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Effect of subzero incubation on fluoride binding by laccase

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Abstract

We have refined a useful incubation method for preparing adducts of tree laccase with inhibitor anions; however, the technique should have wider application. The procedure involves incubating a previously frozen aqueous solution at subzero temperature. Factors influencing the amount of adduct that forms include the nature of the buffer, pH changes that occur at subzero temperatures and the presence of glycerol. In the absence of a glassing agent like glycerol, the phase separation and solute pooling that occur with ice formation help drive adduct formation. The results reveal several important factors to consider in designing or interpreting low-temperature spectroscopic investigations.

Keywords: EPR of type 2 copper; Fluoride inhibitor; Freezing effects, Glycerol; Inhibitor; Subzero incubation method; Tree laccase

1. Introduction

Laccase is a blue copper oxidase that catalyzes the oxidation of a variety of electron donors by molecular oxygen [1–3]. In the process, laccase orchestrates the four-electron reduction of molecular oxygen without releasing hydrogen peroxide or other partially reduced forms. The focus in the following is on the enzyme known as tree laccase which comes from the oriental lacquer tree (*Rhus vernicifera*). As regards physiological function, tree laccase probably has a role in wound healing processes [1,4] and possibly in lignification as well [5,6]. The native enzyme contains four copper ions, originally assigned to three spectroscopically distinct binding sites [2,7]. Recent crystallographic work on a related enzyme, ascorbate oxidase, suggests a function-oriented classification of metal centers [8,9]. Within each subunit of ascorbate oxidase, one of the copper ions, type 1 copper, acts as an electron transfer station that relays electrons to the dioxygen reduction site several angstroms away [8,9]. The latter site is actually a trinuclear cluster composed of the other three copper ions of the catalytic unit. They correspond to type 2 copper and the type 3 pairs in the original spectroscopic classification scheme [1,2]. Even before the X-ray results appeared, studies of binding interactions with inhibitor anions established the integral character of the type 2 and the type 3 copper centers [10].

Certain inhibitors, including fluoride ion, operate by binding within the dioxygen reduction site [7,10–13]. These adducts are of interest in part because

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they may mimic physiologically relevant intermediates in which the enzyme binds partially reduced forms of dioxygen. The TlHg derivative of laccase serves as an ideal vehicle for studies of inhibitor complexes because the dioxygen reduction site is the only EPR-active center due to the presence of $Hg^{(II)}$ in the type 1 site [14,15]. With fluoride as the inhibitor, EPR studies show that up to two anions can bind to the EPR-active copper center in the trinuclear cluster, although formation of the difluoride adduct usually requires treatment with 100 or more eq. of fluoride [12,16]. However, Severns and McMillin made the interesting discovery that they could form the difluoride adduct essentially quantitatively by incubating TlHg laccase with only two eq. of the anion at −20°C in a sodium phosphate buffer [17]. With the view that diffusion would not be important at −20°C, they interpreted the results in terms of an intramolecular shift of the anion from one type of binding site to another within the enzyme.

Two recent reports have led us to revisit this method of adduct formation. First, Hill and Buckley called attention to the fact that proteins dissolved in sodium phosphate buffers tend to experience a sharp drop in pH as freezing occurs [18]. This effect could be quite important because laccase binds anions in a pH-dependent fashion [17,19]. Second, an incisive paper by Yang and Brill showed that a similar incubation method facilitated the uptake of fluoride by methemoglobin [20]. In light of previous work by Luyet [21], they pointed out that diffusion is possible within fluid domains that persist at temperatures as low as −20°C. For our follow up studies, we have chosen to focus on fluoride binding because laccase readily forms both a monofluoride and a difluoride adduct.

2. Experimental

2.1. Materials

Saito and Co., Osaka, Japan, supplied us with acetone powder of latex harvested from the Chinese lacquer tree (*Rhus vernicifera*). Oak Ridge National Laboratories, Oak Ridge, TN, supplied ^{65}CuO. The glycerol was redistilled, MB Grade from Boehringer Mannheim. The CM Sepharose CL-6B cation-exchange resin came from Pharmacia Fine Chemicals, Piscataway, NJ. Buffer salts and other miscellaneous chemicals were reagent grade materials obtained from standard suppliers.

2.2. Methods

Unless otherwise noted, we used all materials as received. To remove adventitious metal ions, we passed buffer solutions through Chelex 100 columns. Except when diluted with glycerol, the buffers had an ionic strength of 0.1 M. For the isolation of laccase, we followed the method of Reinhammar [22]. The ratio of the absorbance at 280 nm to that at 614 nm was 16 for the purified product. Published methods allowed conversion to ^{65}Cu-enriched TlHg laccase [14,17,23]. To obtain high quality product, we loaded the crude TlHg laccase onto a CMCL-6B column and eluted with an ionic strength gradient (0.02–0.2 M pH 6 potassium phosphate). We then pooled fractions with a copper-to-protein ratio between 2.8 and 3.0.

We estimated the protein concentration from the absorbance at 280 nm and $\epsilon_{280} = 87600\ M^{-1}cm^{-1}$, the molar absorptivity determined in separate experiments on the assumption that the native enzyme contained 4.00 Cu per molecule. For a given protein solution, we determined the copper concentration colorimetrically by the method of Felsenfeld with 2,2′–biquinoline [24]. For EPR studies the protein concentration was 0.6 mM in a volume of 150–200 μl. We introduced the anion as an aliquot of a stock solution, prepared from the sodium or potassium salt as needed. Before running the EPR spectrum, we froze each sample in a standard 4 mm O.D. quartz tube by immersion in a methycyclohexane/liquid nitrogen slurry. For spectral measurements we used a modulation frequency of 100 kHz, a power of 40 mW and a temperature of 123 K. We simulated EPR spectra as before [17].

We inferred the pH of a frozen solution from the color of a corresponding solution containing a universal indicator [18,25]. Control studies showed that no significant change in pH occurred with the freezing of a solution of the indicator in deionized water. To prepare color standards, we added 15 drops of indicator to 30 ml of a series of buffers from pH 1 to

pH 8. In each case we verified the pH reading with the pH meter. We froze the standards in polystyrene cells by immersion into a bath of liquid nitrogen. From the color, we could estimate the pH to within about 0.5 units.

2.3. Instrumentation

Some of the EPR spectra came from a Varian E-109 X-band spectrometer equipped with a E-935 data system. A Varian variable temperature controller regulated the temperature of the sample holder which we measured with an Air Products APD-T1 thermocouple. A Bruker ESP300E spectrometer with an ER 4121 VT liquid nitrogen variable temperature system yielded the other EPR data. A Perkin-Elmer lambda 4C spectrophotometer provided the UV-visible spectra. Other equipment included a Radiometer model PHM 64 pH meter, for room-temperature readings, and an Amicon ultrafiltration device.

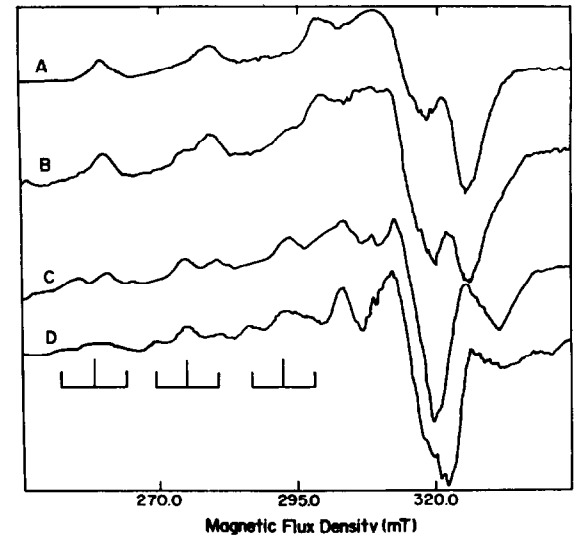

Fig. 1. EPR spectra of TlHg laccase in a 0.1 M pH 6 citrate buffer at 123 K. (A) untreated, (B) with 3 eq. fluoride immediately after freezing, (C) sample in B after exhaustive incubation at 253 K, (D) with 10 eq. fluoride after exhaustive incubation at 253 K. The microwave frequency was 9.08 GHz, and the modulation amplitude was 0.5 mT. The stick diagrams illustrate the hyperfine pattern of the difluoride adduct.

3. Results

3.1. Effect of freezing on pH

For most of the buffers used, there were only minor changes in pH upon freezing. For example, with the potassium phosphate buffer, the drop was about one unit. With the acetate and citrate buffers the drop was about half a unit on freezing. However, we confirmed that there was a dramatic decrease of about 3 pH units upon freezing of the sodium phosphate buffer. On the other hand, with 25% glycerol present, the pH of the sodium phosphate buffer dropped only a unit or so.

3.2. Effect of pH

In a pH 6 citrate buffer, TlHg laccase gave the EPR spectrum presented in Fig. 1A. When the solution also contained three eq. of fluoride, we obtained the spectrum shown Fig. 1B. After several hours incubation at −20°C, the signal evolved into Fig. 1C. During the incubation, each line of the metal hyperfine pattern in the $g_{\|}$, or low flux density, region of the spectrum developed the doublet structure characteristic of the monofluoride adduct. Comparison with Fig. 1B shows that the monofluoride adduct was also present immediately after freezing, but only as a minor species. Experiments carried out in pH 6 potassium phosphate buffer produced a similar sequence of spectra.

In contrast, prolonged incubation of a sample in a pH 6 citrate buffer solution containing ten eq. of fluoride yielded the spectrum in Fig. 1D. In this case the superhyperfine structure corresponds to a binomial triplet which indicates formation of the difluoride adduct. The intensity enhancements at 275 and at 303 mT in Fig. 1C suggest that some difluoride adduct also forms at lower anion-to-protein ratios.

At pH 5, incubation had less of an effect, and no single species was dominant. Fig. 2A is a control spectrum of TlHg laccase in a pH 5 sodium acetate buffer. When the solution contained three eq. of fluoride, we obtained the spectrum shown in Fig. 2B. A comparison with Fig. 1 shows that the latter reflects a mixture of the monofluoride and difluoride adducts. Incubation at −20°C produced only minor

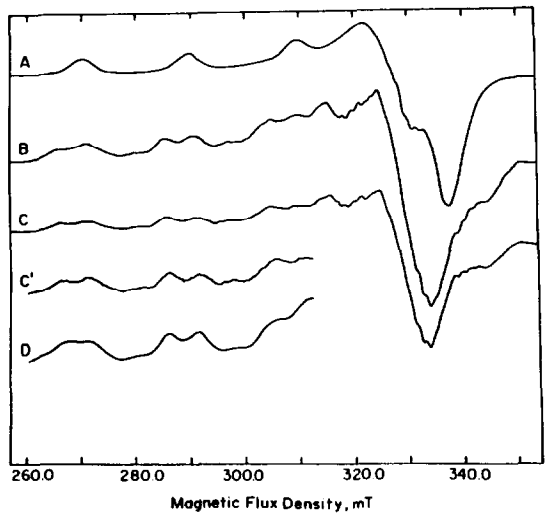

Fig. 2. EPR spectra of TlHg laccase in a pH 5 sodium acetate buffer at 123 K. (A) untreated protein, (B) sample in A with 3 eq. of fluoride immediately after freezing, (C) sample in B after exhaustive incubation at 253 K, (C′) enlargement of the parallel region of C, (D) simulation of C′; see text for details. The microwave frequency was 9.44 GHz, and the modulation amplitude was 0.8 mT.

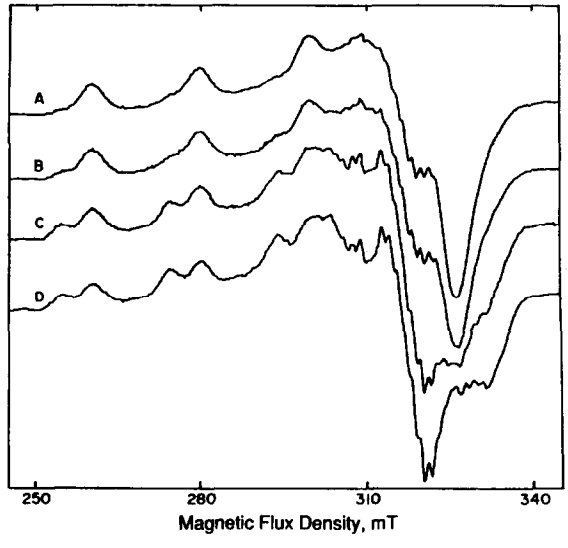

Fig. 3. EPR spectra of TlHg laccase exposed to 3 eq. F^- in a 0.1 M sodium phosphate buffer diluted to contain 25% glycerol. We recorded each spectrum at 123 K after incubation at 253 K for (A) 0 h, (B) 4 h, (C) 23 h, (D) 48 h. The microwave frequency was 9.08 GHz and the modulation amplitude was 0.5 mT.

Table 1
EPR parameters for the type 2 cooper of tree laccase

Species	$g_{\|}$	$A_{\|}$(Cu) [a] (cm^{-1})	A (F) (cm^{-1})	Ref.
TlHg	2.24	0.0207 [b]		This work
Monofluoride	2.26	0.0209 [b]	0.0060	This work
	2.26	0.0190		1990 [17]
	2.27	0.0187	0.0058	1985 [12]
Difluoride	2.29	0.0189 [b]	0.0060	This work
	2.30	0.0170	0.0060	1990 [17]
	2.29	0.0170	0.0058	1985 [12]

[a] ^{63}Cu except as noted.
[b] ^{65}Cu in sample.

spectral changes (Fig. 2C), and experiments carried out in a pH 5 citrate or ammonium acetate buffer gave similar results.

3.3. Effect of glycerol

The influence of glycerol varied with the buffer. In most cases the introduction of glycerol had no marked effect on the incubation of samples of TlHg laccase that contained 3 eq. of fluoride. However, the presence of the glycerol profoundly altered the results obtained in the sodium phosphate buffer. In previous work, Severns and McMillin found that TlHg laccase readily evolved into the difluoride adduct upon incubation at −20°C in the presence of only 2 eq. of fluoride [17]. Their experiments involved a sodium phosphate buffer prepared at room temperature to buffer at pH 6. A similar experiment carried out in 25% glycerol gave the results shown in Fig. 3. With the mixed solvent only the monofluoride adduct formed as indicated by the doublet structure that emerged during incubation.

4. Discussion

4.1. Basis of the incubation method

In previous EPR studies of TlHg laccase, Severns and McMillin assumed that a diffusive encounter with the anion would not be possible in frozen solution at −20°C [17]. They proposed that, during

the incubation period, the anion transferred to the EPR-active copper center from another binding site within the type 2/type 3 cluster. Reports that the presence of fluoride influences the protein absorbance, but not the room-temperature EPR spectrum [12,26], helped shape the original model. However, Luyet's freezing experiments [21] suggest a more straightforward explanation of the incubation effect. Those studies and others show that, with the onset of freezing, the developing ice phase excludes solutes and creates pools enriched in protein and anions [20,21]. These domains remain liquid at temperatures at least as low as −20°C due to freezing point lowering. As a consequence, the freezing process itself promotes inhibitor binding via sheer mass action, not to mention the fact that the equilibrium constant for adduct formation may increase as the temperature decreases. However, during the freezing process the kinetics of anion uptake may be such that little adduct formation occurs before the temperature drops so low that the sample becomes highly viscous or completely rigid. When this is the case, warming the sample back to ca. −20°C may promote the reaction due to the reappearance of the fluid phase. Figs. 1B and 1C give graphic evidence of successful incubation at −20°C.

4.2. pH effects

The results are, however, very pH dependent. In contrast to what happens at pH 6, Fig. 2 shows that significant adduct formation is apparent immediately after freezing at pH 5 and that extended incubation at −20°C does not significantly alter the composition. The other complication is that there is a considerable amount of the difluoride adduct as well as the monofluoride adduct present. To gain some idea about the distribution of species, we expanded the $g\|$ region of the spectrum and simulated with the spin hamiltonian parameters listed in Table 1. For the sake of comparison, the Table also contains values previously reported in the literature. Figs. 2C′ and 2D present the experimental and the calculated spectra. The results indicate there are approximately equal amounts of the two types of fluoride adducts as well as 10–15% unreacted laccase in the sample. (The presence of free laccase accounts for the anomalous signal intensity at 270 mT.) The fact that bound fluoride is evident immediately after freezing suggests that the rate of fluoride binding may be more rapid at pH 5. This would be consistent with the work of Morpurgo et al. who reported higher formation constants for adducts formed at pH 4.5 compared with pH 6 [19]. At an even lower pH, however, Severns and McMillin found that fluoride binding was incomplete immediately after freezing and that incubation at −20°C spurred adduct formation [17]. They actually used a pH 6 sodium phosphate buffer that experiences a pH drop of 2–8 units in frozen solution due to the precipitation of the disodium monohydrogen phosphate component [27]. The other interesting point is that there was little, if any, of the difluoride adduct present immediately after the freezing in the sodium phosphate buffer, despite the fact that this is unequivocally the dominate species in solution after incubation [17]. It is evident that the kinetics and the equilibria are both complex functions of the pH. One important consideration may be that at pH's as low as 4, fluoride forms an ion pair with hydronium ion [28]. This could influence the kinetics of fluoride binding and, hence, the species distribution immediately after freezing. Another effect that could influence the kinetics is the fact that the pH changes *during* the freezing process with the sodium phosphate buffer.

4.3. Glass formation

The function of a glassing agent like glycerol is ordinarily to improve the optical properties of low-temperature aqueous solutions, but our results show that glycerol has an impact on the speciation as well. Some time ago, Spira-Soloman et al. found that they formed only the monofluoride adduct of laccase in a 50% glycerol solution containing at least 10 eq. of fluoride ion, whereas the difluoride adduct was evident in a 0.1 M potassium phosphate buffer [29]. To rationalize these observations, they suggested that glycerol might bind competitively at the type 2 copper or have a nonspecific effect on the tertiary structure of the protein. In the context of what we have seen, it seems more likely that the difference is due to the influence glycerol has on the microenvironment of the protein.

More specifically, the presence of glycerol alters the medium in at least two important and interrelated

ways. The first is to increase the viscosity and hence to slow down diffusion. Secondly, the presence of the glassing agent inhibits the formation of long-range ice structure. As a consequence, upon cooling the medium retains better homogeneity, and there is less pooling of solutes. Yang and Brill discuss these effects in detail [20]. With glycerol present precipitation is less favorable in the case of our sodium phosphate buffer, and there is a much smaller drop in the pH. Without the benefit of the pH change, Fig. 3 shows that the system does not progress beyond the monofluoride adduct, even after exhaustive incubation. The results of Spira-Soloman et al. suggest that glycerol has a similar effect with a potassium phosphate buffer [29]. On the positive side, the thermodynamic analysis is more straightforward for data obtained in glassing media. Moreover, the incubation method is still useful for equilibrating the sample and encouraging adduct formation to the extent that uptake is an exothermic process.

5. Conclusions

Incubating a previously frozen aqueous solution containing laccase and inhibitor anions at −20°C can be a useful method of promoting uptake for EPR studies. The key to the method appears to be the natural phase separation that occurs with the onset of freezing. Although we cannot discount a role for the EPR-nondetectable intermediate proposed earlier [17], a model that incorporates diffusion provides a much simpler explanation of the fact that increasing the anion concentration increases the probability that the adduct is present immediately after freezing. The diffusion model is also attractive because it implies that the method should be more broadly applicable. Indeed, Yang and Brill have independently found that a similar procedure facilitates anion binding to methemoglobin [20]. With enough binding agent in solution, of course, incubation at a subzero temperature will not be necessary for adduct formation. However, the incubation method will be advantageous when there are solubility limitations or there is a need to minimize the ionic strength.

By the way of summary, we list several important considerations for low-temperature spectroscopic studies of adducts in aqueous media: (1) Mass action can be an important driving force for adduct formation as a result of solute pooling during the freezing process. (2) Incubation at −20°C allows for the exploitation of this effect. The suggested incubation temperature is typical for a laboratory freezer, but even lower temperatures are quite feasible [30]. (3) Precipitation of electrolyte and/or protein aggregation is a possible side effect of the solute pooling phenomenon. (4) Introduction of glycerol improves the homogeneity of the solution and reduces the tendency for precipitate formation; however, without solute pooling there is less of a driving force for adduct formation. (5) Finally if one does not use the incubation method, the method of freezing may have some influence on the observed species distribution. A slower method of freezing should allow more time for equilibration when the sample is in the critical two-phase regime.

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