Circular dichroism and fluorescence studies to probe the conformational properties of *Rhus vernicifera* laccase

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Abstract

The conformational properties of native, apo- and type-2 copper depleted laccase from *Rhus vernicifera* have been investigated by circular dichroism and fluorescence spectroscopies. Circular dichroism experiments reveal a high prevalence of random-coil structure in all laccase derivatives, the content of α-helix and β-sheet not exceeding 8% and 21%, respectively. Nevertheless, the microenvironment of the tryptophan residues is deeply shielded from the external medium and exhibits a marked stability against pH-denaturation. Fluorescence data suggest that a class of tryptophan residues close to type-2 site contributes 50% to the overall fluorescence emitted by apo- and type-2 copper depleted laccase. These residues are masked in the native enzyme, due to quenching effects by copper ions and adjacent amino acid side chains. The interaction of 1-anilino-8-naphthalene sulfonate (ANS) with laccase has been studied by following the fluorescence changes of the dye upon binding. Native laccase does not bind ANS. The type-2 copper depleted derivative and apo-laccase bind one and three moles of dye per mole of protein, respectively. These findings suggest that the three ANS binding sites correspond with the three different copper binding sites in laccase. These can be distinguished on the basis of their dissociation constants (K_D) for ANS. The type-2 copper site exhibits the highest affinity for ANS.

Introduction

Laccases ($MW = 65\,000$–$140\,000$) are copper containing proteins widely distributed in higher plants and fungi [1]. They catalyze the oxidation of a variety of aromatic phenols, amino-phenols and diamines by full reduction of dioxygen to water. The enzymatic activity is due to four tightly bound copper ions distributed in three different redox sites (namely: type-1, type-2 and type-3 copper sites).

Structural models for the three copper sites of laccase have been proposed on the basis of the X-ray analysis of other copper proteins and from comparative studies of their spectroscopic and magnetic properties [2, 3]. Thus, the type-1 Cu(II), deeply embedded in the protein matrix, is supposed to coordinate two hystidines, one cysteine and, probably, one methionine in a distorted tetrahedral geometry [1]. Only limited information is available about the protein ligands of the type-2 copper site which is located in a solvent-accessible protein pocket. Some spectroscopic properties of the type-2 Cu(II) suggest a metal coordination to three nitrogen ligands and one oxygen, probably H_2O or OH^- [4, 5]. NMR relaxation studies, however, provide evidence for the coordination of one water molecule and one protein hydroxyl group (serine or threonine) to the type-2 Cu(II) [5]. The type-3 Cu(II) site consists of a binuclear center with two Cu^{2+} ions strongly magnetically coupled. The spectroscopic and magnetic properties are consistent with nitrogen coordination and with oxygen probably as a bridging ligand, analogously to hemocyanin and tyrosinase [6].

Laccase is one of the best understood oxidases in terms of catalytic properties [1] and the primary structure of the protein isolated from *Neurospora crassa* has been recently elucidated [7]. Only limited information is, however, available on its three-dimensional structure as well as on its conformational properties in solution.

This paper reports a spectroscopic investigation on *Rhus vernicifera* laccase by using fluorescence and circular dichroism (CD) techniques. The results of such measurements would help to gain some insight into the conditions necessary to maintain the protein conformational stability, the response of the protein to

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different media and the role of copper in preserving the native conformation. The different influences of the three copper sites on laccase fluorescence as well as their capability to bind fluorescent probes such as 1-anilino-8-naphthalene sulfonate (ANS) have been investigated as basic knowledge to the study of the site environmental properties and site–site interactions.

Materials and methods

Laccase derivatives and solutions

Rhus vernicifera laccase ($MW = 110\,000$) from the Japanese lacquer tree was kindly provided by Professor J. Peisach, Albert Einstein College of Medicine, New York. The purity was checked by the absorbance ratio $A_{280}/A_{615} = 15$ [1]. Type-2 Cu(II) depleted (T2D) enzyme derivative was prepared by the method of Graziani *et al.* [8]. Apo-laccase was prepared by dialysis of the native protein against 0.1 M Tris/HCl pH = 8.0 containing 20 mM KCN, 10 mM ascorbic acid and 5 mM dithionite followed by several changes against pure buffer.

Protein concentration was calculated from the absorbance at 280 nm, by using the extinction coefficient $\epsilon = 93\,500\ M^{-1}\ cm^{-1}$.

All experiments were performed in 0.1 M phosphate buffer pH 6.0, unless otherwise specified. In the measurements as a function of pH, phosphate buffer was used in the pH range 5.5–8.0, 0.1 M Tris/HCl buffer above pH 8.0. Below pH 5.5, suitable solutions of HCl were used.

Spectroscopic measurements

CD measurements were performed in the range 190–250 nm with a Cary 65 dichrograph by using a fused quartz cylindric cell with 0.05 nm optical path. The mean residue ellipticity (in deg. $cm^2\ dmol^{-1}$) was calculated as:

$$[\Theta] = \frac{\theta}{10}\,\frac{M_r}{dc} \qquad (1)$$

where θ is the ellipticity measured in degrees, M_r the molecular weight per mean residue (i.e. 112 from amino acid analysis), d the optical path of the solution in cm and c the protein concentration in g/ml. The α-helix and β structure content was evaluated by the method of Chen *et al.* [9].

Copper content was measured by a Perkin-Elmer model 4000 atomic absorption spectrometer.

Fluorescence measurements were performed with a Perkin-Elmer MPF 4 spectrophotofluorimeter. Quantum yields (Q) were calculated by comparing the integrated fluorescence intensities of the sample, F_x, and of a solution of *N*-acetyl-tryptophanamide (NATA), F_s, as reference standard ($Q = 0.13$) in 0.1 M phosphate buffer at pH 7.0:

$$Q = (F_x/F_s)(A_s/A_x) \times 0.13 \qquad (2)$$

A_s and A_x are the absorbances of the standard and of the sample at the excitation wavelength (295 nm).

Fluorescence quenching measurements

Fluorescence quenching experiments were carried out by using acrylamide, iodide and caesium as external quenchers. When ionic quenchers were used, the ionic strength of the solutions was kept constant by addition of suitable amounts of KCl. The fluorescence quenching parameters, f_a (fraction of fluorescence accessible to the quencher) and K_Q (apparent quenching constant), were obtained following the procedure described by Lehrer [10].

Thermal quenching experiments were carried out by following the temperature dependence (in the range 2–40 °C) of the fluorescence emission (excitation $\lambda = 295$ nm). The experimental data were analyzed according to the Arrhenius equation:

$$(Q^{-1} - 1) = k \times \exp(-E_a/RT) \qquad (3)$$

where Q is the fluorescence quantum yield, E_a is the activation energy for the radiationless deactivation of the excited singlet state, T is the absolute temperature and R is the gas constant [11].

Binding of ANS to laccase

The binding of ANS to apo- and T2D derivatives was studied by titration experiments of the protein following the increase of ANS fluorescence (F) at 480 nm (excitation $\lambda = 310$ nm) with increasing ANS concentrations. The data were analyzed by the Scatchard equation [12]:

$$\nu/c = n/K_D - \nu/K_D \qquad (4)$$

where ν is the molar ratio of ANS bound to the protein, c (in M) is the concentration of free ANS (total ANS added minus bound ANS) and K_D (in M) is the dissociation constant of the ANS–protein complex; n is the number of ANS binding sites per protein molecule. The concentration of ANS bound (ANS_b) to the protein at each point of the titration curves was calculated by the relationship:

$$[ANS]_b = F/F^{\circ}_{max} \qquad (5)$$

The value of F°_{max} was obtained by titrating a fixed amount of ANS (5×10^{-6} M) with variable quantities (from 9×10^{-6} to 4.5×10^{-5} M) of protein. The reciprocal of ANS fluorescence intensity at 480 nm was then plotted against the reciprocal of protein concentration ($1/P$), the intercept for $1/P = 0$ giving $1/F_{max}$. The ratio between F_{max} and the molar ANS concen-

tration corresponds to the fluorescence intensity F°_{max} of 1 M ANS solution completely bound to the protein. All titration experiments were carried out at $T=20\pm0.1$ °C. Further details on the treatment of the data are reported elsewhere [13].

When the Scatchard plots were non-linear, we have reinterpreted the titration data on the basis of a model involving three classes of non-interacting binding sites (see 'Discussion'). Thus, the total concentration of bound ligand is the sum of the concentrations of ligand bound to each class of sites:

$$\nu=\nu_1+\nu_2+\nu_3$$

$$=\frac{n_1c}{(K_{D1}+c)}+\frac{n_2c}{(K_{D2}+c)}+\frac{n_3c}{(K_{D3}+c)} \quad (6)$$

In our case, with $n_1=n_2=n_3=1$ (see 'Discussion'), the three dissociation constants can be easily calculated by a best fit procedure. A model involving two classes of ANS binding sites was also considered.

Results

Circular dichroism studies

The CD spectra of laccase derivatives in the far UV at different pH show a negative Cotton effect occurring at 212 nm for native and 207 nm for apo- and T2D laccase (Fig. 1A). The analysis of the CD spectra by the method of Chen *et al.* [9] reveals the prevalence of random-coil structure for all laccase derivatives, the content of α-helix and β-sheet ranging between 4–8% and 12–21%, respectively.

The pH-stability profiles followed by CD in the far UV are shown in Fig. 1B. The data are reported as mean residue ellipticity ratios taking as reference the values of native protein at pH 5.5. Native laccase exhibits the maximum $[\Theta]$ value at pH 5.5; above pH 8.0 and below pH 5.5 the ellipticity gradually decreases. On the contrary, apo and T2D derivatives display higher ellipticities at acidic pH (3.5–5.5).

The effect of 0.1 M concentration of perturbant ions such as Mg^{2+}, Ca^{2+}, SO_4^{2-} and ClO_4^- on the dichroic properties of laccase has also been investigated. Only minor effects on the ellipticity values are observed; in the presence of these ions, however, the pH-stability region extends up to pH 9.6 for all laccase derivatives (data not shown).

Fluorescence studies

The fluorescence emission spectra of native, apo- and T2D laccase at pH 6.0 are shown in Fig. 2A. Native laccase is characterized by a lower emission quantum yield, roughly half that of the apo and T2D derivatives in the pH range 4.0–7.5. Above pH 7.5, the native protein shows a sharp increase of fluorescence until pH 9.0 (Fig. 2B).

In the 4.0–9.2 pH region, the emission maximum of native laccase occurs at 328 nm (Fig. 2A), indicative of tryptophan (Trp) residues deeply buried in the protein matrix. Below pH 4.0, a small red shift of the emission λ_{max} (2 nm) is observed. Apo and T2D derivatives show emission maxima at 330 nm (Fig. 2A) in the whole pH range examined.

Fluorescence quenching studies

The Trp microenvironment was further investigated by fluorescence quenching experiments at pH 6.0. The results obtained by using Cs^+ and I^- as quenchers of surface fluorophores [10] confirm that Trp residues are not exposed to the external medium. Actually, no fluorescence quenching is observed with Cs^+ while only 10% of fluorescence is accessible to I^-. Upon full removal of copper, about 50% of fluorescence is quenched by both I^- and Cs^+. Acrylamide, which is a quencher of both exposed and buried Trp, extinguishes

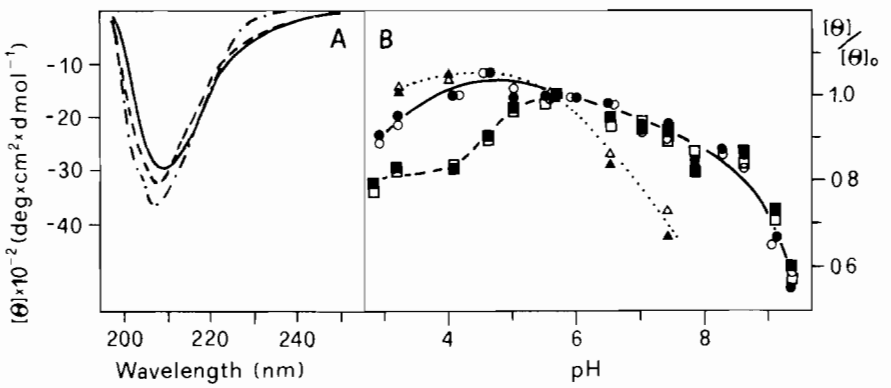

Fig. 1. Circular dichroism properties of *Rhus* laccase. A: CD spectra of (——) native, (– – –) T2D and (–·–) apoprotein in 0.1 M phosphate buffer, pH 6. B: pH-dependence of the mean residue ellipticity of (■ — □) native, (● — ○) apo- and (▲ ··· △) T2D laccase. Filled and empty symbols represent the ellipticities at 205 and 215 nm, respectively. $[\Theta]_0$ is the mean residue ellipticity of native laccase at pH 5.5.

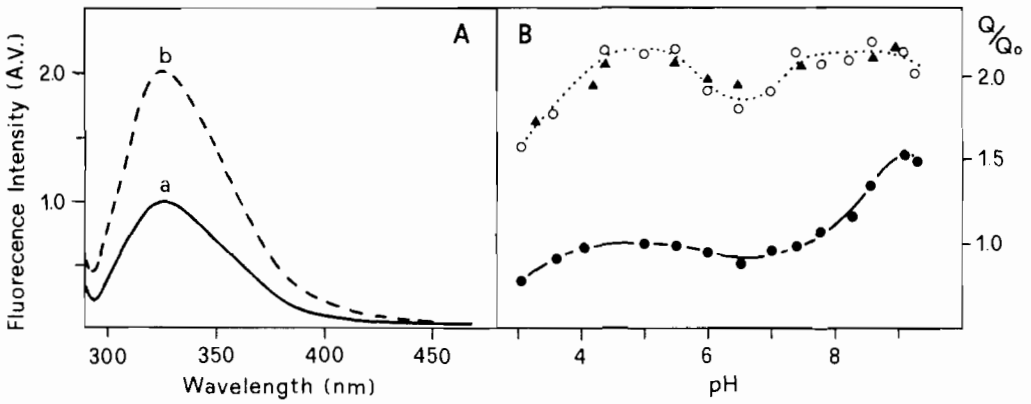

Fig. 2. Fluorescence properties of laccase. A: fluorescence emission spectra of (——) native, (– – –) T2D and apoprotein. The excitation λ was at 295 nm. B: pH dependence of the fluorescence quantum yield of (● — ●) native, (○ · · · ○) apo- and (▲ · · · ▲) T2D laccase. Q_0 (0.1) is the fluorescence quantum yield of native laccase at pH 5.5.

60% of the emission in native laccase with $K_Q = 4.0$ M^{-1} and 50% in apo-laccase with $K_Q = 8.0$ M^{-1}.

The general burial of Trp residues in all laccase derivatives is evidenced also by the low values of the activation energies E_a [14], calculated from the slopes of the Arrhenius plots at low temperatures (data not shown): 2.30, 2.40, 3.10 Kcal/mol for native, apo- and T2D laccase.

Binding of ANS to laccase

Fluorescent dyes proved to be useful tools to probe the hydrophobic character and the conformational flexibility of active sites in proteins [15]. We have, therefore, undertaken a study on the binding properties of native, T2D and apo-laccase with ANS.

Native laccase does not bind ANS. Apo-laccase and T2D derivative bind ANS, as indicated by the strong increase of the ANS quantum yield and the blue shift of its emission maximum, from 520 to 465 nm (data not shown) [15]. In Fig. 3, the titration curves of apo-laccase with ANS, both in pure buffer and buffer containing 0.1 M SO_4^{2-}, are shown. In all conditions, apo-laccase binds up to 3 ANS molecules, as calculated by the measurement of the total protein-bound dye according to relationship (5). In the presence of SO_4^{2-} (or ClO_4^-), the titration curves show two inflections (Fig. 3) corresponding to $n = 1$ and $n = 2$, probably reflecting a stepwise saturation of the ANS binding sites. The reproducibility of these latter curves is rather high, the variability of the data being within ±3%. The ANS titration of T2D laccase (Fig. 3) is consistent with the presence of a single ANS binding site. Accordingly, linear Scatchard plots with no indication for weaker sites are obtained for the T2D derivative, in all experimental conditions (Fig. 4). The Scatchard plots obtained with apo- laccase in phosphate buffer are reported in Fig. 5(A) (for the case of three binding

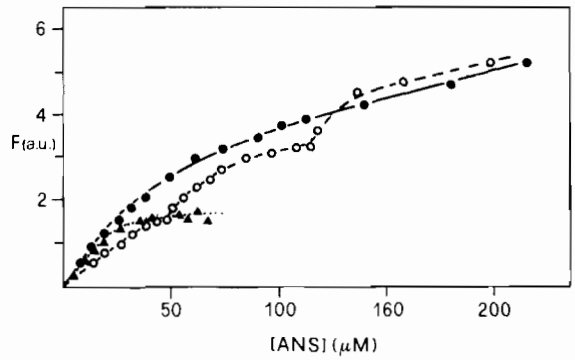

Fig. 3. Fluorescence titration curves of 0.5 μM laccase with ANS. Apolaccase in (● — ●) 0.1 M phosphate buffer pH 6.0 and (○ – – ○) in buffer containing 0.1 M SO_4^{2-}. In the presence of 0.1 M ClO_4^-, the titration curve (not shown) shows a trend similar to that obtained with SO_4^{2-}. (▲ · · · ▲) T2D derivative in buffer (similar curves are obtained in buffer containing 0.1 M SO_4^{2-} or ClO_4^-).

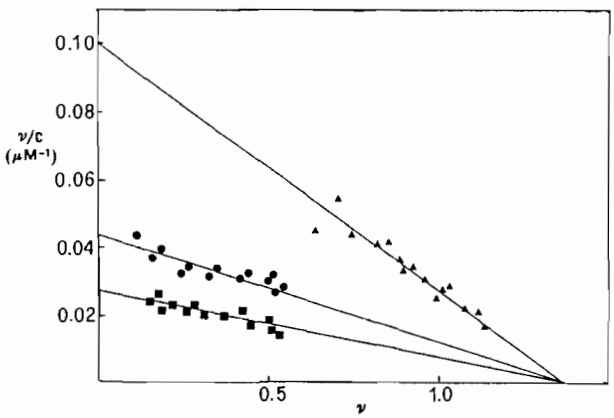

Fig. 4. Scatchard plots obtained from the ANS titration data of T2D laccase. T2D in: (●) 0.1 M phosphate buffer pH 6.0, (▲) in buffer plus 0.1 M SO_4^{2-}, (■) in buffer plus 0.1 M ClO_4^-.

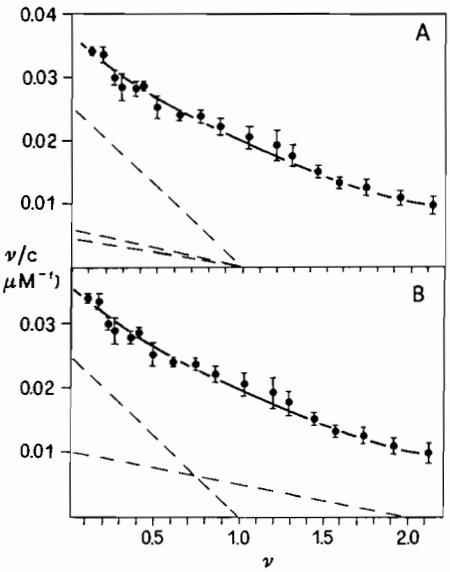

Fig. 5. Scatchard plots obtained from the ANS titration data of apo-laccase in 0.1 M phosphate buffer pH 6.0. In A the dashed lines refer to the deconvolution of the titration data on the basis of ANS binding to three distinct and non-interacting sites ($n_1=n_2=n_3=1$). The solid line is the theoretical plot calculated from eqn. (6) and the K_D values listed in Table 1 (● represents the experimental points which are the average of three separate experiments). In B the same data were fitted on the basis of two classes of ANS binding sites ($n_1=1$, $n_2=2$).

TABLE 1. Dissociation constants (K_D) of the complexes between ANS and apo- and T2D laccase (in 0.1 M phosphate buffer pH 6.0)

Sample	K_{D1} ($\times 10^4$ M)	K_{D2} ($\times 10^4$ M)	K_{D3} ($\times 10^4$ M)
Apo-laccase in			
buffer	0.42 ± 0.04	1.65 ± 0.35	2.10 ± 0.60
$+SO_4^{2-}$	0.20[a]		
$+ClO_4^-$	0.48[a]		
T2D in			
buffer	0.30 ± 0.02		
$+SO_4^{2-}$	0.14 ± 0.05		
$+ClO_4^-$	0.50 ± 0.03		

[a]Approximated values of K_{D1}, obtained by extrapolating the linear initial portion of the Scatchard plots (see 'Results and Discussion').

sites) and Fig. 5(B) (for the case of two classes of binding sites), together with the theoretical curves deconvoluted for each class of ANS binding site. The K_D values, calculated from the Scatchard plots by means of the best fitting method are reported in Table 1. In the presence of 0.1 M SO_4^{2-} or 0.1 M ClO_4^-, the ANS titration data of apo-laccase did not fit the model described in eqn. (6), for any value of n (data not shown).

Discussion

Circular dichroism studies indicate that the conformation of laccase is characterized by a high prevalence of aperiodic structure. The microenvironment of Trp residues, however, appears to be shielded from the external medium in the three derivatives, as indicated by the blue-shifted emission maxima and by the low values of the activation energy for the deactivation of the first excited singlet state [14, 16]. Partial or total copper removal induces a difference in the conformational stability of the protein at low pH. Above pH 7.0, the conformational stability of native and apo-laccase is very similar. The unusual behaviour of laccase in comparison with other metalloproteins can be ascribed to a very high content of carbohydrates (40% of the total weight): likely, these moieties play a major role in the organization of the tertiary structure of laccase as well as in preventing the effects of denaturing agents. The addition of neutral salts such as $KClO_4$, $(NH_4)_2SO_4$, $MgCl_2$ and $CaCl_2$ causes an increase of the pH range of conformational stability. While this is expected in the case of $(NH_4)_2SO_4$, $MgCl_2$ and $CaCl_2$, the effect of $KClO_4$ on laccase conformation is in contrast with that observed for many proteins: it is known that $KClO_4$ serves as very potent structural destabilizer of proteins while $(NH_4)_2SO_4$, $CaCl_2$ and $MgCl_2$ strongly stabilize the native conformations [17]. The observed findings could be accounted for by the proposed role of the sugar moiety in controlling the protein conformation: actually, carbohydrates are less sensitive than polypeptide chains to the medium composition. Unfortunately, no detailed information is so far available about the structure of the carbohydrate moieties in laccase.

Fluorescence data suggest heterogeneity in the Trp environment. A first discrimination between various classes of fluorophores can be made after analysis of the pH-dependence of the fluorescence quantum yields of the three laccase derivatives. In particular, native laccase displays a sharp transition between pH 7.5–9.0, with midpoint at pH 8.6. Since no major change in the Trp microenvironment is likely to occur in this pH range, as indicated by the constancy of the emission λ_{max}, this effect may be attributed to a local modification probably implying the ionization of an amino acid residue located in a critical position with respect to the fluorophores. Actually, ^1H and ^{17}O NMR relaxation studies on *Rhus* laccase [5] indicated that an anomalous aliphatic –OH group (Ser or Thr), which is a ligand of Cu(II)

in the type-2 site, dissociates with pK=8.6. On this basis, the low fluorescence quantum yield of native laccase below pH 7.5 can be, at least in part, ascribed to a quenching effect of this –OH group, probably through a hydrogen bond with one tryptophan located in close proximity of type-2 copper site.

The removal of type-2 copper induces a further increase of protein fluorescence: since no changes in the conformational properties of the Trp microenvironment are observed between native and T2D laccase, this fact can be attributed to the demasking of some fluorophores whose fluorescence is quenched in the native protein by 'heavy atom' or/and 'paramagnetic ion' effect. Type-1 and type-3 copper ions do not exert any influence on laccase fluorescence, as indicated by the identity in the fluorescence quantum yields of T2D and apo derivatives and by the fact that the fluorescence of the former does not change after reduction of type-1 copper [18].

The above considerations lead to assess that the two-fold fluorescence increase in apo- (and in T2D laccase), in comparison with the native protein originates from Trp residues close to type-2 site. This class of Trp probably corresponds to that accessible to the quenching action by Cs^+ and I^- in apo-laccase (f_a=0.5, see 'Results'). These quenchers are known to preferentially interact with external fluorophores, their large hydration sphere precluding penetration to the protein matrix. This finding is somewhat in contrast with the general burial of Trp residues as deduced by other fluorescence parameters. The apparent discrepancy can be explained on the basis of the properties of the type-2 site. According to Goldberg *et al.* [5], the type-2 site is accommodated into a cavity of the protein matrix, connected to the bulk solvent via an orifice which, in the native enzyme, is not penetrable by exogeneous ligands but protons. Water and extrinsic molecules can penetrate the cavity when the enzyme is reduced, after a rearrangement to a conformational state controlling the access to the site. A similar change of the active site conformation is probably occurring also upon removal of copper: this is confirmed by the absence in apo and T2D derivatives of the sharp transition with pK 8.6 in the pH-titration curves (Fig. 3), typical of the native protein, and by the appearance of two small reversed transitions with pK 5.9 and 7.2.

The residual fluorescence observed in the native enzyme can be further discriminated as due to at least two classes of very buried Trp: only one (corresponding to 60% of the residual, i.e. 30% of the overall fluorescence) is accessible to acrylamide quenching.

We attempted to further differentiate the three metal active sites of laccase in terms of their reactivity toward ANS, a probe of hydrophobic sites in proteins [15, 19]. The coincidence of the ANS binding sites in laccase with the copper sites is supported by the fact that the native protein does not bind ANS while T2D and apo derivatives bind one and three ANS molecules, respectively. This justifies the values given to n in the elaboration of the ANS titration data for the Scatchard analysis applied to the multisite protein.

The K_D values obtained depend on the nature of the site. Tentatively, the strongest ANS binding site can be identified with type-2 copper site since the K_{D1} value obtained for apo-laccase in pure buffer is very similar to that found for T2D derivative. The analysis of the ANS binding sites, according to eqn. (6), cannot discriminate between the K_{D2} and K_{D3} values. Actually, a good fit of the titration data in pure buffer is obtained both on the basis of two or three classes of binding sites (Fig. 5). Likely, type-1 and type-3 copper depleted sites exhibit very similar microenvironments for the bound ANS.

The situation for apo-laccase in the presence of ClO_4^- or SO_4^{2-} is more complex. Actually, the model describing the ANS binding (see eqn. (6)), is not consistent with the titration data. Probably, as is also suggested from the trend of the titration plots (Fig. 3), some cooperativity in the binding occurs, due to conformational changes of the three ANS binding sites of laccase induced by the perturbing ions. Thus, an essential condition for eqn. (6) to be valid, i.e. the presence of non-interacting binding sites, is not fulfilled. A roughly approximated value of K_D for the site with highest affinity (K_{D1}) can be obtained by extrapolating the linear initial portion of the Scatchard plots (data not shown). K_D values of 0.2 and 0.48×10^{-4} M are obtained in the presence of SO_4^{2-} and ClO_4^-, respectively. These values are similar to the corresponding values obtained for T2D laccase (Table 1), thus confirming the identification of the strongest ANS binding site with the type-2 copper site.

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