The Electronic Spectrum of Co(II) in the Type 1 Site of *Rhus vernicifera* Laccase

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Abstract

The absorption bands of Co(II) bound in the type 1 site of *Rhus vernicifera* laccase have been resolved by difference spectroscopy in conjunction with a laccase sample containing Hg(II) in the type 1 site. At pH 8 d–d band maxima occur at 540 and 595 nm, and the charge transfer maximum occurs at *c.* 303 nm. The results suggest that the type 1 site in laccase may have a distinctive axial ligand field. In particular, the spread in the visible bands of Co(II) is relatively narrow by comparion with other derivatives, and the charge transfer absorption is hypsochromically shifted as well.

Introduction

The laccase enzyme is the simplest of the blue copper oxidases which include ceruloplasmin and ascorbate oxidase [1–3]. While molecular oxygen is reduced to the level of water, laccase catalyzes the oxidation of *o*- and *p*-diphenol derivatives, phenylenediamine, and numerous other substrates [1]. Laccase contains four copper ions distributed in three different binding sites. When oxidized, type 1 copper is EPR active and exhibits a strong visible absorption which maximizes at 614 nm. The fine structure (EXAFS) in the X-ray absorption spectrum of Hg(II) bound in the type 1 site [4] indicates that the principal donors are two imidazole nitrogens from histidine side chains and a sulfur from a cysteine side chain, analogous to the type 1 sites in several other proteins [5] including ascorbate oxidate [6]. The type 2 copper is EPR active, but it exhibits at best a very weak visible absorbance. No absorption bands have been identified for this site as yet. In contrast, the binuclear type 3 site exhibits an absorption band at around 330 nm when oxidized but is EPR silent in the resting enzyme. This site acts as a two-electron acceptor and contains a pair of antiferromagnetically coupled copper(II) ions. In ascorbate oxidase a deprotonated form of water appears to act as a bridge between the two ions, and possibly the type 2 copper as well [6].

In order to characterize many of the small blue copper proteins, Co(II) has been incorporated as a replacement for Cu(II) because Co(II) can exist in a variety of coordination geometries, and the d–d transitions vary with the ligand environment. Proteins for which Co(II) derivatives are known include stellacyanin [7], plastocyanin [8], plantacyanin [9], and the pseudoazurin from *A. cycloclastes* [10]. About 10 years ago, Larrabee and Spiro [11] reported that Co(II) could be introduced into the type 1 site of laccase because they found that characteristic charge transfer absorption bands developed in the UV when Co(II) was combined with apolaccase. The visible spectrum could not be so readily interpreted because of the possibility that Co(II) might be bound at the type 2 and/or type 3 site as well. Recently, Sakurai and coworkers [12] have disputed this result. When they combined apolaccase with Co(II), they found no evidence of the UV bands, but they did observe d–d bands in the visible which were attributed to Co(II) bound at the type 3 site. The findings of Sakurai and co-workers seemed surprising in view of the vast literature pertaining to the binding of Co(II) to type 1 copper sites in a variety of proteins. We have reinvestigated this problem by using difference spectroscopy in conjunction with a laccase derivative which contains Hg(II) in the type 1 site [13]. Our results establish that Co(II) does in fact bind to the type 1 site, although the affinity is reduced by comparison with the low molecular weight proteins previously investigated.

Experimental

Materials

Laccase was extracted according to the method of Reinhammar [14] from the acetone powder of *Rhus vernicifera* latex which was harvested near Chu Shi, China, and supplied by Saito and Company, Osaka, Japan. The purified laccase had an absorbance ratio A_{280}/A_{614} = 16.4 where A_i is the absorbance at wavelength i in nanometers. All reagent grade chemicals were used without further purification.

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Methods and Instrumentation

All buffers were prepared from deionized water and were passed through columns of (Bio Rad) Chelex 100 resin as needed in order to remove trace metal ions. Glassware was soaked for at least 12 h in strong acid and plastic containers were soaked at least 24 h in an EDTA solution to avoid contamination by adventitious metal ions.

In order to prepare apolaccase, laccase was dialyzed against 0.1 M imidazole/acetate pH 7 buffer containing 50 mM potassium cyanide and 5 mM ascorbate as before [13]. Hg-laccase was prepared by combining the apoprotein with 1.0 eq. of $Hg(O_2CCH_3)_2$ dissolved in 0.05 M pH 5.2 ammonium acetate. After dialysis the protein analyzed [15] for 1.0 ± 0.1 mercury atom per molecule of protein. Protein concentrations were determined by the biuret method [16], calibrated with native laccase on the assumption that freshly isolated laccase contains exactly four copper ions. All dialyses were carried out in a custom-designed hollow fiber device at 5–10 °C. For the spectral studies, aliquots of a stock solution of $CoCl_2 \cdot 6H_2O$ in 0.025 M pH 7.4 tris·HCl were added to a solution of Hg-laccase in tris buffer. The sample was then allowed to incubate at room temperature for several hours.

All absorbance measurements were carried out with a Perkin-Elmer Lambda 4C spectrophotometer. The pH meter was a Radiometer Model PMH 64.

Results

First, we confirmed the observations of Sakurai *et al.* who suggested that Co(II) binds at the type 2 and type 3 sites of laccase to give chromophores that absorb in the visible [12]. When 3 mol eq. of Co(II) were combined with Hg-laccase at room temperature in pH 7.4 tris buffer, band maxima developed at 534 and 560 nm, and they are plausibly associated with Co(II) at the type 3 and/or type 2 sites since Hg(II) occupies the type 1 site, *vide infra*.

Evidence of binding at the type 1 site was obtained at higher cobalt levels in studies involving apolaccase and difference spectroscopy. Figure 1(A) is a difference spectrum at pH 8 and was obtained by subtracting the spectrum of Hg-laccase equilibrated with 30 mol eq. Co(II) from that of apolaccase equilibrated with the same amount of Co(II). Band maxima are resolved at *c.* 540 and 595 nm with a shoulder at 630 nm. Figure 1(B) is the same except that only 0.6 eq. Hg(II) were present in the reference sample. Bands are also apparent in the near UV regions of Fig. 1(A) and (B). When calculated on the basis of the protein concentration, the apparent molar extinction coefficients are 2700 M^{-1} cm^{-1} at 303 nm and 200 M^{-1} cm^{-1} at 540 nm in the pH 8 spectrum. Figure 1(C) is analogous to

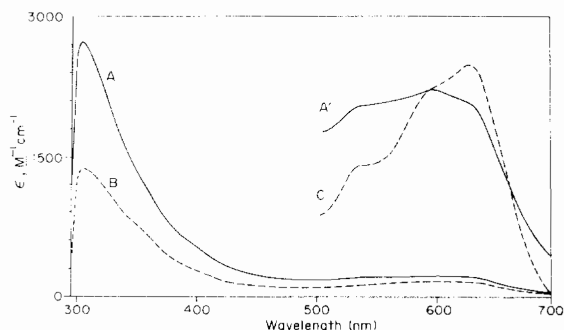

Fig. 1. Difference spectra. (A) Spectrum of apolaccase in 0.1 M pH 8 tris buffer treated with 30 mol eq. Co(II) minus spectrum of apolaccase treated with 1 mol eq. Hg(II) followed by 30 mol eq. Co(II). (A′) Spectrum (A) multiplied by 10. (B) Same as (A) except that 0.6 mol eq. Hg(II) was used. (C) Same as (A′) except in 0.1 M pH 7.4 tris buffer.

(A′), except that the spectrum was measured at pH 7.4. All spectra reported in Fig. 1 were recorded after the absorbance had stopped changing, approximately 20 h after the addition of cobalt. The time dependence of the development of the difference spectrum was somewhat complicated. Although the ΔA_{303} value grew essentially monotonically, the ΔA_{630} profile was complex. After the first spectrum was taken (approximately 10 min after addition of cobalt), the ΔA_{630} reading decreased and reached a minimum after about 4 h. It then began to increase again; after about 15 h, the changes began to parallel those observed at 303 nm, when the difference in molar absorptivities was accounted for.

Discussion

The available data suggest that the type 1 copper site in *Rhus* laccase has a structure similar to those found in a number of small blue copper proteins which have been characterized crystallographically. The intense visible absorption of the type 1, or 'blue', copper chromophore is attibutable to a S(Cys) → Cu(II) charge transfer transition, originally assigned as a ligand-to-metal charge transfer transition from the σ donor orbital of the cysteine sulfur [8, 17]. More recently, Solomon and co-workers have suggested that the transition may originate from a $p\pi$-like orbital which overlaps the $d_{x^2-y^2}$ acceptor orbital in a bilobal fashion [18]. Either way, the existence of the sulfur ligand is indicated. Two nitrogen ligands, assumed to be from histidine side-chains, have been implicated by ENDOR measurements [19]. The Hg EXAFS spectrum of the mixed-metal derivative of laccase also suggests that the primary donors in the type 1 site of laccase are two imidazole nitrogens and a cysteine sulfur [4]. Because of the low effective coordination number of this site and because of the thiol group, the type 1 site

TABLE 1. Electronic spectral data for Co(II) derivatives of type 1 copper centers

Protein	λ^{CT} (nm)[a]		λ^{dd}_{max} (nm)				Remarks
Stellacyanin (*Rhus vernicifera*)	310	365	540		625(sh)	655	pH 8.0[b]
			540		630	655	pH 9.5[c]
Laccase (*Rhus vernicifera*)	~303	~360	540	595(sh)	630		pH 7.4
			540	595	630(sh)		pH 8.0
Azurin (*P. aeruginosa*)	330	375	521		637		[b]
Azurin (*Alcaligenes* sp.)	329	374	522		638	655(sh)	[d]
Plastocyanin (*P. vulgaris*)	333	385	508			658	[b]
Plantacyanin (cucumber)	331	390	510		640(sh)	676	[e]
Pseudoazurin (*A. cycloclastes*)	335	390	505		640(sh)	673	[d]

[a]The lower energy CT transition is usually a shoulder on the higher energy, more intense CT transition. [b]Ref. 8. [c]Ref. 7. [d]Ref. 10. [e]Ref. 9.

exhibits an extremely high affinity for Hg(II) [4, 20].

In the present study we have used Hg(II) to block the binding of Co(II) to the type 1 site in a reference sample in order to subtract out contributions from cobalt bound anywhere except the type 1 site. The relevant difference spectrum is depicted in Fig. 1(A). Comparison of Figs. 1(A) and 1(B) establishes that Hg(II) and Co(II) are competing for the same site, hence the bands can be attributed to Co(II) bound in the type 1 site. This conclusion is confirmed by the observation of CT absorption in the near UV. These bands can unambiguously be assigned as S(Cys) $\rightarrow$ Co(II) transitions by analogy with results from a variety of Co(II) derivatives of other blue copper (Table 1). The sharp rise just below 300 nm in the onset of the CT absorbance in Fig. 1 may reflect a contribution from the Hg site in the reference sample. For this reason the position of the UV absorption maximum of the cobalt chromophore is presented as an approximate value in Table I.

A triangular arrangement of the donor atoms from the sidechains of cysteine and histidine residues is the hallmark of the type 1 copper site [5]. Differences in the 'axial' ligation may explain variations within the chemical and physical properties of the series. For example, Suzuki *et al.* have suggested that a change in the distance from copper to the methionine sulfur may influence the intensity of the charge transfer absorption band that occurs at *c.* 450 nm in the spectrum of type 1 copper [10]. They have also noted that Co(II) derivatives of azurins exhibit distinctive visible spectra which may reflect the presence of a second distant axial ligand – a peptide oxygen identified in the crystal structures of the native proteins [21, 22]. In idealized tetrahedral symmetry, the visible absorption would correspond to the ${}^4T_1(P) \leftarrow {}^4A_2(F)$ transition; however, in the proteins, this transition is split by low symmetry components of the ligand field, and the splitting is less pronounced in the azurin derivatives. An even narrower splitting, symbolized by the horizontal spacing between the λ_{max} values in Table 1, occurs in the visible spectra of the Co(II) derivatives of stellacyanin and *Rhus* laccase. This suggests that these two proteins may form a unique structural class. In accordance with this hypothesis the λ_{max} values for the CT transitions are also significantly hypsochromically shifted for the Co(II) derivatives of stellacyanin and laccase (Table 1). (Table 1 and Fig. 1 also reveal the visible spectra of the same two derivatives to be pH dependent. This may or may not be a unique characteristic of these systems; the pH dependence does not appear to have been investigated for the other members of the series.) If the existence of a distant fifth ligand is the distinctive feature of the azurins, it is possible that an analogous group makes an even closer approach to the metal in stellacyanin and *Rhus* laccase.

Finally, we should consider the reasons why characterization of the Co(II) derivative of the type 1 site has proven to be more difficult in laccase than in systems studied previously. One problem is that laccase is a relatively large protein with many aromatic residues. As a consequence the background absorbance of the protein is quite significant in the near UV, and a difference technique must be used to resolve the CT transitions. Similarly, it has been necessary to resolve the d–d bands from those of cobalt bound at the type 3 and/or type 2 sites [12]. The other major problem is that the type 1 site in laccase appears to have a relatively low affinity for Co(II). Indeed, a large excess of cobalt must be added in order to complete the occupancy of the type 1 site.

The low affinity for Co(II) binding may be a reflection of conformational flexibility within the protein. In case of plastocyanin, crystallographic results suggest that the ligands at the type 1 site assume almost the same spatial disposition whether or not a metal ion is present [23]. Thus, very little of the free energy associated with metal binding is sacrificed in the organization of the donor framework. This may not be the case in laccase. One inter-

pretation of the complex time dependence observed in the absorbance at 630 nm is that the binding of Co(II) in the type 1 site requires a change in the protein conformation.

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