Low-temperature resonance-Raman spectra of Japanese-lacquer-tree (*Rhus vernicifera*) laccase, type-2-copper-depleted laccase and H₂O₂-treated type-2-copper-depleted laccase

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Resonance-Raman spectra of Japanese-lacquer-tree (*Rhus vernicifera*) laccase, type-2-copper-depleted laccase and the latter form treated with H₂O₂ were measured in liquid and frozen solution, on excitation into the 600 nm absorption band. Significant changes in intensity and/or frequency of the bands lying in the 370–430 cm⁻¹ region were observed on freezing, indicating local structural rearrangements taking place at the blue copper site. These findings corroborate previous suggestions based on e.p.r. measurements and redox data [Morpurgo, Calabrese, Desideri & Rotilio (1981) *Biochem. J.* **193**, 639–642]. They show the strong dependence of the physical properties of blue copper centres on local symmetry. Some conclusions on the origin of the Raman bands are also drawn.

The blue copper oxidases are enzymes characterized by the presence of three spectroscopically different types of copper in their molecule (Fee, 1975): type-1 or 'blue' copper displays an e.p.r. signal with a small hyperfine splitting constant ($A_{\parallel}$) and a strong absorption in their electronic spectra at approx. 600 nm ($\epsilon = 5000 \text{ M}^{-1} \cdot \text{cm}^{-1}$). This band is assigned to a $\sigma(\text{S}(\text{Cys})) \rightarrow \sigma^*(\text{Cu}(\text{II}))$ charge-transfer transition (Solomon *et al.*, 1976). The X-ray-diffraction structure of this centre has been determined in two single-copper-type-1-containing proteins, namely plastocyanin (Colman *et al.*, 1978) and azurin (Adman *et al.*, 1978), indicating a distorted tetrahedral structure with the metal atom bound to two imidazole nitrogen atoms of histidine, one sulphur atom of methionine and one sulphur atom of cysteine.

Type-2 copper is also paramagnetic, but its e.p.r. signal has a larger $A_{\parallel}$ value; it does not contribute appreciably to the electronic absorption spectra. Type-3 copper, on the other hand, does not show any e.p.r. signal, and is assumed to be an

antiferromagnetically coupled [Cu(II)–Cu(II)] pair (Fee, 1975).

Morpurgo *et al.* (1981) have found that, among the blue copper proteins, Japanese-lacquer-tree (*Rhus vernicifera*) laccase shows different e.p.r. behaviour at room temperature and in the frozen (77 K) state, since the parameters of both the e.p.r.-detectable types of copper change on freezing. Type-1 copper, in particular, shows $A_{\parallel}$ and redox potential values approaching those of blue copper proteins containing no other copper types, namely stellacyanin and umecyanin, when the sample is frozen. On the other hand, LuBien *et al.* (1981) showed that in type-2-copper-depleted (T2D-) laccase structural changes in the type-1-copper site may be detected. They also demonstrated that in this derivative the type-3 copper is, in fact, reduced but can be re-oxidized by treatment with H₂O₂.

Resonance-Raman (r.R.) spectroscopy on excitation within an absorption band of the molecule under study enables the analysis of the vibrational spectra of the chromophore responsible for that particular absorption. It is therefore a very sensitive and useful probe to detect local structural changes selectively. In the present paper we give the results of

Abbreviation used: r.R., resonance Raman.

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an r.R.-spectral study of laccase, T2D laccase (treated or not treated with H_2O_2), stellacyanin and caeruloplasmin at room temperature and on frozen samples, which give additional information on local changes in geometry.

Materials and methods

Laccase and stellacyanin, in the form of acetone-dried powder of the latex of the Japanese lacquer tree (*Rhus vernicifera*), were obtained from Saito and Co., Osaka, Japan. They were purified as previously described (Reinhammar, 1970). T2D-laccase was prepared as described by Morpurgo *et al.* (1980), and the oxidation of type-3 copper by H_2O_2 was achieved by following the method of LuBien *et al.* (1981). Human caeruloplasmin was prepared as previously described (Pejaudier *et al.*, 1970). The r.R. spectra were recorded on a DH 800 Coderg spectrometer, equipped with holographic gratings. The detector used a d.c. amplifier and a cooled RCA C31034 photomultiplier tube. A quartz tube for e.p.r. was used as cell. The samples were cooled in a cryostat by means of a flow of N_2 regulated by a temperature controller. The 647.1 nm exciting line of a Spectra Physics Kr^+ laser was used throughout. Band intensities were measured by the trace, cut and weight method of the gaussian components of the Raman peaks obtained by means of a 310 Dupont de Nemours curve analyser. Intensities at 380 and 404 cm^{-1} are measured relative to 415 cm^{-1} .

Results and discussion

Fig. 1. shows the r.R. spectra, in the $350\text{--}450\text{ cm}^{-1}$ region, of holo-laccase at room temperature and in the frozen state (200 K). As can be noticed, there is an increase of about 20% in the relative intensity of the 379 cm^{-1} band, whereas that at 403 cm^{-1} remains unchanged. There is a concomitant shift of the three bands to higher frequencies, this being stronger for the peak at 379 cm^{-1} (see Table 1). This spectrum remains unchanged on further lowering of temperature (130 K), and the modifications observed are strictly reversible. The 262 cm^{-1} band (not seen in Fig. 1) is not affected by temperature changes.

As previously reported (LuBien *et al.*, 1981), in the r.R. spectrum of T2D-laccase the 379 cm^{-1} band is considerably less intense than in that of the holoenzyme and is shifted to higher frequencies. In our room-temperature spectra of this laccase derivative, however, this band is split in two peaks lying at 375 and 382 cm^{-1} (see Fig. 2). As a matter of fact, the area lying below these bands is equivalent to the area lying below the one at 379 cm^{-1} in the

Fig. 1. Resonance-Raman spectra of *Rhus vernicifera* laccase (0.25 mm) in H_2O at 300 K (upper spectrum) and 200 K (lower spectrum)

Experimental conditions: laser power, 130 mW; slit width, 5 cm^{-1} ; time constant, 0.5 s; scanning rate, $25\text{ cm}^{-1}/\text{min}$.

spectra of the holoenzyme. In addition, the other two peaks are also slightly shifted to higher frequencies. In the r.R. spectrum of the frozen samples all these bands move slightly to higher frequencies and again their relative intensities are modified (see Table 1). These spectra are of considerably lower quality than those taken at room temperature. Hence intensity measurements and frequencies are less accurately measured.

The r.R. spectra of the H_2O_2 -treated T2D-laccase at room temperature and in the frozen state are shown in Fig. 2. As indicated by LuBien *et al.* (1981), the addition of H_2O_2 restores, although not completely, the original r.R. spectrum of the holoenzyme. The two peaks at 375 and 383 cm^{-1}

Table 1. Relative intensities of the 350–430 cm⁻¹-region bands of laccase, T2D-laccase and H₂O₂-treated T2D-laccase at room temperature (300 K) and in the frozen state (200 K)

	300 K		200 K		$\Delta\bar{\nu}$ (cm ⁻¹)
	$\bar{\nu}$ (cm ⁻¹)	Relative intensity	$\bar{\nu}$ (cm ⁻¹)	Relative intensity	
Holo-laccase	379.2 ± 0.2	1.22	381.7 ± 0.2	1.45	2.5
	403.5 ± 0.1	1.33	404.7 ± 0.3	1.33	1.2
	417.5 ± 0.2	1.00	418.9 ± 0.2	1.00	1.4
T2D-laccase	383.0 ± 0.1		384.1 ± 0.2		
	375.0 ± 0.2	0.84	?	0.90	1.1
	404.1 ± 0.1	1.27	405.3 ± 0.3	1.33	1.2
	419.4 ± 0.3	1.00	420.9 ± 0.3	1.00	1.5
H ₂ O ₂ -treated T2D-laccase	379.3 ± 0.3	1.07	382.2 ± 0.3	1.16	2.9
	405.1 ± 0.2	1.16	407.0 ± 0.2	1.08	1.9
	417.3 ± 0.2	1.00	418.2 ± 0.3	1.00	0.7

coalesce in a rather symmetric band at 379 cm⁻¹, but the relative intensities at 379 and 405 cm⁻¹ are slightly lower than in the native enzyme (see Table 1). Lowering of temperature shifts the Raman bands to higher frequencies, in particular at 379 cm⁻¹ (3 cm⁻¹), and modifies their relative intensities, which differ appreciably from those of the holo-enzyme (compare Figs. 1 and 2). Finally, a fourth form of the enzyme, the native one treated with 30 equiv. of H₂O₂, displays r.R. spectra indistinguishable from those of the native enzyme at all temperatures tested.

The r.R. spectra of the single-copper-type-1-containing protein stellacyanin and of human caeruloplasmin (containing all three types of copper and two blue copper centres) have been taken for comparison. In both cases no changes were observed on freezing.

From the foregoing results it is clearly apparent that the local symmetry of the type-1 copper centre of laccase and its derivatives is modified on freezing, presumably owing to some re-arrangement of the polypeptide backbone. Local structural changes in the case of the holoenzyme were inferred by Morpurgo and co-workers on grounds of the e.p.r. and redox data referred to above (Morpurgo *et al.*, 1981). The present Raman measurements are in perfect agreement with their assumptions and demonstrate the strong dependence of the spectral and redox properties of blue centres on co-ordination geometry.

At this stage it is of interest to compare the room-temperature r.R. spectra of laccase with those of the two blue copper proteins containing no other copper types whose X-ray structure is known, namely plastocyanin and azurin. They are roughly similar to that of laccase, presenting three bands at 379, 407 and 426 cm⁻¹ (0.7:0.9:1 relative intensities) in that of plastocyanin, and at 372, 407 and

425 cm⁻¹ (0.8:1.9:1 relative intensities) in that of azurin (Siiman *et al.*, 1976; Ferris *et al.*, 1979). These spectral similarities suggest a similar distorted tetrahedral ligand environment in the blue copper centre of laccase, with minor differences in copper–ligand distances and angles.

Inasmuch as the chromophore responsible for r.R. enhancement is the σ S(Cys)→ σ^* Cu(II) charge-transfer transition (Solomon *et al.*, 1976), the vibration most strongly enhanced is expected to be the Cu–S stretching mode, with some contribution of deformation modes, since the ligand environment should be mostly distorted along the Cu–S bond during the electronic transition (Hirakawa & Tsuboi, 1975). Deformation along the other bonds are by no means precluded, and, although the σ N(His)→ σ^* Cu(II) charge-transfer transition lies in the u.v. region (Fawcett *et al.*, 1980), enhancement of the Cu–N stretching modes are also plausible. In fact all the metal–ligand vibrations (stretches and deformations) must be strongly coupled, and this explains why the r.R. spectra of blue copper centres are rather rich. Presumably the Cu–S stretch is contained in more-or-less strong proportions in the three bands lying in the 350–430 cm⁻¹ region (Ferris *et al.*, 1979). Furthermore, any change in local symmetry must be mainly reflected in the vibrations strongly coupled to the electronic transition. Since, of the three bands affected in our experiments, the one at 379 cm⁻¹ shows the strongest variations in intensity accompanying the strongest shifting, it seems reasonable to attribute this peak mainly to a Cu–S stretch. Similar assignment has been made by two of us on grounds of r.R. measurements of square-planar Cu(II)–thiolate complexes (Tosi & Garnier, 1979). However, there must be some contribution of the Cu–S mode to the two other bands that are also affected.

That none of these bands corresponds to a pure

Fig. 2. Resonance-Raman spectra of T2D-laccase at 300 K (lower spectrum), H_2O_2 -treated T2D-laccase at 300 K (upper spectrum) and at 200 K (middle spectrum). All protein samples were 0.25 mM in H_2O . Experimental conditions were the same as indicated in Fig. 1 legend.

vibration becomes clearly apparent by the splitting of the 379 cm^{-1} peak in the r.R. spectrum of the T2D-laccase derivative (Fig. 2). Three pure metal-ligand stretching modes are expected in this region, namely two Cu-N(His) and one Cu-S(Cys), inasmuch as the Raman bands are all polarized. The Cu-S(Met) mode has been assigned to the band at 260 cm^{-1} (Ferris *et al.*, 1978), and should not be strongly coupled to the former ones since the

Cu-S(Met) bond distance is unusually long in the blue copper centre of plastocyanin (Colman *et al.*, 1978). The lack of sensitivity of this vibration to temperature changes confirms this assignment. The bands at 382 and 376 cm^{-1} that coalesce in the spectra of the holoenzyme and H_2O_2 -treated T2D-laccase may be then assigned primarily to the Cu-S mode that is more or less coupled to ligand, or metal-ligand deformations.

Finally, one interesting observation to be drawn from our data is that the geometry of the type-1-copper site of laccase is dependent not only on the oxidation state of type-3 copper, as has been stated previously (LuBien *et al.*, 1981), but on the presence of type-2 copper as well, as the room-temperature spectrum in Fig. 2 demonstrates.

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