X-ray Absorption Study of *Rhus* Laccase: Evidence for a Copper–Copper Interaction, Which Disappears on Type 2 Copper Removal†

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ABSTRACT: X-ray absorption spectra are reported for the multi-Cu oxidase *Rhus vernicifera* laccase in oxidized and fully reduced forms and for laccase from which the type 2 Cu has been depleted (T2D). The structure of the Cu K edge for both preparations shows the presence of Cu^{II} and Cu^{I} in the oxidized and reduced states, respectively. As previously reported by LuBien et al. (1981), removal of the type 2 Cu leads to reduction of the type 3 center, which can be reoxidized with H_2O_2. Fourier transforms of the extended X-ray absorption fine structure (EXAFS) give well-defined first and outer shell scattering peaks. Analysis of the first shell peak is complicated by the heterogeneity of the Cu sites. When $(imidazole)_4Cu^{II}SO_4$ is used as a model of the average Cu–ligand interactions, it is shown that all of the first shell peaks contain 2.7–3.5 near neighbors per Cu, at an average distance of 1.97–1.98 Å. For T2D laccase, the fit is improved by inclusion of one-third of a sulfur atom at 2.19 Å, corresponding to the presumptive cysteine ligand of the type 1 Cu, which remains in the preparation containing three Cu atoms per molecule. The outer shell region shows two peaks characteristic of scattering from distant imidazole atoms. For T2D laccase the filtered outer shell contribution can be satisfactorily fit by scattering from an average of 2.1–2.4 imidazole groups. For native laccase, however, imidazole alone cannot satisfactorily model the outer shell contribution. Scattering is required from an additional half-atom, at a distance 3.4 Å, presumably the Cu at the type 3 binuclear site. Thus EXAFS gives evidence for a binuclear site in native laccase that is significantly perturbed by type 2 Cu removal. This perturbation might involve a substantial increase in the Cu–Cu distance of the type 3 site or, alternatively, a substantial decrease in rigidity of the site (increased Debye–Waller factor), perhaps by disruption of a bridging group.

Laccase from the lacquer tree *Rhus vernicifera* has been much studied for the insight it may give into the mechanisms that biology has evolved for O_2 reduction to water via transition metal ion catalysis (Reinhammer & Malmstrom, 1981; Farver & Pecht, 1981). This oxidase contains three different kinds of copper, which have been designated types 1, 2, and 3. Type 1 Cu is characterized by a strong optical absorption band near 600 nm and an EPR spectrum with an unusually small hyperfine coupling constant, characteristics shared with other "blue" copper proteins. Examples of these include plastocyanin (Colman et al., 1981) and azurin (Adman & Jensen, 1981), in which the Cu is bound to two histidines, a N atom, a cysteine S atom, and (very weakly) a methionine S atom. The type 2 Cu in laccase has EPR parameters (Peisach & Blumberg, 1974) typical of tetragonal Cu^{2+} (the expected weak visible absorption is masked by the type 1 Cu) and can bind a variety of anionic ligands, known to inhibit enzymatic activity. The type 3 site contains two Cu ions, which do not contribute to the magnetism and are believed to be antiferromagnetically coupled ($-2J > 500$ cm^{-1}; Dooley et al., 1978) in the oxidized enzyme. This binuclear site is believed to be the point at which O_2 binds, whereas the type 1 and type 2 Cu ions are thought to transfer electrons to the O_2 bound at the type 3 site. The situation is analogous to that of the O_2-reducing terminus of the mitochondrial respiratory chain, cytochrome oxidase, in which a binuclear site, containing cytochrome a_3 antiferromagnetically coupled to a Cu^{2+} ion, is believed to be the site of O_2 binding, to which electrons are transferred via cytochrome *a* and an additional Cu (Malmstrom, 1980). The type 2 Cu of laccase can be depleted selectively (Graziani et al., 1976), leaving a form of the enzyme (T2D) with substantially altered kinetics.

The type 3 site of laccase is reminiscent of the binuclear Cu sites of the O_2-carrying protein hemocyanin, and there is much interest in the extent of the analogy of these binuclear sites. Solomon and co-workers have carried out extensive ligand binding studies for methemocyanin and laccase (Solomon, 1981; Spira et al., 1982; Winkler et al., 1982). While the same ligands bind to the binuclear sites in both proteins, the binding constants and spectral effects are substantially different. It has been suggested (Winkler et al., 1982) that whereas peroxide and azide bind to both Cu ions at the hemocyanin site, they bind to only one Cu at the type 3 site of laccase. Moreover, T2D laccase has been found to bind peroxide and azide much less strongly than native laccase, suggesting a role for the type 2 Cu in ligand binding at the type 3 site (Winkler et al., 1982).

X-ray absorption spectroscopy has been useful in defining the structure of the binuclear sites in hemocyanin (Hc) (Brown et al., 1980; Co & Hodgson, 1981a,b; Co et al., 1981; Woolery et al., 1984). The shape of the Cu K absorption edge established that the oxidation state was Cu^{I} for deoxy-Hc and Cu^{II} for oxy- and met-Hc. The extended X-ray absorption fine structure (EXAFS) showed that the near-neighbor (first shell) scattering was due exclusively to first row (N, O) atoms and that there was a decrease in average coordination number from four in oxy- and met-Hc to two in deoxy-Hc. In addition, the distant-neighbor (outer shell) scattering was attributable in part to the distant atoms of the imidazole ring of histidine ligands. In all cases, however, an additional scattering atom was found in the outer shell EXAFS, at a distance of 3.4 Å for deoxy- and met-Hc and 3.6 Å for oxy- and metazido-Hc (Woolery et al., 1984). This atom was assigned to Cu in oxy-Hc for which the imidazole contribution to the outer shell

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scattering is low. For the other forms, a first row atom (not part of imidazole) could be made to fit the data, but Cu, which gave better fits, is more plausible on chemical grounds.

In the present study we have applied the same techniques to laccase and its T2D form. As with Hc, the K-edge structure shows clear evidence for the presence of Cu^{II} and Cu^{I} in oxidized and reduced laccase, respectively. As reported previously by LuBien et al. (1981), removal of type 2 Cu induces a Cu^{I} contribution to the K edge, which is diminished by the addition of H_2O_2, indicating reduction of Cu during the T2D preparation and reoxidation of Cu, presumably at the type 3 site, by peroxide. EXAFS analysis is more difficult for laccase than for Hc because all three types of Cu (two for T2D laccase) contribute simultaneously. There is little that can be said about the first shell scattering, which contains contributions from all of the various Cu ligand atoms, except that there is no evidence for appreciable lowering of the coordination number upon reduction of laccase, as there is for met-Hc. The outer shell scattering, however, potentially contains a unique contribution from the Cu scattering in the binuclear site, along with the outer atom scattering from imidazoles. For T2D laccase and its H_2O_2-treated form, we find that imidazole alone is sufficient to account for the outer shell scattering. The absence of detectable Cu scattering in T2D laccase has also been reported by Spira et al. (1983), during the preparation of this paper. We find positive evidence, however, for Cu scattering in native laccase in both oxidized and reduced forms. In neither case is imidazole sufficient to account for the outer shell scattering; an additional atom is needed, at a distance of 3.4 Å, as in the case of met-Hc, which we infer to be the second Cu atom at the binuclear type 3 site. The removal of type 2 Cu from the native laccase apparently alters the nature of this site and eliminates the Cu scattering. A substantial increase in the Cu–Cu distance or, alternatively, in the Debye–Waller factor is implied. Either of these changes is likely to involve a substantial rearrangement of the type 3 Cu ligands including, in all probability, the bridging ligand, which is believed to account for the antiferromagnetic interaction.

Experimental Procedures

Protein Samples. Rhus vernicifera laccase was prepared from Japanese lacquer acetone powder (Saito and Co., Osaka, Japan) and purified by the method of Reinhammar & Oda (1979). A final ultrafiltration step, to remove low molecular weight impurities, yielded an absorption ratio $A_{259}/A_{614} = 15$. Reduced laccase was produced by addition of solid sodium ascorbate. The type 2 depleted form was prepared by a modification (Reinhammar & Oda, 1979) of the procedure of Graziani et al. (1979), and the loss of type 2 copper was monitored with electron paramagnetic resonance (EPR). Reoxidation of the type 3 site in T2D laccase, by addition of H_2O_2, was monitored via the 330-nm absorption shoulder with a Cary 118 absorption spectrophotometer in the derivative mode. For X-ray analysis, all protein samples were concentrated in 0.1 M phosphate buffer, pH 6.0, to 2–4 mM.

X-ray Absorption Measurements and Data Analysis. X-ray data collection was conducted during dedicated operation of the SPEAR storage ring (3.0 GeV, 40–80 mA) at the Stanford Radiation Laboratory. Data were collected on beam line I-5 according to methods described previously (Powers et al., 1981). Approximately 16 single scans were averaged together to improve signal to noise. K-edge and EXAFS data were analyzed by the procedures used previously for hemocyanin (Woolery et al., 1984). The samples were held at −100 °C to minimize radiation damage. Their integrity was checked before and after irradiation via EPR.

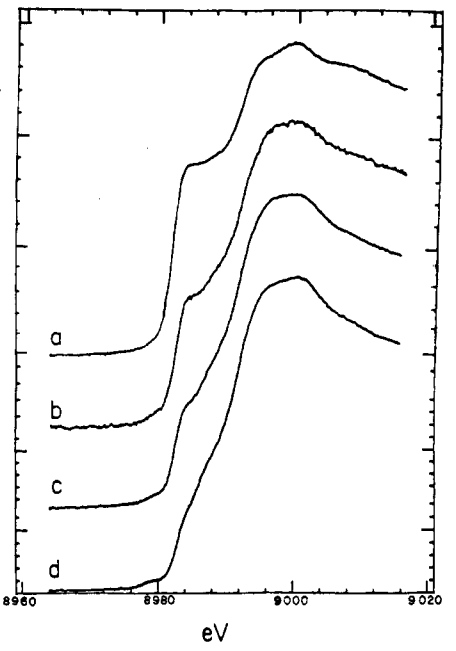

FIGURE 1: K-edge X-ray absorption spectra for laccase deviatives: (a) native, reduced; (b) T2D; (c) T2D, H_2O_2 treated; (d) native, oxidized.

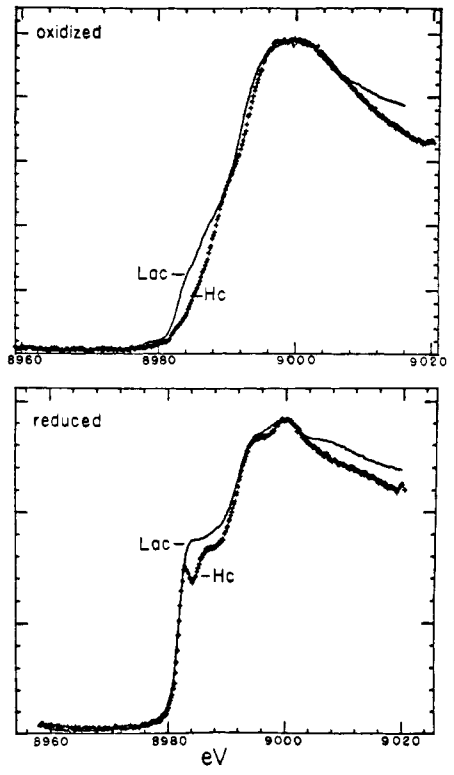

FIGURE 2: K-edge spectra comparing oxidized laccase (—) with oxyhemocyanin (+) (top) and reduced laccase (—) with deoxyhemocyanin (+) (bottom).

Results

Edges. Figure 1 shows the K-edge absorption spectra for the four samples used in the study: native oxidized (d), native reduced (a) laccase, T2D (b), and H_2O_2-treated T2D (c) laccase. Figure 2 compares the K-edge spectra of native and reduced laccase with those of oxy- and deoxy-Hc from *Busycon canaliculatum* (Brown et al., 1980). The similarity of the spectra is readily apparent, although significant differences in detail can be observed. Thus deoxy-Hc and reduced laccase both show the strong low-energy shoulder (8982 eV) that is characteristic of Cu^{I} (Brown et al., 1980), but the shoulder

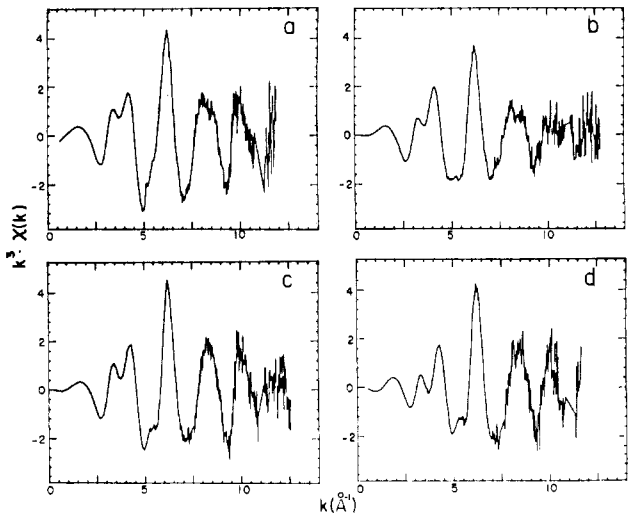

FIGURE 3: Background-subtracted, k^3-weighted EXAFS modulations for laccase derivatives, normalized to one Cu atom: (a) native, oxidized; (b) native, reduced; (c) T2D, H_2O treated; (d) T2D.

is less structured (no doubt reflecting the heterogeneity of the three types of Cu in the protein) and slightly stronger (10%) for laccase. The low-energy shoulder is much weaker for Cu^{II} but is nevertheless distinctly seen for native laccase, in contrast to oxy-Hc. Upon type 2 Cu removal, the low-energy shoulder is again prominent (spectrum b in Figure 1), indicating autoreduction of the type 3 site, as documented by LuBien et al. (1981). (The shoulder is not as strong as in the reduced native laccase, since the type 1 Cu remains oxidized.) Addition of H_2O_2 to T2D laccase (spectrum c in Figure 1) diminishes the low-energy shoulder substantially. It still appears to be stronger than in native laccase (spectrum d in Figure 1), but this may be due to the averaging in of the type 2 Cu contribution to the latter.

EXAFS. The background-subtracted, k^3-weighted, EXAFS modulations, normalized to one Cu atom (Powers et al., 1981), are shown in Figure 3 for the laccase samples. The data are of good quality, comparable to those obtained for hemocyanin (Woolery et al., 1984), and extend to $k \sim 12.5$ Å^{-1}. The EXAFS Fourier transforms (FTs) are shown in Figure 4. Just beyond the dominant first shell peak, there is a secondary peak (marked with an asterisk) of variable amplitude, depending upon the length of the data set being transformed. This is attributed to a side lobe due to truncation errors, although it may also contain a real scattering contribution. There follow two outer shell peaks, which are seen when imidazole (ImH) is bound to the absorbing atom (Brown et al., 1980; Co & Hodgson, 1981b) and are attributable, in part, to the scattering from the second (C2, C5) and third (N3, C4) shells of the rigid imidazole ring. Beyond these features, only noise peaks are seen, their low amplitudes reflecting the quality of the data.

Figure 5 compares FTs calculated with k^2 vs. k^3 weighting of the EXAFS modulation. This comparison was introduced in our study of hemocyanin (Woolery et al., 1984) to discriminate heavy (Cu) vs. light (N, O) atom contributions to the outer shell scattering. Heavy atom scattering maximizes at higher k than light atom scattering and is more strongly emphasized by k^3 than by k^2 weighting. Thus k^2 weighting lowered the outer shell amplitude, relative to k^3 weighting, for oxy-Hc, reflecting the dominant Cu scattering contribution, but it raised the amplitude for mononuclear Cu–ImH model compounds, reflecting the C, N scattering. For deoxy- and met-Hc, the outer shell amplitude was the same for k^2 and k^3 weighting, suggesting counterbalancing effects of light atom (ImH) and heavy atom (Cu) scattering (Woolery et al., 1984).

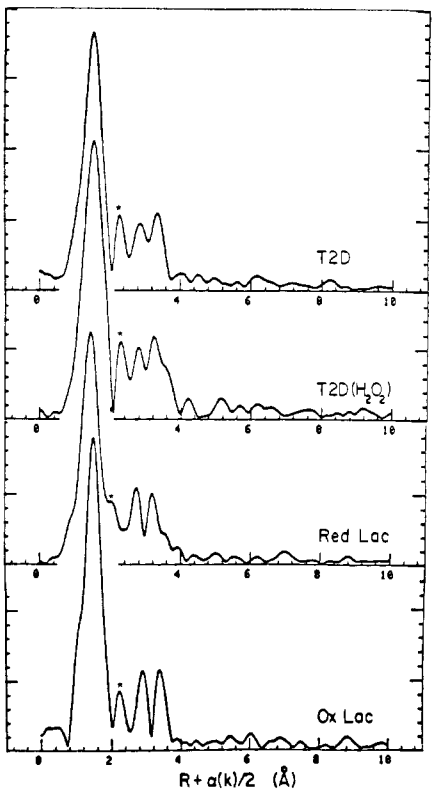

FIGURE 4: Fourier transforms of the k^3-weighted EXAFS modulations for laccase derivatives. From top to bottom: T2D; T2D, H_2O_2 treated; native, reduced; native, oxidized.

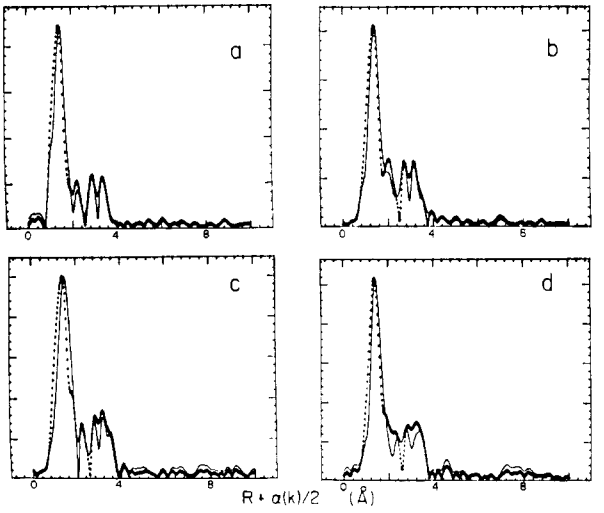

FIGURE 5: Fourier transforms of the laccase EXAFS modulations, with k^3 (—) and k^2 (+) weighting: (a) native, oxidized; (b) native, reduced; (c) T2D, H_2O_2 treated; (d) T2D.

For T2D laccase (panels c and d in Figure 5), the outer shell amplitude *increases* for k^2 weighting, indicating that ImH scattering is the dominant contribution. For native laccase (panels a and b in Figure 5), the outer shell scattering amplitude is the same for k^2 and k^3 weighting, suggesting, as for deoxy- and met-Hc, that both ImH and Cu contribute to the scattering.

Outer Shell Analysis. Figure 6 shows the outer shell EXAFS, isolated by Fourier filtration [$R + \alpha(k/2) = 2.4$–4.4 Å] and back-transformation. It is notable that the T2D samples have significantly lower scattering amplitudes at high values of k, where heavy atom scattering is emphasized, than do the native laccase samples. The outer shell EXAFS modulations were then fit with Cu–ImH model compounds, either

Table I: Parameters[a] from Fitting Filtered Outer Shell EXAFS to Imidazole and Copper

	ImH				Cu[b]		
	R (Å)	N	$\Delta\sigma^2 \times 10^3$	χ^2	R (Å)	N	$\Delta\sigma^2 \times 10^3$
native laccase, oxidized	3.45[c]	1.5	4.3	1.6	[e]		
	3.44[c]	2.2	1.3	0.13	3.36	0.5	−4.2
native laccase, reduced	3.44[d]	1.7	0.6	1.1	[e]		
	3.44[d]	2.1	1.5	0.16	3.43	0.5	−4.1
untreated T2D laccase	3.43[c]	2.2	0.2	0.41	[e]		
	3.47[d]	2.1	−1.0	0.85	[e]		
H_2O_2-treated T2D laccase	3.42[c]	2.4	0.6	0.56	[e]		
	3.47[d]	2.4	−0.6	0.85	[e]		

[a] R, N, and $\Delta\sigma^2$ are the distance, the number of scatters, and the change in Debye–Waller factor (model − sample), all referenced to the model compounds [R (Å) ± 0.05; N ± 20%; $\Delta\sigma^2$ (Å^2) ± 1.5 × 10^3]. χ^2 is the goodness of fit parameter, representing the sum of the squares of the residuals. [b] Referenced to [(*N,N,N′,N′*-tetramethylethylenediamine)Cu^{II}OH]$_2$Br$_2$. [c] Referenced to (ImH)$_4$Cu^{II}SO$_4$ solid. [d] Referenced to aqueous Cu^{II} imidazole. [e] Not included in the fitting.

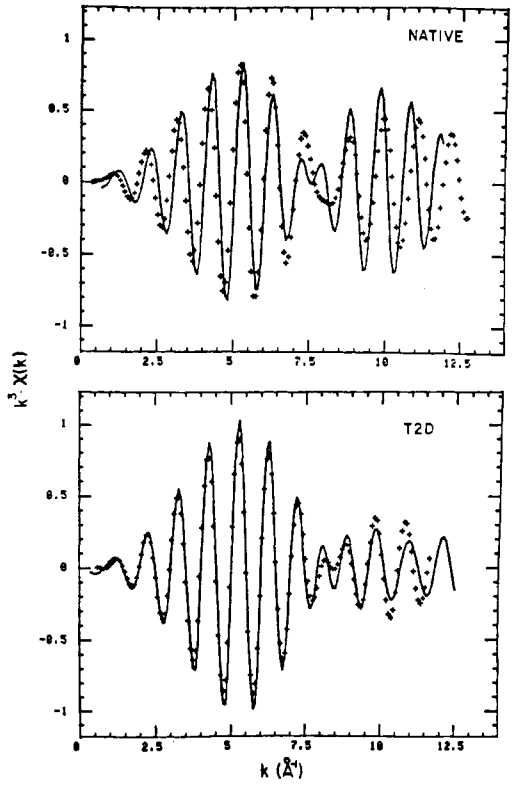

FIGURE 6: Fourier-filtered and back-transformed outer shell EXAFS modulations for oxidized (—) and reduced (+) native laccase (top) and for H_2O_2-treated (—) and untreated (+) T2D laccase (bottom).

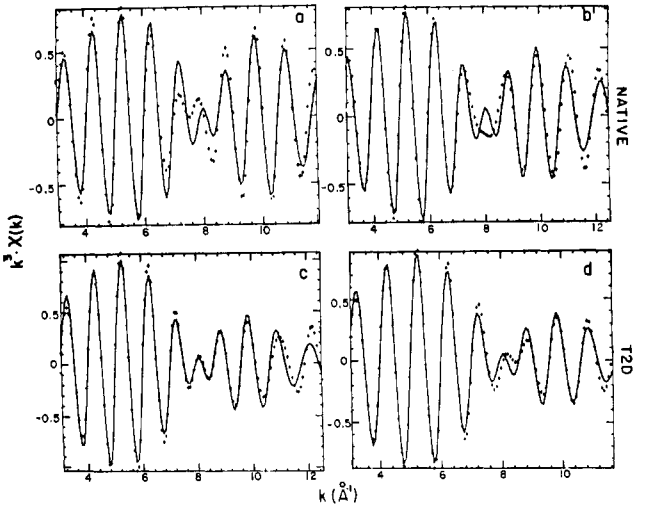

FIGURE 7: Comparison of laccase Fourier filtered outer shell EXAFS modulations (+) with best-fit calculations (—) using a single Cu–imidazole model, (ImH)$_4$CuSO$_4$: (a) native, oxidized; (b) native, reduced; (c) T2D, treated; (d) T2D.

(ImH)$_4$Cu^{II}SO$_4$ or aqueous CuSO$_4$ in excess imidazole. The ImH ligands were treated as rigid groups, with a single average distance for the outer atoms (~3.5 Å). This distance and also the number of ImH's, the change in Debye–Waller factor (model − sample), and the difference in the value of the edge energy between model and sample, E_0, were allowed to vary in the fitting procedure (Powers et al., 1981).

The results are shown in Figure 7, where the EXAFS modulations (crosses) are compared with the curves calculated from the best sets of parameters; these are given in Table I along with the goodness of fit parameter, χ^2 (sum of the residuals, squared). The data for the T2D samples were fit satisfactorily with 2.1–2.4 ImH per Cu. Although chemically reasonable, the ImH number is not particularly accurate, since the relative amplitude of the outer shell peaks differs as much as 30% for various Cu–ImH model compounds having the same number of ImH's. These compounds can, however, be fitted satisfactorily to one another (Woolery et al., 1984) since the difference in distance between the two outer peaks is always unchanged, pinning the beat node of the filtered data in k space. The amplitude difference in the fitting procedures is accounted for by variances in N (<20%) and $\Delta\sigma^2$ (>50%), which are correlated. Examination of the residuals for the ImH model − model fits shows slight differences over the entire k range, but the phase is identical. The differences are probably due to orientational differences among the ImH's. Bunker et al. (1982) have given an interesting discussion on orientational and disorder factors that might influence the relative amplitudes of the ImH outer shell peaks.

For the native laccase samples, however, the optimal fit with ImH alone is unsatisfactory. Not only is the value of χ^2 appreciably higher than that for the T2D samples but also the calculated curves differ markedly from the data in the critical beat note region ($k \sim 8$ Å^{-1}), where the different scattering contributions show a destructive interference. Improvement in the fit requires introduction of an additional scattering atom, which, on the basis of the k dependence of the outer shell amplitude (see above), is most likely a heavy atom. Figure 8 shows the dramatic improvement in the native laccase fits when a half Cu atom (taking account of the fact that there are four Cu's, only two of which are at the binuclear site) is introduced by use of the model compound [(*N,N,N′,N′*-tetramethylethylenediamine)Cu^{II}OH]$_2$Br$_2$ (Wasson et al., 1968). For the best fits, the required Cu–Cu distances are 3.36 and 3.43 Å for oxidized and reduced laccase, respectively (see Table I). As in the case of the oxy- and met-Hc (Woolery

Table II: Parameters[a] for Fitting Filtered First Shell EXAFS

	R (Å)	N	$\Delta\sigma^2 \times 10^3$	χ^2	reference material
native laccase, oxidized	1.97[b]	3.5	0.5	1.8	$(ImH)_4Cu^{II}SO_4$
	1.97[c]	3.0*	0*	1.7	H_2O_2-treated T2D plus
	2.03	1.0*	0*		$(ImH)_4Cu^{II}SO_4$
native laccase, reduced	1.96[b]	3.2	−3.0	3.5	$(ImH)_4Cu^{II}SO_4$
untreated T2D laccase	1.98[b]	2.7	0.5	6.0	$(ImH)_4Cu^{II}SO_4$
	1.98[c]	2.0*	0*	8.8	deoxy-Hc plus
	2.0	1.0*	0*		oxidized azurin
	1.98[c]	2.0	0*		metaquo-Hc plus
	2.0	1.0*	0*		oxidized azurin
H_2O_2-treated T2D laccase	1.97[b]	3.0	1.0	4.3	$(ImH)_4Cu^{II}SO_4$
	1.95[c]	2.6	0.5	1.4	$(ImH)_4Cu^{II}SO_4$ plus
	2.19	0.3	5.0		Cu–S_4[d]
	1.98[c]	0.3	5.0		metaquo-Hc plus
	2.00	1.0*	0*		oxidized azurin

[a] Symbols the same as in Table I. [b] Single-model fitting. [c] Two-model fitting; parameters marked with an asterisk are held constant—coordination number and $\Delta\sigma^2$ the same as for the model. [d] Cu(diethyldithiocarbamate)$_2$.

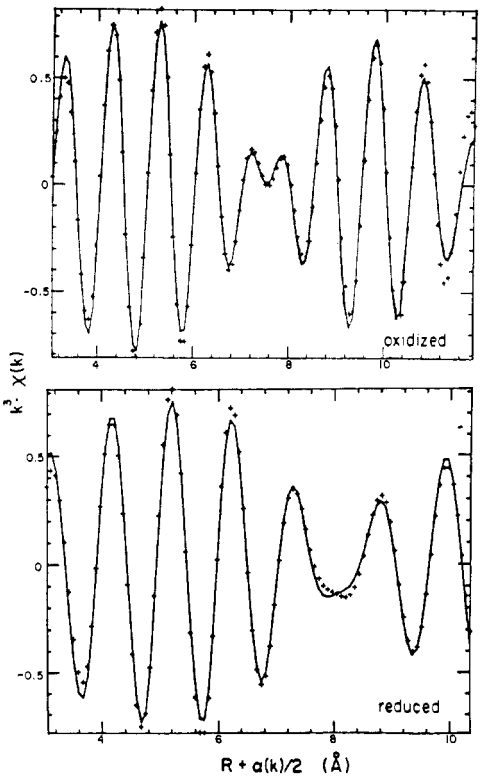

FIGURE 8: Comparison of laccase Fourier filtered outer shell EXAFS modulations (+) with best-fit calculations (—) using both Cu–imidazole and Cu–Cu contributions (see the text and Table I): native, oxidized (top) and native, reduced (bottom).

et al., 1984), it would no doubt be possible to substitute N or O for Cu and obtain a satisfactory fit by increasing the number of scatters. Arguing against this is the k^2 dependence of the outer shell scattering amplitude, as noted above, and the chemical implausibility of a significant scattering contribution at 3.4 Å from first row atoms that are not part of the rigid imidazole rings.

Inner Shell Analysis. Since the first shell EXAFS contains contributions from all of the ligands of the three kinds of Cu (two for T2D laccase), only a limited analysis was attempted. The first shell peak was filtered, back-transformed, and fitted to $(ImH)_4Cu^{II}SO_4$, on the assumption that most of the ligand atoms are N (or O). A fit was obtained (Table II) with 2.7–3.5 scatterers, at an average distance of 1.96–1.98 Å. The least-squares deviation of the fits was relatively high (χ^2 = 1.8–6.0), and the smaller than expected average coordination number is suggestive of interference effects due to heterogeneity of the sites. The lack of any significant variation in the average number of scatterers, however, indicates that there is no major change in the coordination number upon type 3 Cu reduction, as is observed for Hc, in which the number of ligands decreases from four in the oxy and met forms to two for the deoxy protein. If a similar 50% decrease had occurred for the type 3 Cu's in native laccase, then the average coordination number of the four Cu's should have decreased by 25% upon reduction, and this is not observed. The change for T2D laccase, between H_2O_2-treated and untreated preparations, should have been 33%, and this is likewise inconsistent with the data.

The first shell fit for H_2O_2-treated T2D laccase was improved by allowing for a sulfur ligand, with data from the model compound Cu(diethyldithiocarbamate)$_2$ (Powers et al., 1981) (Table II). The number of sulfur atoms, 0.3, is that expected for T2D laccase, containing three Cu atoms, with a single cysteine ligand at the type 1 Cu site. This result, which indicates that there are no sulfur ligands at the type 3 site, is in agreement with that of Spira et al. (1983); their Cu–S distance, 2.09 Å, is slightly shorter than ours, 2.19 Å, but the differences are certainly within experimental error, in view of the low contribution of the Cu–S interaction to the total scattering. The fit for native laccase was not significantly improved by including Cu–S scattering, but a satisfactory fit was obtained by adding the EXAFS of H_2O_2-treated T2D laccase and $(ImH)_4CuSO_4$, with a three to one weighting, allowing the average distances to vary slightly (Table II).

The first shell EXAFS of H_2O_2-treated T2D laccase could be fit to those of met-Hc (Woolery et al., 1984) and oxidized azurin (W. Blumberg and L. Powers, unpublished results) with a two to one weighting (Table II). However, the analogous fit of the untreated T2D laccase data to those of deoxy-Hc (Woolery et al., 1984) plus oxidized azurin was less satisfactory. Indeed, the fit was better if met-Hc was substituted for deoxy-Hc. This behavior again suggests that the coordination number of the type 3 Cu is not halved upon reduction, as it is in Hc.

Discussion

The most interesting result of the present work is that the Cu EXAFS of native laccase, in both oxidized and reduced forms, gives positive evidence for a distant scatterer at 3.4 Å, most plausibly Cu at the type 3 site, whereas the T2D forms do not. The question naturally arises whether this result is reliable, in view of the multiple contributions to the EXAFS

from the three different kinds of Cu in laccase. This interference is substantially ameliorated by the Fourier filtration of the data. The only contribution of the type 1 and 2 Cu's to the outer shell EXAFS should be from bound imidazole [there are probably two for type 1 Cu; the number of type 2 Cu is not known although its presence has been demonstrated (Aviglioni et al., 1981)]; it is unlikely that any other atoms at 3.4 Å are held rigidly enough at the type 1 and 2 sites to contribute significantly to the EXAFS. Thus, the additional dilution of the putative Cu scattering by the extra imidazoles of the type 1 and 2 Cu's is the main source of uncertainty.

We believe that the evidence for Cu scattering in native laccase is compelling. It is similar in character to that demonstrated for met- and deoxy-Hc (Woolery et al., 1984): (A) The outer shell Fourier peak amplitudes do not change when the k weighting is changed from k^3 to k^2, implying counterbalancing effects of imidazole and heavy atom scattering. When imidazole alone is present, these amplitudes increase. (B) The back-transformed outer shell EXAFS cannot be fit satisfactorily with imidazole alone. There are large discrepancies in the beat node regions, which are eliminated by inclusion of an extra scatterer at 3.4 Å. The number of Cu's required by the fit is 0.5, consistent with the type 3 site being the sole locus of the extra scatterer. The average number of imidazoles, 2.1–2.2, is also satisfactory.

The situation is quite different for T2D laccase, in both H_2O_2-treated and untreated forms: k^2 weighting *does* increase the outer shell amplitude, relative to k^3 weighting, and it *is* possible to fit the back-transformed outer shell EXAFS satisfactorily with imidazole alone. The lack of evidence for Cu scattering in T2D laccase is consistent with the results of Spira et al. (1983).

Thus, there appears to be a distinct change in the type 3 site upon type 2 Cu depletion. One possibility is that the type 2 Cu is itself responsible for the Cu scattering in native laccase, but this is unlikely, since both native and T2D laccase have a pair of antiferromagnetically coupled Cu's at the type 3 site and the type 2 Cu has a conventional EPR spectrum of a mononuclear $S = {}^1/_2$ species. Alteration of the Cu scattering in T2D laccase could result either from an increase in the Cu–Cu distance or from a decrease in the rigidity of the type 3 site, leading to a large increase in the Debye–Waller factor. The retention of the antiferromagnetic coupling of the type 3 site in this preparation, again evidenced by lack of an EPR contribution, implies that the Cu ions must be bridged by a common ligand, in order to allow for spin–spin coupling. Such coupling is possible over Cu–Cu distances substantially larger than 3.4 Å, however, provided that the Cu and bridging orbitals are properly aligned (Hatfield et al., 1983). The coupling constant, $-2J$, has been estimated to be greater than 500 cm^{-1} from magnetic susceptibility measurements on native laccase (Dooley et al., 1978). As far as we are aware, such measurements have not been reported for T2D laccase.

The apparent alteration of the type 3 site upon type 2 Cu depletion has significant implications for research on laccase, since it cannot be assumed that the functional effects of type 2 depletion are related only to the role of type 2 Cu. Thus, the well-documented inhibition of electron transfer to the type 3 site of T2D laccase (Reimhammar & Malmstrom, 1981) might be due to an altered conformation of the type 3 site, as well as to the absence of an electron-transferring type 2 Cu. Similarly, the dramatic differences in the binding of peroxide and azide between native and T2D laccase, which have been attributed to stabilization of the bound anions by the type 2 Cu (Winkler et al., 1982), might also result from a reduced intrinsic affinity of the type 3 site, possibly associated with an increase in the Cu–Cu distance, or by a rearrangement of the endogenous ligands.

Another result of interest is the apparent near constancy of the average coordination number in both native and T2D laccase, independent of the Cu oxidation state. In hemocyanin, the coordination number is reduced from four to two between Cu^{II} and Cu^{I} forms. It has been suggested (Woolery et al., 1984) that the dissociation of endogenous ligands in deoxy-Hc might trigger the protein conformation change that is responsible for cooperativity in O_2 binding. In addition, the tendency toward 2-coordination can be expected to favor the Cu^{I} oxidation state. Thus, the variable coordination number of hemocyanin must have an important bearing on the position of the oxygen binding equilibrium and may be necessary to achieve adequate deoxygenation rates. Laccase, being an oxidase, functions unidirectionally in oxygen binding and has no requirement for a significant deoxygenation rate. The maintenance of the coordination number at a value appropriate for Cu^{II} is expected to sustain a high rate of O_2 reduction.

Registry No. Cu, 7440-50-8; $(imidazole)_4Cu^{II}SO_4$, 76633-47-1; laccase, 80498-15-3.

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Separation of Ribosomal Proteins from *Escherichia coli* and Rabbit Reticulocytes Using Reverse-Phase High-Performance Liquid Chromatography†

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ABSTRACT: Reverse-phase high-performance liquid chromatography has been used to fractionate ribosomal proteins from *Escherichia coli* and rabbit reticulocytes. Different column packing materials and solvent systems were compared for their effectiveness with bacterial proteins. A large-pore (300 Å) short alkyl chain support (Altex RPSC) in conjunction with a triethylamine phosphate (pH 2.2)/acetonitrile solvent system was particularly effective and separated mixtures of total protein from each ribosomal subunit into a number of peaks approaching the actual number of proteins present. For example, with the use of the Altex RPSC column, the 21 proteins of 30S subunits were resolved into 18 distinct peaks, and the 33 proteins of the 50S subunits were resolved into 28 peaks. Overall recovery varied from 75% to 90% in different experiments. The composition of each peak was established by two-dimensional gel electrophoresis. Relatively acidic proteins, for example, S1 and L7/L12 of *Escherichia coli*, were bound more tightly to the column and recovered in lower yields than the other more basic proteins. Proteins that were incompletely resolved in a single step could be obtained in pure form by rechromatography on the same column with an altered gradient or with a different type of reverse-phase packing material. Ribosomal proteins from rabbit reticulocytes were also separated with good resolution and yield by using the RPSC column.

The determination of the protein topography of ribosomes from *Escherichia coli* using cross-linking with the reagent 2-iminothiolane is a major goal in this laboratory. The substantial number of cross-linked protein dimers established for 30S, 50S, and 70S particles (Sommer & Traut, 1976; Lambert & Traut, 1981; Lambert et al., 1983) represents an exhaustive investigation of the cross-links formed with this lysine-specific reagent. Identification of sites of cross-link formation within individual proteins can provide topographic information at higher resolution. This approach entails the purification of individual cross-linked dimers from the complex heterogeneous mixture of chemically modified ribosomal proteins. Such mixtures have proven difficult to resolve by using methods applied in the past for purifying ribosomal proteins such as ion-exchange chromatography. Before investigating the utility of reverse-phase high-performance liquid chromatography (HPLC)[1] for fractionating mixtures of cross-linked proteins, we first conducted an investigation of reverse-phase HPLC as a method for purifying non-cross-linked ribosomal proteins, and these results are presented here. We compared different column supports and solvent systems for the fractionation of ribosomal proteins from *Escherichia coli*. A system consisting of TEAP, pH 2.2, and acetonitrile on large-pore (300 Å) short alkyl chain supports (RPSC) gave the best results for most proteins. While this work was in progress, very similar results were published by Kerlavage et al. (1982, 1983). They employed a solvent system consisting of TFA and acetonitrile. We compared the relative efficacy of TFA and TEAP and found the latter slightly superior in some regards. The present work complements that of the other group, and the two systems represent alternative strategies for the purification of individual proteins. In addition, the applicability of the method for fractionating cross-linked proteins and eukaryotic ribosomal proteins is demonstrated.

Experimental Procedures

Triethylamine and TFA were from Aldrich. Triethylamine was redistilled twice from ninhydrin and stored under nitrogen at −20 °C. Trifluoroacetic acid was refluxed over CrO_3 (Allen, 1981) followed by distillation and storage under nitrogen at −20 °C. HPLC-grade acetonitrile, 1-propanol, and 2-propanol were from Burdick and Jackson. 2-Iminothiolane was from Serva. All other chemicals were reagent grade.

Isolation of Prokaryotic Ribosomal Proteins. Ribosomes were prepared from *Escherichia coli* strain MRE 600 as described by Hershey et al. (1977). Zonal centrifugation, using the method of Eikenberry et al. (1970), was used to prepare

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[1] Abbreviations: HPLC, high-performance liquid chromatography; TEAP, triethylammonium phosphate; TFA, trifluoroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Bis-Tris, [bis-(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

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