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PREPARATION AND CHARACTERIZATION OF A STABLE HALF MET DERIVATIVE OF TYPE 2 DEPLETED RHUS LACCASE: EXOGENOUS LIGAND BINDING TO THE TYPE 3 SITE

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SUMMARY: We report the preparation and characterization of a stable half met (Cu(II)Cu(I)) type 2 copper depleted derivative of Rhus laccase. Anion binding studies to this mixed valent type 3 protein form indicate no tight binding of anions nor group 1 - group 2 ligand behavior. This suggests that, in contrast to the well-characterized hemocyanins and tyrosinase coupled binuclear sites, exogenous ligands do not appear to bridge the type 3 binuclear copper ions in laccase.

INTRODUCTION

Rhus vernicifera laccase [1] contains one type 1 (blue), one type 2 (normal), and one type 3 (coupled binuclear) copper site which together catalyze the four-electron reduction of dioxygen to water with concomitant oxidation of substrate. In the native enzyme, the types 1 and 2 cupric centers are paramagnetic and EPR detectable, while the binuclear cupric ions are strongly antiferromagnetically coupled and hence EPR non-detectable; this type 3 site reduces with two electrons at the same potential [2].

While all four copper atoms are oxidized in native laccase, we have recently demonstrated [3] that the type 2 copper depleted (T2D) enzyme derivative, after preparation, contains a reduced type 3 binuclear unit in the pres-

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ence of O_2 (essentially no absorption at 330 nm; $\varepsilon_{614} \sim 5700\ M^{-1}\ cm^{-1}$ associated with the oxidized type 1 center). Upon treatment with excess peroxide, an oxidized T2D form ($\Delta\varepsilon_{330} \sim 2000\ M^{-1}\ cm^{-1}$; $\Delta\varepsilon_{410} \sim 175\ M^{-1}\ cm^{-1}$; $\Delta\varepsilon_{614} \geq -300\ M^{-1}\ cm^{-1}$) with an antiferromagnetically coupled, EPR non-detectable binuclear cupric type 3 site (a spectroscopic analogue to the met hemocyanin derivative) is generated. In this communication, we report the preparation and characterization of a stable, half met (Cu(II)Cu(I)) derivative of this type 3 center in T2D laccase. In addition, the anion binding behavior of this half met T2D derivative has been investigated; these results are presented and used to indicate that, in contrast to the half met hemocyanin derivatives, exogenous ligands do not appear to bridge the two coppers of the type 3 site in laccase.

MATERIALS AND METHODS

Rhus vernicifera laccase from Japanese lacquer acetone powder (Saito and Co., Osaka, Japan) was purified by the method of Reinhammar [4]. T2D and oxidized T2D protein derivatives were prepared by the methods of Graziani [5], (modified) [6], and LuBien [3], respectively. All studies were in 0.1 M potassium phosphate buffer, pH 6.0.

RESULTS AND DISCUSSION

Anaerobic ferrocyanide reduction of oxidized T2D protein results in a new cupric EPR signal whose intensity maximizes with 0.7 - 1.0 protein equivalents of reductant. Exposure to air reoxidizes any reduced type 1 copper for a final double-integrated EPR intensity of ~ 1.6 spin/mole (see Figure 1). The rhombic signal is clearly cupric in nature, with four discernible hyperfine lines ($A_{\parallel} = 135 \times 10^{-4}\ cm^{-1}$, $g_{\parallel} = 2.287$) suggesting no delocalized mixed valence character. In the optical absorption spectrum, the 614 nm band is unperturbed, while 330 nm absorption is decreased relative to oxidized T2D protein. Removal of the ferricyanide by dialysis against buffer does not alter the half met spectral features. These signals are also stable to dialysis against EDTA and chromatography on Chelex 100. Addition of excess peroxide, however, regenerates oxidized T2D, restoring the optical and EPR features associated with this form. Half met T2D therefore is a reversible, and thus valid, protein derivative. X-ray absorption spectroscopy [7] confirms the presence of cuprous copper in half met T2D ([Cu(I)]/[Cu(II)] ≃ 1/6).

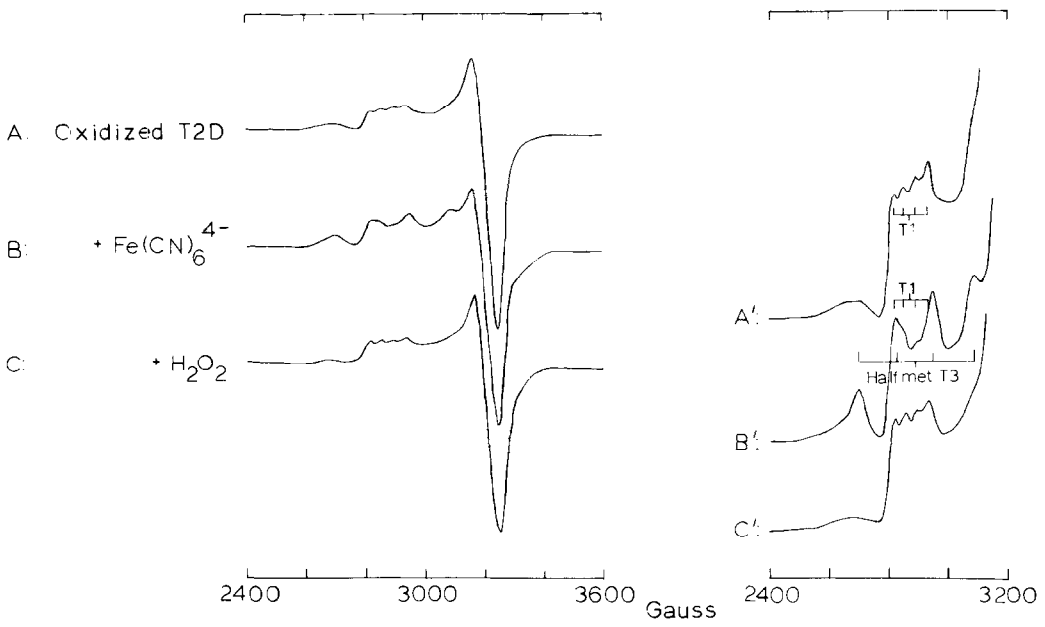

Figure 1. 77K EPR spectra of A) oxidized T2D laccase; B) + 0.7 protein equivalents $Fe(CN)_6^{4-}$, dialyzed; C) +30 protein equivalents H_2O_2. Microwave frequency was 9.25 GHz and microwave power was 10 mW. Primed spectra were recorded at ~ 5x higher gain.

This ratio, together with the double-integrated EPR intensity of ~ 1.5 spin/mole, characterizes this new derivative as containing T2D laccase in which 50 percent of the protein contains binuclear sites which are mixed valent and the remaining 50 percent contains fully oxidized type 3 sites. While a related type 3 cupric EPR signal may have been observed in T2D Polyporus versicolor laccase [8], only half met type 3 kinetic intermediates during reoxidation have been reported for T2D tree laccase [9].

Ligand binding to this stable, half met T2D laccase has been investigated for a series of anions. With less than 25 protein equivalents of azide, the type 3 $A_{\parallel}$ decreases to 101 x 10^{-4} cm^{-1} and $g_{\parallel}$ shifts to 2.280, still exhibiting little, if any, delocalized character (see Figure 2); oxidized T2D shows no EPR perturbation at these azide concentrations [10]. With additional azide, no further perturbation of the half met T2D EPR signal is seen, suggesting that only one azide binds to the half met site. This is also supported by a single binding constant, K ~ 300 M^{-1} at 330 nm (corrected for 50 percent oxidized T2D also present), in the room-temperature optical absorption spectrum. Short-term dialysis (5 hours) removes the azide and restores the orig-

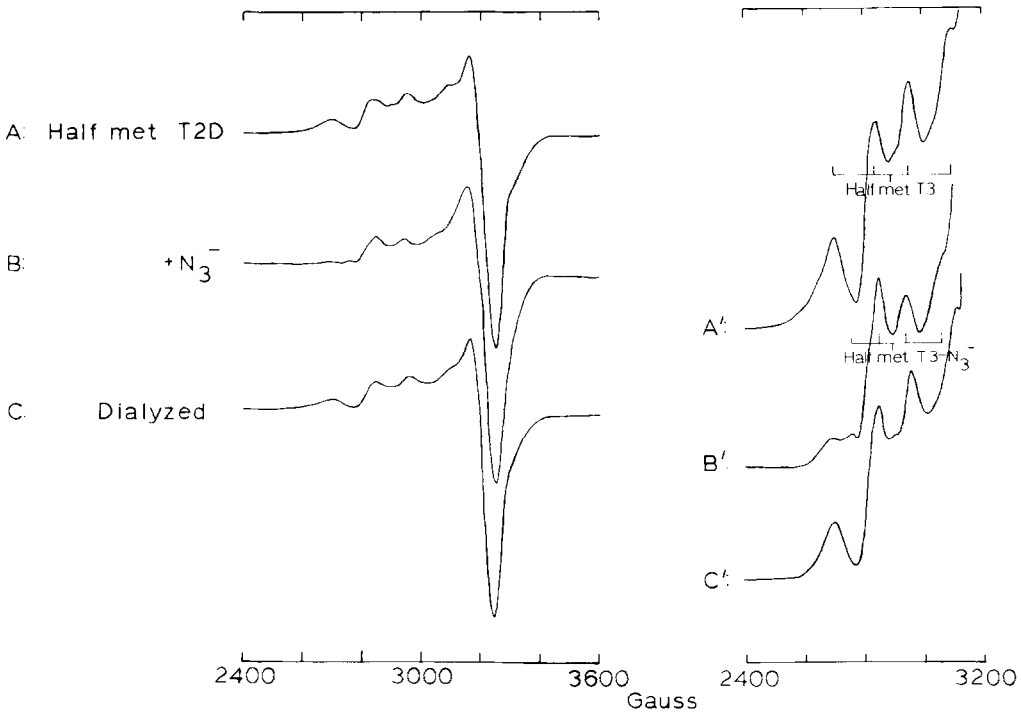

Figure 2. 77K EPR spectra of A) half met T2D laccase; B) +10 protein equivalents N_3^-; C) dialyzed 5 hours against buffer. Microwave frequency was 9.25 GHz and microwave power was 10 mW. Primed spectra were recorded at ~ 6x higher gain.

inal half met aquo EPR features (see Figure 2C). SCN^- also binds to half met T2D, although the EPR features due to half met T2D-SCN^- are complicated by the simultaneous perturbation of oxidized T2D at equivalent SCN^- concentrations [10]; dialysis again removes all exogenous ligand related features. The halogens, at most, only weakly perturb the half met type 3 EPR signal. To summarize, labile ligand substitution chemistry as found for normal tetragonal aqueous Cu(II) complexes seems to predominate [11].

This behavior is in marked contrast to anion binding in half met hemocyanins [12] and tyrosinase [13]. In these proteins, exogenous ligands were determined to bridge the cuprous and cupric ions of the binuclear site based on their especially tight binding at this site, and group 1 - group 2 ligand behavior. Group 1 ligands (F^-, Cl^-, Br^-, I^-, OAc^-, NO_2^-) bind tightly to the binuclear coppers with a < 4 Å Cu-Cu distance and produce no change in EPR signal when present in excess concentrations, while group 2 ligands (N_3^-, SCN^-, CN^-) can bridge with a > 5 Å Cu-Cu distance and produce major EPR

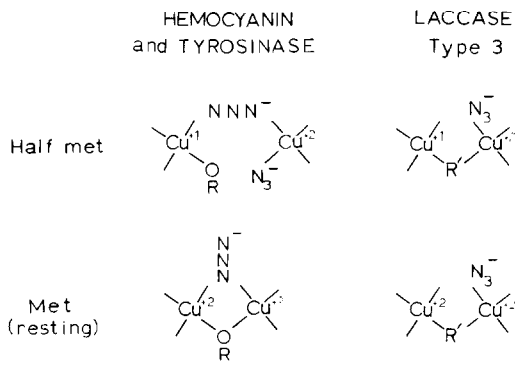

Figure 3. Comparison of spectroscopically effective models for azide binding in binuclear copper-containing protein derivatives. OR and R' denote the endogenous protein bridge in the hemocyanins and laccase, respectively.

charges at high excess, indicating that a second group 2 ligand is bound at the cupric center. The increased Cu-Cu separation breaks the endogenous protein bridge, providing the additional coordination position. Upon dialysis, a new EPR signal is observed, indicating that while the second group 2 ligand is removed, the bridging exogenous ligand remains tightly bound [14].

The lack of group 1 - group 2 behavior, tight binding of anions, or electron delocalization on the EPR time scale in half met T2D laccase indicates that, in contrast to the hemocyanins and tyrosinase, exogenous ligands do not appear to bridge the two coppers (see Figure 3). This absence of bridging behavior extends to the oxidized T2D or met derivative as well (Figure 3). Absorption and CD spectra of azide bound to oxidized T2D [15] show only one N_3^- → Cu(II) CT band (λ_{max} = 450 nm), whereas azide on met hemocyanin clearly exhibits three CT peaks in the optical spectra, indicating that azide must bridge the two coppers [16,17]. It is likely that the inability of exogenous ligands to bridge the type 3 site in half met T2D is intrinsic to the binuclear unit in this multicopper oxidase. Extending this spectroscopically effective type 3 site in Figure 3 to peroxide binding to native laccase would indicate that the μ-dioxo bridging mode of peroxide in oxyhemocyanin [12c,18] is inaccessible in laccase; this suggests a mono-oxo binding of peroxide (likely in the form of HO_2^- near neutral pH) to only one of the coppers in the type 3 Cu(II) center (see next communication).

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