Stability of Japanese-lacquer-tree (*Rhus vernicifera*) laccase to thermal and chemical denaturation: comparison with ascorbate oxidase*

Enzo AGOSTINELLI, Laura CERVONI, Anna GIARTOSIO and Laura MORPURGO†

Dipartimento di Scienze Biochimiche 'A. Rossi Fanelli' and Centro di Biologia Molecolare del Consiglio Nazionale delle Ricerche, Università di Roma 'La Sapienza', 00185 Roma, Italy

The thermal denaturation of laccase from the Japanese lacquer tree (*Rhus vernicifera*) was studied by differential scanning calorimetry. The endotherms of holo-laccase, type 2-Cu-depleted laccase and apo-laccase were deconvoluted into two independent two-state transitions, providing evidence for a domain structure of the protein. The correlation of the two transitions with the bleaching of copper optical bands and the decrease of the transitions' enthalpy on Cu removal show that the process involves the denaturation of Cu sites. No detectable unfolding of secondary structure was observed, since the thermal transitions, characterized by low overall specific enthalpy, did not modify either the laccase c.d. spectra in the β -fold region or the maximum wavelength of the fluorescence emission. On chemical denatura-

tion, however, the emission was red-shifted by about 20 nm. The laccase behaviour is substantially different from that of stellacyanin, a protein containing a single blue Cu ion, in which the thermal transition had higher specific enthalpy and induced a large change of the c.d. spectrum in the β -fold region. The laccase denaturation behaviour is similar to that of ascorbate oxidase from zucchini (courgette; *Cucurbita pepo*) [Savini, D'Alessio, Giartosio, Morpurgo and Avigliano (1990) Eur. J. Biochem. 190, 491–495], suggesting a structural analogy. In both proteins heating may cause a change of tertiary structure through modifications of Cu co-ordination with loosening of the bonds between the structural domains at the interface of which the trinuclear Cu cluster is located.

INTRODUCTION

Laccase, together with ascorbate oxidase and caeruloplasmin, belongs to the group of blue oxidases, enzymes widespread in Nature that catalyse the four-electron reduction of molecular oxygen to water by a variety of substrates [polyphenols, aromatic polyamines, ascorbate, Fe(II), hexacyanoferrate(II)]. They contain several copper ions of three distinct types: the intensely blue type 1 Cu, with $\epsilon = 4000\text{--}6000\text{ M}^{-1}\cdot\text{cm}^{-1}$ around 600 nm and with narrow $A_{||}$ in the e.p.r. spectrum (3.5–9.0 mT), the type 2 Cu with undetectable visible absorption and normal e.p.r. spectrum [$A_{||}$ (hyperfine splitting constant) = 18–20 mT] and the type 3 Cu, a pair of antiferromagnetically coupled Cu(II) ions, undetectable by e.p.r., with an absorption band at 330 nm [1]. Laccase contains a single set of Cu ions of the three types [2], while two sets are present in the dimeric ascorbate oxidase [3,4]. The Cu content of caeruloplasmin is less well defined [5]. Six Cu ions, approximately three blue and three e.p.r.-undetectable, were recently reported for human caeruloplasmin [6].

According to the X-ray crystal structure, each subunit of ascorbate oxidase from green zucchini (courgette; *Cucurbita pepo*) [4,7] is made up of three domains, all showing similar β -barrel folding and distantly related to small mononuclear blue copper proteins such as plastocyanin and azurin. Type 1 Cu is located in domain 3, while type 2 and type 3 Cu form a cluster at the interface between domain 1 and 3. An alignment of the known amino acid sequence of ascorbate oxidase from cucumber (*Cucumis sativus*), laccase from the fungus *Neurospora crassa* and human caeruloplasmin led Messerschmidt and Huber [8] to propose a three-domain structure for laccase, as in the ascorbate oxidase monomer, and a six-domain structure for caeruloplasmin, in which two domains are contributed by each of the

three homologous units of about 350 amino-acids [9]. Suitable type 1 Cu sites were found for caeruloplasmin in domains 2, 4, and 6, and for the trinuclear cluster between domain 1 and 6 [8].

Ascorbate oxidase thermograms, obtained by differential scanning calorimetry (d.s.c.), show a single endotherm that can be deconvoluted into three two-state transitions, corresponding to calorimetric domains, i.e. cooperatively unfolding units. These domains were suggested to correlate with the unfolding of the three crystallographic domains of the protein [10]. The endotherm of sheep caeruloplasmin was also resolved into three calorimetric domains, which were correlated with the unfolding of the three homologous structural units of the protein [11]. The overall mean enthalpy (550 kcal/mol) matches well that of dimeric ascorbate oxidase (520 kcal/mol) since the two proteins have a similar M_r . [All enthalpy data are reported in units of kcal/mol (or cal/g) and can be converted into kJ/mol (or J/g) by multiplying by the factor 4.184]. Turtle and chicken caeruloplasmins exhibit a single endotherm of lower overall enthalpy, around 350 kcal/mol, encompassing three calorimetric domains.

The present paper shows the results of an investigation on the stability to denaturation, in particular by heat, of laccase from the latex of the Japanese lacquer tree (*Rhus vernicifera*). The work was intended as a test of the structural analogy between laccase and ascorbate oxidase [8], based on the correspondence of calorimetric with crystallographic domains. The investigation was extended to stellacyanin, a small blue copper protein from the same source, which could provide a reasonable model for a single crystallographic domain of the multiglobular oxidases. Molecular-modelling calculations assigned to stellacyanin a three-dimensional structure similar to plastocyanin [12] or cucumber basic protein [13]. The analogy between laccase and ascorbate oxidase was confirmed. The thermal process was

Abbreviations used: d.s.c., differential scanning calorimetry; ΔH_c , calorimetric enthalpy change; Δh_c , specific enthalpy change; ΔH_n , calculated enthalpy change for a two-state transition ($\Delta H_c = \Delta H_n$, van't Hoff enthalpy of denaturation); GdmCl, guanidinium chloride.

* This paper is dedicated to the memory of Maria Teresa Graziani.

† To whom correspondence should be sent.

found to produce only limited unfolding around the metal sites, probably loosening the tertiary structure of the two proteins.

MATERIALS AND METHODS

All chemicals were of reagent grade unless otherwise indicated. Laccase, a glycoprotein with an M_r of 110000 and 45% carbohydrate content, and stellacyanin, a glycoprotein with an M_r of 20000 and similar carbohydrate content, were purified from the acetone-dried powder of the latex of the Japanese lacquer tree (*Rhus vernicifera*), obtained from Saito and Co. (Osaka, Japan), by the method of Reinhammar [14]. Type-2-Cu-depleted and apo-laccase were prepared as described by Morpurgo et al. [15] and Savini et al. [16] respectively. For some experiments the reduced type 3 Cu of type-2-Cu-depleted laccase was reoxidized with excess H_2O_2 [17]. A 50 μM enzyme solution was made 1.5 mM in H_2O_2 , left to react for a few minutes until the 330 nm band developed, and then dialysed against 0.1 M phosphate buffer. Native and type-2-Cu-depleted laccase concentrations were measured with identical results from the 280 and 614 nm absorption band using ϵ 93500 $M^{-1}\cdot cm^{-1}$ and ϵ 5700 $M^{-1}\cdot cm^{-1}$ respectively [18]. A 10–20% lower value was obtained at 614 nm for the H_2O_2 -treated sample. The native ϵ_{280} was also used for the apoprotein. Copper content was measured by the biquinolyl method [19]: 4.0 ± 0.1 atoms of Cu/molecule were obtained for native laccase, 3.0 ± 0.1 atoms of Cu/molecule for the type-2-Cu-depleted protein. In the apoprotein the copper content was undetectable by the above procedure. The oxidase activity of laccase was measured at 25 °C by monitoring the increase in A_{420} of a 3.0 mM solution of potassium hexacyanoferrate(II) in 0.1 M sodium acetate buffer, pH 5.3. Less than 2% residual activity was measured for type-2-Cu-depleted and apoproteins.

Apo-stellacyanin was prepared as described by Morpurgo et al. [20]. It contained 6% residual native protein, as measured from its A_{604}/A_{280} ratio. Absorption-coefficient (ϵ) values of 4080 $M^{-1}\cdot cm^{-1}$ and 23500 $M^{-1}\cdot cm^{-1}$ were respectively employed at the two wavelengths [18].

Optical spectra were recorded on a Lambda-9 UV/VIS/NIR Perkin–Elmer spectrophotometer, or on a Cary-14 spectrophotometer, computer-controlled and equipped with an automatic-temperature-control device to set the rate of temperature increase in the water-jacketted cell holder (updated by AVIV). Far-u.v. c.d. spectra were recorded on a Jasco J-500 spectropolarimeter equipped with a Jasco J-500 processor. Mean residue ellipticities ($[\theta]_{m.r.w.}$) were calculated by using, for laccase, a mean residue weight of 112, that is, the value obtained for both *Neurospora* laccase and cucumber ascorbate oxidase from the data reported in [8], and for stellacyanin a value of 106 obtained from the data reported in [21]. Chemical denaturation by guanidinium chloride (GdmCl) was monitored by emission fluorescence spectroscopy, using a Shimadzu Rf-5001 PC spectrofluorimeter and excitation at 280 nm.

Microcalorimetric experiments were carried out using a high-sensitivity MicroCal MC-2D differential scanning calorimeter (MicroCal, Northampton, MA, U.S.A.). The reference cell was filled with the buffer in which the protein sample had been dialysed, the scan rate was 60 °C/h, the protein concentration 2–5 mg/ml. Thermograms were corrected by subtracting the instrumental baseline obtained with buffer in both cells. Calculation of ΔH_c (calorimetric denaturation enthalpy), ΔH_{vH} (van't Hoff enthalpy of denaturation), T_m (denaturation temperature), and deconvolution of the thermal profiles were performed with the DA-2 software package for data acquisition and analysis (provided by MicroCal) after normalizing the data for

protein concentration and subtracting as baseline the straight line connecting the initial and final temperatures of the overall transition [22]. Only in a few cases did the quality of the curve permitted the calculation of the change of denaturation heat capacity ($\Delta C_p = 5\text{--}8 \text{ kcal}\cdot mol^{-1}\cdot K^{-1}$ for laccase and its derivatives) and the use of an appropriate progress curve as the baseline. Since the thermodynamic parameters were not substantially different with the two methods, the former type of calculation was used throughout.

RESULTS

Heat denaturation monitored by d.s.c.

A single calorimetric peak was obtained in the d.s.c. scan of holo-laccase, type-2-Cu-depleted laccase and apo-laccase (Figure 1 and Table 1). None of these samples precipitated, even at 100 °C, but no endotherm was obtained in a second heating run. The optical spectrum due to Cu ions and the enzymic activity of native laccase were irreversibly lost after heating. The graphic deconvolution of the endotherms into the sum of curves representing two-state transitions ($\Delta H_c = \Delta H_{vH}$) was attempted, although the process did not exhibit any reversibility [23]. Two transitions, hereafter called peak I and peak II, were found in all cases (Table 1). The only exception was the thermogram of holo-laccase in 2.0 M GdmCl, in which a single two-state transition with lower T_m and ΔH_c was observed. The incubation with 1.0 M GdmCl did not induce such a change.

A moderate T_m decrease occurred in the thermograms of fully-Cu-depleted laccase (Table 1). Small differences were also produced by changing the pH over the range 5.6–7.0. The T_m of holo-laccase was higher at acidic than at neutral pH, while the apoprotein T_m showed an opposite trend. As a consequence, the ΔT_m between holo- and apo-laccase was 10–12 °C at pH 5.6 and only 4–5 °C at neutral pH. ΔH_c did not show appreciable changes with pH, except in the apoprotein, but showed a substantial decrease in the series holo-laccase > type-2-Cu-depleted laccase > apo-laccase. More precisely, the removal of type 2 Cu decreased the ΔH of peak I (ΔH_1) in samples with reduced type 3 Cu and the ΔH of peak II (ΔH_2) in samples with reoxidized type

Figure 1 Laccase thermograms

The Figure shows native (a), type-2-Cu-depleted (b) and apo-laccase (c) at pH 5.6 (upper), 6.0 (middle) and pH 7.0 (lower) scans

Table 1 Thermodynamic data from laccase and stellacyanin d.s.c. curves

Protein/pH	Experimental data			Deconvolution in two-state transitions			
	T_m (°C)	ΔH_c (kcal/mol)	Δh_c (cal/g)	T_1 (°C)	ΔH_1 (kcal/mol)	T_2 (°C)	ΔH_2 (kcal/mol)
Holo-laccase							
pH 5.6	53.5	238		50.0	117	54.7	120
pH 6.0	51.9 ± 0.5	239 ± 11		48.5 ± 1.0	104 ± 11	53.1 ± 1.3	118 ± 23
pH 6.0*	44.6	82		43.5	87		
pH 7.0	50.7 ± 0.7	244 ± 9	2.2	47.9 ± 1.6	104 ± 10	51.3 ± 0.7	142 ± 10
Type-2-Cu-depleted laccase							
pH 5.6	55.3	201		50.9	79	54.8	135
pH 6.0	53.9 ± 1.0	210 ± 16		50.3 ± 1.4	92 ± 14	55.1 ± 1.1	117 ± 16
pH 6.0†	50.3	207		49.1	109	52.9	97
pH 7.0	51.5 ± 0.7	200 ± 17	1.8	47.6 ± 1.7	80 ± 17	51.6 ± 1.0	134 ± 6
Apo-laccase							
pH 5.6	43.8	184		37.8	72	44.7	98
pH 6.0	45.5 ± 1.1	144 ± 35		44.3 ± 1.9	66 ± 19	46.3 ± 0.7	84 ± 13
pH 7.0	46.6 ± 2.1	133 ± 36	1.2	44.6 ± 3.2	64 ± 18	48.0 ± 2.1	78 ± 30
Apo-stellacyanin							
pH 7.2	52.7	115	5.7	52.7	107		

* In 2 M GdmCl.

† Reoxidized by H₂O₂. **Figure 2** Thermograms of type-2-Cu-depleted laccase at pH 6.0

(a) Reduced form (trace a) and reoxidized form (trace b). (b) Deconvolution in two-state transitions of the scans in (a).

3 Cu, while the overall ΔH_c was identical in the two cases. The different relative intensity of the two peaks resulted in a shift of T_m in the corresponding thermograms (Figure 2 and Table 1). The complete removal of Cu decreased the ΔH of both peaks (Table 1). The large scattering of data relating to apo-laccase is due in part to the low ΔH_c value and in part to the use of samples from different preparations. The thermograms of native and type-2-Cu-depleted laccase were much less affected by the use of samples from different stocks or preparations.

The calorimetric scans at pH 7.2 of native and apo-stellacyanin are shown in Figure 3. The corresponding scans of holo- and

Figure 3 A comparison of laccase (traces a) and stellacyanin (traces b) thermograms(a) Native proteins; (b) apoproteins. Note that ordinate units of heat capacity are cal · g⁻¹ · °C⁻¹.

apo-laccase are also inserted in the Figure in order to emphasize the large difference of specific enthalpy change (Δh_c) existing between the two proteins. An accurate measurement of ΔH_c of holo-stellacyanin was prevented by the presence of exothermic features occurring after T_m (≥ 78.8 °C), which may indicate aggregation of the protein. Precipitation, however, did not occur, so that the optical and c.d. spectra could be recorded on heated samples. A flat line was obtained in a second heating run, although the heated protein preserved about 15% of the blue absorption. The scan of apo-stellacyanin showed, instead, a symmetric endotherm of $T_m = 52.7$ °C, well resolved from an

Figure 4 Bleaching of laccase visible-near-u.v. spectrum with temperature

The absorbance was monitored at 614 nm (●) and 330 nm (○). The rate of temperature increase was 60 °C/h; the ΔA was 4900 M⁻¹·cm⁻¹ at 614 nm and 1800 M⁻¹·cm⁻¹ at 330 nm.

Figure 5 Far-u.v. c.d. spectra at 25 °C of holo-laccase (trace a) and apo-laccase (trace b)

The cell contained 0.7 μ M protein in 0.1 M potassium phosphate buffer, pH 5.6. $[\theta]_{m.r.w.}$ represents the mean residue ellipticity in degrees · cm² · dmol⁻¹.

exothermic feature at higher temperature. In Table 1 are only reported thermodynamic data relating to this endotherm, which exhibited a single two-state transition.

Heat denaturation of laccase monitored by visible absorption

Figure 4 shows a plot of the intensity of native laccase optical bands at 614 nm and 330 nm as a function of the temperature. Both bands are bleached in parallel with peak II of the calorimetric experiment. At the same heating rate of the calorimetric scan (60 °C/h), the inflection point of the 330 nm sigmoid curve occurs at a temperature close to T_2 ; that of the 614 nm curve occurs at a temperature about 1 °C lower. In the type-2-Cu depleted laccase, with either reduced or reoxidized type 3 Cu, the bleaching of the blue band occurred within peak I. The 330 nm band, only present in the reoxidized sample, lost intensity ($\Delta \epsilon = 700$ M⁻¹·cm⁻¹) at a temperature 2–3 °C lower than the 614 nm band (result not shown).

Figure 6 Far-u.v. c.d. spectra of stellacyanin at 25 °C

Spectra of holo-stellacyanin before (trace a) and after (trace b) the calorimetric run are shown. The spectra of apo-stellacyanin are superimposable on the corresponding spectra of the holoprotein. $[\theta]_{m.r.w.}$ represents the mean residue ellipticity in degrees · cm² · dmol⁻¹.

Figure 7 Shift of the laccase maximum fluorescence emission wavelength by chemical denaturation

○, Native laccase; □, type-2-Cu-depleted laccase; and △, apo-laccase. The solution monitored contained 1.0 μ M protein in 0.1 M potassium phosphate buffer, pH 6.0, after 24 h incubation with GdmCl.

Heat denaturation monitored by c.d.

The c.d. spectrum of laccase in the 200–250 nm region (Figure 5) shows a predominance of β -structure like that of ascorbate oxidase [24]. It was unaffected by pH changes in the pH 5.6–7.0 range or by removal of the type 2 Cu. Complete removal of Cu in the apoenzyme only caused the disappearance of the small shoulder at about 240 nm and a slight shift of the band to a shorter wavelength. The spectrum of the heated protein, measured at 25 °C on samples recovered from the calorimetric cell after heating up to 100 °C, showed only a small increase of the Cotton effect.

In Figure 6 are reported the c.d. spectra at 25 °C of holo-stellacyanin recorded before and after the d.s.c. experiment. The spectra of the apoprotein, recorded under the same conditions, were superimposable on the corresponding spectra of the holo-

protein. The predominant β -structure is lost in both heated samples.

Chemical denaturation of laccase

Figure 7 shows the effect of denaturant (GdmCl) on the laccase fluorescence emission maximum. A 24 h incubation was required in order to obtain stable fluorescence readings. Holo-laccase and type-2-Cu-depleted laccase display a very similar shift of the maximum to longer wavelength, while the apo-laccase maximum is already shifted toward the red by 5 nm before the addition of denaturant. Removal of type 2 Cu increased the emission intensity by 50 %, as previously reported [20], but reoxidation of the type 3 Cu with H_2O_2 approximately restored the native lower intensity. In the apoprotein the intensity of the fluorescence emission was 2.5 times that of native laccase. A similar increase, without shift of the maximum wavelength, was observed in samples of holo-laccase recovered from the calorimetric cell after heating up to 100 °C.

GdmCl at 1 M had no appreciable effect on the 614 nm absorbance or thermal behaviour. Higher concentrations decreased the absorbance on incubation (25 % in 2 M GdmCl) and lowered the bleaching temperature (result not shown) down to that of the single co-operative d.s.c. transition (Table 1).

DISCUSSION

Thermal denaturation

Laccase thermal profiles were always described by the sum of two independent two-state transitions (Table 1). The presence in the protein of two independent co-operative units (calorimetric domains) shows that it consists of at least two structural domains. A striking feature of the laccase thermograms is the low specific Δh_c (2.2 and 1.2 cal·g⁻¹ in the holoprotein and apoprotein respectively). This is not attributable to the presence of a large carbohydrate moiety, since stellacyanin, which has a similar glycidic content, shows a much higher specific Δh_c (5.7 cal·g⁻¹ in the Cu-depleted derivative). Furthermore, the c.d. spectrum of heated laccase was unchanged in the β -fold region, while that of heated stellacyanin, in either holo or apo form, was considerably modified (Figure 6). The wavelength of the laccase fluorescence emission maximum was unaffected by thermal denaturation, in spite of the increased intensity, while it was 20 nm red-shifted by chemical denaturation. It is reasonable to conclude that the laccase secondary structure is not appreciably affected by heat.

A further step towards the understanding of the laccase thermal denaturation process is provided by the correlation of the bleaching of copper optical-absorption bands at high temperature with the two independent two-state transitions. In native laccase the lack of optical variations in the temperature range of peak I might suggest that the transition does not involve the copper sites. However, ΔH_1 substantially decreased in the type-2-Cu-depleted derivative containing reduced type 3 Cu, while the ΔH and T_m of both peaks decreased in the fully-Cu-depleted protein (Table 1). Thus it is possible to conclude that both d.s.c. transitions involve the copper sites.

The persistence of secondary structure in the heated protein, after thermal transitions involving copper, may be explained by assuming that the *R. vernicifera* laccase structure, like that of *Neurospora crassa* laccase, is similar to that of an ascorbate oxidase monomer [8], in which two domains are linked through the copper of the trinuclear cluster, by means of histidine co-ordination [7,8]. In ascorbate oxidase, domains 1 and 3 symmetrically contribute eight histidine residues, from four His-Xaa-His sequences, to Cu ion co-ordination. The type 2 Cu is

bound to a water molecule and two histidine residues from different domains, and each type 3 Cu is co-ordinated to three histidine residues from two domains. One of the four His-Xaa-His sequences (where Xaa = Cys) connects both type 3 Cu ions to the type 1 Cu through the thiolate group. This stretch was also found in fungal laccase B [25]. A fourth ligand of the type 3 Cu is a bridging water molecule or hydroxyl ion. In reduced ascorbate oxidase the bridging ligand is lost and the structure of the cluster is released, with increased average distance between the Cu ions [26]. These features can reasonably account for the decreased enthalpy of laccase thermal transitions when either type 2 Cu or all Cu types are removed (Table 1), for the redistribution of the calorimetric domains enthalpy in the type-2-Cu-depleted protein following the reoxidation of type 3 Cu (Figure 3), and for the limited heat effects on folding of the domains. It can be noted that, in both laccase and ascorbate oxidase, the reduction of type 3 Cu is a prerequisite for the access of chelating agents to type 2 Cu site [27]. The type 1 Cu is also rather close to the interface between domains 1 and 3 of ascorbate oxidase, so that the thermal denaturation of the site does not affect the c.d. spectrum nor the fluorescence emission wavelength.

The differences between the thermal-denaturation processes of ascorbate oxidase and laccase may raise some criticism of the above assignment. Ascorbate oxidase shows an endotherm of higher T_m and higher specific denaturation enthalpy, encompassing at least three independent two-state transitions, which is not modified by type 2 Cu removal [10]. However, a closer inspection of the data shows that at pH 7.0, where ascorbate oxidase does not precipitate during the calorimetric scan [10], the ΔH_c of the ascorbate oxidase dimer (520 kcal·mol⁻¹) is approximately twice that of laccase, and that specific Δh_c values are similar in the two holoproteins (about 4 cal·g⁻¹) and apoproteins (2.2 and 2.6 cal·g⁻¹ for laccase and ascorbate oxidase respectively) by neglecting in calculations the glycidic moieties. Moreover, the endotherm of apo-(ascorbate oxidase) is deconvoluted into two independent transitions and has a T_m almost identical with that of apo-laccase. Since the c.d. spectrum of ascorbate oxidase was only slightly modified by heating [10], it seems likely that, in this protein also, d.s.c. transitions do not cause 'melting' of the secondary structure. This conclusion agrees with the high number of ion-pair interactions found in the ascorbate oxidase X-ray structure, a feature indicative of protein thermostability [7]. The similar behaviour of laccase and ascorbate oxidase towards heating may be compatible with either a loosening of the tertiary structure or even the formation of a molten-globule state [28].

The difference in the number of independent two-state transitions between the two holo- or type-2-Cu-depleted proteins may be ascribed to the ascorbate oxidase quaternary structure, which gives rise to additional transitions in the presence of copper. The lack of effect of type 2 Cu removal on ascorbate oxidase endotherm might be due to a location of the Cu cluster closer to the protein surface than in laccase [29], a conclusion also suggested by comparative studies of the reactivity of these proteins with chelating agents, showing that copper ions are more easily removed from ascorbate oxidase than laccase [16,27].

The thermal behaviour of caeruloplasmins is similar in some respects to that of laccase and ascorbate oxidase. The loss of spectroscopic and catalytic properties occurs in parallel with the calorimetric transitions; only two independent transitions are present in the apoprotein, with decreased overall specific ΔH_c ; Δh_c values were rather low [11]. However, some caeruloplasmins denature at higher temperatures than laccase and ascorbate oxidase, as do stellacyanin and other mononuclear blue copper proteins, which unfold at high temperature [30]. The relatively

high content of blue copper may stabilize caeruloplasmins against heat.

Chemical denaturation

Laccase is more stable to chemical denaturation than ascorbate oxidase, since a higher denaturant concentration and a longer incubation time were required in order to produce comparable shifts of the fluorescence emission maximum [10]. The similarly high resistance to denaturation of holo, type-2-Cu-depleted and apo forms suggests a stabilizing effect of the laccase by the very large carbohydrate component.

The depletion of type 2 Cu increased the intensity of laccase fluorescence emission, without shifting the emission wavelength. The quenching of tryptophan fluorescence, occurring in the native protein because of non-radiative energy transfer, was removed in absence of the 330 nm band [15]. The quenching was restored, together with the 330 nm band, by the reoxidation of type 3 Cu. These effects were not observed in ascorbate oxidase [10], suggesting, again, that the trinuclear Cu cluster is in closer contact with bulk solvent in this protein than in laccase. The complete copper depletion caused instead in both proteins a 5 nm red shift of the emission wavelength and a large increase of the fluorescence intensity. Analogous effects produced by copper removal in stellacyanin were assigned to solvent exposure of a tryptophan close to the blue copper site [20]. In all proteins the backbone c.d. was not modified by copper removal. Therefore the large increase of fluorescence intensity in heated laccase, without shift of the emission wavelength, relates to the bleaching of copper optical bands without exposure to solvent of tryptophan residues.

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