A Mixed-Metal Derivative of Laccase Containing Mercury(II) in the Type 1 Binding Site

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Oxidase enzymes which mediate the reduction of dioxygen to water generally contain multiple, redox-active metal centers. Understanding the properties of these enzymes is a significant challenge (1) because of the vital role(s) played by oxidases in aerobes,[1] (2) because of the technological importance of finding ways to circumvent the kinetic barriers associated with the reduction of molecular oxygen,[2] and (3) because of the fundamental interest in understanding noncomplementary redox reactions. *Rhus vernicifera* laccase is one of the more tractable oxidases that has been identified in that it is readily available and water soluble. Still, it contains four copper ions distributed among three different binding sites,[3] and due to the overlap among the spectral signals and the multiple electron transfer paths available, many structural and functional properties of the enzyme have yet to be established.[4] With the goal of bringing new light to bear on these issues, we have sought to prepare mixed-metal derivatives of laccase, and here we describe one such modification. The derivative contains both mercury and copper, and the evidence is that Hg(II) is bound specifically to the type 1 site while Cu(II) is bound to the type 2 and 3 sites.[5]

Chinese laccase has been extracted by the method of Reinhammar[6] from acetone powder supplied by Saito and Co., Ltd., Osaka, Japan. The mercury derivative is prepared from apolaccase, demetalated as before[7] except the temperature is now maintained at 10 °C. First the apoprotein is dialyzed against a 0.5 M pH 5.5 acetate buffer that is 0.1 M in NaCl, and 2 site equiv of Hg(II) are added. Approximately $^1/_2$ h later, 3.5–4 site equiv of Cu(I) are added. After incubating for 1–2 h, the protein is dialyzed against a 0.025 M pH 7 morpholinopropanesulfonate buffer and then eluted from a CM-Sephadex C-50 column. Finally, the sample is dialyzed into a 0.1 M pH 6 phosphate buffer.

The metal/protein stoichiometry (Table I) and the protein fluorescence, which is discussed below, argue that the metal ions locate in the normal copper-binding sites. The key point to determine, then, is the metal distribution. The close agreement among the EPR parameters (Table II) obtained for the mercury derivative and those of Reinhammar for the type 2 site of Japanese laccase demonstrates that the derivative contains copper in the type 2 site. Furthermore, as noted in Figure 1, double integration reveals that within the experimental error copper binding at this site is quantitative. As a further check on the integrity of type 2 copper, we have confirmed that the spectrum of the mercury derivative and the type 2 signal of native laccase suffer the same changes when fluoride or azide is added.[3] The ligand hyperfine splitting, which is resolved in Figure 1C, strongly suggests at least one nitrogen ligand is present in this site, a more precise count being precluded by the convolution of the ligand hyperfine splitting with that of the metal. The ligand hyperfine structure is barely perceptible in Figure 1B where severe overlap with the type 1 signal occurs. The imidazole moiety from a histidine residue has previously been implicated as a ligand of the type 2 site by spin-echo methods.[8]

Table I. Cu[a] and Hg[b] Analyses of Several Preparations of the Mercury Derivative[c]

sample	N_{Cu}[d]	N_{Hg}[d]	A_{280}/A_{333}[e]
I	3.4	0.7	20
II	3.2	0.8	18
III	2.7	0.9	21
IV	2.9	1.1	22
V	2.9	1.0	18

[a] Felsenfeld, G. *Arch. Biochem. Biophys.* **1960**, *87*, 247–251. [b] Jacobs, M. B. *Chem. Anal. (N. Y.)* **1967**, *22*, 798–802. [c] When apolaccase is remetalated by using only Cu(I), 3.8 ± 0.3 equiv of copper are taken up and an A_{280}/A_{333} absorbance ratio of 22 ± 3 is obtained. [d] Metal to protein ratios; total protein was determined by a modification of the biuret method using native laccase as a standard. [e] Absorbance ratio.

The absorbance data at 333 nm establish that the mercury derivative also contains copper in the type 3 site. Since the oxidized type 3 site absorbs at 333 nm[3] whereas the aromatic side chains absorb strongly at 280 nm, the A_{280}/A_{333} absorbance ratio gives an indication of the amount of type 3 copper that is bound to protein. As can be seen from Table I the absorbance ratio of the derivative accords well with that of the native enzyme. To obtain a more quantitative handle on the type 3 copper present, we subtracted the spectrum of reduced laccase from that of sample V in Table I and observed $\Delta\epsilon = 2800 \pm 300$ M^{-1} cm^{-1}. The value obtained from the difference spectrum between oxidized and reduced laccase[9] has been reported to be 2800 M^{-1} s^{-1}, in excellent agreement with our results.

Several independent lines of evidence lend convincing support for the hypothesis that Hg(II) is bound at the type 1 site. In the first place type 1 copper sites are known to exhibit a high affinity for Hg(II),[10,11] as would be expected for sites involving a thiolate ligand and a pseudotetrahedral coordination geometry.[12-14] Second, thiol titrations confirm that apolaccase exhibits only one accessible thiol,[7] that this group is blocked when apolaccase is treated with 1 equiv of mercury, and that the group is also blocked in the fully metalated mercury derivative. Significantly, the latter does not exhibit the characteristic charge-transfer absorption band associated with the coordination of thiolate sulfur to Cu(II).[15,16] Moreover, the binding of mercury is competitive with the binding of copper to the type 1 site in the sense that the remetalated protein begins to show the characteristic spectroscopic signatures of type 1 copper as the number of site equivalents of Hg(II) added to the apoprotein is decreased. A final point worth noting is the fact that the protein fluorescence of the mercury derivative accords well with that of native protein. This provides at least indirect support for the contention that Hg(II) binds at one of the copper binding sites. While laccase fluorescence is known to be sensitive to metal content,[7] the roles the individual binding sites play in determining the overall intensity are unknown. However, it can be noted that Hg(II) exerts a significant effect on protein fluorescence when it binds at the type 1 site of other copper proteins and that it closely mimics copper in this regard.[17]

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Table II. EPR Parameters

protein	site	$10^4A_{\parallel}$, cm^{-1}	$10^4A_{\perp}$, cm^{-1}	$g_{\parallel}$	$g_{\perp}$	$w_{\parallel}$,[a] mT	$w_{\perp}$,[a] mT	ref
^{65}Cu enriched	type 1	43	15	2.298	2.048	40	30	this work
Chinese laccase	type 2	201	18	2.250	2.050	40	35	this work
Japanese laccase	type 1	43	17	2.298	2.047	35	35	*b*
	type 2	200	18	2.237	2.053	42	42	*b*

[a] Line-width parameters. [b] Malmström, B. G.; Reinhammar, B.; Vänngard, T. *Biochim. Biophys. Acta* **1970**, *205*, 48-57.

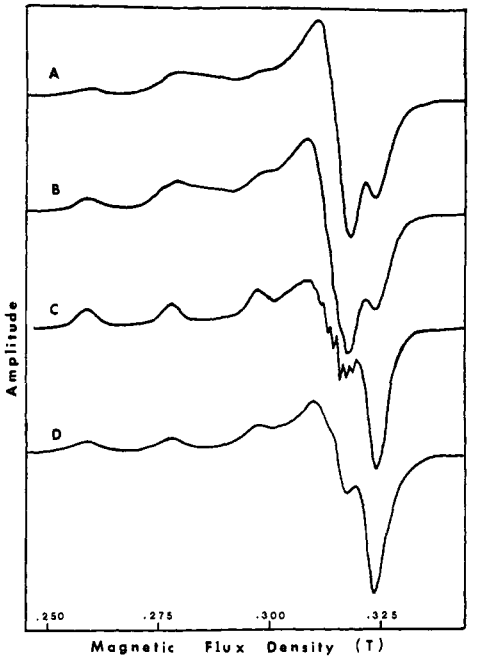

Figure 1. EPR Spectra of native laccase and mercury derivative. (A) The simulated spectrum of native laccase using the parameters presented in Table II. (B) The corresponding experimental spectrum obtained with a 2.17 mM sample of protein at -150 °C with a modulation amplitude of 1.0 mT, a power of 40 mW, and a frequency of 9.080 GHz. (C) The experimental spectrum of a 1.22 mM sample of the mercury derivative from sample V in Table I under the conditions of B except the gain was increased by a factor of 3.1. (D) The simulated spectrum of the mercury derivative using the parameters for the type 2 copper from Table II and ignoring the ligand hyperfine interaction. The ratio of the double integral of spectrum B to that of C is 2.16. All samples have been remetalated with isotopically pure ^{65}Cu. The buffer is 0.1 M pH 6 phosphate.

The results presented above argue strongly that the mercury derivative is fully metalated and that Hg(II) is bound specifically at the type 1 site. Consistent with Reinhammar's kinetic results, which indicate that the type 1 copper plays a key role in accepting electrons and funneling them to the type 3 site,[18] we estimate that the activity of the mercury derivative is less than 5% that of native laccase.[19] The precise activity is difficult to measure because of the presence of trace amounts of underivatized protein. Detailed kinetic studies of the oxidation and the reduction of the mercury derivative would be less sensitive to trace impurities and could shed new light on the role of the type 2 site in the functioning enzyme. Moreover, by analogy with native laccase[20] it may be possible to selectively remove type 2 copper from the mercury derivative. If so, it should be possible to probe the type 3 site of laccase in the absence of other copper centers using EXAFS and other techniques. In conclusion, the availability of a mixed-metal form offers new avenues to the study of laccase; the scope and limitations of the approach will only become clear as new experiments unfold.

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Nitric Oxide Dissociation from Trioxodinitrate(II) in Aqueous Solution

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The decomposition of sodium trioxodinitrate(II), $Na_2N_2O_3$ (Angeli's salt), in aqueous solution has been the subject of considerable interest since Angeli first proposed the existence of the elusive nitroxyl (HNO or NOH) as a reaction intermediate.[1] Although stable as a crystalline solid, $Na_2N_2O_3$ decomposes in neutral or alkaline media to nitrous oxide and nitrite ion stoichiometrically according to eq 1, but at pH <3 nitric oxide is the

$$2HN_2O_3^- \rightarrow N_2O + 2NO_2^- + H_2O \quad (1)$$

sole nitrogen-containing product.[2,3] First-order kinetic dependence on $[HN_2O_3^-]$ is observed for decomposition in alkaline and acidic media,[3-6] and labeling experiments have established that nitrous oxide is formed exclusively from the nitrogen bound to one oxygen[5] whereas nitrite is generated only from the nitrogen that is bound to the two oxygens[7] of the planar[8] $(ONNO_2)^{2-}$ ion. The composite data have been interpreted uniformly from the time of Angeli's first proposal by a mechanism (eq 2 and 3) in which the formation of nitroxyl and nitrite is rate limiting.

$$HN_2O_3^- \rightarrow HNO + NO_2^- \quad (2)$$

$$2HNO \rightarrow N_2O + H_2O \quad (3)$$

We have previously established that hemoglobin is a particularly sensitive reagent for the detection of free nitric oxide generated from nitric oxide donors.[9] Association of deoxyhemoglobin (Hb) with nitric oxide occurs with a rate constant of 2×10^7 M^{-1} s^{-1} and is essentially irreversible.[10] If the pathway discribed by eq 2 and 3 is an accurate accounting of $HN_2O_3^-$ decomposition, the presence of Hb should not alter the reaction process. However, treatment of Hb with $HN_2O_3^-$ in 0.05 M phosphate buffer at pH 7.0 results in an initial production of nearly equivalent amounts of nitrosylhemoglobin (HbNO) and methemoglobin (Hb^+), and

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