Reactivity of the Laccase Trinuclear Copper Active Site with Dioxygen: An X-ray Absorption Edge Study

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Abstract: The multicopper oxidases (laccase, ascorbate oxidase, ceruloplasmin) catalyze the four-electron reduction of dioxygen to water. Laccase contains four Cu atoms: a type 1, a type 2, and a coupled binuclear type 3 center. Low-temperature MCD studies of laccase have demonstrated that the type 2 and type 3 centers comprise a trinuclear Cu cluster site (Allendorf, M. D.; Spira, D. J.; Solomon, E. I. *Proc. Natl. Acad. Sci. U.S.A.* **1985**, *82*, 3063-3067. Spira-Solomon, D. J.; Allendorf, M. D.; Solomon, E. I. *J. Am. Chem. Soc.* **1986**, *108*, 5318-5328), and this model has been supported in a recent X-ray crystal structure of ascorbate oxidase (Messerschmidt, A.; et al. *J. Mol. Biol.* **1989**, *206*, 513-529). In the present study, X-ray absorption edge spectroscopy has been used to determine Cu oxidation states following reaction of reduced laccase derivatives with dioxygen, leading to a description of which of the Cu centers is required for reactivity. In a fully reduced type 2 depleted derivative, the type 1 Cu(I) oxidizes very slowly in a nonphysiological reaction and the type 3 center does not react with dioxygen. In contrast, fully reduced type 1 Hg^{2+}-substituted (T1Hg) laccase, which contains a valid type 2-type 3 trinuclear Cu cluster site, reacts readily with dioxygen, resulting in complete reoxidation of the type 2 and type 3 centers. These results demonstrate that the type 2-type 3 trinuclear site represents the minimal active site required for the multielectron reduction of dioxygen. An intermediate in the reaction of reduced T1Hg laccase with dioxygen has been observed, which provides insight into the oxygen intermediate present in the reduction of dioxygen by native laccase.

The multicopper oxidases[1,2] laccase, ascorbate oxidase, and ceruloplasmin catalyze the four-electron reduction of dioxygen to water. Laccase, the simplest of these enzymes, contains four Cu atoms that have been classified[1] according to their EPR features: type 1 or blue ($A_{\parallel} \leq 95 \times 10^{-4}$ cm^{-1}), type 2 or normal ($A_{\parallel} > 140 \times 10^{-4}$ cm^{-1}), and type 3 or coupled binuclear (EPR undetectable). The oxidized type 3 site is a two-electron acceptor[3] and displays a broad near-UV absorption band maximizing at 330 nm, $\Delta\epsilon = 2800$ M^{-1} cm^{-1}. Through low-temperature magnetic circular dichroism studies of *Rhus vernicifera* laccase, it has been determined that the exogenous anionic inhibitor N_3^- binds as a bridging ligand between the type 2 and type 3 sites, thereby defining a novel trinuclear active site.[4] This trinuclear site model has been confirmed very recently in a crystal structure of ascorbate oxidase.[5]

The reduction and reoxidation reactions of laccase have been extensively studied. Kinetic measurements have demonstrated that type 1 Cu(II) reduction precedes type 2 Cu(II) reduction, followed by intramolecular electron transfer to the type 3 site.[6,7] The mechanism and intermediates involved in the four-electron reduction of dioxygen to water are not well understood. The following model for the reaction of fully reduced native laccase with dioxygen has been proposed:[8] (1) Dioxygen is bound at the type 3 center. (2) Three electrons are rapidly transferred from the type 1 and type 3 sites to dioxygen ($k = 5 \times 10^6$ M^{-1} s^{-1}), resulting in formation of an "oxygen intermediate" associated with an optical absorption band near 360 nm[8a,9] and a broad liquid-He temperature EPR signal near $g = 1.7$.[10] (3) The oxygen intermediate is reduced by either the type 2 site or the rereduced type 1 site, generating water. Because electron transfer from the type 2 site to the oxygen intermediate is too slow to be catalytically relevant ($t_{1/2} \sim 1$-15 s),[8a,11] it is thought that the rereduced type 1 site donates the fourth electron in the presence of excess reductant. Although the above mechanism does not include a role for the type 2 center in the reduction of dioxygen, several lines of evidence suggest that it may be a component of the dioxygen binding site. Definition of the trinuclear Cu active site based on N_3^- binding studies suggests that a bridged type 2-type 3 binding mode could contribute to the multielectron reduction of dioxygen to water at this site.[4] Indeed, one of the two water molecules formed in the reduction of dioxygen by laccase remains bound to the type 2 Cu.[12] In addition, recent kinetic studies have led to the suggestion that the type 2 Cu(I) plays a catalytic role in the reduction of the oxygen intermediate.[13]

Graziani et al. have reported reversible preparation of a type 2 depleted (T2D) laccase derivative,[14] and several groups have used T2D laccase to probe the involvement of the type 2 Cu in the reduction of dioxygen. As isolated, T2D laccase contains an oxidized type 1 site,[14] but the 330-nm band is absent.[15] With the use of X-ray absorption edge spectroscopy to directly probe oxidation states of the type 3 site (vide infra), it was determined that T2D laccase contains a reduced type 3 site.[16] In contrast to the reduced binuclear Cu site in hemocyanin, the reduced type 3 site in T2D is stable to reaction with dioxygen; thus, this derivative is referred to as deoxy T2D laccase. However, the type 3 site can be oxidized by H_2O_2, resulting in an absorbance increase

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centered at 330 nm ($\Delta\epsilon \sim 2000$ M^{-1} cm^{-1}).[16] In native laccase, oxidation of type 1 Cu(I) occurs concomitantly with generation of the oxygen intermediate,[8a] possibly indicating that electron donation from the type 1 site is required for reaction with dioxygen. Thus, to accurately assess the effect of type 2 Cu depletion, it is necessary to characterize the reaction of both deoxy T2D and *fully reduced* T2D laccase with dioxygen. In a study of the reactions of fully reduced T2D laccase with dioxygen, Reinhammar and Oda[17] reported that the type 3 and type 1 sites oxidize at the same rate as in native laccase and that similar oxygen intermediate optical and EPR signals are generated. It was concluded that the reoxidation reactions proceed in the absence of the type 2 Cu. These results imply that rapid electron transfer from both the type 1 and type 3 sites is necessary for dioxygen reactivity. Native laccase may be reconstituted from T2D by treatment with Cu(I),[14] and we have observed that under reducing conditions native laccase spontaneously regenerates from T2D, presumably via migration of Cu(I).[18] Thus, we have reexamined the reactions of fully reduced T2D laccase with dioxygen under conditions that minimize the regeneration of native enzyme using optical absorption, EPR, and X-ray absorption edge spectroscopy to directly quantitate Cu redox states. A preedge 1s → 4p transition near 8984 eV is present in the X-ray absorption edge spectra of Cu(I) but is absent in Cu(II) spectra. We have developed a normalized difference edge analytical method that uses the amplitude of this 8984-eV feature to quantitate the amount of Cu(I) in proteins.[16,19] In the case of the coupled type 3 site in laccase, which does not give rise to an EPR signal, this method is more reliable than optical measurements, which, at best, only indirectly probe the copper ion oxidation state.

In addition to the T2D derivative, a laccase derivative containing Hg^{2+} substituted into the type I site (T1Hg) has been developed by McMillin and co-workers.[20] As prepared, T1Hg contains an oxidized type 2 Cu, and the presence of a 330-nm absorption band[20] suggests that the type 3 site is also oxidized. Fluoride binds to the type 2 Cu in T1Hg in a manner similar to that observed in native laccase.[21] In addition, low-temperature MCD studies on T1Hg reveal that N_3^- binds as a bridging ligand between the type 2 and type 3 sites.[22] Thus, the trinuclear Cu site appears to be intact in this derivative. Because Hg^{2+} is redox-inactive, the T1Hg laccase derivative represents an excellent system to test whether the reduced type 2–type 3 trinuclear Cu center is capable of reacting with dioxygen in the absence of type 1 Cu. Through a detailed comparison of the reoxidation of T2D and T1Hg laccase, we have determined that an intact type 2–type 3 trinuclear center is necessary and sufficient for reactivity with dioxygen. In addition, an intermediate in the reaction of T1Hg laccase with dioxygen has been identified with optical and EPR spectroscopy.

Experimental Section

R. vernificera laccase was isolated[23] from the acetone powder (Saito and Co., Osaka Japan) to a purity ratio A_{280}/A_{614} of 14.5–15.5, as modified in ref 24. Laccase activity was assayed spectrophotometrically with *N,N*-dimethyl-*p*-phenylenediamine as the substrate.[23] The T2D derivative of laccase was prepared by the procedure of Graziani et al.[14] as modified in ref 24. The T1Hg derivative of laccase was prepared according to published procedures[20,25] with the following modifications: ^{65}Cu(II) stock solution was prepared by dissolving ^{65}CuO (Oak Ridge National Laboratory, Oak Ridge, TN) in a small volume of concentrated HCl followed by dilution into 0.5 M acetate and 100 mM NaCl, pH 5.5. Prior to use, the ^{65}Cu(II) was reduced to ^{65}Cu(I) by addition of 10 equiv of ascorbate. Hg(II) was prepared by dissolving the acetate salt in 25 mM tris(hydroxymethyl)aminoethane, pH 7.0. The T1Hg laccase was purified by chromatography on CM50 Sephadex (Sigma, St. Louis, MO), with a step elution from 10 to 200 mM potassium phosphate, pH 7.0. Adventitiously bound metal ions were removed by passing T1Hg through a 1 cm × 1 cm column of Chelex 100 (Biorad, Richmond, CA). As determined by atomic absorption spectroscopy,[24] the T1Hg contains 2.99 ± 0.21 Cu/mol of protein (average of seven determinations). EPR double integration of T1Hg, with Cu(II)-EDTA as a standard, reveals 1.13 ± 0.15 spin/mol of protein (average of two determinations). The residual enzymatic activities of the T2D and T1Hg laccase preparations were variable; the preparations used for the X-ray edge measurements exhibited 7 ± 2% and 5 ± 2%, respectively, of the specific activity of native enzyme. Deionized water (Barnstead Nanopure) was used for all buffer solutions. All chemicals were reagent grade and were used without further purification.

Unless otherwise noted, all experiments were performed in 0.1 M potassium phosphate, pH 6.0, and protein was maintained at 4 °C. Samples for X-ray edge analysis were concentrated to ~1 mM with either an Amicon ultrafiltration cell (Amicon Corp., MA) or Immersible-CX ultrafilters (Millipore Corp., MA). A 30% solution of hydrogen peroxide was standardized against potassium permanganate.[26] UV–visible absorption spectra were recorded at 298 K on either a Cary 17 or a Hewlett-Packard HP8452A diode array spectrophotometer. EPR spectra were obtained at 77 K with a Bruker ER 220-D-SRC spectrometer. Reduction of T2D laccase was carried out in a N_2-purged cuvette by addition of 2 equiv of ascorbate. T1Hg laccase was reduced by anaerobic dialysis against 5 mM sodium dithionite. Excess dithionite was removed by subsequent anaerobic dialysis against buffer, and reduced T1Hg laccase was transferred to a N_2-purged cuvette for optical absorption measurements. For the X-ray absorption spectroscopy experiments, an aliquot of fully reduced protein was transferred under argon into 150-μL lucite sample holders with a glovebag and frozen in liquid N_2. Reoxidation was initiated by exposing the cuvette to air, and at the indicated time points, UV–visible spectra were recorded and aliquots of protein were frozen in lucite sample holders. Following X-ray absorption measurements at Stanford Synchrotron Radiation Laboratory, the EPR spectra were rerecorded, the samples were thawed to 298 K, and the UV–visible spectra were rerecorded.[16b] For kinetic studies of the optical intermediate and the type 2 EPR signal, reoxidation of reduced T1Hg laccase was initiated by addition of dioxygen-saturated buffer. At the indicated time points, UV–visible spectra were recorded and aliquots were frozen to 77 K in 4-mm-o.d. quartz EPR tubes.

X-ray absorption edge spectra were recorded under dedicated conditions at the Stanford Synchrotron Radiation Laboratory with a Si(220) double-crystal monochromator on beam line 7-3 (unfocused). A Lytle detector was used to monitor X x-ray fluorescence.[27] Sample temperature was maintained at 85 K in an Oxford Instruments CF1208 continuous-flow He cyrostat. Between one and seven scans were measured for each sample and averaged together. A Cu foil internal energy calibration was measured simultaneously with each scan,[28] and energy referencing was accomplished by assigning the first inflection point in the Cu foil spectrum to 8980.3 eV. The edge spectra were normalized by fitting a smooth first-order polynomial to the EXAFS region and extrapolating back to the edge region. Similarly, a second-order polynomial was fit to the preedge region and extrapolated forward to the edge region, and the normalization was adjusted to give a unit difference at 9000 eV between these fitted curves.[16b] The difference edge quantitation of Cu(I) was performed as previously described.[16b] The contribution of a small amount of native laccase to the difference edges of the T2D and T1Hg laccase samples were removed by subtracting appropriately weighted edge spectra of reduced or resting native laccase and normalizing before performing the quantitation of Cu(I).

Results and Analysis

A. Reaction of T2D Laccase with Dioxygen. Resting, deoxy T2D laccase contains a reduced type 3 site and an oxidized type 1 site. The type 1 site was reduced with ascorbate to generate fully reduced enzyme. Loss of the 614-nm absorption band ($\Delta\epsilon = -4600$ M^{-1} cm^{-1}) and the type 1 EPR signal indicates complete reduction of the type 1 Cu following 60-min incubation with 2 equiv of ascorbate (Figures 1 and 2). T2D laccase is <5% active

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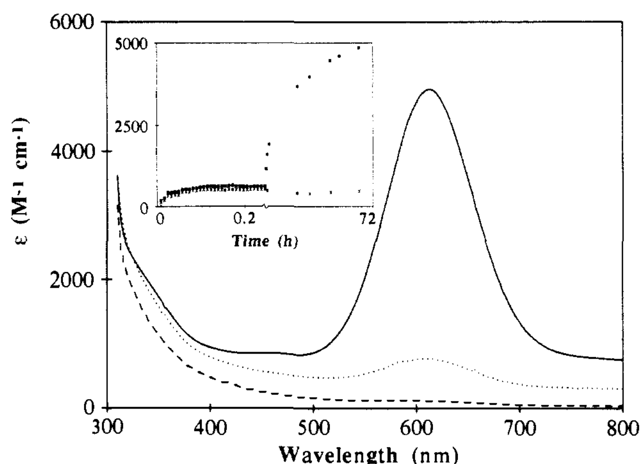

Figure 1. 298 K UV-visible absorption spectra of reduced and reoxidized T2D laccase: (---) fully reduced; (···) exposed to air for 15 min; (—) exposed to air for 63 h. Inset: Kinetics of T2D reoxidation upon exposure to air. Key: (×) 330 nm; (■) 614 nm.

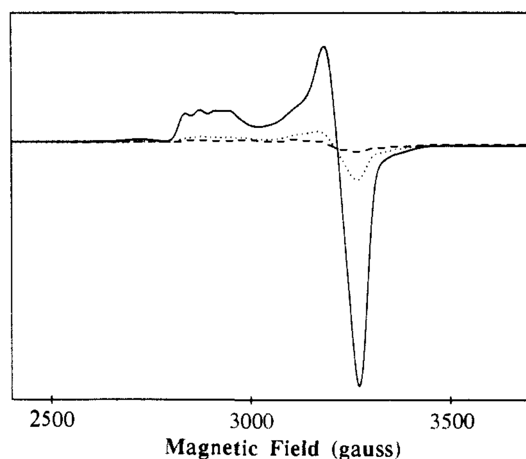

Figure 2. 77 K EPR spectra of reduced and reoxidized T2D laccase: (---) reduced; (···) exposed to air for 15 min; (—) exposed to air for 63 h. Spectrometer conditions: microwave frequency, 9.312 GHz; microwave power, 10 mW; scan range, 2000–4000 G; field modulation, 16 G; scan time, 20 s; time constant, 0.2 s.

in turnover assays,[14,17] and thus enzymatic activity could be used to assay regeneration of native enzyme under reducing conditions. Under conditions used to prepare the reduced X-ray edge samples (2 equiv of ascorbate, 1-h incubation), the specific activity of the T2D preparation increased from 7 to 14 ± 2% relative to native laccase.

Upon exposure of fully reduced T2D laccase to air the type 1 absorption and EPR spectra return (Figures 1 and 2). As indicated by the 614-nm absorption band, the type 1 Cu(I) reoxidizes with biphasic kinetics (Figure 1, inset); a small proportion of the sites rapidly reoxidize ($t_{1/2}$ = 70 ± 20 s), whereas the bulk of the sites reoxidize slowly ($t_{1/2}$ = 11.4 ± 1.5 h). Morpurgo and co-workers have reported similar slow reoxidation kinetics of T2D laccase under similar conditions.[15c] A small absorbance increase is also observed at 330 nm, and the kinetics ($t_{1/2}$ = 90 ± 20 s) parallel the fast phase of the type 1 reoxidation (Figure 1, inset). The amplitudes of the rapid absorbance changes at 614 and 330 nm correspond to reoxidation of 10 ± 2 and 15 ± 4% of the type 1 and type 3 sites, respectively. In order to directly quantitate the type 3 Cu oxidation states associated with the rapid and slow reoxidation reactions, samples for X-ray absorption edge and EPR spectroscopy were frozen 15 min and 63 h after exposure to air.

The 10–15% of the laccase molecules that rapidly reoxidize directly corresponds to the native laccase present in the T2D preparation as defined by enzymatic activity following reduction. The rate of reoxidation of the native laccase is governed by the rate of consumption of the residual unreacted ascorbate remaining

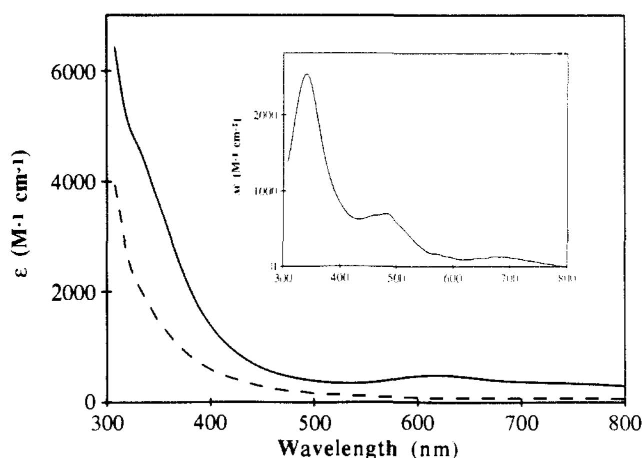

Figure 3. UV-visible absorption spectra of reduced and reoxidized Tl Hg laccase: (---) reduced; (—) exposed to air for 30 min. Inset: Transient absorption diffrence spectrum of Tl Hg reoxidized for 5 s minus Tl Hg reoxidized for 1.5 h. Conditions: Tl Hg laccase concentration, 50 μM; dioxygen concentration, 0.13 mM.

in solution. In contrast to the native laccase contaminant, the bulk of the type 1 sites in T2D reoxidize very slowly, and this reoxidation is not accompanied by changes in the 330-nm band. In order to test whether any oxygen intermediate is transiently generated in T2D laccase, separate EPR reoxidation experiments were performed under the conditions described by Reinhammar and Oda[17] for observation of the oxygen intermediate EPR signal in T2D laccase. Samples of native laccase and T2D in 100 mM potassium phosphate, pH 7.4, were fully reduced by reaction with 2 equiv of ascorbate/Cu for 15 min and reoxidized by injection of 7 mol of dioxygen/mol of protein in the same buffer. Both samples were frozen in liquid nitrogen after 20 s. A broad negative EPR signal centered near g = 1.7 was observed at 10 K in the native laccase sample but was absent in the T2D sample (data not shown). The line shape of the EPR signal in native laccase is similar to the previously reported oxygen intermediate signal in native[10,11] or T2D laccase at pH 7.4.[17]

B. Reactions of Tl Hg Laccase with Hydrogen Peroxide and Dioxygen. Our earlier studies demonstrated that H_2O_2 is capable of oxidizing the type 3 center in deoxy T2D laccase and the fraction (22%) of reduced type 3 centers present in native laccase.[16,19] By analogy to these other laccase derivatives, H_2O_2 reactivity and X-ray absorption spectroscopy were used to assess the redox state of the type 3 center in resting Tl Hg. Upon treatment of Tl Hg with 30-fold excess of H_2O_2, only a small stable increase is observed in the 330-nm absorption band ($\Delta\epsilon$ = 230 ± 60 M^{-1} cm^{-1}). With a differential extinction coefficient of 3600 M^{-1} cm^{-1} for the 330-nm band,[16b] this increase corresponds to oxidation of 6 ± 2% of the type 3 centers.

Ascorbate is capable of facile reduction of both native and T2D laccase. However, reaction of Tl Hg laccase with 5 mM ascorbate for 2 h under anaerobic conditions results in only a partial decrease in the 330-nm absorption band ($\Delta\epsilon \sim -1100$ M^{-1} cm^{-1}). In contrast, Figure 3 shows that dithionite, an inner-sphere small-molecule reductant, completely reduces the 330-nm absorption band ($\Delta\epsilon$ = −3300 M^{-1} cm^{-1}). In addition, the EPR spectrum of the dithionite-reduced sample shows that 90% of the type 2 centers is reduced (Figure 4). Upon exposure of the dithionite-reduced Tl Hg sample to air, the 330-nm absorption band returns ($\Delta\epsilon$ = 2550 M^{-1} cm^{-1}) and the type 2 Cu reoxidizes. The sample for X-ray edge analysis was frozen after 30-min exposure to air.

In separate experiments, we have employed optical and EPR spectroscopy to investigate the kinetics of reoxidation of Tl Hg laccase at 298 K. Upon reaction of reduced protein with 2.5 mol of dioxygen/mol of protein, the absorbance increase near 330 nm is completed within the time of mixing (5 s), indicating that k > 10^3 M^{-1} s^{-1}. Measurement of the true rate constant for this reaction will require stopped-flow techniques; however, it is clearly much faster than reoxidation of the type 1 Cu(I) in T2D laccase.

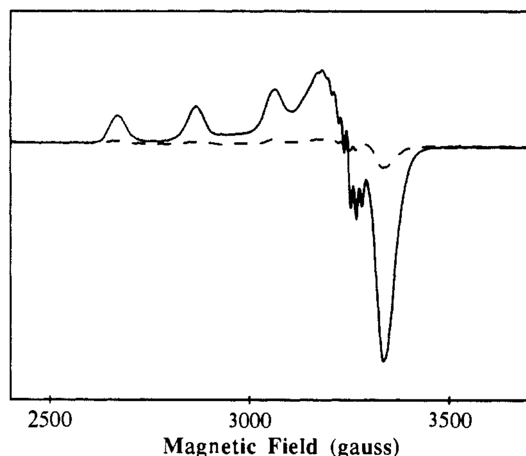

Figure 4. 77 K EPR spectra of reduced and reoxidized T1Hg laccase: (---) reduced; (—) exposed to air for 30 min. Spectrometer conditions: microwave frequency, 9.315 GHz; microwave power, 10 mW; scan range, 2000–4000 G; field modulation, 5 G; scan time, 200 s; time constant, 0.2 s.

The magnitude of the prompt absorbance change near 330 nm ($\Delta\epsilon$ = 4600 ± 500 M^{-1} cm^{-1}) is greater than may be accounted for by oxidation of the type 3 site (maximum $\Delta\epsilon$ = 3600 M^{-1} cm^{-1}),[16b] suggesting that an additional spectral feature contributes intensity in this region. Indeed, a portion of this UV absorbance increase is not stable and decays with $t_{1/2}$ = 6.4 ± 2 min, such that after 30 min the amplitude at 330 nm has decreased to that of the original resting T1Hg. The difference spectrum of 5-s reoxidized T1Hg laccase minus 1.5-h reoxidized T1Hg laccase (Figure 3, inset) reveals that, in addition to the transient absorbance change at 340 nm ($\Delta\epsilon$ = 2600 M^{-1} cm^{-1}), weaker transient features are also observed at 470 nm ($\Delta\epsilon$ = 700 M^{-1} cm^{-1}) and 670 nm ($\Delta\epsilon$ = 140 M^{-1} cm^{-1}). These absorbance changes are similar to those associated with the oxygen intermediate observed in native laccase reoxidation experiments ($\Delta\epsilon_{340} \sim$ 1300 M^{-1} cm^{-1}, $\Delta\epsilon_{470} \sim$ 300 M^{-1} cm^{-1}).[8a,9,29] Also, in both systems the 330-nm band associated with the oxidized type 3 site remains following the decay of the transient signal. The rise kinetics of the type 2 Cu EPR signal are significantly slower than the absorbance increase observed near 330 nm. Upon addition of 2 mol of dioxygen/mol of protein, the type 2 EPR signal shows a monophasic increase ($t_{1/2}$ = 4.3 ± 1 min). The decrease of the intermediate absorption features occurs on the same time scale as the appearance of the type 2 EPR, suggesting that these phenomena are correlated (Figure 5). A similar inverse correlation was reported between the oxygen intermediate signals and the type 2 EPR signal in native laccase,[8a,11] thus strengthening the identification of the T1Hg absorbance changes with an oxygen intermediate similar to that reported in native laccase. With the use of the EPR conditions appropriate for observation of the oxygen intermediate in native laccase (20–200-mW microwave power, 5 K),[10] no oxygen intermediate EPR signal is observed in samples of T1Hg frozen 30 s after reoxidation. Thus, in contrast to native laccase, observation of a transient optical change associated with an oxygen intermediate is not accompanied by a He temperature EPR signal in T1Hg laccase.

C. X-ray Absorption Edge Spectroscopy of T2D laccase. The normalized Cu edge spectra of reduced and 15-min and 63-h reoxidized T2D laccase samples are shown in Figure 6. In the edge spectrum of the fully reduced sample, the intense shoulder at 8984 eV indicates that the bulk of the Cu in the preparation is in the +1 oxidation state. The amplitude of this feature is

(29) Much weaker absorbance changes at the same energies as in T1Hg laccase have also been assigned to an unliganded metastable form of native laccase following reoxidation (C state): (a) Goldberg, M.; Farver, O.; Pecht, I. *J. Biol. Chem.* **1980**, *255*, 7353–7361. (b) Farver, O.; Goldberg, M.; Pecht, I. *Eur. J. Biochem.* **1980**, *104*, 71–77. However, the C state was observed to be stable for several days, whereas the T1Hg laccase absorption features decay completely within 30 min, suggesting these are more closely identified with the oxygen intermediate absorption bands in native laccase.

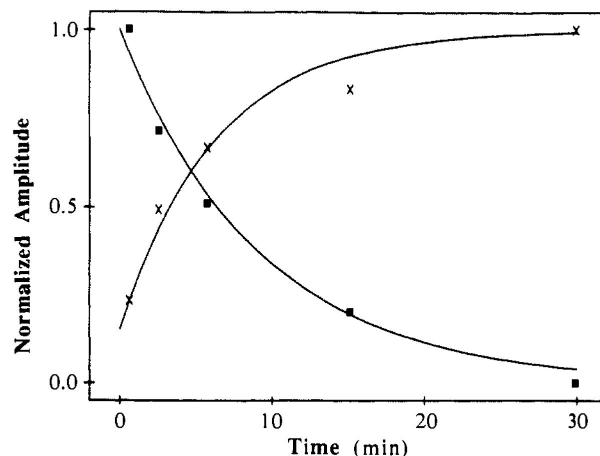

Figure 5. Kinetics of decay of the T1Hg laccase intermediate optical absorption signal and formation of the type 2 77 K EPR spectrum: (■) absorption at 330 nm; (×) type 2 EPR. The absorption spectrum at 30 min was subtracted from the earlier time points. Conditions: T1Hg concentration, 0.15 mM; dioxygen concentration, 0.3 mM. The type 2 EPR signal was quantitated by the amplitude of the low-field $\Delta m_s = -^3/_2$ Cu hyperfine line in the $g_\parallel$ region. The solid lines are single exponential fits to the data. Rate constants are 1.8 × 10^{-3} s^{-1} ($t_{1/2}$ = 6.4 min) for the 330-nm absorption band and 2.7 × 10^{-3} s^{-1} ($t_{1/2}$ = 4.3 min) for the type 2 EPR.

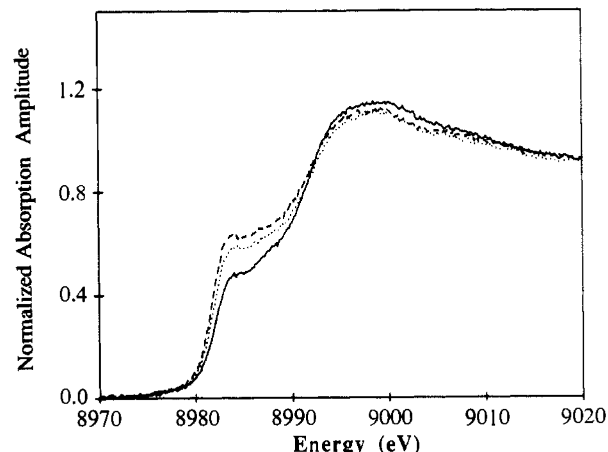

Figure 6. Normalized X-ray absorption edge spectra of reduced and reoxidized T2D laccase: (---) reduced; (···) exposed to air for 15 min; (—) exposed to air for 63 h.

slightly decreased in the sample exposed to air for 15 min, indicating a small fraction of the Cu is oxidized. After 63-h exposure to air, there is a more substantial decrease in the 8984-eV feature, but a large 8984-eV peak remains. Thus, upon exposure of fully reduced T2D to air, there is a small fraction of the Cu(I) that rapidly oxidizes, a larger fraction that slowly oxidizes, and a third component that remains reduced after 63-h exposure.

For some Cu proteins, X-ray-induced photoreduction has been observed during the course of X-ray absorption measurements,[30] even at temperatures as low as 10 K.[31] The optical and EPR spectra of the T2D samples used for X-ray edge studies did not change following exposure to X-rays, except for the appearance of a small g = 2.0 radical signal in the EPR spectrum. Also, the difference edge spectrum calculated between the first and last scans on a given sample does not contain any features near 8984 eV associated with an increase in Cu(I) concentration.

We have employed a normalized difference edge analysis to quantitate the fraction of Cu(I) present in the T2D and T1Hg laccase samples. Subtraction of the normalized edge of the T2D

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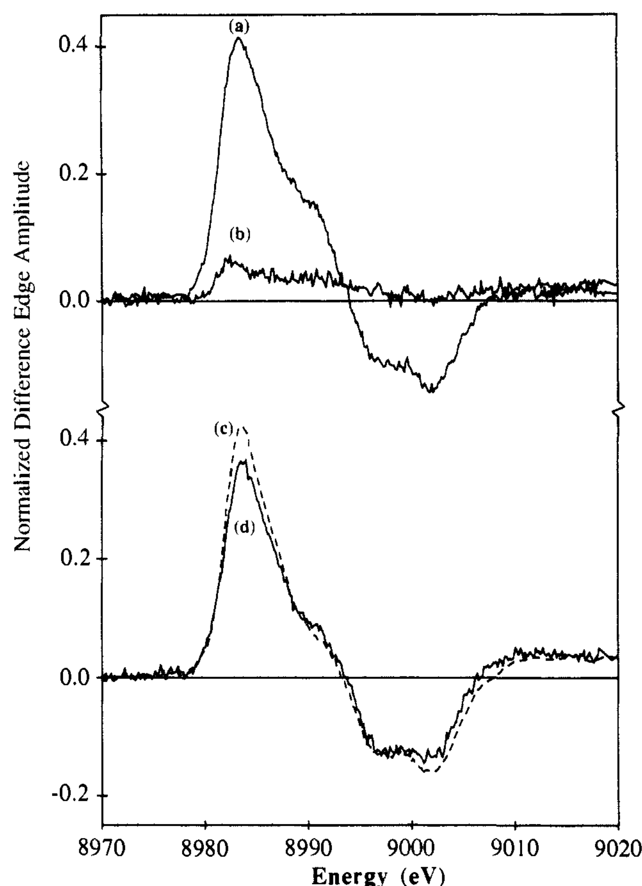

Figure 7. Normalized difference edge spectra of T2D laccase: (a) reduced native laccase − resting native laccase; (b) reduced T2D − T2D exposed to air for 15 min; (c) deoxy T2D − (T2D + 60 × H_2O_2); (d) T2D exposed to air for 63 h − (T2D + 60 equiv of H_2O_2). Spectra c and d have been corrected for the presence of ~10% native laccase contaminant in the T2D preparations.

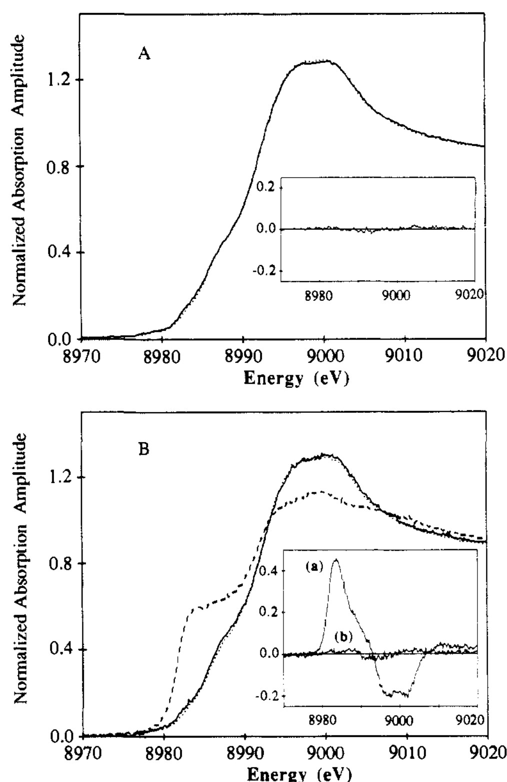

Figure 8. Normalized edge spectra of T1Hg laccase. (A) (—) resting enzyme; (···) resting + 30 × H_2O_2. Inset: difference edge spectrum of resting − (resting + 30 × H_2O_2). (B) (---) reduced; (—) exposed to air for 30 min; (···) resting + 30 × H_2O_2. Inset: difference edge spectra. Key: (a) reduced − (resting + 30 × H_2O_2); (b) exposed to air for 30 min − (resting + 30 × H_2O_2). All difference spectra have been corrected for the presence of 5–10% native laccase in the T1Hg preparations.

laccase sample exposed to air for 15 min from the edge of the fully reduced sample gives rise to a characteristic signal near 8984 eV that may be used to quantitate the extent of air oxidation (Figure 7b). On the basis of the optical and EPR data (vide supra), the oxidation observed after 15-min exposure is likely associated with the component of native laccase regenerated from T2D under reducing conditions. Thus, the amplitude of the T2D difference edge at 8984 eV may be referenced against a native laccase amplitude standard generated by subtracting the normalized edge of resting native laccase from that of fully reduced native laccase (Figure 7a). With this standard, the oxidation observed after 15 min corresponds to 10 ± 4% native laccase. Within the error of the measurement, this agrees with the fraction of native laccase present in the T2D preparation as determined by enzymatic activity and optical absorbance changes. For subsequent quantitative analysis, 10% of a reduced or oxidized native laccase edge has been subtracted from the edges of reduced or oxidized T2D laccase, respectively, and the resultant spectrum renormalized. After exposure of reduced T2D laccase to air for 63 h, optical and EPR data indicate that the type 1 center becomes fully oxidized. Previously, we have shown that a standard for complete reduction of the laccase type 3 site may be generated by subtracting the edge of T2D in which the type 3 site has been oxidized by addition of 60 × H_2O_2 (met T2D) from the edge of deoxy T2D (Figure 7c).[16b] To use this reference, the edge spectrum of met T2D laccase was also subtracted from the edge of the 63-h reoxidized sample. The amplitude of the resultant difference edge (Figure 7d) corresponds to 86 ± 8% reduced type 3 sites in the 63-h reoxidized sample.

D. X-ray Absorption Edge Spectroscopy of T1Hg Laccase. The Cu X-ray edge spectra of resting and H_2O_2-treated T1Hg laccase are shown in Figure 8A. There is no prominent 8984-eV feature in the spectrum of resting T1Hg, indicating that the type 2 and type 3 sites are primarily oxidized. In contrast to T2D, treatment of T1Hg laccase with 30-fold excess of H_2O_2 does not significantly change the shape of edge, confirming that little if any Cu(I) is present in the resting preparation (Figure 8A, dotted line). As expected, the reduced T1Hg edge exhibits a large shoulder near 8984 eV (Figure 8B, dashed line). However, when the reduced sample is exposed to air for 30 min, the 8984-eV feature decreases and the edge becomes essentially superimposable with that of the H_2O_2-treated sample, suggesting that the trinuclear Cu site has completely reoxidized.

Except for the generation of a small $g = 2.0$ radical EPR signal, no changes were observed in the EPR and optical spectra of the T1Hg laccase samples following exposure to the X-ray beam. However, in the resting and H_2O_2-treated samples there were some increases in the edge amplitude near 8984 eV during successive scans. The total increase in the 8984-eV region corresponded to the photoreduction of 3–5% of the Cu(II) present in these samples. Thus, only the first scans on these samples were employed for difference edge analysis. Because the rate of photoreduction was only ~1%/scan, the errors associated with X-ray-induced photoreduction will not significantly affect the determination of Cu redox states in these samples.

In order to quantitate the amount of Cu(I) present in the resting and reoxidized T1Hg samples without reference to Cu(I) models, a T1Hg laccase difference edge standard for complete reduction of the type 2–type 3 trinuclear site was generated by subtracting the edge of the fully oxidized H_2O_2-treated sample (Figure 8B, dotted line) from the edge of the reduced sample (Figure 8B, dashed line). This produces the difference edge in Figure 8B, inset a. From the EPR data, 10% of the type 2 sites remain oxidized in the reduced sample. Since this fraction most likely represents reoxidation of protein during transfer into the lucite sample holder, an equivalent fraction of the type 3 sites is also likely oxidized, and the reduced T1Hg laccase edge has been corrected for this 10% oxidized T1Hg. The edge of the H_2O_2-treated T1Hg sample

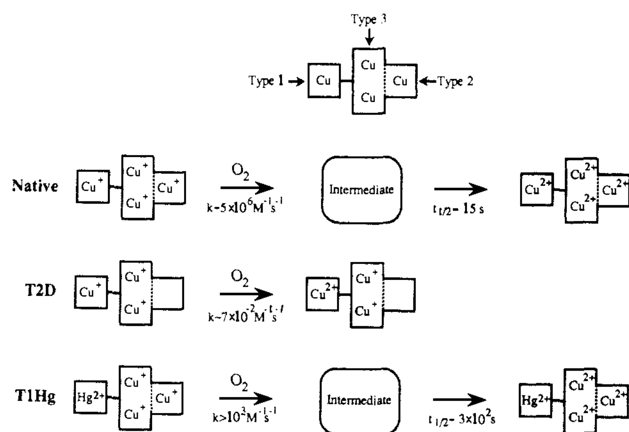

Figure 9. Reactivity of laccase derivatives with dioxygen.

was also subtracted from the reduced/reoxidized (Figure 8B, inset b) and resting (Figure 8A, inset) T1Hg laccase samples. With the use of the amplitude at 8984 eV in these difference edges to quantitate Cu(I), the resting T1Hg sample was determined to contain 1 ± 8% Cu(I) and the reduced T1Hg laccase sample exposed to air for 30 min was determined to contain 2 ± 11% Cu(I).

Discussion

The reactivity of fully reduced T2D and T1Hg laccase with dioxygen has been probed with X-ray edge spectra to directly quantitate Cu redox states. The results of this study are summarized in Figure 9. The type 3 center in fully reduced T2D does not react with dioxygen. The ~10% of the type 3 sites that does react with dioxygen is associated with contaminant native laccase, which is regenerated from T2D under reducing conditions. Although the type 1 site in fully reduced T2D laccase does oxidize, the bimolecular rate constant for the reaction with dioxygen is $\sim10^8$ less than for native laccase ($k \sim 0.07$ vs 5×10^6 M^{-1} s^{-1}), and the oxidation of the type 1 Cu is not accompanied by oxidation of the type 3 site or formation of an oxygen intermediate EPR signal. Thus, this reaction is not related to the normal catalytic dioxygen reactivity of native laccase. Stellacyanin contains one Cu atom in a type 1 site, and it is more reactive with dioxygen ($k = 1.8$ M^{-1} s^{-1}) than the type 1 site in fully reduced T2D, whereas another single type 1 site protein, plastocyanin, does not react with dioxygen at a measurable rate.[32] The origin of this variation in reactivity likely relates to the different redox potentials of these blue Cu sites (*Rhus* laccase, 394 mV;[3] plastocyanin, 370 mV;[33] stellacyanin, 184 mV[33]) and the different accessibilities of the Cu sites to solvent.[34]

The previous observations[17] of facile reactivity of fully reduced T2D laccase with dioxygen must be reevaluated in light of the present results. It is likely that the prolonged period of ascorbate reduction used in that study (20 h) would lead to significant regeneration of native laccase from T2D and that the rapid oxidation reactions observed were, in fact, associated with a significant native laccase contaminant. They reported that 40% of the type 1 and type 3 sites reoxidize rapidly ($k \sim 5 \times 10^6$ M^{-1} s^{-1}), whereas the remaining type 1 reoxidation is very slow and is not accompanied by further absorbance increases at 330 nm associated with the type 3 site. The present observations suggest that the 40% of the type 1 and type 3 that readily react with dioxygen correspond to native laccase. This large amount of native laccase presumably also accounts for the observation of an oxygen intermediate EPR signal in their study, whereas we have not observed this signal in our T2D preparations.

T1Hg, as prepared, is fully oxidized, suggesting that this laccase derivative contains an intact type 2–type 3 trinuclear Cu site. EPR studies have shown that fluoride binds with extremely high affinity to the laccase type 2 Cu(II),[35] and similar binding to T1Hg has been used to establish the integrity of its type 2 center.[21] Low-temperature MCD studies[22] show that N_3^- binds as a bridging ligand between the type 2 and type 3 sites in a manner similar to that observed in native laccase,[4] providing further support for the existence of an intact trinuclear site in T1Hg. Thus, T1Hg represents a valid derivative of laccase, and it is a suitable system for further investigations of the structure and reactivity of the trinuclear Cu active site.

Reduced T1Hg laccase is completely reoxidized upon exposure to dioxygen (Figure 9). In contrast, the type 3 site in T2D laccase does not react with dioxygen independent of the redox state of the type 1 Cu. Thus, the presence of the type 2 Cu is an absolute requirement for dioxygen reactivity. Although in native laccase the type 1 Cu(I) is oxidized concomitant with the formation of the oxygen intermediate, this redox center is clearly not required for reactivity, and the trinuclear Cu site represents the minimal structural unit for dioxygen reduction. These results are consistent with previous suggestions that the type 2 Cu is a component of the dioxygen reduction site.[4]

In native laccase and T1Hg, intermediates are detected in the reaction of reduced enzyme with dioxygen that exhibit interesting similarities and differences. In native laccase, a rapid UV absorbance increase occurs upon exposure of fully reduced protein to dioxygen ($\Delta\epsilon_{340} \sim 3900$ M^{-1} cm^{-1}).[8a] Subsequently, the intermediate decays, causing an absorbance decrease in the region from 300 to 500 nm ($\Delta\epsilon_{340} \sim -1300$ M^{-1} cm^{-1}, $\Delta\epsilon_{470} \sim -300$ M^{-1} cm^{-1}), producing fully oxidized laccase that exhibits a type 3 absorption band ($\Delta\epsilon_{340} \sim +3600$ M^{-1} cm^{-1} relative to reduced laccase). The transient absorbance changes were assigned to an unstable oxygen intermediate in the reaction of laccase with dioxygen. A very similar set of UV–visible absorbance changes are observed in T1Hg laccase (initial absorbance increase, $\Delta\epsilon_{330} = 4600 \pm 500$ M^{-1} cm^{-1}; subsequent decrease, $\Delta\epsilon_{340} = -2600$ M^{-1} cm^{-1}, $\Delta\epsilon_{470} = -700$ M^{-1} cm^{-1}; remaining type 3 absorption band, $\Delta\epsilon_{330} = 2100 \pm 400$ M^{-1} cm^{-1} relative to reduced T1Hg). Also, in both systems the decay of the intermediate absorption features is coupled to appearance of the type 2 Cu(II) EPR signal, and in native laccase this reaction has been attributed to electron transfer from the type 2 Cu(I) to the oxygen intermediate, forming water.[8a,11] The similarities in the transient absorption features and their inverse correlation with the type 2 Cu(II) EPR signal suggest that the trinuclear site oxidation state changes that accompany formation and decay of the oxygen intermediate are similar in the two systems. A type 3 330-nm absorption band is presumed to be present in both intermediates, suggesting that the type 3 is oxidized at this stage of the reaction. However, absorption spectroscopy is at best an indirect probe of the type 3 oxidation state. Also, the absence of the type 2 Cu(II) signal in the intermediate state is not definitive evidence that the type 2 Cu is reduced; for example, it may be broadened due to magnetic interaction with a nearby paramagnetic center. Thus, in both systems X-ray edge data at early reaction times are necessary to define the redox state of the type 3 and the type 2 sites when the oxygen intermediate is present.

In native laccase, it is clear from the distinctive EPR and optical features that oxidation of the type 1 Cu(I) accompanies formation of the oxygen intermediate. However, in T1Hg laccase electron donation from the type 1 Cu(I) has been eliminated. Thus, the intermediates that are observed in the two systems differ by one reducing equivalent. In native laccase the decay kinetics of the oxygen intermediate varies from $t_{1/2} = 13$ s at pH 7.4 to $t_{1/2} = 1$ s at pH 4.0 (298 K).[11] Under the conditions used in the present study (pH 6.0, 298 K), the rate of decay of the intermediate in T1Hg is ~20 times slower than in native laccase. This difference in kinetics may be associated with the different intermediates and products in the two systems. Because donation from the type 1 Cu(I) has been prevented, the intermediate that is detected in T1Hg may represent a precursor that to the oxygen intermediate

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in native laccase. The similar optical absorption spectra associated with the two intermediates (vide supra) despite the difference in their reduction state suggests that the absorption features are not directly associated with the oxygen intermediates per se but arise from a similarly perturbed trinuclear Cu site, i.e., with respect to Cu oxidation states or ligand binding mode.

Another difference between the two intermediates is that native but not T1Hg laccase exhibits a broad EPR signal observable at liquid He temperature. This is consistent with the extra reducing equivalent derived from the type 1 Cu(I) in native laccase. The signal is further broadened when reduced laccase is oxidized by [^{17}O]dioxygen,[10b] suggesting that it is associated with an oxygen radical, and it has been assigned to $O^{\bullet-}$ on the basis of its rapid electron spin relaxation, the assumed transfer of three electrons to dioxygen,[8] and the uptake of two protons concomitant to its formation.[36] However, note that the significant divergence of the lowest g value of this signal (1.7) from 2.0 is not consistent with this assignment to an $O^{\bullet-}$ species, which would not have sufficient orbital angular momentum due to the low spin–orbit coupling constant of oxygen. Also it cannot arise from a magnetically isolated tetragonal Cu(II) ion,[37] because a system with a $d_{x^2-y^2}$ ground state cannot exhibit g values below 2.0. However, it does show some similarity to the triplet state EPR spectra associated with a pair of dipolar-coupled $S = {}^1/_2$ centers;[38] such signals can arise from Cu(II) dimers and have been observed upon N_3^--induced uncoupling of the type 3 site in native[4] and met T2D laccase.[24] Thus, although the absence of the EPR signal in T1Hg is consistent with the formulation of this intermediate as an even-electron species, the assignment of the intermediate EPR signal in native laccase to $O^{\bullet-}$ requires detailed spectral study.

In summary, it is clear that the type 2 Cu is required for dioxygen reactivity in laccase and that dioxygen reduction occurs in the absence of the type 1 Cu. This demonstrates that the type 2–type 3 trinuclear Cu site represents the active site for the binding and multielectron reduction of dioxygen. Previous observation that N_3^- can bind as a bridging ligand between the type 2 and type 3 sites[4] suggests that a similar binding mode may be relevant to the reactivity of the trinuclear site with dioxygen. The type 1 Cu is clearly not necessary for reactivity with dioxygen, and in its absence, an intermediate is formed that shares some properties with the oxygen intermediate previously described in native laccase. Spectroscopic characterization of the oxygen intermediates in native and T1Hg laccase and X-ray edge determination of the associated Cu redox states should provide a detailed description of the mechanism of the irreversible multielectron reduction of dioxygen to water catalyzed by the multicopper oxidases.

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Registry No. Cu, 7440-50-8; O_2, 7782-44-7; H_2O_2, 7722-84-1; laccase, 80498-15-3.

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Heterocyclic 1,2,4,6-Thia- and 1,2,4,6-Selenatriazinyl Radicals. Spin Distributions and Modes of Association

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Abstract: Synthetic routes to the heterocyclic 1-chloro-1,2,4,6-thia- and -selenatriazines $[(Ph_2P)_{2-x}(PhC)_xN_3ECl]$ ($x = 0$, 1; E = S, Se) have been developed. For $x = 0$ the method involves the coupling of $[ClPh_2PNPPh_2Cl]^+Cl^-$ with the N,N′-disilylated diimides $Me_3SiNENSiMe_3$ (E = S, Se), while for $x = 1$ the preparation uses the condensation of $(Me_3Si)_2NPN(Ph)CNSiMe_3$ with either SCl_2 or $SeCl_4$. Reduction of the 1-chloro derivatives with triphenylantimony affords the thia- and selenatriazinyl radicals $[(Ph_2P)_{2-x}(PhC)_xN_3E]^\bullet$, all of which have been characterized by ESR spectroscopy. Hyperfine coupling constant data are interpreted in the light of MNDO calculations on model structures. X-ray structural analysis of the radical dimers $[Ph_2(tol)PCN_3S]_2$ and $[Ph_3PCN_3Se]_2{\cdot}CH_3CN$ reveals significantly different modes of association; for the sulfur-based radical, association occurs through a long (average (range) = 2.489 (9) Å) sulfur–sulfur bond, while in the selenium species the dimer pair is coupled by a selenium–nitrogen linkage, the structural parameters within the two rings resembling those expected for a charge-transfer interaction.

Many of the recent advances in the chemistry of sulfur nitrogen compounds have stemmed from the study of radical species.[2] Interest has focused on the use of such compounds in the design of synthetic metals,[3] and also on how radical association can

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