>
<td>614 (5200)</td>
<td>[8]</td>
</tr>
<tr>
<td></td>
<td>330</td>
<td>614 (4700)</td>
<td>[9]</td>
</tr>
</tbody>
</table>

* Expected error is ±30 M⁻¹·cm⁻¹.
same as that of the native enzyme (Table 1). Unlike our previous preparation [11], the difference spectrum of the MDL showed minimal absorption in the region of 614 nm. The probable reason why replacement of Cu(II) by Hg(II) was incomplete in earlier preparations is that we failed to control the pH carefully enough during metal addition.

In the absence of interference from absorption by type 1 copper, the oxidized MDL clearly displayed an absorption band at around 740 nm with a molar absorption coefficient of $380 \pm 30 \text{ M}^{-1}\text{cm}^{-1}$. Within experimental error, the same increase in the molar absorption coefficient was observed after the T2D enzyme was treated with peroxide. When ascorbate was added anaerobically to the MDL, the 740 nm absorbance was bleached at the same rate as the 330 nm absorbance (Fig. 2). On the other hand, reduction of the type 2 copper occurred at a much faster rate, as determined by room-temperature e.p.r. measurements.

**E.p.r. data**

The low-temperature e.p.r. spectrum of the MDL was pH-dependent in the range pH 5.0–8.5, with the major changes occurring between pH 6 and pH 7 (Figs. 3 and 4). Small but significant shifts in the peaks due to the $m_i = \pm \frac{1}{2}$ transitions are apparent in the parallel region as well as in the overshoot line at high flux density (results not shown). Plotting the position of the $m_i = - \frac{1}{2}$ transition versus pH yields the titration curve depicted in Fig. 4. Table 2 contains the $g_z$ and $A_z$ values obtained from simulations of the spectra at the pH extremes. Note that the changes in $g_z$ and $A_z$ as a function of pH are known more precisely than the absolute values. We also attempted to simulate the perpendicular region of the spectrum, but the agreement between the experimental and calculated spectra was insufficient to guarantee an unambiguous spectral interpretation. A contribution from a small amount of an e.p.r. active form of type 3 copper may confuse this spectral region, as could a deviation from axial symmetry.

As a comparison, we also studied the pH-dependence of the e.p.r. spectrum of the Cu(Et$_4$dien)$^{2+}$ system. This complex has a pseudotetragonal co-ordination geometry with an N$_4$O donor set in the x–y plane, and the
Fig. 5. X-band e.p.r. spectra of Cu(Et4dien)2+ in aqueous solution

The pH was measured at room temperature, and spectra were run at \(-150^\circ\text{C}\) with a copper concentration of 0.2 nm. Conditions: microwave frequency, 9.08 GHz; microwave power, 40 mW; modulation amplitude, 0.1 mT. A line is drawn through the \(m_I = -\frac{1}{2}\) line as a visual aid. Spectrum A, at pH 5.06; spectrum B, at pH 5.6; spectrum C, at pH 6.04; spectrum D, at pH 6.75; spectrum E, at pH 6.97; spectrum F, at pH 7.51.

capable of conformational variability such that the type 3 pair can be oxidized without exhibiting the 330 nm absorbance [22]. Although the orbital parentage of the transition has not been established to date, at least some of the suggested possibilities could probably be reconciled with arguments put forward by Frank & Pecht. For example, if the absorption reflects a simultaneous pair transition [23], the intensity could be sensitive to the relative displacement of the type 3 copper centres. On the other hand, if the absorption corresponds to His→Cu(II) charge transfer [24], the intensity could vary with the relative orientations of the imidazole moieties [25]. However, in our preparations of both T2D laccase and the MDL the 330 nm absorption intensity is strictly correlated with that of the weak absorption band that is centred around 740 nm. In the following we argue that the 740 nm absorption can be attributed to ligand-field transitions, and hence that a gross structural alteration of the type 3 site would be required to explain the loss of the ligand-field absorption intensity in terms of a conformational transition. Our results are more easily explained if the type 3 copper is assumed to be partially reduced in T2D laccase before the addition of peroxide. This interpretation is in accord with independent e.x.a.f.s. measurements [18,19] and with a direct spectrophotometric assay of Cu(I) in T2D laccase [26].

On the basis of band position and intensity, the 740 nm absorbance can unambiguously be assigned to a ligand-field transition(s) of the MDL. \textit{A priori}, the band could belong either to the type 2 or to the type 3 copper centres, but several lines of evidence indicate that at least the major part of intensity is associated with the type 3 copper. In the first place, e.p.r. data have shown that the type 2 copper is essentially fully oxidized before the addition of peroxide [11,13]. Secondly, in our anaerobic reduction experiments the 740 nm absorbance bleaches at the same rate as the 330 nm absorbance (Fig. 2) whereas the type 2 copper centre is reduced much more rapidly. Finally, corresponding absorbance changes occur at 740 and 330 nm when peroxide is added to the T2D protein, where, of course, no absorption from the type 2 copper is possible. Unfortunately, the ligand-field absorption bands in themselves tell us little about the coordination geometry at the type 3 site, especially if low-symmetry distortions are present. It is worth noting, however, that the met form of haemocyanin, which also contains a binuclear copper(II) pair, exhibits a similar ligand-field spectrum, displaced somewhat to shorter wavelengths [27].

Interpretation of e.p.r. data

The e.p.r. data clarify some seemingly contradictory observations regarding laccase that have been reported over the years. Thus the kinetics of the anaerobic reduction of laccase [4] have been explained in terms of the deprotonation of a water ligand of type 2 copper at approx. pH 7. Hydrolysis of the water ligand (pK 6.2), as well as a hydroxyligic ligand (pK 8.6) supplied by the protein, have been also invoked to explain solvent relaxation data from \(^{1}H\)-n.m.r. studies involving laccase [28]. Yet, years ago, Malmsröm \textit{et al.} determined that the e.p.r. spectrum of the type 2 copper is virtually independent of pH in the range pH 6–12 [29].

By studying the MDL, we have been able to resolve the pH-dependence of the e.p.r. spectrum of the type 2

DISCUSSION

Interpretation of absorption data

The 330 nm absorption band in laccase is known to be a signature of the oxidized type 3 site [14,21]. As can be seen from the data in Fig. 1 and Table 1, the band is fully developed in the spectra of the T2D and MDL species as well as in that of the native enzyme after treatment with a small excess of peroxide. There is therefore little doubt but that the type 3 copper centres are present in the MDL. Frank & Pecht have argued that T2D laccase is
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REFERENCES


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