as observed for the 5-butyl hydroperoxy adduct of tetrakis(4-methoxyphenyl) iron(III) porphyrin.[29] The latter region has not yet been studied carefully for compound 0 and should be diagnostic for this type of species.

Turning attention to species **5**, an iron(V) porphyrin similar to the p-type manganese(III) hyperporphyrins should have a number of sharp, well-defined bands in the 500–800-nm region.[22] These are not observed for compound 0; however, since there is no precedent for species **5**, its optical spectrum would be difficult to predict. Finally, the ferric π-dication radical (**7**) of the Zn(II) and Mg(II) complexes of OEP exhibit bands near 350 nm in the ultraviolet and have a weak featureless band near 550 nm.[33] These features are close to those observed for compound 0. However, a structure of this type is hard to rationalize with the thermodynamic and activation parameters unless the RO portion of RO_2H remains bound. One intriguing possibility is that compound 0 is a ferric porphyrin π-dication radical produced by an initial, selective oxidation of the porphyrin ligand and that k_{obs}^{max} reflects its conversion to compound I through an intramolecular electron transfer from the ferric atom to the porphyrin radical. More detailed studies will be required to resolve these questions.

Registry No. EtO_2H, 3031-74-1; t-BuO_2H, 75-91-2; AcO_2H, 79-21-0; H_2O_2, 7722-84-1; peroxidase, 9003-99-0.

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Evidence for Temperature-Dependent Changes in the Coupling within the Type 2/Type 3 Cluster of Laccase

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Abstract: The type 2 depleted form of isotopically enriched laccase has been prepared and subsequently reconstituted with a different copper isotope. The results indicate that the copper ion which is removed and reinserted is *not* the same copper ion that is responsible for the type 2 EPR signal observed at low temperature. However, S-band EPR data show that the reactive copper center is EPR active at room temperature. These findings are explained in terms of a temperature-dependent structural reorganization and a change in the nature of the antiferromagnetic coupling that occurs within the type 2/type 3 cluster of laccase. This phenomenon may be connected with a previously identified conformational transition of the enzyme.

Introduction

The copper-containing proteins laccase, ascorbate oxidase, and ceruloplasmin form a class of enzymes known as the blue oxidases which catalyze the reduction of dioxygen to water without releasing potentially harmful intermediates such as H_2O_2 and the hydroxyl radical. A minimum of four copper ions appears to be required for efficient catalysis, and they are distributed in three spectroscopically distinct binding sites.[1,2] The type 1, or blue, copper center gives rise to an EPR signal and exhibits a strong visible absorbance near 600 nm that is responsible for the enzyme's blue color. The type 2 copper is also EPR active, but as yet no absorption bands have been resolved for this site. In contrast, the pair of coppers bound in the type 3 site exhibit absorption bands in the visible[3] and near-ultraviolet, but they are EPR silent due to a strong antiferromagnetic coupling interaction. Of all the blue oxidases, laccase is probably the easiest to investigate from the mechanistic viewpoint because in this enzyme each type of copper-binding site occurs only once. However, ascorbate oxidase, which contains two copies of each site, has been crystallographically characterized.[4]

Chemical modification studies have contributed to our understanding of laccase, and the preparation of the type 2 depleted (T2D) form of tree laccase can be considered to be a hallmark in the modification work.[5-7] T2D laccase contains three coppers and when oxidized exhibits characteristic spectroscopic signals of the type 1 and type 3 copper sites.[6-8] Evidently, the type 2 copper can be removed without significantly modifying the other copper sites. However, recent anion-binding studies involving laccase as well as structural studies of ascorbate oxidase suggest that the type 2 and type 3 coppers are so intimately related that they cannot necessarily be construed as separate sites.[4,9,10] Some workers have even speculated that all three of the copper ions within the type 2/type 3 cluster can be magnetically coupled.[11,12] The isotope-labeling studies described below provide striking new evidence of cooperative interactions within the cluster.

Experimental Section

Materials. Acetone powder of the latex of the Chinese lacquer tree (*Rhus vernicifera*) was harvested near Chu Shi, China, and supplied by Saito and Co., Osaka, Japan. Laccase was extracted and purified by the method of Reinhammar.[13] Isotopically pure ^{63}CuO and ^{65}CuO were

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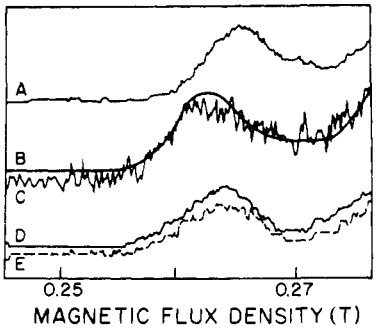

Figure 1. $m_I = -3/2$ transition of the type 2 copper in various laccase derivatives: A, ^{63}Cu-enriched laccase; B, ^{65}Cu-enriched laccase (smooth curve); C, ^{65}Cu-enriched T2D laccase reconstituted with ^{63}Cu; D, T2D laccase with natural-abundance copper reconstituted with excess ^{65}Cu; E, native laccase (- - -). All samples are in phosphate buffer at −150 °C. Microwave frequency 9.13 GHz.

obtained from Oak Ridge National Laboratory, Oak Ridge, TN. All other chemicals were reagent grade and were used without further purification.

Methods. All buffer solutions were prepared from deionized water and were passed through columns of (Bio Rad) Chelex 100 resin as needed in order to remove trace metal ions. Stock solutions of the metal ions were prepared by dissolving the metal oxide in aqueous HCl, diluting with acetate buffer, and adjusting to pH 6.0 by the addition of 6 M NaOH. All glassware was soaked at least 12 h in 50% H_2SO_4, and all plastic containers were soaked at least 24 h in an EDTA solution to minimize contamination by adventitious metal ions.

Apolaccase, i.e., completely demetalated laccase, was prepared by dialyzing the protein against 0.1 M pH 6.25 imidazole acetate buffer containing 50 mM potassium cyanide and 5 mM ascorbate.[14] (The pH was measured prior to addition of the cyanide.) Samples of type 2 depleted laccase were prepared as before.[7] The T2D product was further purified by elution from a cation-exchange column with an ionic strength gradient (0.015–0.15 M pH 6.0 phosphate). Typically, the first 30–50% of the protein eluent was discarded. The reconstitution of apolaccase or T2D laccase was carried out under anaerobic conditions by treating the protein with excess copper(I) and then incubating overnight at 4 °C in 0.1 M pH 6.25 imidazole acetate buffer. Excess metal ions were removed by dialyzing the sample against 0.015 M pH 6.0 phosphate buffer or 0.05 M pH 4.5 acetate. Unless otherwise indicated, a 4- to 8-fold excess of copper(I) was employed in the reconstitution of T2D laccase. All dialyses were carried out at about 5 °C under N_2(g) in custom-made fiber dialysis units. The fiber bundles contained 88 tubes with a molecular mass cutoff of 6000 and were supplied by Spectrum Medical Co.

Instrumentation. The X-band EPR spectra were recorded in standard 3-mm tubes with a Varian E-109 spectrometer equipped with a Varian E-935 data system. The cavity temperature was regulated at −150 °C by a Varian variable-temperature controller and verified using an Air Products APO-T1 thermocouple. Whether the phosphate or acetate buffer was used, the frozen solution was between pH 4 and 5 according to standard indicator dyes. Except as noted, X-band EPR spectra were run with a modulation frequency of 100 kHz, a modulation amplitude of 0.5 mT, and a power of 40 mW. The S-band data were collected at the National Biomedical ESR Center in Milwaukee with a modulation amplitude of 0.5 mT, a power of 20 mW, and a spectrometer that operates between 2 and 4 GHz and utilizes a loop-gap resonator circuit to excite the sample. The pH readings of buffer solutions were measured at room temperature with a Radiometer Model PHM 64 pH meter.

Results and Discussion

The only well-resolved transition of the type 2 copper in laccase occurs at the low flux density end of the EPR spectrum and represents the $m_I = -3/2$ transition. (This assignment assumes a negative coupling constant for the copper nucleus.) The $m_I = -3/2$ transitions of the frozen-solution, X-band EPR spectra of ^{63}Cu-enriched and of ^{65}Cu-enriched laccase are presented in Figure 1, parts A and B, respectively. Note that the resonance occurs at lower flux density in the ^{65}Cu derivative because of the larger nuclear magnetic dipole moment. As illustrated in Figure 1C, this effect can be used to show that the EPR-active site still

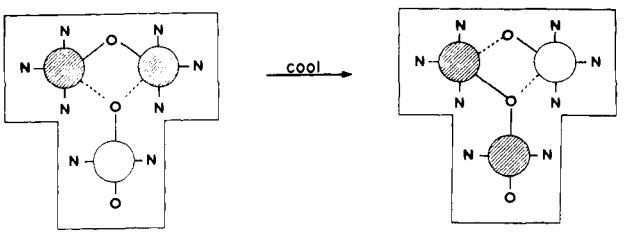

Figure 2. Schematic view of the type 2/type 3 cluster in ascorbate oxidase. The structure on the left is the room-temperature structure with the dominant antiferromagnetic coupling pathway depicted as solid lines connecting coppers through a bridging oxygen. The structure on the right is a possible structure for the low-temperature form of the enzyme wherein a different pair of coppers are antiferromagnetically coupled. EPR-silent coppers are shaded.

contains ^{65}Cu even after ^{65}Cu-enriched T2D laccase has been reconstituted with ^{63}Cu. By the same token, when ^{63}Cu-enriched T2D laccase is reconstituted with ^{65}Cu, the low-temperature EPR spectrum shows that ^{63}Cu is retained in the type 2 site. To explore these results further, we prepared a sample of T2D laccase containing the natural abundance of copper isotopes (69.1% ^{63}Cu, 30.9% ^{65}Cu) and reconstituted it with a 12-fold excess of ^{65}Cu. The $m_I = -3/2$ transitions of native laccase and the reconstituted sample are compared in parts D and E of Figure 1. Once again, the frozen-solution spectrum reveals that there is no significant enrichment of the isotope in the type 2 site. All of these experiments indicate that the copper center which is taken up during the reconstitution of T2D laccase is not EPR active in the low-temperature form of the enzyme.

Previous studies have shown that the type 2 copper exhibits distinct EPR spectra at room temperature and at low temperatures in frozen solution.[10,15] These findings as well as the present isotope data can be neatly explained if we postulate that different copper centers are EPR active in the two temperature regimes. The structure of the type 2/type 3 cluster in ascorbate oxidase provides a clue as to how this could happen. In the crystal, the type 2 copper has been tentatively assigned as the copper ion with two nitrogen ligands and a terminal water or hydroxide ligand which is directed away from the other copper centers in the cluster.[4] Although not found in the electron density map, a second water or deprotonated water ligand is probably directed toward the type 3 pair and could bridge the two sites.[12] The bridging oxide (or hydroxide) ligand that is evident in the electron density map links the type 3 coppers and is far removed from the type 2 site. Superexchange via this oxygen undoubtedly influences the magnetic coupling within the type 3 site.[4] In addition, the type 3 coppers are bound by a trigonal-prismatic array of imidazole nitrogens supplied by the protein. A schematic view of the room-temperature structure of the oxidized type 2/type 3 cluster in ascorbate oxidase appears at the left of Figure 2 where the solid lines to the bridging oxygen designate a pathway for antiferromagnetic coupling of the type 3 coppers. Laccase probably contains the same basic cluster in view of the sequence homology.[4,16] To explain the isotope data, we propose that another antiferromagnetic coupling pathway is dominant within the low-temperature form of the protein (Figure 2). The low-temperature coupling pathway would logically be mediated by the deprotonated water ligand that is proposed to reside between the type 2 and the type 3 sites.[12] With the proposed redirection of the dominant coupling pathway, a copper center which is EPR silent at room temperature becomes the EPR-active type 2 copper at low temperature.

In principle, the hypothesis can be tested by studying a mixed-isotope sample at room temperature; however, the line widths are too broad at X-band frequencies to distinguish the isotopes. Fortunately, the resolution is improved at S band. Figure 3 contains S-band EPR spectra of ^{63}Cu-enriched and ^{65}Cu-enriched

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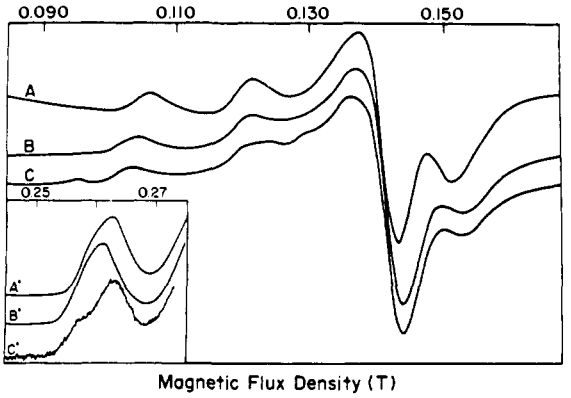

Figure 3. S-band EPR spectra of laccase derivatives recorded at room temperature in acetate buffer: A, ^{63}Cu-enriched laccase; B, ^{65}Cu-enriched laccase; C, ^{63}Cu-enriched T2D laccase that has been reconstituted with ^{65}Cu. Microwave frequency 3.92 GHz. Inset: Lowest flux density peaks in the corresponding X-band EPR spectra measured at 9.13 GHz and at -150 °C in acetate buffer. See text for a discussion of the shoulder in spectrum C'.

laccase along with the spectrum of a mixed-isotope sample prepared by reconstituting ^{63}Cu-enriched T2D laccase with the ^{65}Cu isotope. As predicted by the model, the room-temperature EPR spectrum of the mixed-isotope derivative shows that the type 2 copper site contains ^{65}Cu.

For completeness the $m_I = -3/2$ transitions of corresponding samples monitored at X-band frequency at -150 °C are presented in the inset in Figure 3. These data show that a different site is EPR active at low temperature. Signals 3C and 3C' were actually obtained with different solutions, but both samples were prepared in the same way. The extra feature on 3C' is assigned to splitting from ^{19}F due to a fluoride impurity which is bound to a fraction of the laccase molecules in solution.[17]

Even though the structural reorganization that has been identified occurs in frozen solution under nonphysiological conditions, the results are of considerable interest. In the first place, they indicate one type of reorganization that is feasible within the type 2/type 3 cluster. This is relevant because significant reorganization is likely to occur under active turnover, e.g., during the binding of reduced-oxygen intermediates or inhibitor anions.[10] In addition, this work adds credence to the recent proposal that a deprotonated water ligand can act as a bridge between the type 2 and the type 3 coppers in blue oxidases.[12] Finally, the results may explain why low-temperature EPR data implicate three nitrogens, rather than two, in the coordination sphere of the type 2 copper in tree laccase.[14]

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Registry No. Cu, 7440-50-8; hydroxide, 14280-30-9; laccase, 80498-15-3.

(17) Simulations as well as the results in parts D and E of Figure 1 reveal that the splitting *cannot* be explained by invoking a mixture of ^{63}Cu and ^{65}Cu in the type 2 site.

Genetically Directed Syntheses of New Polymeric Materials. Expression of Artificial Genes Encoding Proteins with Repeating -(AlaGly)$_3$ProGluGly- Elements

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Abstract: The goals of this work were to develop reliable methods for the bacterial expression of artificial repetitive polypeptides and to examine the connection between primary sequence and solid-state conformation in such materials. DNA fragments encoding variable numbers of repeating (AlaGly)$_3$ProGluGly elements (**1**) were constructed via chemical synthesis and cloning of short oligonucleotides followed by self-ligation. The resulting multimer population was size fractionated and cloned into an adapter plasmid that provides methionine residues at positions flanking the repetitive sequences. DNA multimers modified in this way, and encoding 10, 18, 28, or 54 repeats of sequence **1**, were transferred to an expression vector that features a T7 phage promoter, and protein expression in *Escherichia coli* was monitored via incorporation of [^{3}H]glycine. Purification of the target proteins was accomplished by selective precipitation with ammonium sulfate and subsequently with dilute acetic acid, followed by ion-exchange chromatography on DEAE-Sephadex. Typical yields of purified protein were ca. 10 mg/L of fermentation medium. The structure of the longest chain length variant was confirmed by amino acid compositional analysis, N-terminal protein sequencing (through 58 residues), matrix-assisted laser desorption mass spectrometry, ^{1}H and ^{13}C NMR spectrometry, cyanogen bromide cleavage, and combustion analysis. This polymer can be cast into coherent, optically clear films and displays a reversible glass transition at 170 °C. Thermal decomposition starts at ca. 250 °C and results ultimately in the loss of 70% of sample weight. Evidence from differential scanning calorimetry, X-ray scattering, and Fourier transform infrared spectroscopy suggests that this polymer and its CNBr cleavage product form amorphous glasses at room temperature.

Introduction

The solid-state and solution properties of polymeric materials are determined by five critical architectural variables: molecular size, topology, composition, sequence, and stereochemistry. Of these, topology alone is subject to control in an absolute sense; conventional polymerization processes allow only statistical control of each of the remaining structural variables. As a result, the polymeric materials currently in use—even in fundamental studies—are not pure substances but instead are mixtures characterized by substantial molecular heterogeneity.

Continuing progress in the synthesis, cloning, and expression of artificial genes has provided a powerful method for the preparation of structurally homogeneous "synthetic" polypeptides. This

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