Temperature and Anation Studies of the Type 2 Site in *Rhus vernicifera* Laccase†

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Received February 27, 1990; Revised Manuscript Received May 22, 1990

ABSTRACT: Temperature-dependent structural changes involving the type 2 site in laccase are probed by EPR studies of a derivative of laccase in which the type 1 Cu has been replaced by Hg(II) [Morie-Bebel, M. M., Morris, M. C., Menzie, J. L., & McMillin, D. R. (1984) *J. Am. Chem. Soc. 106*, 3677-3678]. At the temperature extremes (123 and 299 K), single well-defined species are present, but at intermediate temperatures (between 213 and 253 K), the presence of multiple structures is indicated. For the first time, the room temperature EPR spectrum of the type 2 copper has been resolved. Azide binding and fluoride binding have also been studied as a function of temperature. The results suggest that each anion preferentially interacts with the type 3 site in fluid solution and that these adducts can be trapped by rapidly cooling the sample to 123 K. Annealing the adducts at 253 K permits rearrangement and binding at an equatorial position of the type 2 Cu. This pathway to anation at the type 2 site contrasts sharply with previous studies which required a large excess of anions, and it reveals important insight into the flexibility of the type 2/type 3 cluster in laccase.

Laccase is a member of the family of blue copper oxidases which includes ascorbate oxidase and ceruloplasmin. The enzymes contain copper in distinctly different environments, referred to as the type 1 (blue), type 2 (normal), and type 3 (coupled binuclear) sites. All four copper ions in laccase are believed to be involved in the catalytic reduction of dioxygen to water (Reinhammar, 1984). By analogy with the binuclear copper site in tyrosinase, the type 3 coppers might be considered likely to form the site for dioxygen binding and reduction. However, azide binding studies (Morpurgo et al., 1982; Allendorf et al., 1985; Spira-Solomon et al., 1986) indicate that the azide ion may be capable of bridging the type 2 and type 3 copper centers. This realization led Solomon and co-workers to propose that the dioxygen reduction "site" should really be regarded as trinuclear (Allendorf et al., 1985; Spira-Solomon et al., 1986). The proximity of the type 2 and type 3 sites has subsequently been established by the solution of the crystal structure of ascorbate oxidase (Messerschmidt et al., 1989).

The structure and reactivity of the type 2/type 3 site in laccase appear to depend on a number of factors. For example, the binding affinity for N_3^- at the type 2 copper is dependent upon the oxidation state of the type 3 copper (Morpurgo et al., 1982; Allendorf et al., 1985). The conformational state of the protein is also important. Thus, the rate of exchange of a water ligand of the type 2 copper appears to vary depending on whether the "open" or the "closed" form of the enzyme is present (Goldberg et al., 1980). The kinetics of anation may also be affected by this conformational transition (Holwerda et al., 1982). In addition, EPR data suggest that the structure of the type 2 copper center is temperature dependent (Morpurgo et al., 1981, 1983, 1985) and that the presence of fluoride only affects the signal of the type 2 copper in the low-temperature form (Morpurgo et al., 1985).

However, structural information about the type 2 site is hard to obtain because of the lack of any resolved electronic absorbance bands and because of the overlap with the signal from the type 1 copper in the EPR spectrum. Fortunately, the EPR spectrum of the type 2 copper is clearly resolved for the mercury derivative of laccase (T1Hg-L) (Morie-Bebel et al., 1984). Results obtained from EXAFS (Klemens et al., 1989), UV-visible (Tamilarasan & McMillin, 1989), and EPR methods (Tamilarasan & McMillin, 1989; Morie-Bebel et al., 1986) have demonstrated that Hg(II) is bound selectively at the type 1 site in this derivative while copper is bound at the type 2 and type 3 sites. The purpose of the present study is to explore the effects that temperature and anion binding have on the type 2 copper in T1Hg-L.

Experimental Procedures

Materials and Methods. An acetone powder of the latex of the Chinese lacquer tree (*Rhus vernicifera*) was obtained from Saito and Co., Osaka, Japan. Isotopically pure ^{63}CuO was supplied by Oak Ridge National Laboratories, Oak Ridge, TN. All other materials were of reagent grade and were used without further purification. Buffer solutions were passed through Chelex 100 (Bio-Rad, Richmond, CA) columns to remove trace metals.

Laccase was extracted and purified by the method of Reinhammar with minor modifications (Reinhammar, 1972). The mercury derivative of laccase was prepared by the reported procedure (Morie-Bebel et al., 1984; Tamilarasan & McMillin, 1989) with the following changes: (1) A stock solution of $^{63}Cu(II)$ was prepared by dissolving ^{63}CuO in concentrated HNO_3, diluting with acetate buffer, and adjusting the pH to ~6.0 by the addition of NaOH(aq). (2) After apolaccase was combined with metal ions, the protein was eluted from a CM-Sepharose CL-6B column (Pharmacia Fine Chemicals, Uppsala, Sweden) by appliction of an ionic strength gradient (0.02–0.20 M) in phosphate at pH 6.0. Fractions with a copper-to-protein ratio between 2.9 and 3.0 were collected and concentrated. T1Hg-L samples analyzed for 1.0 ± 0.1 Hg per protein molecule.

The protein concentration was determined from the absorbance at 280 nm and $\epsilon_{280} = 86\,700\ M^{-1}\ cm^{-1}$, where the molar absorptivity was experimentally obtained on the assumption that native laccase contained 4.0 Cu per molecule. Copper and mercury were analyzed spectrophotometrically by using the biquinoline (Felsenfeld, 1960) and dithizone (Yamamura, 1960) methods, respectively. EPR spectra were

† This work was supported by Grant GM 22764 from the U.S. Public Health Service.
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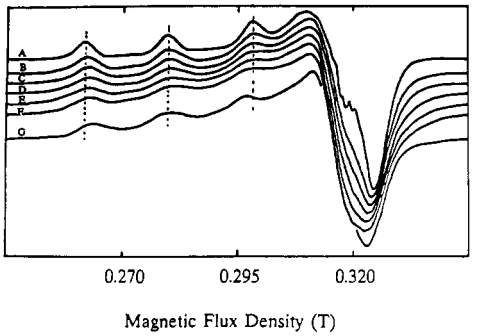


FIGURE 1: EPR spectra of T1Hg-L at pH 6.0 recorded as a function temperature: (A) 123, (B) 173, (C) 193, (D) 213, (E) 233, (F) 253, and (G) 299 K. Protein concentration ca. 1 mM. Spectrometer settings: ν = 9.10 GHz, modulation amplitude = 0.5 mT [except 1 mT for (G)], power = 40 mW. Spectra were scaled to a constant amplitude. Equally spaced vertical lines added as visual aids.

scaled and base line corrected by using the program "intspec" provided by Dr. Chris Felix of the National Biomedical ESR Center, Milwaukee, WI.

Anions were introduced into the protein solution from buffered stock solutions of the sodium salts. Unless otherwise indicated, the solutions were mixed and equilibrated for 10 min prior to freezing. The samples were frozen by immersing the EPR tube in a methylcyclohexane/liquid nitrogen slurry. All experiments were conducted in μ = 0.1 M pH 6.0 phosphate buffer, except as indicated. EPR simulations (Toy et al., 1971) were performed with the assumption of axial symmetry and without the inclusion of ligand superhyperfine coupling, except in the case of the fluoride adducts.

Instrumentation. The EPR spectra were recorded in standard 3-mm tubes or in quartz capillary tubes with a Varian E-109 spectrometer operated at X band and equipped with a Varian E-935 data system. The cavity temperature was regulated by a Varian variable-temperature controller and verified with an Air Products APD-T1 thermocouple. Spectrophotometric data were obtained from a Perkin-Elmer Lambda 4C spectrophotometer. A Radiometer Model PHM 64 pH meter was used to determine pH at room temperature.

RESULTS

Temperature Dependence. X-band EPR spectra of the mercury derivative (T1Hg-L) were recorded at seven different temperatures (Figure 1). In the parallel region of the spectrum, the $m_I = -3/2$ transition, which occurs at lowest flux density, gradually moved to higher flux density as the temperature was increased from 123 K. In contrast, the position of the $m_I = -1/2$ peak hardly changed, and the $m_I = +1/2$ feature shifted to lower flux density as the temperature increased. The complex temperature dependence was most evident in the $g_\perp$ region of the spectrum. The deep minimum in the derivative spectrum initially shifted to lower flux density as the frozen solution was warmed to 253 K. Then, in the room temperture spectrum, the minimum shifted back to higher flux density. Interestingly, the line width is broadest in the intermediate temperature range. Similar spectra were obtained when the sample was cooled slowly from room temperature.

The spectra of 123, 173, 193, and 299 K could be satisfactorily fit by a spin Hamiltonian under axial symmetry. Sample fits of the 123 and 299 K spectra are shown in Figure 2, and the spin Hamiltonian parameters for the parallel region are summarized in Table I. In contrast, the experimental spectra at 213, 233, and 253 K could not be fit with the assumption of only one species. The spectrum measured at

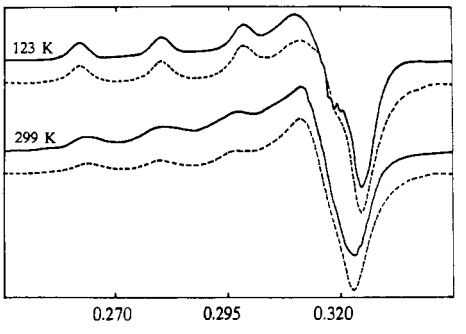


FIGURE 2: Comparison between experimental (—) and simulated (---) EPR spectra of T1Hg-L at 123 and 299 K. Spectrometer settings same as in Figure 1.

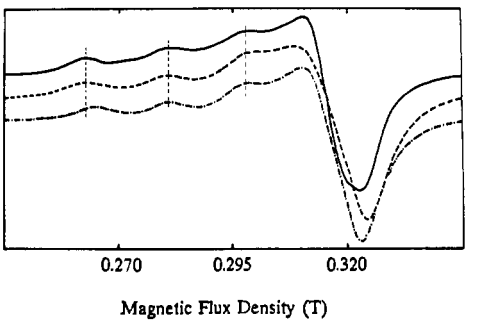


FIGURE 3: EPR spectra of T1Hg-L at 253 K: experimental (—); best simulation with a single spin Hamiltonian (–·–); composite simulation generated by adding 1.0 part 123 K simulation and 0.15 part 299 K simulation (---) from Figure 2. Equally spaced vertical lines added as visual aids.

Table I: Spin Hamiltonian Parameters for the Type 2 Copper in MDL and Native Laccase at Various Temperatures

protein	T (K)	$g_\parallel$	$A_\parallel$ ($cm^{-1} \times 10^4$)	$W_\parallel$[a] (mT)
native[b]	110	2.24	190	
T1Hg-L	123	2.247	190	2.8
T1Hg-L	173	2.246	186	3.5
T1Hg-L	193	2.247	186	3.8
T1Hg-L	299	2.258	167	4.5
native[b]	298	2.28	144	
native[c]	298	2.26	153	

[a] Width parameter. [b] Enzyme from Japanese lacquer tree (Morpurgo et al., 1985). [c] Enzyme from Vietnamese lacquer tree (Morpurgo et al., 1985).

233 and 253 K could not be accurately simulated because the hyperfine lines are not equally spaced in the parallel region. The use of rhombic parameters did not improve the fit of the simulation. Better agreement with the experimental spectrum was obtained when the calculated spectrum for 123 K was added to that calculated for 299 K with weights of 1.0 and 0.15, respectively (Figure 3). However, the composite simulation still gave a poor fit in the perpendicular region.

Effect of Anions. In line with previous results pertaining to native laccase (Allendorf et al., 1985; Morpurgo et al., 1985; Brändén et al., 1973), we verified that the presence of small amounts of azide or fluoride anion had no detectable effect on the room temperature EPR spectrum of the type 2 copper in T1Hg-L but that the UV absorbance of the protein was altered. More interesting effects occurred at lower temperatures.

When a solution of T1Hg-L was combined with 6 mol equiv of azide and then rapidly cooled to 123 K, the EPR signal of the type 2 copper was identical with that of untreated T1Hg-L. Incubation at 4 °C for up to 24 h before cooling to 123 K did not alter the result. On the other hand, if the sample was kept

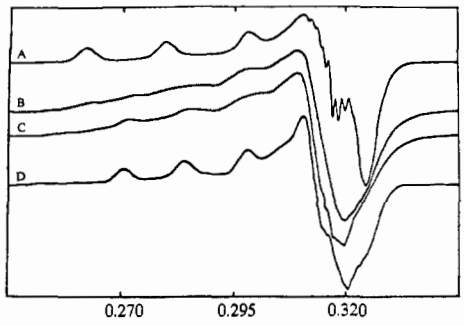


FIGURE 4: EPR spectra of T1Hg-L in the presence of 6 mol equiv of azide at pH 6.0: (A) 123 K, immediately after freezing; (B) after warming to 253 K; (C) after 1 h at 253 K; (D) after recooling to 123 K. Instrument settings same as in Figure 1. The protein concentration was 0.47 mM.

Table II: Spin Hamiltonian Parameters for Low-Temperature Adducts of Type 2 Coppers

sample	$g_\parallel$	$A_\parallel$ ($cm^{-1} \times 10^4$)	T (K)
T1Hg-L			
azide (6 equiv)	2.238	141	123
monofluoride	2.264	190	123
difluoride	2.295	170	123
native laccase			
azide[a] (large excess)		141	100
monofluoride[b]	2.27	187	110
difluoride[b]	2.29	171	110

[a] Laccase from Japanese lacquer tree (Morpurgo et al., 1982). [b] Laccase from Japanese lacquer tree (Morpurgo et al., 1985).

frozen but was warmed to 253 K in the EPR cavity, the signal broadened, and a new species evolved (Figure 4). After 1 h, the sample was cooled back to 123 K to improve the spectral resolution whereupon $g_\parallel$ and $A_\parallel$ for the azide adduct were determined from the best-fit simulation to be 2.238 and 141 $\times 10^{-4}$ cm^{-1}, respectively. These values agree with those reported for the native protein (Table II) (Morpurgo et al., 1982). The spectral changes were almost, but not completely, reversed by warming the sample back to room temperature. Thus, when the sample in Figure 4D was thawed at room temperature for a few minutes and refrozen at 123 K, the "unperturbed" type 2 EPR pattern returned, but some signal from the azide adduct was present as well. Development of the azide adduct was clearly a thermally activated process. When a frozen solution was incubated at 193 K, the signal of the azide adduct appeared over a period of a few days, but at 77 K, only minimal conversion took place after 2 weeks. As a control study, a sample of native laccase (which had been pretreated with 1 equiv of H_2O_2) was also incubated with 6 equiv of azide. Difference spectra showed that the type 2 copper signal of the native protein showed the same changes.

The effect of varying the amount of azide was investigated, and 6 equiv was found to be the optimum amount to effect the development of the adduct pattern cleanly. At less than 4 equiv of N_3^-, the adduct pattern failed to fully evolve, even after extensive incubation at 253 K. When 10 or more equiv of N_3^- was added, the spectrum recorded just after freezing also showed no evidence of adduct formation. However, at these higher azide concentrations, the spectrum evolved beyond the $A_\parallel = 141 \times 10^{-4}$ cm^{-1} species. Yet another signal, which was found by simulation to have $g_\parallel = 2.289$ and $A_\parallel = 136 \times 10^{-4}$ cm^{-1}, appeared after further annealing at 253 K.

Somewhat more complicated behavior was observed when 2 mol equiv of fluoride ion was combined with T1Hg-L (Figure 5). Immediately after being cooled to 123 K, a mixture of

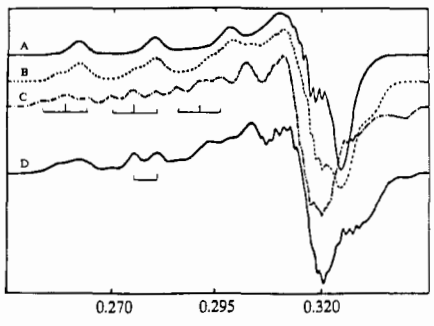


FIGURE 5: EPR spectra of T1Hg-L at pH 6.0: (A) untreated; (B) +2 equiv of fluoride at 123 K, just after freezing; (C) sample from (B) at 123 K after 3-h incubation at 253 K; (D) sample from (C) at 123 K after incubation at room temperature for ca. 5 min followed by recooling to 123 K. Instrument settings same as in Figure 1. The protein concentration was 0.60 mM.

the unperturbed type 2 signal and another signal which showed hyperfine splitting from a single fluoride was evident. The relative amounts of the signals were found to be independent of the equilibration time prior to freezing. If the sample was warmed to 253 K for 1 h and then recooled to 123 K, the relative intensity of the unperturbed type 2 signal was reduced, and evidence for a species that showed splitting from two approximately equivalent fluorine nuclei was present. The spectrum in Figure 5C was obtained after the sample was subsequently incubated at 253 K for a total of 3 h. In this spectrum, each transition in the parallel region is split into a triplet due to coupling with two fluorine nuclei. In contrast to what was seen with azide, the unperturbed low-temperature type 2 EPR signal did not reappear when the sample was thawed and refrozen; rather, the monofluoride species was dominant after the sample was refrozen (Figure 5D). Subsequent incubation at 253 K resulted in the return of the difluoride adduct. Both the monofluoride and difluoride adduct EPR spectra were simulated. Spin Hamiltonian parameters are included in Table II, and it can be seen that they agree with literature values.

Unlike azide, varying the amount of fluoride present in solution changed the nature of the species present initially after freezing. While 2 equiv of F^- resulted in a mixture of the unperturbed type 2 and the monofluoro adduct, addition of 10 equiv of fluoride resulted in a predominantly monofluoro signal, with a small amount of difluoro present. Upon increasing to 20 equiv of F^-, the difluoro adduct was nearly fully developed just after freezing. The spectra recorded after annealing of each of these samples were indistinguishable.

A slight excess of fluoride was also combined with T1Hg-L in $\mu = 0.1$ M pH 4.5 acetate buffer. In contrast to the result of pH 6.0, the fully evolved triplet pattern of the difluoride adduct was observed in the EPR spectrum at 123 K immediately after freezing. Incubation of the sample at 253 K overnight did not result in any change in the spectrum. Also, in contrast to the pH 6.0 results, thawing and refreezing of the sample did not result in the loss of any fluorine superhyperfine structure.

Discussion

Room Temperature EPR Spectrum. The room temperature EPR spectrum that we have observed for the type 2 copper in the mercury derivative of laccase, T1Hg-L, is quite different from the spectrum that was previously reported for the same copper of the native protein (Table I) (Morpurgo et al., 1981). The previously reported spectrum was obtained by a difference

method; in particular, the spectrum of a type 2 depleted laccase derivative was subtracted from the spectrum of native laccase. A pitfall of this method is that the type 1 signal may not be identical in both forms. Indeed, LuBien et al. (1981) have shown that the spectral properties of the type 1 copper vary with the redox state of the type 3 copper, and this is relevant because the type 3 copper tends to be reduced in preparations of type 2 depleted laccase (Hanna et al., 1988; LuBien et al., 1981). Apparently, Morpurgo et al. (1985) also simulated the spectrum of native protein; however, the inherent difficulty of accurately resolving the type 2 signal under these conditions has already been discussed. Accordingly, we regard the spectrum in Figure 1 as more representative of the type 2 copper in laccase. In principle, the presence of Hg(II) in the type 1 site could alter the structure of the type 2 copper, but this does not appear to be a problem because published data show that mercury has no effect on the type 2 copper at low temperature where the EPR signals of both coppers in laccase are better resolved (Morie-Bebel et al., 1984, 1986). Furthermore, our EPR parameters compare favorably with the room temperature values reported for Vietnamese laccase (Table I) for which the type 2 signal is better resolved (Morpurgo et al., 1985).

Temperature Dependence. Morpurgo et al. (1981) first reported that the EPR signals from both type 1 and type 2 coppers in laccase were different at room temperature and at 77 K, and they suggested that a structural change occurred upon freezing. In particular for the type 2 copper signal, they found that $g_{\|}$ decreased whereas $A_{\|}$ increased upon cooling (Table I). A later report from the same laboratory interpreted this to mean that the coordination geometry changes from trigonally distorted square pyramidal at room temperature to a more tetragonal geometry at low temperature (Morpurgo et al. 1983). Subsequent studies showed that the $m_I = -3/2$ transition of the type 2 copper steadily shifted position over a wide temperature range and therefore that the conformational transition was not simply a consequence of the onset of freezing (Morpurgo et al., 1985).

Similar behavior is observed with T1Hg-L, but the improved resolution allows us to examine the spectral changes of the type 2 site in more detail. The fact that we have been able to simulate the spectrum of the type 2 copper in T1Hg-L at 299 K and at or below 193 K suggests that the site has a well-defined, albiet distinct, structure in these temperature regimes. At intermediate temperatures (213–253 K), the signal cannot be modeled by a single species because of the uneven hyperfine splitting in the parallel region. Irregular spacing between hyperfine lines can be caused by strain phenomena (Hagen, 1981; Giugliarelli & Cannistraro, 1985; Cannistraro & Giugliarelli, 1986), but strain effects in other copper systems have been much smaller. A simple "A → B" transition between the low-temperature and room temperature structures is also ruled out because in the intermediate temperature regime the spectrum does not correspond to a summation of signals from a high-temperature and a low-temperature form. The most likely explanation is that the structure changes incrementally over a range of temperatures; i.e., there is at least one intermediate form.

Effect of Temperature on Anion Binding. As early as 1974, the complex nature of the interaction of laccase with azide was becoming evident (Morpurgo et al., 1974). Two different types of binding were identified and were dubbed high- and low-affinity binding on the basis of their respective equilibrium constants. High-affinity binding was observed with near-stoichiometric concentrations of azide and was characterized by an increase in the visible absorption spectrum at around 500 nm. No corresponding perturbation in the low-temperature EPR spectrum was observed; hence, the high-affinity binding was tentatively ascribed to the type 3 copper. At higher azide concentrations, a new band was observed in the room temperature visible absorption spectrum at round 400 nm. A corresponding change in the low-temperature EPR spectrum of the type 2 copper strongly implicated the type 2 Cu as the site of low-affinity azide binding. The binding of azide was also shown to be dependent upon the oxidation state of the enzyme as well as the pH (Morpurgo et al., 1974, 1983). Later work by Solomon and co-workers indicated that the presence of type 2 copper had an influence on the anion binding at the type 3 site (Allendorf et al., 1985; LuBien et al., 1981). In particular, they showed that oxidized T2D laccase only exhibited a low-affinity binding of azide with a λ_{max} at 450 nm.

An important breakthrough came when Morpurgo et al. (1982, 1983) showed that the high- and low-affinity binding of azide reflected an inherent heterogeneity in the native enzyme. Their results indicated that the high-affinity binding of azide could be traced to laccase molecules in which the type 3 site was reduced. Since the bulk of the resting laccase molecules have an oxidized type 3 site, the low-temperature EPR spectrum was unperturbed except at high azide concentrations, in which case a new signal with an $A_{\|} = 13.5$ mT was observed. On the other hand, in the presence of a reducing agent such as hydroquinone, the EPR spectrum of the type 2 copper could be observed in the high-affinity adduct form of the enzyme with $A_{\|} = 10.0$ mT. On the basis of comparisons with studies of half-met-hemocyanin and half-met-tyrosinase, Morpurgo et al. (1982) suggested that high-affinity azide binding occurred when azide was a bridging ligand between the oxidized form of the type 2 site and the reduced form of the type 3 site. In line with this proposal, Solomon and co-workers subsequently used a low-temperature MCD technique to obtain direct evidence that the low-affinity form of azide binding involved an interaction with both a diamagnetic and a paramagnetic metal center (Allendorf et al., 1985; Spira-Solomon et al., 1986). They also showed that azide could bind to the oxidized type 3 site in either a coupled or an uncoupled form (Spira-Solomon et al., 1986). Furthermore, their anion competition studies suggested that F^- displaced N_3^- from type 2 Cu only in those molecules with oxidized type 3 sites.

The binding of fluoride with laccase has also proven to be complicated. Winkler et al. (1982) have demonstrated that at pH 6 the low-temperature EPR signal of the type 2 copper is partially split into doublets when 1 equiv of fluoride is present. Morpurgo et al. (1983) found the same to be true when 7 equiv of fluoride was added. On the other hand, a difluoride adduct with a characteristic triplet superhyperfine pattern is apparent when 150–1000 equiv of fluoride is added (Brändén et al., 1973; Morpurgo et al., 1983). Despite the fact that fluoride does not perturb the room temperature EPR spectrum, UV difference spectra demonstrate that a strong interaction with 2 equiv of fluoride occurs in room temperature solution (Winkler et al., 1982). As we shall see, our studies with T1Hg-L show that these results can be understood in terms of anion binding to two different copper centers.

First note that at room temperature we see no evidence of *high-affinity* binding of azide to T1Hg-L in the visible spectrum. This is consistent with X-ray absorption data (Cole et al., 1990) which have shown that the type 3 coppers are oxidized in T1Hg-L. Nevertheless, the data in Figure 3 reveal

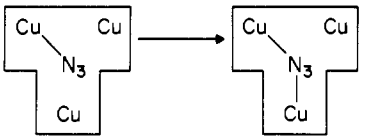


FIGURE 6: Proposed structural model for formation of the azide adduct. The type 2/type 3 site is schematically depicted as a triangular cluster with the type 2 copper lying below the type 3 pair.

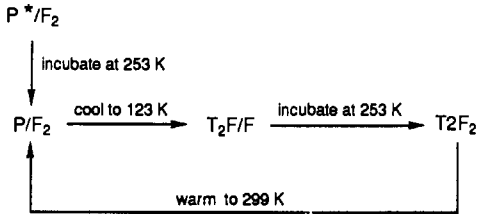
that the protein binds azide avidly at temperatures as high as 253 K. Since it is not clear that the anion is mobile in the frozen matrix, our working hypothesis is that the initial binding of the anion to the protein occurs prior to freezing. At least for azide, the anion need not be bound at room temperature since the equilibrium may be temperature dependent and could become more favorable at a reduced temperature. The most likely site for formation of a coordinate covalent bond is the type 3 site of laccase since the EPR spectrum of the type 2 copper is unperturbed. Furthermore, the initial adduct cannot be the result of a simple electrostatic interaction because the buffer anion is present at a much higher concentration than azide.

The initial adduct is obviously a kinetic product because with time and sufficient thermal energy the EPR data show that the azide ion migrates to an equatorial position of the type 2 copper. Preferential formation of the kinetically controlled product probably indicates that in fluid solution the type 3 copper has a higher affinity for azide than the type 2 copper. An alternative explanation is that the kinetic product involves azide binding in an axial position of the type 2 copper center. Since the valence orbitals of the anion would have minimal overlap with the $d_{x^2-y^2}$ orbital of the copper, it is possible that formation of this adduct would have minimal effect on the EPR spectrum. Although this explanation cannot be rigorously excluded, the previous model is preferred because binding at the type 3 copper is more consistent with the relatively high affinity which is required for azide binding.

The key transformation of our proposed model is shown in Figure 6 where, by analogy with ascorbate oxidase, the type 2/type 3 site is depicted as a triangular cluster with the type 2 site next to the type 3 pair. Actually, our results do not require that the azide remain attached to the type 3 site once the anion is attached to the type 2 copper. The structure offered for the final state is based on the results of Solomon and co-workers (Spira-Solomon et al., 1986). By analogy with fluoride binding (Morie-Bebel et al., 1986), the azide is assumed to bind in an equatorial position of the type 2 site. The other spectrum, which was observed when >10 equiv of azide was added to T1Hg-L, may be indicative of a diazide adduct, perhaps one that is analogous to the difluoride adduct discussed below. However, in the absence of resolved ligand hyperfine splitting in the EPR spectrum, we cannot be certain that the second azide binds at the type 2 site. The results are nevertheless quite interesting because, to our knowledge, this species has not been previously reported in the literature.

A further extension of the model is required to explain fluoride binding at pH 6. Four different species are required by the spectral data. First, the room temperature UV difference spectrum reveals that there is a species, denoted P/F_2 in Scheme I, in which two fluorides are bound to laccase but neither is bound at the type 2 copper, at least in an equatorial position. In line with the interpretation of the azide data, it seems likely that the fluoride binds preferentially to the type 3 copper at room temperature. However, immediately after cooling to low temperature, there is evidence from the EPR data for a species with one fluoride bound at an equatorial

Scheme I



position of the type 2 copper. This species will be denoted as T2F/F. Finally, after incubation at 253 K, the difluoride adduct of the type 2 copper ($T2F_2$) is formed. Thawing returns the sample to the P/F_2 state, as in Scheme I. We have also included in Scheme I a P^*/F_2 state which must also be present in the original resting form of the protein. This species is required because T2F/F is not formed quantitatively when the sample is initially frozen. However, the P^*/F_2 form does convert to $T2F_2$ upon incubation at 253 K. Whether P/F_2 and/or T2F/F is an intermediate in this process is unclear. Note that all of the protein reverts to the P/F_2 state when the sample is warmed to room temperature, at least for some period of time, because the T2F/F adduct forms quantitatively when the sample is refrozen. In this case, when enough fluoride is present, the difluoride adduct with type 2 copper can be observed directly in the initially frozen solution. Interestingly, the results of the F^- binding study at pH 4.5 demonstrate that the barrier(s) to formation of $T2F_2$ is (are) sharply reduced at lower pH. This suggests that a protonation step involving a group in the vicinity of the T2/T3 site influences the fluoride binding. Morpurgo et al. (1982) have previously shown that decreasing the pH enhances azide binding.

Thus far, we have established that when the annealing method is used, 6 equiv of N_3^- is the minimum amount needed to cleanly generate the azide adduct, while 2 equiv of F^- is sufficient to give the difluoride adduct with the type 2 copper. We have discussed the way in which we believe the anions interact with the copper sites during the annealing process. We have as yet, however, not reconciled our results with previous reports in which the azide (Morpurgo et al., 1981) and difluoride (Morpurgo et al., 1985) adducts were observed immediately after freezing. Evidently, addition of a large excess of the anions provides an alternate pathway to adduct formation. To accommodate these results, we must postulate the association, if only transiently, of a third anion with the type 2/type 3 site. The site at which the third anion binds is unknown; it could be the type 2 site or it could be the type 3 site. The only requirement is that formation of this adduct must facilitate coordination of the anion at an equatorial position of the type 2 copper during the freezing process. Furthermore, the data in Table II suggest that the final product is the same whether laccase is treated with a large excess of anion or annealed at 253 K in the presence of a slight excess of anion.

Relevance to Function. Most of the processes described above occur at low temperature, far from physiological conditions. However, it is clear that the dioxygen reduction "site" in the enzyme must be very flexible. During catalysis, several different forms of the substrate must be bound including diatomic and monoatomic intermediates/reduction products. Machinery for funneling protons to these intermediates must be present, and as mentioned in the introduction, "open" and "closed" forms of the enzyme are believed to exist under physiological conditions. The flexibility in the protein that has been demonstrated in the temperature studies and the

cooperativity between the type 2 and type 3 sites that has been observed in the anion binding studies may ultimately provide clues for understanding how the enzyme carries out the reduction of O_2 to H_2O so efficiently.

ACKNOWLEDGMENTS

We thank R. Tamilarasan and W. E. Antholine for valued assistance.

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