Dependence on freezing of the geometry and redox potential of Type 1 and Type 2 copper sites of Japanese-lacquer-tree (*Rhus vernicifera*) laccase

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The room-temperature e.p.r. spectrum of the Japanese-lacquer-tree (*Rhus vernicifera*) laccase shows $A_{\parallel}$ (the hyperfine splitting constant) and $g_{\parallel}$ values of both the Type 1 and Type 2 Cu appreciably different from those measured at liquid-N₂ temperature. The geometry of the sites, as inferred from the room-temperature e.p.r. parameters, is more consistent with their redox properties. A rough correlation is found between $A_{\parallel}$ and $g_{\parallel}$ values and redox potential of the blue copper in several enzymes.

The blue copper oxidases are characterized by the presence of two distinct types of e.p.r.-detectable Cu(II), namely the intensely blue Type 1 Cu with small $A_{\parallel}$ (hyperfine splitting constant) and the Type 2 Cu with large $A_{\parallel}$ and no apparent colour (Fee, 1975). The redox potentials of the two sites are rather close to each other in the Japanese-lacquer-tree (*Rhus vernicifera*) laccase. Values ranging from 390 to 434 mV are reported for the Type 1 Cu and from 365 to 390 mV for the Type 2 Cu under different experimental conditions (Reinhammar, 1972). This is quite surprising, since the strongly tetragonal Type 2 Cu would be expected to have a much lower redox potential than the Type 1 Cu (Vallee & Williams, 1968) for which a distorted tetrahedral geometry is accepted and the evaluation of the redox potential from optical parameters gave a value in good agreement with the experimental one (Dooley *et al.*, 1979). On the other hand, more recent reports suggest that the properties of each copper type may be considerably modified when some other copper is either reduced or bound to inhibitors (Morpurgo *et al.*, 1980b). For instance, azide- or cyanide-bound Type 2 Cu has a co-ordination geometry considerably different from that of the unbound site (Desideri *et al.*, 1979), and binding of the anions is favoured by reduction of the Type 3 Cu.

Changes of the redox state of the Cu ions were observed to occur on freezing of the enzyme for e.p.r. experiments (Morpurgo *et al.*, 1980b; Desideri *et al.*, 1979). This may be due to a temperature-dependence of redox potentials (O'Reilly, 1973) or to conformational changes of the protein, affecting the geometry of the metal sites. To test such a possibility, the e.p.r. spectra of the lacquer-tree

laccase and of the Type 2 Cu-depleted enzyme were recorded at room temperature.

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), and the Type 2 Cu-depleted enzyme was prepared as previously described (Morpurgo *et al.*, 1980a). The preparation contained about 7% residual Type 2 Cu, determined as the difference between e.p.r.-detectable Cu and Type 1 Cu measured from $\epsilon_{614} = 5700 \text{ M}^{-1} \cdot \text{cm}^{-1}$. Caeruloplasmin was prepared from bovine plasma by a novel procedure involving batchwise absorption from fresh plasma on QAE-Sephadex at pH 7.4 and (NH₄)₂SO₄ fractionation of the eluate. The purified protein was electrophoretically homogeneous and displayed optical and e.p.r. properties typical of the purest preparations (Deinum & Vänngård, 1973). In particular, it was free from e.p.r.-detectable copper-containing impurities.

Room-temperature e.p.r. spectra were measured on 1.5–1.0 mM-protein solutions by using flat silica e.p.r. cells. The instrument was a Varian E-9 spectrometer. Optical-absorption spectra were obtained with a Beckman model-5230 u.v. spectrophotometer.

Results and discussion

Type 1 Cu

Fig. 1 shows the e.p.r. spectra of lacquer-tree laccase and of the Type 2 Cu-depleted enzyme

Fig. 1. Room-temperature e.p.r. spectra

(a) 1.5 mM Lacquer-tree laccase; (b) 1.5 mM Type 2 Cu-depleted enzyme; (c) smoothed difference spectrum (a–b); (d) 3.0 mM-superoxide dismutase. Potassium phosphate buffer (0.1 M, pH 6.0) was used. 'Primed' curves (a', b' and c') were recorded at double instrument gain. Setting conditions: 9.51 GHz microwave frequency, 200 mW microwave power.

(curves *a* and *b* respectively) measured at room temperature at two different instrument gains. The hyperfine splitting constant ($A_{\parallel}$) of the Type 1 Cu is apparently larger than in the spectra measured on the same frozen samples (Fig. 2 and Table 1). Values of $60 \times 10^{-4} \text{ cm}^{-1}$ and $38 \times 10^{-4} \text{ cm}^{-1}$ were obtained at room temperature and in the frozen state respectively for both the native and Type 2 Cu-depleted enzyme. The respective $g_{\parallel}$ values were 2.19, and 2.30.

In Table 1 are collected the redox potentials, the $g_{\parallel}$ and $A_{\parallel}$ values of the Type 1 Cu of several blue oxidases and blue copper proteins. It is immediately apparent that the values measured for lacquer-tree laccase at room temperature are similar to the values reported for all other oxidases, and that the very small $A_{\parallel}$ and high $g_{\parallel}$ values measured on the frozen samples are only found in some blue proteins containing a single type of copper, the Type 1 Cu, such as stellacyanin and umecyanin. The latter proteins also show a redox potential lower than the oxidases, and this may explain why the blue copper of lacquer-tree laccase, reduced at room temperature by ferrocyanide, a relatively weak reducing

Fig. 2. Low-temperature e.p.r. spectra in the low-field region

(a) Lacquer-tree laccase; (b) the Type 2 Cu-depleted enzyme; (c) smoothed difference spectrum (a–b). Samples were the same as those in Fig. 1. Setting conditions: microwave frequency 9.15 GHz; microwave power 20 mW.

agent, with a redox potential (E_0) of about 360 mV (O'Reilly, 1973) reoxidizes on freezing (Morpurgo *et al.*, 1980b). The redox potential of the Type 1 Cu

Table 1. Redox potential and e.p.r. parameters of Type 1 Cu

Protein	Redox potential (E_0) (mV)	$g_{\parallel}$	$10^4 \times A_{\parallel}$ (cm^{-1})	References*
Lacquer-tree laccase				
Room temperature	434–390	2.19	60	Reinhammar, 1972; the present work
77 K	<360†	2.30	38	The present work
Caeruloplasmin‡	580	2.21	92	Deinum & Vänngård, 1973;
	490	2.20	76	Gunnarsson <i>et al.</i> , 1973
Fungal laccase	785	2.19	90	Reinhammar, 1972; Malmström <i>et al.</i> , 1968
Ascorbate oxidase	—	2.23	58	Deinum <i>et al.</i> , 1974
Stellacyanin	184	2.29	35	Reinhammar, 1972; Malmström <i>et al.</i> , 1970
Umecyanin	283	2.32	35	Stigbrand <i>et al.</i> , 1971
Plastocyanin	370	2.23	63	Blumberg & Peisach, 1966

* When two references are given, the former refers to the redox potential, the latter one to e.p.r. parameters.

† Not reduced by ferrocyanide.

‡ The relative order of redox potentials and e.p.r. parameters of the two Type 1 Cu of the enzyme is arbitrary.

Table 2. E.p.r. parameters of the Type 2 Cu

Protein	$g_{\parallel}$	$10^4 \times A_{\parallel}$ (cm^{-1})	References
Lacquer-tree laccase			
Room temperature	2.28	146	The present work
77 K	2.24	195	The present work
Superoxide dismutase			
Room temperature	2.26	138	The present work
pH 3.6	2.27	159	Fee & Phillips, 1975
CN-adduct	2.21	194	Fee & Gaber, 1972
Cu(II)-diethylenetriamine	2.23	193	Morpurgo <i>et al.</i> , 1973

roughly correlates with $g_{\parallel}$ and $A_{\parallel}$ values in the various enzymes, i.e. it is higher in proteins with larger $A_{\parallel}$ and smaller, $g_{\parallel}$. The e.p.r. parameters, in turn, are a measure of the copper ligand field deviation from a regular tetrahedral geometry (Sakaguchi & Addison, 1979) and suggest a substantial rearrangement of geometry of the Type 1 Cu site of laccase on freezing. On the other hand, caeruloplasmin did not show significant changes in e.p.r. spectra recorded at room temperature or at 77 K (Fig. 3), in line with the expectation based on the above considerations.

Type 2 Cu

The general features of the room-temperature Type 2 Cu e.p.r. spectrum were graphically evaluated from the difference spectrum between the native and Type 2 Cu-depleted enzyme (Curves *c* and *c'* of Fig. 1). The spectrum has smaller $A_{\parallel}$ and higher $g_{\parallel}$ values than the difference spectrum obtained at low temperature (Fig. 2), which, in turn, agrees perfectly with that obtained by computer simulation of the low-temperature spectrum (Malmström *et al.*, 1970). The room-temperature parameters of lacquer-tree laccase Type 2 Cu are

Fig. 3. E.p.r. spectra of bovine caeruloplasmin at 77 K (a) and at room temperature (b).

Enzyme was 1.0 mM in 0.1 M-potassium phosphate buffer, pH 7.4. The spectra are presented on a *g*-scale for better comparison, as they were run at different frequency: 9.51 GHz the room-temperature one, 9.15 GHz the low-temperature one; the microwave powers were 200 mW and 20 mW respectively.

very similar to those of superoxide dismutase (Fig. 1 and Table 2), the copper ions of which have a redox potential around 400 mV (Fee & DiCorleto, 1966) and have been assigned a distorted square pyramidal structure (Richardson, 1977; Morpurgo *et al.*, 1973). The low-temperature parameters are rather similar to those of the tetragonal species, none of which is reduced by ferrocyanide (Morpurgo *et al.*, 1973, 1976), i.e. have a redox potential lower than 360 mV. On this basis the Type 2 Cu reduced by ferrocyanide at room temperature would be expected to reoxidize on freezing, as the Type 1 Cu does. This does not occur, but it may be for kinetic reasons, since the reoxidation of the Type 2 Cu is reported to be a slow reaction (Brändén & Reinhammar, 1975). It is noteworthy that the Type 1 Cu reoxidizes only in part on freezing, after room-temperature reduction by ferrocyanide, in Type 2 Cu depleted laccase, i.e. under conditions in which its reoxidation is also a slow process (Morpurgo *et al.*, 1980a).

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