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EXAFS INVESTIGATION OF THE BINUCLEAR CUPRIC SITE IN MET T2D RHUS LACCASE AND ITS AZIDE BOUND DERIVATIVE

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SUMMARY EXAFS analysis of met T2D Rhus laccase and its azide bound derivative indicates an average of 0.33 S at 2.09 Å and 3-4 N (or O) atoms at 2.00 Å per copper atom for the three copper centers. Using the plastocyanin Cu(II) EXAFS spectrum to model the type 1 site in laccase, a difference EXAFS spectrum for the type 3 site is generated; this spectrum enables assignment of the one S ligand in met T2D to the type 1 site and indicates no evidence of a detectable copper scatterer for the coupled binuclear copper site. Implications regarding type 3 optical features and related studies on the hemocyanins are also discussed.

As the coupled binuclear copper site in hemocyanin and tyrosinase has become well defined [1], it is important to determine how this site relates to the type 3 (T3) site in the more complicated proteins, the multicopper oxidases [2]. Rhus vernicifera laccase [3], the simplest of the multicopper enzymes, contains one T1 (blue), one T2 (normal) and one T3 (coupled binuclear) copper site which together catalyze the four-electron reduction of dioxygen to water. In the native enzyme, the T1 and T2 cupric centers are paramagnetic and EPR detectable, while the binuclear cupric centers, like those of the hemocyanins [4] and tyrosinase [5], are strongly antiferromagnetically coupled and hence EPR non-detectable. In the optical absorption spectrum, the T3 site has been associated with a 330 nm feature, which in the native enzyme ($\varepsilon \sim 2800\ M^{-1}cm^{-1}$) reduces with two electrons at the same potential [6]. An important simplification particularly suited to study the T3 site in laccase has been achieved through reversible preparation of a type 2 copper depleted (T2D) protein derivative, first reported for Rhus laccase by Graziani et al [7]. Our

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initial x-ray absorption edge studies [8] demonstrated that this T2D protein form, after preparation, contains a reduced T3 binuclear unit in the presence of O_2 (essentially no absorption at 330 nm; $\varepsilon_{614} \sim 5700$ $M^{-1}cm^{-1}$ associated with the oxidized T1 center) which can be oxidized with excess peroxide to generate met T2D ($\Delta\varepsilon_{330} \sim 2000$; $\Delta\varepsilon_{410} \sim 175$; $\Delta\varepsilon_{614} \geq -300$; $\Delta\varepsilon_{745} \sim 150$ $M^{-1}cm^{-1}$) with an antiferromagnetically coupled, EPR non-detectable binuclear cupric site. Through the collection and interpretation of EXAFS spectra on met T2D and its N_3^- bound derivative, we now investigate further the nature of the T3 site in T2D . With plastocyanin as a model for the T1 site, we have generated a difference EXAFS spectrum which is the average environment of the two coppers in the T3 site. Herein, we report these EXAFS results, their implication regarding potential assignment of the 330 nm absorption feature in native and met T2D laccase, and their comparison to related studies on the hemocyanins and tyrosinase coupled binuclear copper active sites.

MATERIALS AND METHODS

Laccase was purified [9,10] from the acetone powder (Saito and Co., Osaka, Japan) of the Japanese lacquer tree, Rhus vernicifera. Selective removal of the T2 copper was achieved by the modified [11] method of Graziani [7] and oxidized to met T2D as reported by LuBien [8]. All studies were in 0.1 M potassium phosphate, pH 6.0.

X-ray absorption data were collected at the Stanford Synchrotron Radiation Laboratory (beamlines 1V-2 and II-3) using Si[220] monochromator crystals. All data were recorded (at -60°C) as fluorescence excitation spectra by using an array of NaI(Tl) scintillation counters with Ni filters. The energy scale was calibrated relative to a Cu foil, with the first preedge inflection of the Cu K-absorption edge being assigned to 8980.3 eV. EXAFS data reduction and analysis have been described previously [12]. EXAFS data are normalized per copper atom and are reported as average values per atom present. First and the final scan comparison of the X-ray spectra, as well as optical and EPR characterization after irradiation ascertained protein integrity.

RESULTS AND DISCUSSION

Visual comparison of the EXAFS data of met T2D laccase and its azide complex and their Fourier transforms (Figure 1) shows that the environment of the copper sites is not greatly perturbed by N_3^- coordination. As each copper atom generates an EXAFS spectrum which is dependent on the ligands coordinated to it, the EXAFS spectra of the T2D laccase derivatives are average spectra of the three absorbing copper atoms.

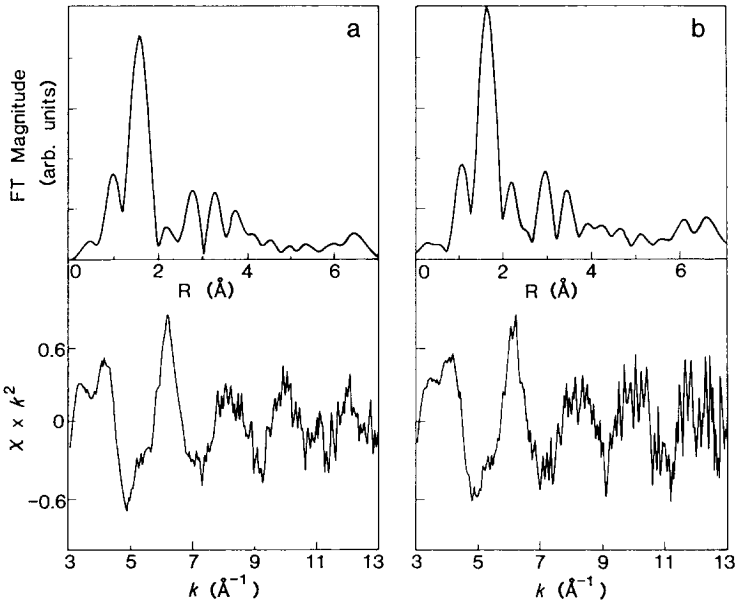

Figure 1: The Fourier transforms, $\underline{k}$ = 4-12 $Å^{-1}$, $\underline{k}^3$ weighted (top) and EXAFS (bottom) of ~ 2 mM Rhus laccase in 0.1 M potassium phosphate, pH 6.0: (a) met T2D, (b) + 75x N_3^-.

The major peaks in both transforms are filtered and backtransformed to $\underline{k}$-space for least-squares curve-fitting analysis, as summarized in Table 1. In met T2D, the first shell can be fit with a shell of 3.5 nitrogen (or oxygen) atoms at an average Cu-N (or -O) distance of 1.99 Å. Inclusion of

Table 1. Laccase EXAFS first shell curve-fitting results

Protein derivatives	Function value[13]	Cu-N Å	Cu-N #	Cu-S Å	Cu-S #
Met T2D					
T1 + T3	0.30	1.99	3.5		
	0.22	2.00	3.0	2.09	0.33
T3	0.20	2.01	3.2		
	0.19	2.01	3.2	2.26	0.06
Azide treated met T2D					
T1 + T3	0.38	1.99	3.5		
	0.32	2.00	3.2	2.07	0.34
T3	0.43	2.01	3.5		
	0.42	2.02	3.5	2.08	0.13

Errors are estimated to be about ± 0.02 Å in distances and about ± 25 % in coordination numbers.

an additional shell of sulfur improved the fit significantly, reducing the function value [13] from 0.30 to 0.22. An average of 0.33 sulfur at 2.09 Å and 3.0 nitrogens (or oxygens) at 2.00 Å is obtained in the two shell fit. As the T2D derivative contains three copper atoms, this result suggests that one sulfur atom is coordinated to one of the copper atoms while 3-4 nitrogen (or oxygen) atoms will complete the coordination spheres of all the copper atoms. As all copper atoms are defined to be Cu(II) from the absorption edge studies [8,14] and Cu(II) ions are usually coordinated by 4-6 ligands, the indicated average of 3-4 N (or O) atoms is somewhat low. However, static disorder (as a consequence of averaging heterogenous copper sites) will cause destructive interference which will lower the prediction of coordination numbers, keeping the Debye-Waller factor constant. Analogous analysis of the azide derivative gives similar results (see Table 1) for the first shell fit.

Quantitative chemical and spectroscopic studies have established that T2 copper is ≥90% removed in T2D [7] and that met T2D laccase contains an oxidized Blue copper center [8,14]. Further, as shown in Table 2, the optical and EPR properties of the laccase T1 Cu(II) closely relate to those of the simpler Blue copper proteins, plastocyanin and azurin (but not stellacyanin) [2a,15]. Hence, we have used the plastocyanin copper EXAFS spectrum to model that of the T1 site in T2D laccase and generate a difference EXAFS spectrum for the T3 site. Specifically, we multiply the average T2D laccase spectrum by a factor of three and subtract the Blue copper site (using normalized plastocyanin EXAFS [16]) from it; the division of this difference spectrum by two then generates an average spectrum of the two coppers in the T3 site. The major peak in the transform is again filtered and backtransformed for curve-fitting analysis. The results (see Table 1) indicate 3.2 nitrogen (or oxygen) atoms at 2.01 Å. Sulfur atoms were again included in a two-shell fit, but no improvement in function value was observed and the amplitude of the resulting sulfur atom was no more than the level of noise. The difference EXAFS analysis is

Table 2 Comparison of Laccase Tl Copper(II)[a] to the Blue Copper Proteins[b]

	Rhus met T2D	Plastocyanin[c]	Pseudomonas aeruginosa Azurin	Rhus Stellacyanin
EPR				
$g_{\parallel}$	2.300	2.226	2.26	2.287
$g_{\perp}$	2.054	2.053	2.05	*2.077, 2.025*[d]
$A_{\parallel}$ (cm^{-1})	42.9×10^{-4}	63×10^{-4}	60×10^{-4}	35×10^{-4} (29, 57)
Optical				
λ_{max} (nm)	614	597	625	608
ε ($M^{-1}cm^{-1}$)	∿5400	4900	3500	4030
CD[e]				
λ_{max} (cm^{-1}) $\Delta\varepsilon$ ($M^{-1}cm^{-1}$)		24,000 (+1.26)	24,500 (+0.3)	
	22,100 (-2.8)	21,200 (-1.32)	21,400 (-1.8)	22,400 *(-7.35)*
	18,900 (+1.4)	19,000 (+0.4)	19,000 (+1.2)	19,000 (+0.75)
	16,300 (+1.5)	16,500 (+4.08)	16,100 (+6.5)	16,500 (+3.6)
	∿13,600 (-3.0)	12,800 (-3.78)	12,500 (-5.9)	12,800 (-5.0)
rR (cm^{-1})[f]	258	262	262	267
			340 (sh)	
	382	379	372	*350*
	407	407	407	388
	421	426	425	410
			460 (sh)	422, 424
E° (mV)	447	370	300-328	*184*

(a) From LuBien, C.D. et al. (1981) J. Am. Chem. Soc. 103, 7014-7016.
(b) Adapted from Fee, J.A. (1975) Struct. Bonding (Berlin) 23, 4-5.
(c) CD for bean plastocyanin; all other data refers to spinach plastocyanin.
(d) Italicized data most significant in differentiating stellacyanin from the other Blue Copper containing proteins.
(e) All Blue Copper protein CD data from Solomon, E.I. et al. (1980) J. Am. Chem. Soc. 102, 168-177.
(f) All Blue Copper protein rR data from Gray, H.B. and Solomon, E.I. (1981) Copper Proteins, Chapter 1, Academic Press, New York (Spiro, T.G., ed.).

thus consistent with the results stated above that a sulfur atom is coordinated to one copper - in the type 1 site - and that there is no

strong sulfur ligation at the T3 site in laccase. We note that while the Cu-S distance of 2.09 Å for the T1 site in T2D is slightly short for thiolate coordination to Cu(II), a similar distance of 2.11 Å is indicated by EXAFS treatment of plastocyanin, our T1 model system. The average coordination environment of 3-4 nitrogen (or oxygen) atoms at 2.00 Å in met T2D is essentially unaltered by subtraction of the plastocyanin contribution to the EXAFS spectrum. The EXAFS difference data for met-N_3^- T2D is quite similar to that of met T2D (Table 1) and indicate no large change in site geometry upon coordination of N_3^-.

To further probe the T3 site in laccase, we compare this calculated difference spectrum with the hemocyanin EXAFS spectrum [17,18]. The outer peak pattern in the transform of the laccase difference spectrum shows the familiar imidazole two-peak pattern but no evidence of a heavy atom scatterer. Curve-fitting analysis of the outer peaks further shows no indication of a copper scatterer at a distance $\lesssim$ 3.8 Å. Detection of a copper scatterer at $\gtrsim$ 3.8 Å is not possible at this noise level and is further complicated by multiple scattering effects and unknown Debye-Waller factors. In oxyhemocyanin [17,18] a large peak is present at ~ 3.6 Å and is associated with the second copper. Alternatively, for methemocyanin which also contains an antiferromagnetically coupled binuclear cupric site at a Cu-Cu distance of < 4 Å, it is less clear whether scattering from a second copper can be observed. Thus, while EXAFS does indicate the T3 site in met T2D to be different from the coupled binuclear copper site in oxyhemocyanin (despite their similar lack of paramagnetism), it is unclear whether this difference is associated with T2D having a larger Cu-Cu distance or larger Debye-Waller factor, the latter potentially relating to the lack of exogenous ligand bridging [19-21] at the T3 site in constrast to hemocyanin. Analysis of the EXAFS data from the met-N_3^- T2D derivative shows analogous results (see Table 1), in contrast to met-N_3^- hemocyanin where an outer shell peak at ~ 3.6 Å is distinctly present [18] and N_3^- has been shown to bridge the two coppers [4].

These results depend on how well the type 1 site is represented by plastocyanin. Computer simulation and metal complex studies of difference EXAFS techniques have shown that inappropriate subtraction of one site leads to erroneous analysis of the other site [22]. While the lack of x-ray crystallographic data for the T1 site in laccase precludes absolute evaluation of model propriety, the similar spectroscopic properties, as summarized in Table 2, would suggest no more appropriate model has been reported.

The exclusion of strong S ligation (to < 2.4 Å) at the T3 site in laccase indicates that the major differences in binuclear copper properties of laccase and hemocyanin, e.g. the apparent inability of exogenous ligands to bridge the binuclear coppers and irreversible oxygen binding, are not due to fundamental differences in this first shell ligation. An average of 3-4 N (or O) atoms at a distance of 2.00 Å in met T2D laccase and 3-5 N (or O) at 1.98 - 2.02 Å in met-aquo hemocyanin is the only ligation evidenced by EXAFS studies. The lack of S at the T3 site in laccase further suggests that absorption in the 330 nm region, present in native and met but not the untreated T2D, is not S → Cu(II) CT [23]. Based on model studies [24], this transition likely derives from a composite of His → Cu(II) CT transitions with potential contribution of RO^- → Cu(II) CT from the endogenous protein bridge. While a similar absorption feature is observed in met hemocyanin [25], the band is significantly broader, perhaps due to greater variation in the orientations of the imidazole planes relative to the equatorial plane (hence $d_{x^2-y^2}$ orbital) of each copper (II).

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