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NITRITE REACTIVITY OF THE BINUCLEAR COPPER SITE IN T2D RHUS LACCASE: PREPARATION OF HALF MET-NO_2^- T2D LACCASE AND ITS CORRELATION TO HALF MET-NO_2^- HEMOCYANIN AND TYROSINASE

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SUMMARY: Through chemistry directly comparable to that of the hemocyanins and tyrosinase, half met-NO_2^- T2D laccase derivatives have been prepared; this NO_2^- reactivity entails both two electron oxidation of the cuprous binuclear site in deoxy T2D laccase and one electron reduction of the coupled cupric site in the met derivative. However, the labile ligand substitution chemistry and lack of dimer formation in half met-NO_2^- T2D are in marked contrast to behavior of the simpler binuclear copper containing proteins under analagous conditions. This chemistry supports and extends our earlier studies on the ferrocyanide-generated half met T2D which first indicated an inability of exogenous ligands to bridge the binuclear copper site in laccase.

A significant number of metalloproteins and enzymes contain a coupled binuclear copper unit [1] at their active site. While the copper centers in each of these systems interact with oxygen, reduce with two electrons at the same potential, and are strongly antiferromagnetically coupled and hence EPR nondetectable, great diversity exists in binuclear copper active site function. Through a series of protein derivatives which allow the active site to be systematically varied, a "spectroscopically effective" picture of the binuclear copper unit in the hemocyanins [2] and tyrosinase [3] has been generated. This work is now being extended [4-6] to the simplest multicopper oxidase [7], Rhus vernicifera laccase, for the correlation of differences in geometric structure with variation in biological function.

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Native laccase [8] contains four coppers at its active site (one Blue, T1; one normal, T2; one coupled binuclear copper, T3). Reversible removal of the T2 copper [9], however, to prepare a valid type 2 copper depleted (T2D) protein derivative has greatly simplified this system and enabled study of the binuclear copper in laccase without complication by the T2 center. While optical and EPR data clearly demonstrate that the T1 copper is oxidized in the prepared T2D laccase, X-ray absorption edge studies [4] were necessary to define this derivative (untreated, or deoxy T2D) as containing T3 sites which are > 90% reduced in the presence of oxygen, but which can be reoxidized by excess peroxide to generate a protein form (met T2D) [4] containing an antiferromagnetically coupled binuclear cupric site. It was further shown [5] that ferrocyanide partially reduces the EPR nondetectable site in met T2D to generate a stable, EPR detectable half met binuclear copper derivative which is also peroxide-reoxidized to met T2D.

In contrast to the anaerobic $Fe(CN)_6^{4-}$ reduction of met T2D to prepare half met T2D laccase, half met hemocyanin [10,11] is accessible only through the action of nitric oxide [1]:

$$\text{(i)}\quad \underset{[Cu(I)Cu(I)]}{\text{deoxy}} + 2NO \rightarrow \underset{[Cu(II)...Cu(II)]}{\text{met}} + N_2O$$

$$\text{(ii)}\quad \underset{[Cu(II)...Cu(II)]}{\text{met}} + NO \rightarrow \underset{[Cu(I)Cu(II)]\text{-}NO_2^-}{\text{half met-}NO_2^-}$$

as can be derived from aqueous nitrite chemistry at pH < 7.0:

$$3HNO_2 \rightarrow 2NO + HNO_3 + H_2O.$$

The extension of this reactivity to the T2D laccase derivatives is reported in this communication. Through chemistry directly comparable and consistent with that found for the hemocyanins and tyrosinase, new routes to (i) oxidation of the reduced T3 site in deoxy T2D to form met T2D and (ii) generation of half met T2D laccase forms have been developed. Alternately, significant differences exist in the nitrite reactivity of these proteins; the labile ligand substitution chemistry of half met-NO_2^- T2D and an apparent lack of dimer formation further substantiate our earlier studies [5] on the ferrocyanide-generated half met T2D laccase which indicated that, in strong contrast to the hemocyanins and tyrosinase, exogenous ligands do not bridge the laccase binuclear copper site, as depicted in Figure 1.

MATERIALS AND METHODS

Rhus vernicifera laccase was purified from the acetone powder of the Japanese lacquer tree according to published procedures [12]. The T2 copper was selectively removed by the modified [13] method of Graziani [9]; met and half met T2D derivatives were prepared as reported in [4] and [5], respectively. All studies utilized reagent grade chemicals in 0.1 M potassium phosphate buffer, pH 6.0. A Brüker ER 220D-SRC EPR spectrometer monitored frozen solution samples at 9.8 mW power and 20 G modulation.

RESULTS AND DISCUSSION

The EPR signal of deoxy T2D laccase (T3:[Cu(I)Cu(I)]; T1:[Cu(II)]) (Fig.2A) treated with excess sodium nitrite is shown in Figure 2B; overlapping the peaks normally associated with the distorted tetrahedral Blue copper center (T1) are four additional hyperfine lines characteristic of isolated tetragonal Cu(II) ($g_{\parallel} = 2.31$, $g_{\perp} = 2.06$, $A_{\parallel} = 160 \times 10^{-4}$ cm^{-1}). As the T2 center is $\geq$ 90% removed and double integrated EPR intensity is 1.4 spin/mole, the new cupric signal must originate from the binuclear copper site. Increased $[NO_2^-]$'s (up to 1000 X), lower temperatures (~ 12K) and higher microwave powers (197 mW) result in no apparent g ~ 4, $\Delta m_s = 2$ signal nor broad g ~ 2, $\Delta m_s = 1$ signal indicating that, in contrast to hemocyanin chemistry [16,17], an N_xO_y uncoupled derivative has not been formed. Short term dialysis (< 5 hr) alters the T3-related signal and results in an EPR spectrum (Fig. 2C) which is identical to that of half met-aquo T2D, as generated through ferrocyanide reduction of met T2D. The 330 nm absorption, associated with the T3 site in met T2D [4], is increased relative to the original deoxy T2D but less than that normally observed in the

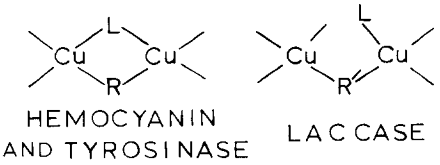


Figure 1: Spectroscopically effective models for exogenous ligand (L) binding at the binuclear copper sites in hemocyanin, tyrosinase and laccase.

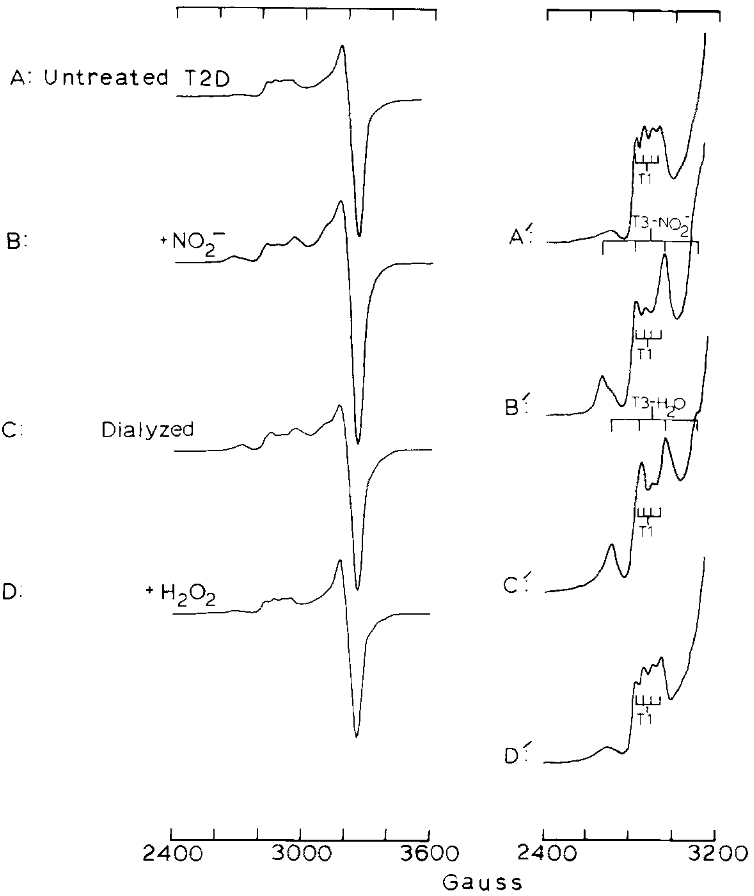


Figure 2: 77 K EPR spectra of (A) untreated T2D laccase [14]; (B) A + 100 protein equivalents $NaNO_2$; (C) B dialyzed into 0.1 M potassium phosphate, pH 6.0; and (D) C + 30 protein equivalents H_2O_2. 9.26 GHz microwave frequency and 1.25 x 10^4 instrument gain. Primed spectra were recorded at 8.0 x 10^4 gain. Protein concentration was 0.15 mM in (A,B), 0.14 mM in (C,D).

peroxide-oxidized T2D; little or no perturbation in the 600 nm region is observed. As is found for the $Fe(CN)_6^{4-}$-generated half met T2D derivative, peroxide removes the T3 EPR signal, decreases the integrated EPR intensity to ~ 1.0 spin/mole and increases 330 nm absorption, indicating oxidation to met T2D (Fig. 2D) and assuring that the NO_2^- treated deoxy T2D is a regenerable protein form. X-ray absorption edge studies [18] indicate 11 ± 5% Cu(I) in the NO_2^--treated deoxy T2D protein, consistent with the double integrated EPR intensity and a mixture of met and half met-NO_2^- binuclear copper sites (optical and EPR data demonstrate the Blue copper is fully oxidized). Thus, the significantly decreased percentage of Cu(I) and

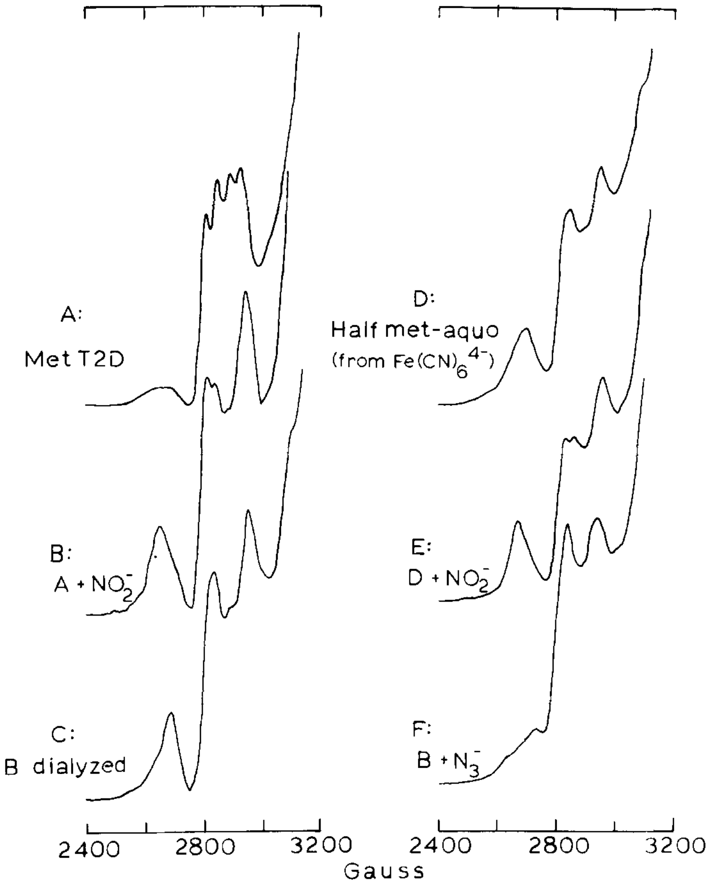


Figure 3: 77 K EPR spectra of (A) met T2D *Rhus* laccase; (B) A + 100 protein equivalents $NaNO_2$; (C) B dialyzed into 0.1 M potassium phosphate, pH 6.0; (D) half met-aquo T2D; (E) D + 200 x $NaNO_2$; (F) B + 10 x N_3^-. All spectra are at 9.25-9.26 GHz microwave frequency; A-C, F represent 0.186 mM protein at 1.25 x 10^5 gain; D,E are 0.107 mM at 10 x 10^4 gain.

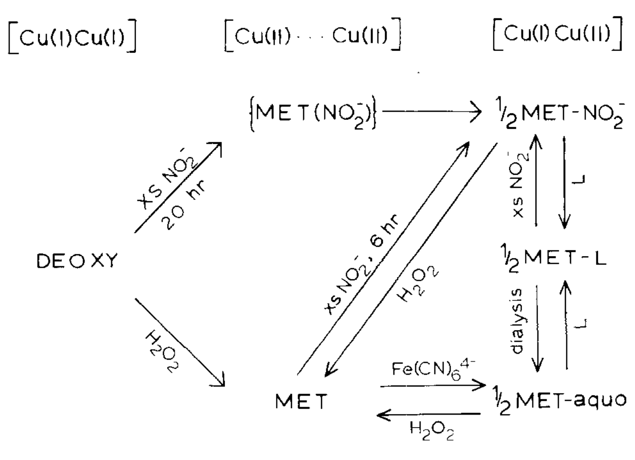
increased 330nm absorption in the NO_2^--treated protein indicate that, analogous to hemocyanin and tyrosinase nitrite chemistry [1], NO oxidizes the cuprous T3 site in deoxy T2D. Further, the new T3 EPR signal associated only with a $\Delta m_s = 1$ transition, its conversion to half met-aquo T2D upon simple dialysis, the Cu(I) in the X-ray absorption edge, and related nitrite studies on met T2D (*vide infra*) together demonstrate that this met intermediate reduces by one electron to generate a stable half met-NO_2^- protein form.

Parallel nitrite reactions on met T2D (T3:[Cu(II)Cu(II)]; T1:[Cu(II)]) (Fig.3A) also result in the 1.4 spin/mole EPR spectrum (Fig. 3B); the spectral and chemical properties associated with this protein are essentially indistinguishable from those of the NO_2^- treated deoxy T2D derivative. As some $Fe(CN)_6^{3-/4-}$ is inevitably present [5,9,15] in the T2D protein prior to NO_2^- addition and this reductant has been shown [5] to generate half met-aquo from met T2D, control experiments monitored the oxidation state stability of met T2D in the absence of nitrite and confirmed the direct involvement of NO/NO_2^- in the one-electron reduction of the binuclear cupric site [19].

Short term dialysis of the NO_2^--treated met T2D again removes the weakly coordinated NO_2^- ligand to produce half met-aquo T2D (Fig. 3C). This lack of tight binding is consistent with the action of lower $[NO_2^-]$'s (10 X) on met T2D which directly result in half met-aquo T2D (e.g., 1e$^-$ reduction but no NO_2^-coordination); 10 X NO_2^- reacted with deoxy T2D results in no significant T3 contribution to the EPR spectrum. The lack of half met-X formation in deoxy but not met T2D at low $[NO_2^-]$'s and the observed longer reaction time of deoxy (20 hr) relative to met (6 hr) with 100 X NO_2^- further point to generation of an oxidized met intermediate (2e$^-$ oxidation) prior to half met formation (1e$^-$ reduction). Finally, while quantitative X-ray absorption edge analyses [18] indicate that the met and deoxy T2D proteins differ by 70 ± 15% in their Cu(I) composition, the derived NO_2^--treated edges are essentially superimposable; these studies corroborate both the oxidation of deoxy to met and the assignment of the aforementioned ~ 11% Cu(I) as being primarily due to half met sites.

The ligand substitution chemistry of half met T2D laccase has also been pursued. The interconversion of the various half met T2D derivatives indicates $Fe(CN)_6^{4-}$ and NO_2^- reactivity lead to equivalent half met protein forms. Nitrite titration of ferrocyanide-generated half met T2D produces half met-NO_2^- T2D (Fig. 3D→3E); this further demonstrates that the original products of the NO_2^- preparations contain a coordinated nitrite ligand which is readily removed upon dialysis. Only one NO_2^- is bound, based on half met-aquo T3 EPR perturbation with increasing $[NO_2^-]$. N_3^- addition to the nitrite-induced half met-NO_2^- (in excess NO_2^-) decreases $A_{\parallel}$ of the T3 signal and results in half met-N_3^- (Fig. 3F) identical to that observed upon N_3^- titration [5] of the $Fe(CN)_6^{4-}$-induced half met-aquo T2D. This chemistry is summarized in Scheme I.

Significantly, short term dialysis readily removes all exogenous ligands and results in half met-aquo T2D; the characteristic tight binding of anions and group 1-group 2 ligand chemistry of the binuclear copper unit in half met hemocyanin and tyrosinase are not observed [11]. In the latter proteins, the exogenous ligand is tightly bound, bridges the cuprous and cupric centers, and is not removed by >100 hr dialysis. The markedly different behavior strongly indicates that exogenous ligands do not bridge in laccase; simple, labile ligand substitution chemistry, similar to that of met apo hemocyanin [11,21] is all that is observed. The inability of exogenous ligands to bridge the T3 site could also account for the apparent lack of dimer [16, 17] formation in native [20,22] and T2D laccases. While NO_2^- reacts with both deoxy hemocyanin and deoxy T2D to generate half met-NO_2^- protein derivatives (through a met intermediate), this chemistry requires only inner sphere electron transfer and exogenous ligand coordination; uncoupling of the site to form dimer hemocyanin, however, is believed [1] to occur through an N_xO_y exogenous ligand bridge which forces Cu-Cu to >6Å and ruptures the endogenous protein bridge. If the coordinated



Scheme 1: Reactivity of the binuclear copper site in T2D Rhus laccase.

exogenous ligand is not able to bridge the binuclear coppers in T2D laccase, the NO_2^- reactivity observed in hemocyanin and tyrosinase, and hence, this route to dimer formation is inaccessible.

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