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Heterogeneity of the Type 3 copper in Japanese-lacquer-tree (*Rhus vernicifera*) laccase

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The two steps of the titration of the Japanese-lacquer-tree (*Rhus vernicifera*) laccase with N_3^- [Morpurgo, Rotilio, Finazzi-Agrò & Mondovi (1974) *Biochim. Biophys. Acta* **336**, 324–328; LuBien, Winkler, Thamann, Scott, Co, Hodgson & Solomon (1981) *J. Am. Chem. Soc.* **103**, 7014–7016] were shown to be two distinct reactions, each involving one different portion of the native enzyme molecules. The difference consists in the oxidation state of the Type 3 Cu, which is reduced in the portion with higher affinity for N_3^- and oxidized in the portion with lower affinity for N_3^-. The difference is eliminated by treatment with oxidizing (H_2O_2) or reducing agents, and a single N_3^- adduct is then formed. The e.p.r. spectra of the H_2O_2-treated enzyme and of its F^- derivatives support this interpretation of the results. The similarity of the spectroscopic properties of the high-affinity N_3^- adduct to those of the N_3^- adducts of half-met-haemocyanins and half-met-tyrosinase is discussed.

The lacase from the Japanese lacquer tree (*Rhus vernicifera*) is a blue oxidase containing one paramagnetic Type 1 Cu(II), one paramagnetic Type 2 Cu(II) and a pair of magnetically coupled Cu(II) ions, the Type 3 Cu (Fee, 1975). It is well established that the copper sites interact with the solvent, since laccase enhances the nuclear magnetic relaxivity of water protons (Rigo *et al.*, 1979*a*; Goldberg *et al.*, 1980) and of ^{19}F ($S = \frac{1}{2}$) nuclei (Rigo *et al.*, 1979*b*) and since its optical and e.p.r. spectra are modified by the presence of inhibitory anions such as N_3^- (Morpurgo *et al.*, 1974; Holwerda & Gray, 1974), CN^- (Desideri *et al.*, 1979) and F^- (Brändén *et al.*, 1973). The Type 2 Cu is the most likely interacting site, but other possibilities exist, in particular the Type 3 Cu. This was suggested to be the binding site for intermediate products of oxygen reduction, such as H_2O_2 (Farver & Pecht, 1981), and it was shown to bind N_3^- in the Type-2-Cu-depleted laccase (LuBien *et al.*, 1981).

In the attempt to clarify the nature of these interactions, we have re-examined the reactions of laccase with N_3^- and F^-. N_3^- is known to react with laccase in two steps (Morpurgo *et al.*, 1974), with equilibrium constants differing by about two orders of magnitude at pH 6.0 (LuBien *et al.*, 1981). The N_3^- that binds with higher affinity produces an increase of optical absorbance at 400 and 500 nm. The intensity of the 500 nm absorbance is further increased on anaerobic addition of ferrocyanide in conditions likely to cause reduction of the Type 3 Cu (Morpurgo *et al.*, 1980). The second reaction step is strongly pH-dependent, being almost absent above pH 7.5, and increases only the absorbance at 410 nm (Morpurgo *et al.*, 1974).

Materials and methods

Laccase, in the form of an acetone-dried powder of the latex of the Japanese lacquer tree (*Rhus vernicifera*), was obtained from Saito and Co., Osaka, Japan. It was purified as described by Reinhammar (1970). E.p.r. spectra were measured with a Varian E-9 spectrometer, and optical spectra with a Beckman model 5230 u.v. spectrophotometer. Experiments in anaerobic conditions were performed as previously described (Morpurgo *et al.*, 1980).

Results and discussion

As mentioned in the introduction, addition of stoicheiometric amounts of N_3^- to laccase solutons causes an increase of absorbance at 400 and 500 nm, due to formation of an adduct with high affinity for N_3^- (Morpurgo *et al.*, 1974). A very careful analysis of the corresponding e.p.r. spectra (Fig. 1, curves *A* and *B*) reveals a change of the shape to be ascribed to modification of a fraction of the Type 2 Cu(II) signal. In fact it is possible to detect a small decrease

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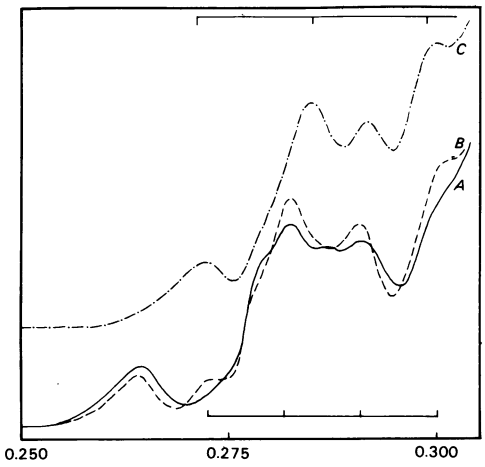

Fig. 1. *E.p.r. spectra in the low-field region of lacquer-tree laccase treated with NaN_3*
Curve *A*, 0.5 mM native enzyme in 0.1 M-sodium acetate buffer, pH 4.2; curve *B*, 1 mol of NaN_3/mol of enzyme was added; curve *C*, NaN_3 concentration was made 0.1 M. Setting conditions: 9.15 GHz microwave frequency, 20 mW microwave power, 100 K temperature. The hyperfine splitting pattern of the N_3^- derivatives with $A_{\parallel} = 0.0100$ T and 0.0135 T is indicated.

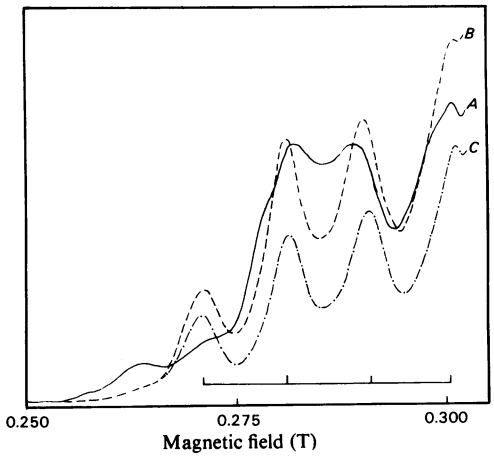

Fig. 2. *E.p.r. spectra in the low-field region of lacquer-tree laccase treated with quinol in the presence of NaN_3*
Curve *A*, 0.4 mM native enzyme in 50 mM-phosphate buffer, pH 6.2, and 12 mM-NaN_3; curve *B*, solution as for curve *A* frozen immediately after anaerobic addition of 1 mol of quinol/mol of enzyme; curve *C*, solution as for curve *B* frozen immediately after anaerobic addition of a further 1 mol of quinol/mol of enzyme. Setting conditions were the same as given in Fig. 1 legend. The hyperfine splitting pattern of the N_3^- derivative with $A_{\parallel} = 0.0100$ T is indicated.

of the lowest-field hyperfine line, with concomitant appearance of a species characterized by a hyperfine splitting constant, $A_{\parallel}$, of approx. 0.0100 T. The integrated intensity of the spectrum did not change. On increasing N_3^- concentration, a second species was formed with $A_{\parallel} = 0.0135$ T, paralleling the formation of the adduct with lower affinity for N_3^-, which absorbs at 410 nm (Morpurgo *et al.*, 1974). In the acidic pH range all Type 2 Cu(II) signal could eventually be converted into the 0.0135 T $A_{\parallel}$ species by high N_3^- concentrations (0.1 M at pH 4.2; Fig. 1, curve *C*), whereas at alkaline pH the second reaction step could not be observed, even in the presence of very high N_3^- concentrations, in agreement with optical data (Morpurgo *et al.*, 1974). However, the inhibition of the second reaction at alkaline pH did not result in a higher yield of the 0.0100 T $A_{\parallel}$ species. At pH 7.8 no further change of the e.p.r. signal was produced by a 200-fold N_3^- excess besides the small effect obtained with 0.6 mol of N_3^-/mol of enzyme, which was very similar to that shown in curve *B* of Fig. 1. Full conversion of the Type 2 Cu(II) signal into the 0.0100 T $A_{\parallel}$ species could only be obtained on anaerobic treatment with a weak reducing agent, such as 1 mol of quinol (hydroquinone)/mol of enzyme (Fig. 2). This modification of the e.p.r. spectrum was observed before any decrease in the integrated intensity could be detected, as when the solution was frozen within a few seconds after mixing with the reductant. This suggests that the reducing equivalents are stored within the e.p.r.-silent Type 3 Cu when the N_3^- adduct with 0.0100 T $A_{\parallel}$ appears. Longer incubation times or higher quinol concentrations resulted in decrease of the e.p.r. signal intensity, mainly of the Type 1 Cu signal (Fig. 2, curve *C*). No 0.0135 T $A_{\parallel}$ species could be detected in the presence of reducing agents, in the pH range 4.2–7.8, at any N_3^- concentration tested, up to 60 mM. On the other hand, when laccase samples were preincubated with 1–3 mol of H_2O_2/mol of enzyme, only the 0.0135 T $A_{\parallel}$ species was formed on addition of N_3^-, the signal shape being very similar to that of Fig. 1 (curve *C*) at saturating N_3^- concentrations. In the presence of H_2O_2 the first reaction step of the spectrophotometric titration, causing absorbance increases at 400 and 500 nm, was also absent.

These results, in agreement with inhibition experiments (Morpurgo *et al.*, 1974) and with the results previously obtained with ferrocyanide as the reducing agent (Morpurgo *et al.*, 1980), support the hypothesis that laccase binds N_3^- more tightly under reducing conditions, giving the 0.0100 T $A_{\parallel}$ species, and that this behaviour is related to the reduction of the Type 3 Cu. Furthermore, the present results indicate that a fraction of partially reduced molecules

is present in the native enzyme, which therefore is not homogeneous as far as the oxidation state of the copper sites is concerned. A minor fraction of molecules that contain reduced Type 3 Cu would be responsible for the first reaction step with N_3^-, whereas the major fraction of fully oxidized molecules is responsible for the second reaction step. The absence of the first reaction step in the presence of H_2O_2 can be explained by its well-known interaction with the Type 3 Cu (Farver *et al.*, 1976), which may be an oxidation of the reduced Type 3 Cu centres, as shown in the Type-2-Cu-depleted enzyme (LuBien *et al.*, 1981). Certainly the reaction with H_2O_2 involves only the minor fraction of molecules. In fact H_2O_2, even in 10-fold excess, did not modify either optical or e.p.r. spectra of samples preincubated with low N_3^- concentrations.

Additional support for this interpretation of the data is that H_2O_2 produces more homogeneous e.p.r. spectra of Type 2 Cu(II) centres in other conditions as well. In the native enzyme the first hyperfine line of the Type 2 Cu(II) e.p.r. signal was affected by H_2O_2 so as to show a more regular bell-shape (Fig. 3, curve *B*). In the presence of fluoride H_2O_2-treated laccase gave a better-resolved ^{19}F superhyperfine pattern (Fig. 3, curves *C* and *D*) than did the native enzyme (Brändén *et al.*, 1973). Binding of 1 (curve *C*) or 2 (curve *D*) mol of F^-/mol, depending on concentration, can be observed, as in fungal laccase (Malkin *et al.*, 1968).

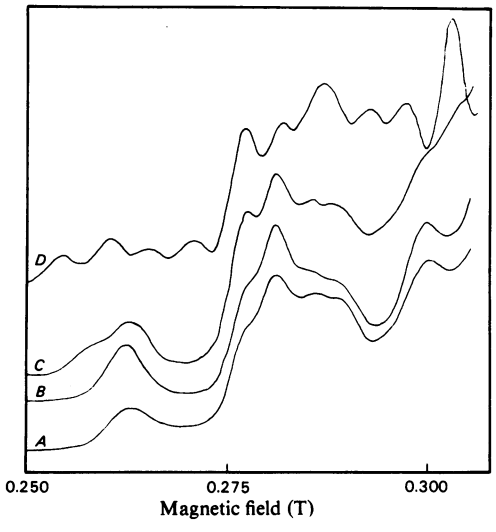

Fig. 3. *E.p.r. spectra in the low-field region of lacquer-tree laccase treated with H_2O_2 and NaF*

Curve *A*, 0.36mM native enzyme in 50mM-phosphate buffer, pH6.0; curve *B*, 1.5mol of H_2O_2/mol of enzyme was added with 10% dilution; curve *C*, NaF was added to the solution used for curve *B* to a final concentration of 2.3mM; curve *D*, NaF was added to the solution used for curve *C* to a final concentration of 330mM. Setting conditions were the same as given in Fig. 1 legend.

It should be pointed out that the N_3^- derivative of the partially reduced enzyme has many common properties with the N_3^- adduct of half-met-haemocyanins (Himmelwright *et al.*, 1980*a*) and half-met-tyrosinase (Himmelwright *et al.*, 1980*b*), i.e. a high affinity for N_3^-, a low $A_\parallel$ value close to 0.0100T, and a relatively low-energy charge-transfer band $N_3^- \rightarrow Cu(II)$ at 500nm. The latter band had been shown to be diagnostic of an N_3^- bridge between oxidized and reduced Cu ions in the half-met (i.e. [Cu(II)Cu(I)]) derivatives (Himmelwright *et al.*, 1980*a*,*b*). In the case of laccase it could indicate the presence of a bridge between oxidized Type 2 Cu(II) and one of the reduced Type 3 Cu(I), and would suggest that the Type 2 and Type 3 Cu sites are close to each other at a distance not exceeding 0.5–0.6nm.

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