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Yeast copper-thionein can reconstitute the Japanese-lacquer-tree (*Rhus vernicifera*) laccase from the Type 2-copper-depleted enzyme via a direct copper(I)-transfer mechanism

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The Type 2-Cu-depleted laccase from the Japanese lacquer tree (*Rhus vernicifera*) can be reconstituted with $CuSO_4$ aerobically and much more rapidly and efficiently under anaerobic reducing conditions. This is to be related to a more favourable conformation of a laccase in the reduced state, rather than to reduction of the metal ion. In fact, reconstitution with Cu(I)-thionein from baker's yeast (*Saccharomyces cerevisiae*) only proceeds under anaerobic reducing conditions, via a direct transfer of Cu(I).

Laccases are copper oxidases containing three different types of copper centres, all apparently involved in the catalytic mechanism (Fee, 1975). Type 1 Cu is intensely blue and displays an e.p.r. spectrum characterized by very narrow hyperfine splitting in the direction parallel to the magnetic field ($A_{\parallel}$). Type 2 Cu has e.p.r. parameters more typical of regular copper complexes. Type 3 Cu is almost certainly an antiferromagnetic pair of Cu(II) ions not detectable by e.p.r. at any temperature between that of liquid helium and ambient. Of the three copper types, only Type 2 Cu was so far selectively and reversibly removed by a procedure involving reducing conditions in both copper-depletion and copper-repletion steps (Graziani *et al.*, 1976). In general, reducing conditions are maintained by using apo-metalloproteins where redox-active residues, such as thiols, are exposed upon removal of the metal (Maret *et al.*, 1980). The Type 2-Cu-depleted laccase, however, is unlikely to belong to this category, because of the spectral properties of the Type 2 Cu. On the other hand, conformational changes have been established to occur in Japanese-lacquer-tree (*Rhus vernicifera*) laccase depending on the state of oxidation. The changes strongly affect the Type 2 Cu site, which becomes more exposed to the solvent (Brändén & Deinum, 1977; Goldberg *et al.*, 1980) and binds more strongly anionic inhibitors, such as azide (Morpurgo *et al.*, 1980*a*, 1982). This led us to reconsider the problem of the reconstitution procedure with a view to the oxidation state of copper. In particular the question was whether the Type 2-Cu-depleted laccase can accept either Cu(II) or Cu(I) and whether the redox state of the protein commands the re-incorporation of copper in either valence state. As a specific donor of Cu(I), to be used irrespective of the redox state of the accepting protein, copper thionein from baker's yeast was employed (Weser *et al.*, 1977), since it has been recently found to be able to transfer Cu(I) directly to apo-stellacyanin (Hartmann *et al.*, 1983). Copper thionein from yeast contains four copper atoms and eight cysteine residues per molecule of mol.wt. 4800 in the form of Cu(I)–S_4 centres (Kimura *et al.*, 1981; Bordas *et al.*, 1982).

Materials and methods

Laccase was purified from the acetone-dried powder from the latex of *Rhus vernicifera*, obtained from Saito and Co., Osaka, Japan, as described by Reinhammar (1970). The Type 2-Cu-depleted protein was prepared as described previously (Morpurgo *et al.*, 1980*b*). The sample used in the present work contained 1.19 atoms of e.p.r.-detectable copper per one Type 1 Cu centre, measured on the basis of $\varepsilon_{614} = 5700\,M^{-1} \cdot cm^{-1}$, and had less than 1% of the enzymic activity of native laccase, determined as the ferrocyanide oxidase activity in 0.05 M-acetate buffer, pH 5.4, at 20°C. An absorption coefficient of $1.0\,mM^{-1} \cdot cm^{-1}$ at 420nm was used to measure the concentration of ferricyanide formed.

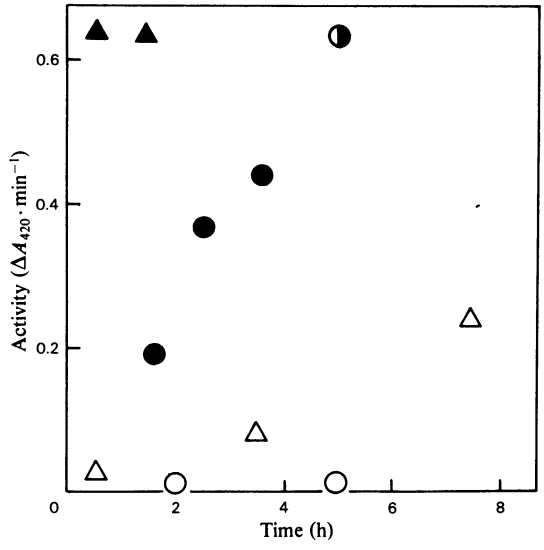

Fig. 1. *Recovery of ferrocyanide oxidase activity of Type 2-Cu-depelted lacase*

The change in absorbance/min at 420nm was measured in an incubation mixture comprising 0.05M-sodium acetate buffer, pH5.4, 3.0mM-ferrocyanide and 0.27μM-laccase (from a 0.27mM stock solution of the Type 2-Cu-depleted enzyme) after different incubation times with: ▲, 0.25mM-$CuSO_4$ and 0.6mM-ascorbate anaerobically; ●, 0.15mM-Cu(I)-thionein and 0.6mM-ascorbate, anaerobically; ◑, 0.30mM-Cu(I)-thionein and 0.6mM-ascorbate anerobically; △, 0.25mM-$CuSO_4$, aerobically; ○, 0.15mM-Cu(I)-thionein, aerobically.

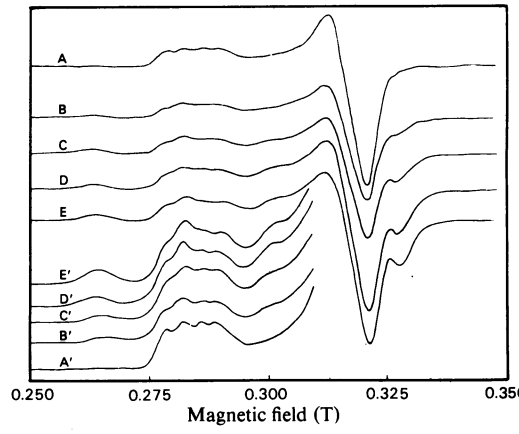

Fig. 2. *E.p.r. spectra of the reconstituted laccase*

A, 0.27mM-Type 2-Cu-depleted laccase and 0.15mM-Cu-(I)-thionein in 0.07M-acetate buffer, pH5.4, immediately after mixing or after 2h incubation in air; B, the same sample as in A, after anaerobic incubation for 1.5h with 0.6mM-ascorbate and re-aeration; C, after anaerobic re-incubation for 1h with 0.6mM-ascorbate and re-aeration; D, recycled as in C; E, 0.27mM-Type 2-Cu-depleted laccase and 0.25mM-$CuSO_4$ in 0.007M-acetate buffer, pH5.4, after anaerobic incubation for 1.5h with 0.6mM-ascorbate and re-aeration. Instrument conditions were: microwave frequency, 9.15GHz; microwave power, 20mW; temperature, 77K. The spectra labelled with primed letters were recorded with a 5-fold higher gain.

S. cerevisiae was grown anaerobically in the presence of 1.0mM-$CuSO_4$ for 48h at 25°C. The isolation of Cu-thionein was performed as described previously (Weser *et al.*, 1977).

Copper was assayed on a Perkin–Elmer atomic-absorption spectrometer (Model 400S) furnished with a HGA-76 unit. Low-temperature e.p.r. spectra at the X-band (9.15GHz) were recorded on an E-9 Varian spectrometer. Electronic absorption spectra were obtained with a Perkin–Elmer model 330 spectrophotometer.

Anaerobiosis was obtained by repeated cycles of evacuation/flushing with purified argon of the samples in a Thunberg apparatus sealed to an e.p.r. tube. Rexoygenation was carried out by the same technique, exchanging argon with air to ensure complete mixing of the sample with oxygen. Distilled deionized water was used throughout the work to avoid contamination by extraneous copper.

Results and discussion

Fig. 1 reports kinetic data on the recovery of ferrocyanide oxidase activity on incubation of Type 2-Cu-depleted laccase with Cu-thionein or $CuSO_4$ under various conditions. It is evident that laccase was reconstituted much more efficiently under reducing anaerobic conditions than in air, with either thionein or inorganic copper, and that Cu(I)-thionein was effective only when the prior incubation with Type 2-Cu-depleted laccase was performed anaerobically in the presence of ascorbate. It is conceivable that the conformational change of laccase related to its reduction is the essential factor for the Cu transfer from thionein to take place. The highest activity values in Fig. 1 are very close to that obtained with the native enzyme. To reach this value a slight excess over 1mol of thionein/mol of laccase was required. Since thionein has four copper ions per molecule, this suggests that either only one copper ion is reactive with Type 2-Cu-depleted laccase, or than an equilibrium is established between the two proteins as in the case of stallacyanin reconstitution (Hartmann *et al.*, 1983).

Fig. 2 shows the e.p.r. spectrum of Type 2-Cu-depleted laccase (curve A), which was unaffected by aerobic incubation with Cu(I)-thionein, in agreement with the activity data of Fig. 1. Curves

B–D show the spectra of the same sample after anaerobic incubation with Cu(I)-thionein in the presence of ascorbate and re-admission of air at different time intervals, as described in the legend to Fig. 2. The integrated intensity of curve B is lower than that of curve A, in spite of the presence of re-incorporated Type 2 Cu. This is very likely due to the fact that the e.p.r. intensity of the sample only accounts for the reconstituted portion of the enzyme, since reoxidation of Type 2-Cu-depleted laccase is a slow process (Morpurgo *et al.*, 1980*b*). The integrated intensity of curves C and D is progressively higher, in a way proportional to the activity of the samples. Curve E shows the spectrum of a sample of Type 2-Cu-depleted laccase after incubation with stoichiometric $CuSO_4$ under reducing conditions. The reaction was faster than with thionein.

These results have two important implications. From the standpoint of laccase conformational states they confirm that the Type 2-Cu-depleted protein reacts with Cu(I) or Cu(II) to incorporate Type 2 Cu, depending on the oxidation state of the Type 1 and Type 3 Cu centres. In other words, reduction of the latter centres preforms the Type 2 Cu binding-site conformation so as to make incorporation of copper faster. The oxidized conformation only appears to take up Cu(II), though at a slower rate than under reducing conditions. In the case of thionein the sum of two unfavourable factors, oxidized laccase and theionein-bound Cu(I), inhibits the reaction completely.

As far as the possible functional role of Cu(I)-thioneins is concerned, it has already been shown that they can exchange copper with Type 3 (Beltramini & Lerch, 1982) and Type 1 (Hartmann *et al.*, 1983) apo-cuproproteins. However, in the former case a prior treatment of the thionein was required, with likely destruction of the Cu(I)–thiolate bonds, whereas in the latter one direct Cu(I) transfer apparently took place. It is now clear that also typical Type 2 Cu centres of proteins, presumably involving nitrogen (Mondovì *et al.*, 1977) and oxygen (Goldberg *et al.*, 1980) ligands, are able to accept copper from thionein. It is noteworthy that the nature of laccase ligands seems to be less important in governing the process than is the geometry of the accepting site imposed by specific conformational strains in the protein.

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