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Cobalt^{II} Substitution in the Type 1 Site of the Multi-copper Oxidase Rhus Laccase

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SUMMARY: Anaerobic dialysis of apolaccase from the Chinese laquer tree against $CoCl_2$ produced an absorption spectrum similar to that of the previously characterized Co^{II} substituted stellacyanin. It includes an intense band at 305 nm, assignable to $S \rightarrow Co^{II}$ charge transfer, and relatively strong visible absorption bands characteristic of non-tetragonal Co^{II}. The splitting of the ligand field transitions is less than for Co^{II} stellacyanin, suggesting a more nearly tetrahedral type 1 site coordination geometry for Co^{II}-laccase The type 1 Co^{II} is more readily removed from laccase than from stellacyanin. Further experiments are needed to determine whether Co^{II} occupies the type 2 and type 3 sites which are not expected to contribute significantly to the absorption spectrum.

INTRODUCTION: We report the first substitution of Co^{II} for copper at the type 1 site of a multi-copper oxidase, Rhus vernicifera laccase. Gray and coworkers (1,2) have demonstrated the feasibility of Co^{II} substitution in type I single-copper proteins, azurin, plastocyanian and stellacyanin, and the utility of the resulting electronic spectra. The relatively high Co^{II} absorptivity in the

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visible region confirms the non-tetragonal character of the binding site, and strong bands near 300 nm are assignable to charge transfer transitions from sulfur ligands, cysteine and/or methionine; analogous charge transfer transitions to Cu^{II} are believed to be responsible for the strong ~600 nm band characteristic of the type I site (1,2). X-ray crystallography indicates that the ligands in plastocyanin (3) and azurin (4) are two histidine a cysteine and a methionine side-chain, in a distorted tetrahedral array.

MATERIALS AND METHODS: Rhus vernicifera laccase was isolated from the laquer acetone powder of the Chinese lacquer tree (Saito and Co., Ltd., Osaha, Japan) by the method of Reinhammer (5). Most previous studies have been on protein obtained from the Japanese laquer tree. We found the Chinese tree laccase to be indistinguishable spectrally; the purity index A_{280}/A_{614} = 15.2 compared favorably with Reinhammer's value, 15.7(5). Copper was removed (6) by anaerobic dialysis (N_2 bubbling through the dialysate in a sealed beaker) against 50 mM ascorbic acid and 20 mM NaCN in 25 mM Tris-HCl buffer, pH 7.4, for two hours, followed by exhaustive anaerobic dialysis against the buffer. $Cobalt^{II}$ substitution was carried out by anaerobic dialysis of 9.5mM apolaccase against 2mM $CoCl_2$ in the same buffer for 36 hours, beyond which the absorption spectrum remained unchanged. Excess cobalt was removed by exhaustive anaerobic dialysis against buffer. The same procedures were used to substitute $cobalt^{II}$ into stellacyanin (1), which had been isolated (6) from the same acetone powder.

Competition between cobalt and copper for the laccase type 1 site was studied by comparing the rate of incorporation of cuprous ion into apolaccase (6) and Co^{II}-laccase. For this purpose aliquots of the two proteins (0.1 mM) were dialyzed simultaneously against 8mM ascorbic acid in 25mM Tris-HCl, pH 7.4. After thorough N_2

purging, $CuSO_4$ and NaCl were added to the dialysate, to concentrations of 4mM and 0.2M, respectively. Under these conditions the copper is reduced to a soluble cuprous chloride complex. Protein samples were removed after 30 minutes and after two hours, dialysed aerobically against buffer (allowing the copper to oxidize in situ) and passed through a Chellex 100 column to remove adventitiously bound Cu^{II} and any remaining Co^{II}. The extent of Cu^{II} incorporation was determined from the absorbance ratio at 614nm (type 1 Cu^{II}) and 280nm (protein). The results are given in Table I.

RESULTS AND DISCUSSION: Figure 1 compares the UV-visible spectrum of Co^{II}-laccase with that of native laccase, and also Co^{II}-stellacyanin. The latter spectrum is in good agreement with the one published by McMillin et. al. (1,2). The Co^{II}-laccase spectrum is shown as a difference spectrum against apolaccase in order to display the 305nm absorption band which is otherwise obscured by the 280nm aromatic side-chain absorption. This band is assignable to a sulfur → Co^{II} charge-transfer transition (1,2). Its absorptivity per mole of protein was estimated to be 4600 and 4920 $M^{-1}cm^{-1}$ for laccase and stellacyanin, respectively, using published values (7) for the 280nm protein absorptivities. The good agreement between the absorptivities suggests that the type 1 site is substantially occupied by Co^{II} in both stellacyanin and laccase. The low-energy UV shoulder, assigned in Co^{II}-substituted stellacyanin, azurin and plastocyanin to a sulfur π orbital component of the charge-transfer process (1,2), is not resolved in the Co^{II}-laccase spectrum.

The visible region of the Co^{II}-laccase spectrum shows relatively strong absorption (ε estimated at ~500 $M^{-1}cm^{-1}$) characteristic of non-tetragonal Co^{II}. The ligand field transitions are less spread out than in Co^{II}-stellacyanin, suggesting a more nearly tetrahedral environment in Co^{II}-laccase. Indeed the visible absorption

Table I: Time course of Type 1 Cu Reincorporation into Apo-and Co^{II}-Lacase via Cuprous Chloride Dialysis.

	Appolaccase		Co^{II}-laccase	
	A_{614}/A_{280}	% Incorporation[a]	A_{614}/A_{280}	% Incorporation
30 Minutes	0.0029	4.6	0.0020	3.2
2 Hours	0.0233	36.6	0.0203	31.9

[a]Based on the native laccase ratio, $A_{614}/A_{280} = 0.0636$

is similar to that of the tetrahedral complex $Co(imidazole)_4^{2+}$(8). The type 1 Cu^{II} site symmetry is believed to be distorted tetrahedral, and analysis of the near-infrared "d-d" transitions by Gray and co-workers (9) suggests that the degree of distortions is comparable in Rhus laccase, azurin and plastocyanin. Although a quantitative analysis is much more difficult for Co^{II}, the available spectral data, summarized in Table II suggest substantial variation among the different proteins, with Co^{II}-laccase showing the least distortion from tetrahedral symmetry. It is conceivable that the Co^{II} ion, or the reconstitution procedure, alters the native binding site geometry.

Table II: Absorption Spectral Data for Co^{II}-Substituted Copper Proteins: ν, kK (ε, $M^{-1}cm^{-1}$).

Laccase	Stellacyanin	Azurin	Plastocyanin
32.8 (4600)[a]	32.3 (4900)[a]	30.3 (2400)[b]	30.0 (2800)[b]
	28.6 (2160)	26.7 (980)	26.0 (950)
20.8 (330)			
18.7 (490)	18.7 (430)	18.9 (200)	19.6 (370)
17.7 (510)			
15.6 (weak)	15.7 (690)	15.6 (350)	14.7 (400)

[a]Estimated by comparison with absorption at 280nm. (ε = 93,500 $M^{-1}cm^{-1}$ for laccase and ε = 35,380 $M^{-1}cm^{-1}$ for stellacyanin (5)), assuming one Co^{II} per protein molecule.

[b]Estimated from data given in reference 2.

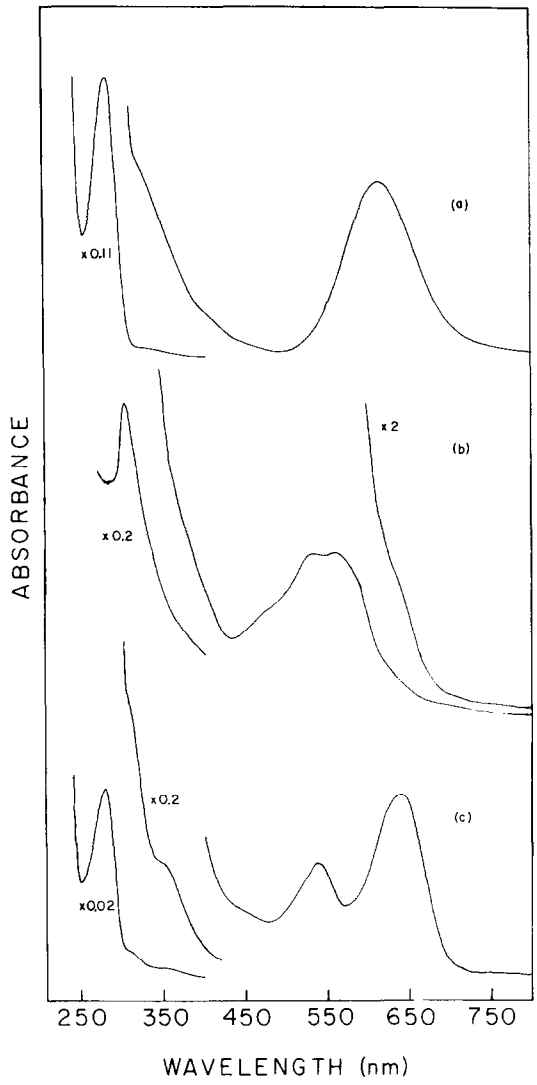

Figure 1: Absorption spectra of (a) native laccase, (0.72mM, 0-1 absorbance, 1mm cell), (b) Co^{II}-laccase run as a difference spectrum against apolaccase, (both protein solutions 0.65mM, 0-0.1 absorbance, 1mm cell), (c) Co^{II}-stellacyanin, (0.13mM, 0-0.2 absorbance, 1 cm cell), all in 25mM Tris-HCl buffer, pH 7.4.

Specific binding of Co^{II} at the type 1 site of laccase was tested by Cu^{II} competition experiments: (1) Treatment of native laccase with $CoCl_2$ under the same conditions used to reconstitute apolaccase produced no detectable change in the native laccase ab-

sorption spectrum, as determined by difference spectroscopy, indicating that the Co^{II}-laccase absorptions cannot be attributed to a adventitiously bound Co^{II}. (2) Cuprous chloride incorporation was slower for Co^{II}-laccase than for apolaccase, as demonstrated in Table I. This inhibition is consistent with Co^{II} being displaced from the type 1 site.

The lag in copper incorporation produced by Co^{II}, although readily detectable, is small enough to suggest that the binding of Co^{II} to the type 1 site is not very firm. This impression is strengthened by the observation that the Co^{II} absorption bands were abolished by passage of Co^{II}-laccase through Chellex 100 resin. This treatment does not affect the absorption spectrum of native laccase, nor of Co^{II}-stellacyanin, plastocyanin or azurin (2). These observations reflect relative rates of metal ion removal from the type 1 sites, and not necessarily the relative equilibrium constants. It may be that the Co^{II} is more accessible to solvent in laccase than in the other proteins.

Laccase contains a type 2 and a pair of type 3 copper atoms, in addition to the type 1 Cu(10). It is possible that these sites are also filled with Co^{II} in the apolaccase substitution procedure, but we have no evidence on this point. The type 2 site is tetragonal (10) and tetragonal Co^{II} ligand-field absorption is expected to be an order of magnitude weaker than for tetrahedral Co^{II}. The coordination geometry of the type 3 site is unknown, since the exchange-coupled Cu^{2+} ions give no epr spectrum and the visible spectrum is dominated by type 1 Cu^{2+} absorption. By analogy with hemocyanin, which has an exchange-coupled Cu^{2+} pair showing ligand field absorptions at normal tetragonal energies (11), we expect the type 3 site to be tetragonal and to contribute little to the Co^{II} absorption spectrum. Further studies are in progress to examine these

sites by other techniques, and to study the details of the substitution process. In connection with the recent observation (12) that the type 2 Cu of laccase can be selectivity removed, the present work suggests that selective substitution of Co^{II}, and perhaps other metals, at the three laccase sites may be feasible.

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