Vol. 85, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 14, 1978 Pages 505-510

ON THE RECONSTITUTION OF LACCASE FROM THE CHINESE LACQUER TREE

Bennett L. Hauenstein, Jr. and David R. McMillin*

Department of Chemistry
Purdue University
W. Lafayette, IN 47907

Received September 21, 1978

Summary: Reconstitution of laccase has been accomplished by the addition of cuprous ion to the cyanide-prepared apo-protein. The apo-protein is characterized by enhanced fluorescence, a disappearance of the 333 nm and 614 nm absorbance features typical of the native protein, and an almost complete disappearance of ferrocyanide activity compared to the native enzyme. A number of different methods of reconstitution are compared with regard to restoration of native properties. Under some conditions a bimodal fluorescence pattern develops which indicates that the metal uptake is linked to conformational changes of the peptide moiety.

Introduction: The laccases are a class of oxidases which catalyze the reduction of O_2 to H_2O. The two major sources of laccase are certain fungi and the latex of oriental lacquer trees. A number of studies have shown that the laccase enzymes contain three distinct copper-binding regions: the type 1 (or "blue") site, the type 2 site, and the type 3 site,[1] the latter being a 2 electron acceptor which probably involves an antiferromagnetically coupled pair of copper atoms in the oxidized enzyme.[1,2] As part of our effort to study these systems, we have been concerned with preparing the apo-protein and characterizing its reaction with metal ions.

To date for both the fungal and the tree systems, reports concerning the extent to which copper can be reversibly removed from laccase have been contradictory. An early report by Tissières, who studied tree laccase derived from Rhus succedanea, stated that cyanide treatment was effective in removing copper and that Cu^{2+} was effective in reactiving the enzyme, but few analytical details were provided.[3] Later, Omura[4] reported that Cu^{+}, but not Cu^{2+}, could

*Author to whom correspondence should be addressed.

0006-291X/78/0851-0505$01.00/0

be used to reconstitute the apo-protein, and these results were confirmed and extended by Ando.[5] Malmström and coworkers, however, subsequently reported that Ando's method of reconstitution was only successful when significant amounts of copper remained in the apo-protein.[6] Experiments similar to those of Omura and Ando were carried out on a fungal laccase (Lactarius piperatus), and a partial recovery of activity was reported.[7] On the other hand, the Swedish group reported that they were unable to reversibly remove copper from fungal laccase (Polyporus versicolor), although they did find that the type 2 copper could be reversibly removed.[8] Here we report on studies of the laccase from the Chinese lacquer tree.

Materials and Methods: All chemicals were reagent grade. Nitrogen was freed of trace oxygen by bubbling through scrubbers containing vanadous ion over zinc amalgam. Buffers were freed from trace metal contamination by elution through chelex columns. Glassware was treated with 50:50 HNO_3:H_2SO_4 and dilute EDTA solution to eliminate trace metal contamination. The protein was extracted according to the procedure of Reinhammar[9] with some modifications. Protein fractions were chromatographed until $A_{280}/A_{614} \leq 21$.

The protein was pretreated by an anaerobic reduction with ascorbate and dialysis against 0.10 M pH 6.0 phosphate buffer. Apo-protein was prepared by anaerobic dialysis against 0.10 M pH 7 imidazole acetate buffer containing 30 mM CN^- and 50 mM ascorbate, then freed of excess cyanide and ascorbate by anaerobic dialysis against 0.10 M pH 7 imidazole acetate before an aliquot was removed for aerobic dialysis against 0.10 M pH 6 phosphate buffer. The protein was reconstituted by anaerobically adding cuprous ion which was dissolved in a 0.10 M pH 7 imidazole acetate buffer that was 0.10 M in NaCl. Following incubation, the protein was dialyzed anaerobically against 0.10 M pH 6 phosphate buffer.

Ferrocyanide activities were measured at 420 nm in a 1 cm cell using 100 µl of protein and 3 ml of 2 mM $K_4Fe(CN)_6$ in 0.10 M pH 5.5 acetate buffer. Enzyme activity is expressed as millimoles of ferricyanide per minute per unit A_{280} of the protein solution in a 1 cm cell. Copper analyses were carried out using a modification of the 2,2'-biquinoline method of Felsenfeld.[10]

Results and Discussion: In our hands purified laccase from the Chinese lacquer tree exhibits an absorbance ratio A_{280}/A_{614} of ~20 and a reciprocal molar absorptivity at 280 nm of 9.3×10^{-6} M cm.[11] Based on the initial rate, the specific activity of the native enzyme was found to be 5.9 ± 0.2 using the ferrocyanide assay at pH 5.5. Treatment with CN^- at pH 7 yielded apo-protein which had negligible activity and, as expected, did not show the absorption bands at 330 nm and 614 nm respectively characteristic of the

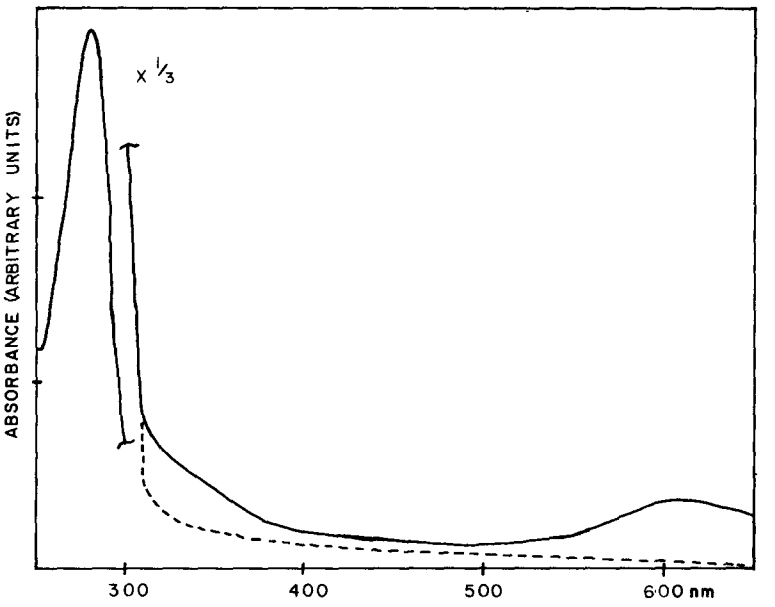

Figure 1. UV-vis absorption spectra for apo (----) and reconstituted (——) laccase.

type 3 and type 1 copper sites of the resting protein. (See Figure 1.) The apo-protein was characterized by increased fluorescence intensity compared to that of the native protein upon exciting in the region of 250-300 nm.

Substantial reconstitution of the laccase enzyme, as judged by the re-appearance of blue color, the uptake of copper, and the regeneration of catalytic activity, was effected by exposing the apo-protein to deoxygenated solutions of Cu^{+} in a pH 7 imidazole buffer. As can be appreciated from Figure 1, nearly complete return of the absorbance at 330 nm and at 614 nm was obtained after exposing the reconstituted protein to oxygen. Several different methods of adding Cu^{+} to the apo-protein were tried, and the results of these experiments are summarized in the Table. In experiment A approximately 3.5 times the stoichiometric amount of Cu^{+} was added at time zero and incubated with the apo-protein, whereas an approximately stoichiometric quantity of Cu^{+} was added at time zero in the case of experiment B. In experiments C and D the Cu^{+} was added in aliquots over a period to time,

Table. Reconstitution Experiments With Apo-Laccase

Experiment		A_{280}/A_{614}	Activity	$10^5 \times [Cu]/A_{280}$
	Native	19	5.8	3.7
A	Apo	-	0.1	0.0
	Reconstituted	27	4.0	4.7
	Native	20	5.6	3.6
B	Apo	-	0.0	0.1
	Reconstituted	25	5.7	2.7
	Native	21	5.9	3.7
C	Apo	-	0.0	0.1
	Reconstituted	24	6.1	3.7
	Native	23	6.1	3.6
D	Apo	-	0.1	0.0
	Reconstituted	23	5.3	2.9

until in both cases a slight excess of Cu^+ was finally achieved. In experiment C initially 0.25 equivalent of Cu^+ was added, the rest of the copper being added 2 hours later, while the copper was added in aliquots of approximately 0.1 equivalent each, at 45 minute intervals, in experiment D.

By all accounts the least successful reconstitution was achieved in experiment A. The reconstituted material was characterized by relatively low activity, and it is evident that excess copper remained bound to the protein, even after extensive dialysis. Interestingly, Ando's results indicate excess copper was bound to protein in some of his experiments with the enzyme from the Japanese lacquer tree[5], and in those experiments excess Cu^+ was usually incubated with the cyanide-treated enzyme. In experiments B, C, and D the reconstituted samples exhibited activities comparable to that

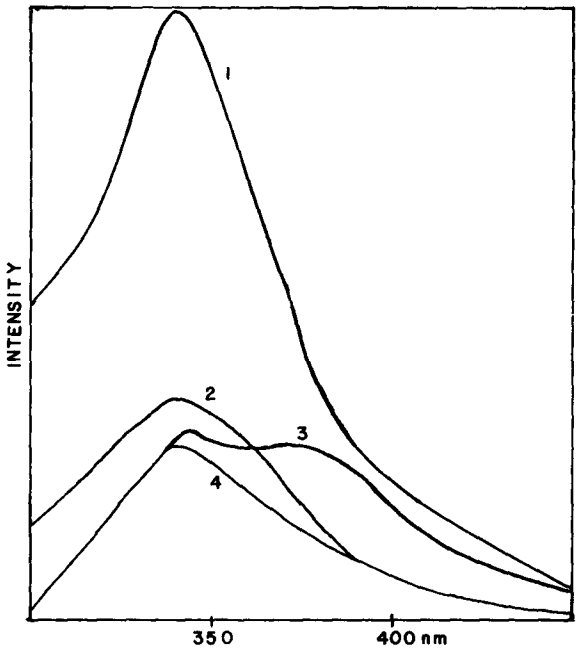

Figure 2. Fluorescence spectra obtained with 300 nm excitation 1: apo laccase 2: native laccase, 3: reconstituted laccase (experiment A); 4: reconstituted laccase (experiment D).

of the native enzyme although the A_{280}/A_{614} ratios and the copper analyses indicated that generally somewhat less than the stoichiometric amount of copper was bound.

A very intriguing effect was observed in the fluorescence spectra obtained with 300 nm excitation, which gives exclusive excitation of tryptophan residues.[12] Representative spectra are presented in Figure 2 which show that the emission spectrum of the reconstituted protein depends upon the method by which Cu^+ was added to the apo-protein. In general, experiments involving the addition of a relatively large quantity of Cu^+ resulted in emission spectra that were distinctly bimodal with an 'extra' maximum on the long wavelength side of the emission spectrum. The long wavelength emission band was not observed for the sample from experiment D and was less prominent in the spectra from experiments B and C than in the spectrum from

experiment A. The long wavelength feature can be assigned to tryptophan(s) located in a relatively hydrophilic environment.[13] A straight forward interpretation of these results is possible if, as seems quite reasonable, conformational changes are associated with certain steps in the metal uptake. If the conformational changes are relatively slow, then too rapid an addition of copper could result in a reconstituted protein having local regions of the enzyme 'frozen' in non-native configurations. Further experiments designed to probe the mechanism of metal uptake are planned.

References:

1. Malkin, R. and Malmström, B. G. (1970) Adv. Enz. 33, 177-244.
2. Dooley, D. M., Scott, R. A., Ellinghaus, J., Solomon, E. I., and Gray, H. B. (1978) Proc. Natl. Acad. Sci. USA 75, 3019-3022.
3. Tissières, A. (1948) Nature 162, 340-341.
4. Omura, T. (1961) J. Biochem. 50, 389-393.
5. Ando, K. (1970) J. Biochem. 68, 501-508.
6. Malmström, B. G., Andréasson, L-E., and Reinhammar, B. (1975) The Enzymes 12, pp. 507-579, Academic Press, New York.
7. Iwasaki, H., Matsubara, T., and Mori, T. (1967) J. Biochem. 61, 814-816.
8. Malkin, R., Malmström, B. G., and Vängård, T. (1969) Eur. J. Biochem. 7, 253-259.
9. Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35-47.
10. Felsenfeld, G. (1960) Arch. Biochem. Biophys. 87, 247-251.
11. Our values agree with those found by Wherland, Goldberg, and Pecht who attribute the increased absorbance at 280 nm of Chinese laccase compared to Japanese laccase to a higher tryptophan count in the Chinese enzyme; private communication.
12. Goldberg, M. and Pecht, I. (1974) Proc. Natl. Acad. Sci. USA 71, 4684-4687.
13. Finazzi-Agrò, A., Rotilio, G., Avigliano, L., Guerrieri, P., Boffi, V., and Mondovi, B. (1970) Biochemistry 9, 2009-2014.