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ON THE SPECTRAL FEATURES ASSOCIATED WITH PEROXIDE REACTIVITY OF THE COUPLED BINUCLEAR COPPER ACTIVE SITE IN TYPE 2 DEPLETED AND NATIVE RHUS LACCASE

James E. Penner-Hahn[1], Britt Hedman[1], Keith O. Hodgson[1,*]
Darlene J. Spira[1,2], and Edward I. Solomon[1,2,*]

[1]Department of Chemistry, Stanford University,
Stanford, California 94305

[2]Department of Chemistry,
Massachusetts Institute of Technology,
Cambridge, Massachusetts 02139

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We report herein an X-ray absorption spectroscopic (XAS) determination of the oxidation state of the copper sites in T2D and native Rhus laccase. The increase in intensity of the 330 nm absorption feature which results from peroxide titration of T2D laccase (T3:[Cu(I)Cu(I)],T1:[Cu(II)]) is found to correlate linearly with the percent of oxidation of the binuclear copper site (determined by XAS analysis). This indicates that peroxide oxidizes but does not bind to the T3 site. We have used this correlation to determine that native laccase, as isolated, contains ~25% reduced T3 sites and that all spectral changes observed upon peroxide addition to native laccase can be accounted for by oxidation of these reduced sites. The importance of this result to previous reports of peroxide binding at the laccase active site is discussed.

Native laccase [1] contains four copper ions, one in a "blue" (T1) site, one in a "normal" T2 site, and two in a "coupled binuclear" (T3) site. The selective removal of the T2 copper to yield the type 2 depleted (T2D) protein form [2] has simplified study of the T3 site.

In native laccase, the T3 copper has been associated with a 330 nm (ε~2800 $M^{-1}cm^{-1}$) absorption feature which reduces as a two-electron acceptor [3]. T2D laccase does not exhibit a 330 nm absorption band [4] and contains a binuclear cuprous [5] site which is stable with respect to aerobic oxidation. Excess peroxide has been shown [5] to oxidize the T3 site leading to the reappearance of the 330 nm feature ($\Delta\varepsilon_{330}$~2000 $M^{-1}cm^{-1}$) and generating the met T2D derivative. Our studies [6,7] of met T2D have demonstrated that in the absence

*To whom correspondence should be addressed at Stanford University.

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of the T2 Cu(II), the oxidized T3 site undergoes a one- rather than a two-electron reduction to yield a stable, mixed valence or half met derivative of the binuclear copper site in T2D laccase. Thus, the T2 Cu(II) is not only necessary for the aerobic oxidation of the reduced T3 site, but also has an important role in enabling the T3 site to function as a two-electron acceptor.

Several studies [8,9] have indicated that during catalytic function, it is the binuclear copper site in the native enzyme which initially binds O_2 for its $4e^-$ reduction to H_2O via a $2e^-$ reduced peroxide intermediate. Addition of H_2O_2 to native laccase is reported to produce spectral changes at ~330mm ($\Delta\varepsilon_{325}$=800 $M^{-1}cm^{-1}$ absorption and $\Delta(\Delta\varepsilon_{l\text{-}r,320})$=-3.15 $M^{-1}cm^{-1}$CD) [10]. From these studies, it was concluded that H_2O_2 binds to the native T3 site and forms a peroxylaccase complex [10,11], producing the above spectral features in analogy to oxyhemocyanin, the peroxide bound derivative of met hemocyanin [12]. However, our chemical and spectroscopic studies of native and T2D laccase indicate significant differences between the hemocyanin and laccase binuclear copper sites. In particular, we note the inability of exogenous ligands to bridge the coupled binuclear copper site in laccase [6] and the significantly different optical changes for these proteins on reaction with peroxide. In contrast with the weak optical changes in laccase, unique and very different spectral features characterize peroxide binding to the hemocyanins [13] and tyrosinase [14] ($\Delta\varepsilon_{345}\approx$20,000 $M^{-1}cm^{-1}$, $\Delta(\Delta\varepsilon_{l\text{-}r,350})\approx$-30 $M^{-1}cm^{-1}$).

Alternatively, the similarity of the spectral changes at 330 nm for H_2O_2 treated native laccase to those associated with oxidation of the T3 site in T2D laccase suggests they might also be due to oxidation. We have therefore further investigated the nature of the changes in the 330 nm absorption which are associated with $O_2^{=}$ reactions of the coupled binuclear copper sites in both T2D and native laccase.

Determination of copper ion oxidation state in both native and T2D laccase is difficult since both an antiferromagnetically coupled binuclear cupric site and a binuclear cuprous site lack characteristic optical and EPR spectral features. Copper ions do, however, have X-ray absorption edges which change

dramatically with metal ion oxidation state. We have used this fact to qualitatively demonstrate [5] that T2D laccase contains a reduced T3 site and an oxidized T1 site. Further, peroxide was shown to oxidize the cuprous binuclear site and restore the 330 nm absorption [5].

Recently, we have utilized differences in the X-ray absorption spectroscopy (XAS) edge structure of Cu(I) and Cu(II) complexes to quantitatively distinguish between these species [15]. The normalized difference edge spectra for the binary differences of a variety of Cu(I) and Cu(II) complexes were found to exhibit a relatively invariant $\Delta\varepsilon_{8984}$, thus allowing quantitative determination of the % of Cu(I) in samples of mixed Cu(I)/Cu(II) composition [15]. T2D laccase was found to contain 70±15 % Cu(I), even after treatment with 25X $Fe(III)(CN)_6^{3-}$. Here we apply this quantitative difference edge analysis to demonstrate that peroxide oxidizes but does not bind to the T3 site in T2D laccase. Further, all of the spectral changes observed upon peroxide reaction of native laccase can be accounted for by oxidation of the binuclear cuprous sites present in native laccase.

MATERIALS AND METHODS

Laccase was purified [16,17] from the acetone powder (Saito and Co., Japan) on the Japanese lacquer tree, Rhus vernicifera; T2 copper was removed by the modified [4] method of Graziani [2]. Reagent grade peroxide (30% aqueous solution) was standardized as described in [18]. Optical absorption spectra were measured at room temperature, before and after X-irradiation, in 1 mm quartz cells on a Cary 14 spectrophotometer. CD spectra for diluted aliquots of protein were recorded on a Jasco J-500C spectropolarimeter. A Bruker ER 220 D-SRC EPR spectrometer (operating at 10 mW power and 20 G modulation) was used to examine the frozen protein solutions in the X-ray sample cells immediately prior to and after X-irradiation.

Fluorescence X-ray absorption data were collected at SSRL (Beam-line IV-2, 1.8 GeV, ~12 mA, 18 kG wiggler field) using an array of NaI(Tl) scintillation detectors with Ni filters. Ten to twenty scans/sample were measured at ~-70°C. The scans were calibrated, using the internal calibration procedure [19] and averaged. The averaged data were normalized as described previously [15]. Since the data are normalized to a Cu K-edge jump of 1.0, the results are independent of concentration. The ε values given below can be converted to molar absortivities using a multiplication factor of 0.57 $M^{-1}cm^{-1}$ [20].

RESULTS AND DISCUSSION

We now correlate the quantitative X-ray technique with optical spectroscopy in order to probe the effects of sequential H_2O_2 addition to T2D laccase. The dependence of the XAS edge structure on added H_2O_2 is shown in Fig. 1A. The isosbestic point indicates changes in only two absorbing species.

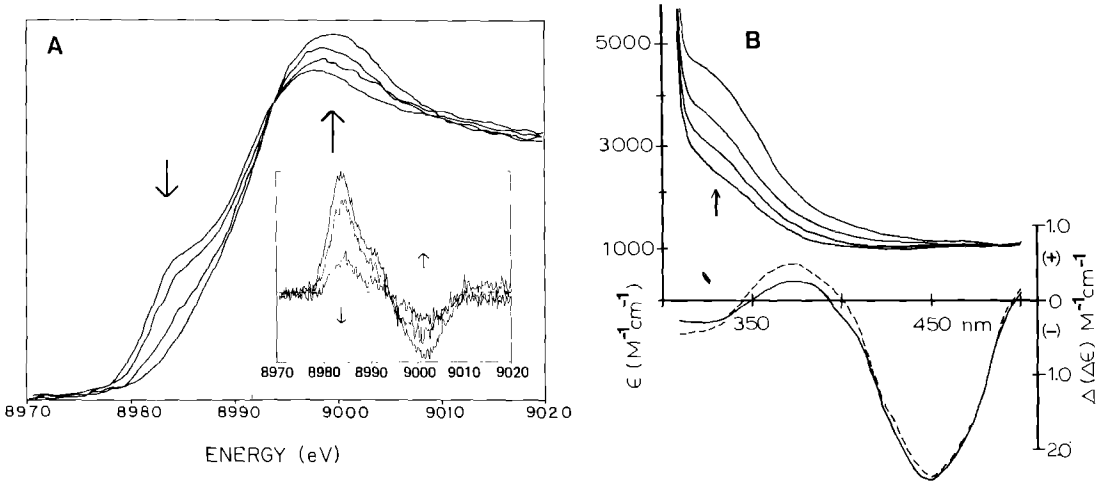

Fig. 1 A. Normalized X-ray absorption edge spectra of T2D laccase (0.1 M potassium phosphate, pH 6.0) in the presence of: 0, 5, 25, and 60 protein equivalents H_2O_2. Data have been smoothed with a Gaussian-weighted running average (Gaussian width = 1.6 eV). Arrows indicate the direction of change on treatment with H_2O_2. Inset: Unsmoothed, normalized difference-edge spectra of these samples, relative to 60X H_2O_2. B. (upper) Absorption spectra for the same T2D samples. (lower) CD spectra of T2D laccase before (——) and after (- - -) treatment with 50X H_2O_2.

We observe no detectable change in the integrated EPR intensity, establishing that the redox state of the remaining (T1) copper in T2D laccase is unaffected by H_2O_2. The difference edges (inset) thus accurately reflect changes in only the T3 copper site [21,23]. In the optical spectrum (Fig. 1B), the peroxide reaction results in a new 330 nm absorption feature [5] which is similar to that observed in native laccase; no significant change occurs in the CD. Within experimental error, the increase in intensity of the absorption band at 330 nm correlates linearly to the decrease in the X-ray absorption feature at 8984 eV [Fig. 2]. Reaction with 60X H_2O_2 yields an overall $\Delta\varepsilon_{330}$ ~2000 M^{-1} cm^{-1} and a 70±15% change in Cu(I); higher [H_2O_2] leads to irreversible protein damage. The linear correlation of these changes indicates that H_2O_2 is acting as an oxidant throughout the titration; no new spectral features are observed. If peroxide further bound to the oxidized T3 site, [as has been proposed by Frank, P., Farver, O., and Pecht, I. (1983) J. Biol. Chem. 258, 11112-11117], one would expect significant additional spectral features, based on the intense $O_2^{=}$→Cu(II) CT transitions observed upon peroxide binding to the coupled

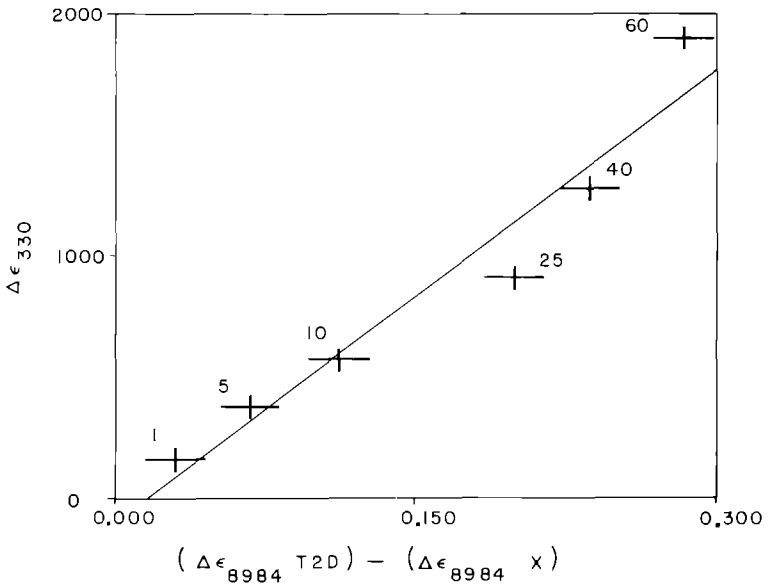

Fig. 2 Correlation of $\Delta\varepsilon_{8984}$ and $\Delta\varepsilon_{330}$ for T2D laccase samples on treatment with H_2O_2. All absorbance changes are referenced to the fully oxidized (60XH_2O_2) sample [23]. The stoichiometric excess of [H_2O_2] is indicated for each data point (X).

binuclear copper sites in hemocyanin and tyrosinase [12]. Such transitions are not observed.

The presence of binuclear cuprous sites in T2D and their stability to oxidation by O_2 but not H_2O_2 suggested that the similar, although weaker, optical changes (Fig. 3B) observed in peroxide reactions of the native enzyme might also be due to oxidation. We therefore extended our XAS edge studies to native laccase, both as isolated and after treatment with excess H_2O_2. EPR and optical spectra demonstrate that the T1 and T2 copper sites are fully oxidized. The difference of their XAS edges (Fig. 3A) must therefore again reflect a change at only the T3 site. Calibrated to a fully reduced T3 site in the untreated T2D laccase (but corrected for the additional T2 Cu(II) absorber present in native laccase), the difference edge corresponds to 12±3% overall Cu(I), or reduction of ~25% of the T3 sites in the native, as isolated, laccase. This reduction has also been suggested from N_3^- binding studies [24].

Using the linear relationship between $\Delta\varepsilon_{330}$ and $\Delta\varepsilon_{8984}$ in T2D laccase (Fig. 2), we calculate that at least $\Delta\varepsilon_{330}$=440±100 is expected when H_2O_2 is

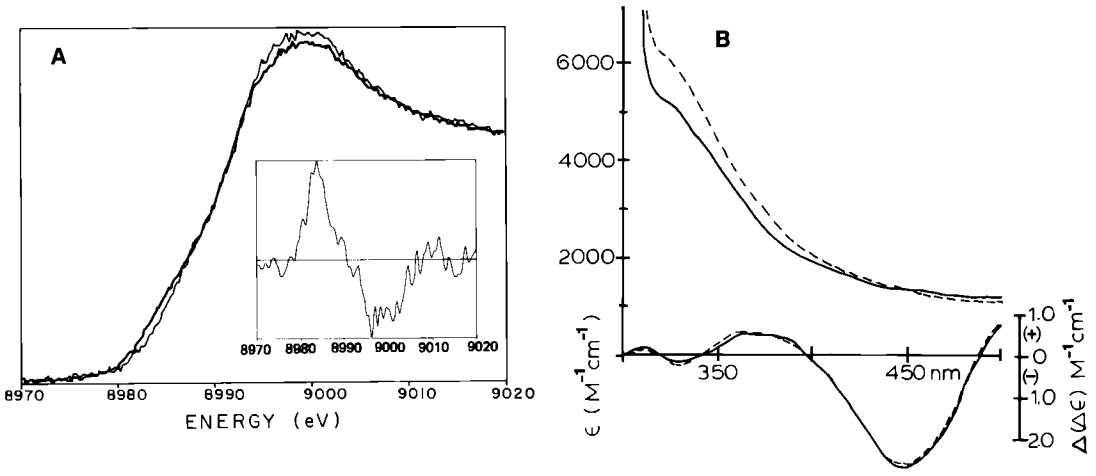

Fig. 3 A. Comparison of normalized X-ray absorption edge spectra of native laccase (dark) and native laccase treated with 30 protein equivalents H_2O_2 (light). Inset: Difference spectrum of native minus (native+30X H_2O_2) laccase edges, smoothed with a gaussian weighted running average (gaussian width = 1.6 eV). B. (upper) Absorption and (lower) CD spectra for the same laccase samples before (——) and after (- - -) treatment with 30X H_2O_2.

reacted with native laccase solely as a result of oxidation of reduced type 3 sites. This calculation depends only on the $\Delta\varepsilon$ values, and is independent of the precise extent of T3 reduction in T2D laccase. Previous studies [10,11] have used $\Delta\varepsilon_{325}$=800 $M^{-1}cm^{-1}$ absorption and $\Delta(\Delta\varepsilon_{1-r,320})$=-3.15$M^{-1}cm^{-1}$ CD features to conclude that peroxide binds to native laccase. After correcting for T3 oxidation, no absorption features remain which could reasonably reflect peroxide->Cu(II) CT. Titration studies [10a, 25] further indicate that the increase at 330 nm represents a single, continuous reaction. The CD change ($|\Delta(\Delta\varepsilon_{320})|$<0.3 $M^{-1}cm^{-1}$) observed in our [25] and other [26] laboratories is also accounted for by the oxidation of ~25% of the binuclear copper sites.

We can use the observed ε_{330}=2000±200 $M^{-1}cm^{-1}$ for the T3 site in T2D and the ratio of the observed (800 $M^{-1}cm^{-1}$) and calculated (440±100 $M^{-1}cm^{-1}$) ε_{330} in native laccase to estimate an ε_{330}=3600±900 $M^{-1}cm^{-1}$ for the fully oxidized native enzyme [27]. A similar value results when the reported ε=2800 $M^{-1}cm^{-1}$ is corrected for T3 oxidation ($\Delta\varepsilon$=800 $M^{-1}cm^{-1}$).

In conclusion, we find that native laccase contains ~25% reduced binuclear copper sites and that all spectral changes observed upon peroxide addition to

native laccase can be accounted for by oxidation of these reduced sites. The lack of additional intense spectral features upon peroxide addition to both T2D and native laccase indicate that peroxide binding is not observed.

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