Fluorescence associated with the type 3 copper center of laccase

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Laccases isolated from the lacquer tree *Rhus vernicifera* and the fungus *Polyporus versicolor* show fluorescence emission near 420 nm and phosphorescence emission in the 440–465 nm region. The fluorescence and phosphorescence excitation spectra for both laccases show maxima in the 315–330 nm range, a spectral region corresponding to the absorbance maxima for the type 3 binuclear Cu centers of the two enzymes. Additional evidence is presented for the association of the newly discovered emissions with the type 3 Cu centers of the two laccases.

Laccase *Type 3 Cu* *Fluorescence* *Phosphorescence*

1. INTRODUCTION

Rhus vernificera laccase (monophenol, dihydroxyphenylalanine:oxygen oxidoreductase, EC 1.14.18.1) catalyzes the 4-electron reduction of molecular oxygen by polyphenols. The enzyme contains 4 copper atoms distributed in type 1, type 2 and type 3 sites [1,2]. The type 3 site appears to contain two anti-ferromagnetically-coupled coppers [2–4] and, under certain conditions, titrates as a 2-electron-carrying center [5,6].

Laccase has been widely studied by a variety of spectroscopic techniques including electron paramagnetic resonance (EPR) spectroscopy [1,2], absorbance [1,2] and circular dichroism [7,8]. Fluorescence studies of the enzyme have been confined to observation of emission from aromatic amino acid residues [8]. In the course of our work with the enzyme, we have observed a previously unreported fluorescence. The characteristics of this fluorescence are described below.

2. METHODS

R. vernicifera laccase was isolated from lacquer tree acetone powder and purified as in [9]. The protein had absorbance ratios of 15.2–15.7 (A_{280}/A_{614}) and 0.80 (A_{330}/A_{614}), similar to those reported for the pure protein [9,10]. Gel electrophoresis [11] of the *R. vernicifera* laccase preparations used for the fluorescence and phosphorescence measurements described below showed a single Coomassie blue-staining protein band, suggesting that the enzyme was at least 95% pure. Type 2 Cu-depleted (T2D) laccase prepared as in [10] had a spectrum and type 2 Cu content similar to those reported in [8,10]. The type 3 Cu center of T2D laccase was oxidized by treatment with H_2O_2 as in [12]. Laccase from the fungus *Polyporus versicolor*, purified as in [13], was a generous gift from Walther Ellis and Professor Harry B. Gray of the California Institute of Technology. Stellacyanin was isolated and purified from lacquer tree acetone powder as in [9]. Plastocyanin, isolated and purified from spinach as in [14], was a generous gift from Richard Chain of the Division of Molecular Plant Biology at the University of California (Berkeley).

Absorbance measurements were made using an Aminco DW-2a UV/visible spectrophotometer. Corrected fluorescence emission and excitation spectra were made using a Perkin-Elmer Model MPF-44B spectrofluorometer equipped with a DCSU-2 corrected spectra accessory. Fluorescence

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lifetime measurements were made using a SLM Model 480 phase-modulation spectrofluorometer interfaced with a Hewlett-Packard 9810 calculator and utilizing sinusoidally modulated light from a 450 W Xenon arc lamp. The calculator was programmed to estimate the amplitude contributions from multiple lifetime components. Lifetimes were measured at modulation frequencies of 10 and 30 MHz. Phosphorescence was measured using an Aminco-Bowman spectrofluorometer with a phosphoroscope accessory. Phosphorescence lifetimes were measured using this instrument coupled to a Tektronix Model 564B oscilloscope equipped with a Model C-12 camera.

3. RESULTS

Fig.1 shows the fluorescence emission (excitation at 330 nm) and excitation spectra (emission at 420 nm) of *R. vernicifera* laccase at 20°C. While excitation at 280 nm resulted in tryptophan fluorescence at 330 nm as reported in [8], excitation in the 330 nm region where the type 3 binuclear Cu center is known to absorb [1,2,5,12] resulted in a previously undetected emission centered at 420 nm (fig.1A). The excitation maximum for the newly-discovered 420 nm emission is at 320 nm (fig.1B). The exact absorbance maximum of the type 3 Cu in laccase is not known, since it is associated with a shoulder near 330 nm rather than a true absorbance maximum [1,2]. Within our instrumental 5 nm spectral resolution, the excitation maximum for the 420 nm emission is probably identical to the type 3 Cu absorbance maximum. The excitation spectrum reveals no contributions to the 420 nm emission from species absorbing in the 260–290 nm region. It thus appears that aromatic amino acids with normal absorbance spectra are not involved in the 420 nm fluorescence. Further evidence for the possible association of the 420 nm emission with the type 3 center came from the observation that bleaching of the 330 nm absorbance band by reduction of the type 3 center with sodium ascorbate abolished the 420 nm emission (not shown).

Native laccase was at least 95% pure and had been dialyzed exhaustively to minimize the possibility that the 420 nm fluorescence could have arisen from any low M_r impurity. Further evidence for the hypothesis that the 420 nm fluorescence is

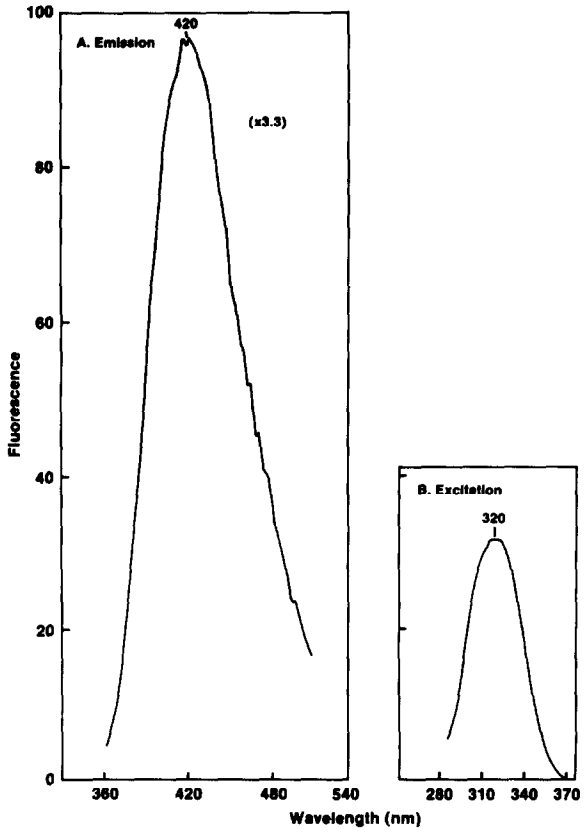

Fig.1. Fluorescence emission and excitation spectra of native *R. vernicifera* laccase. The reaction mixture contained 17.5 μM laccase in 0.5 M ionic strength sodium phosphate buffer (pH 7.0) at 20°C. Slit widths = 5 nm. (A) Excitation wavelength = 330 nm. (B) Emission wavelength = 420 nm.

an intrinsic property of laccases comes from the observation, shown in fig.2A, that a very similar emission was found with laccase isolated from a completely different organism, the fungus *Polyporus versicolor*. An excitation spectrum of this 418 nm emission from the *P. versicolor* enzyme shows maxima at 315 and 330 nm (fig.2B). It should be pointed out that, as is the case for the *R. vernicifera* laccase, the 330 nm absorbance band of the *P. versicolor* protein has been attributed to a binuclear, type 3 Cu center [1,2].

At this point it seemed appropriate to provide additional evidence for the association of the 420 nm fluorescence with type 3 Cu. Samples of plastocyanin and stellacyanin, two proteins that each contain only a single type 1 Cu [1], were ex-

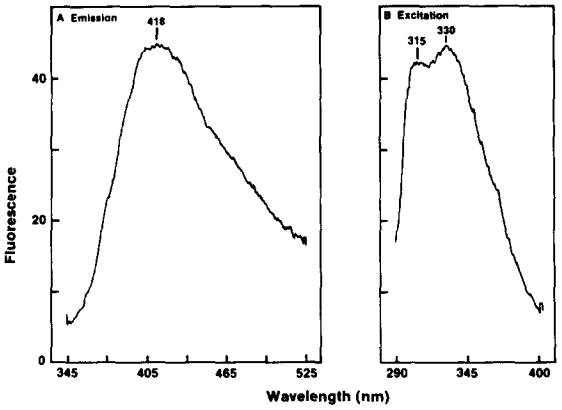

Fig.2. Fluorescence emission and excitation spectra of *P. versicolor* laccase. The reaction mixture contained 13.3 μM laccase in 0.5 M ionic strength sodium phosphate buffer (pH 7.0) at 20°C. Slit widths = 3 nm. (A) Excitation wavelength = 330 nm. (B) Emission wavelength = 418 nm.

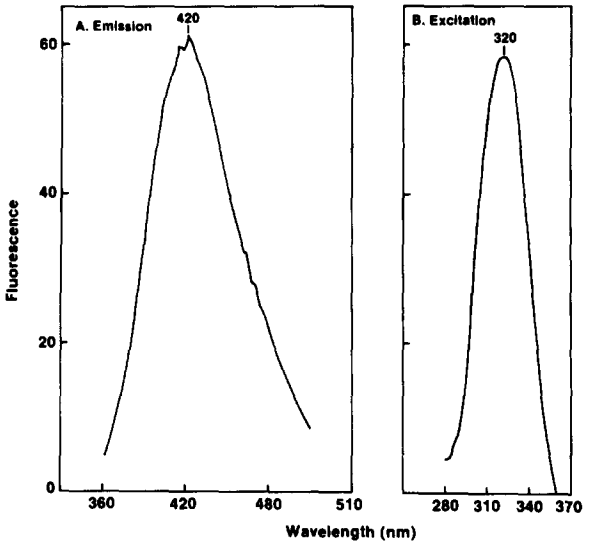

Fig.3. Fluorescence emission and excitation spectra of T2D *R. vernicifera* laccase. Reaction conditions as in fig.1 except that the native laccase was replaced by the H_2O_2-oxidized T2D enzyme.

amined and neither showed any emission in the 420 nm region regardless of excitation wavelength. These results suggest that type 1 Cu centers are not responsible for the newly-observed 420 nm fluorescence seen with the two laccases.

The possibility that the type 2 Cu of laccase was involved in the 420 nm emission was investigated using *R. vernicifera* laccase that had been specifically depleted of 80% of its type 2 Cu using the method in [10]. In agreement with earlier observations [10,12] we found that T2D laccase prepared in this manner shows low absorbance at 330 nm. As might be expected, such preparations of T2D laccase showed little 420 nm fluorescence on excitation at 330 nm. Authors in [12] have proposed that this low absorbance at 330 nm is due to the reduced state of the type 3 Cu in T2D laccase, since the absorbance feature at 330 nm is restored when the T2D enzyme is oxidized by H_2O_2 [12]. We have repeated the experiments reported in [12] and confirmed their observations on the effects of H_2O_2 treatment on the 330 nm absorbance of T2D laccase. Fig.3A shows that H_2O_2-oxidized T2D *R. vernicifera* laccase exhibits the same 420 nm fluorescence seen with the native enzyme. The excitation spectrum (fig.3B) shows the same 320 nm maximum as the native enzyme. Since our T2D laccase preparations contain $\leqslant$20% of their original type 2 Cu but show somewhat greater 420 nm

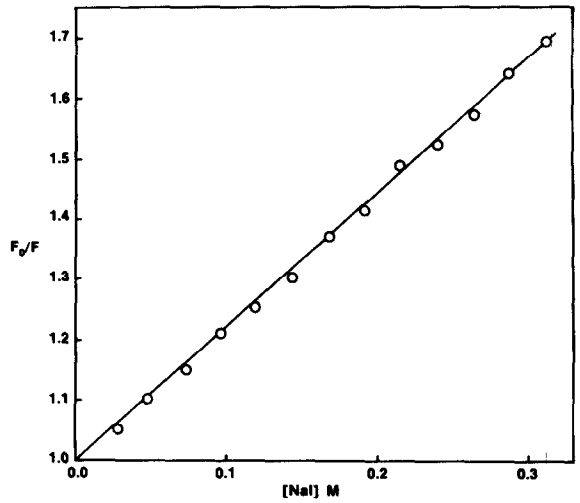

Fig.4. Stern-Volmer plot of *R. vernicifera* laccase fluorescence quenching by I^-. Reaction conditions as in fig.1. Fluorescence was excited at 330 nm and observed at 420 nm. Fifty-μl aliquots of 1.0 M NaI were added to the original 2.0-ml sample of 17.5 μM laccase. Dilution artifacts were corrected for, based on an identical series of additions in which H_2O replaced the NaI solution. The straight line was drawn according to the least-squares best fit to the Stern-Volmer equation: $F_o/F = 1 + K_Q[Q]$ where $[Q]$ is the concentration of the quencher iodide [15].

fluorescence than the native enzyme, it appears likely that type 2 Cu can be ruled out as a significant contributor to the 420 nm fluorescence. In addition, there is no evidence that type 2 Cu contributes significantly to the absorbance of laccase in the 330 nm region.

Fig.4 shows that the fluorescence of native *R. vernicifera* laccase is quenched by I^-. The data for different [I^-] give a good fit to a Stern-Volmer plot [15]. The linear Stern-Volmer plot suggests that the laccase species responsible for the 420 nm fluorescence is freely accessible to the solvent [16]. A Stern-Volmer quenching constant, K_Q, of 2.25 M^{-1} was calculated from the data of fig.4.

Fluorescence lifetime measurements of native *R. vernicifera* laccase at 20°C and pH 6.0 revealed that at least 71% of the 420 nm emission is characterized by decay with a single lifetime of 2.85 ns. The remaining 29% of the emission decayed with considerably longer lifetime(s) ($\geq$7 ns). The origin of this longer lived component(s) is not clear. Fluorescence lifetime measurements at higher pH values exhibited at least two major lifetime components in the 1–5 ns range. The more complicated fluorescence decay observed at these higher pH values is under further investigation.

Laccase also appears to be capable of emission from a triplet state. As shown in fig.5A, native *R. vernicifera* laccase displays phosphorescence at 77 K with an emission maximum at 465 nm. The excitation spectrum for the 465 nm phosphorescence (fig.5B) exhibits a major maximum at 315 nm with a smaller maximum at 278 nm. Phosphorescence was also observed for T2D *R. vernicifera* laccase, although the emission maximum was shifted to 450 nm. This observation suggests that the type 2 Cu is not involved in phosphorescence. A similar phosphorescence was observed at 77 K with the *P. versicolor* laccase, exhibiting emission (fig.5C) and excitation maxima at 440 and 325 nm (fig.5D), respectively. The *P. versicolor* 77 K phosphorescence excitation spectrum also contains shoulders at 288 and 375 nm. Lifetimes of 34.6 and 34.4 ms were measured for the 77 K phosphorescence from the *R. vernicifera* and *P. versicolor* laccases, respectively. Neither stellacyanin nor plastocyanin showed any detectable phosphorescence under the conditions where laccase phosphorescence was observed.

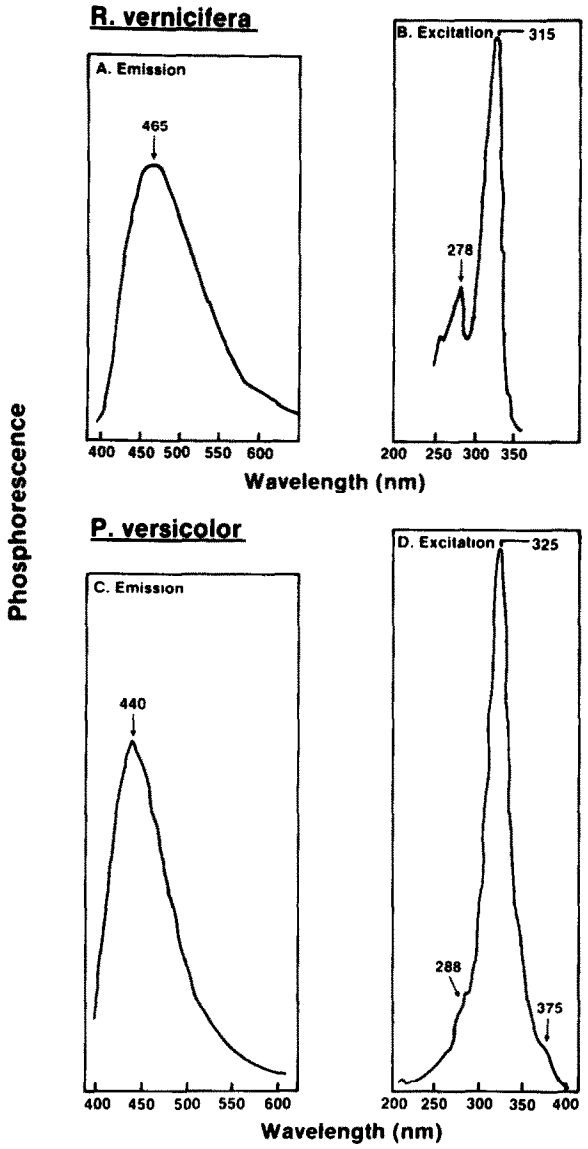

Fig.5. Phosphorescence emission and excitation spectra of native *R. vernicifera* and *P. versicolor* laccases at 77 K. (A,B) The reaction mixture contained 125 μM *R. vernicifera* laccase in 0.5 M ionic strength sodium phosphate buffer (pH 7.0). Slit widths = 8 nm. (A) Excitation wavelength = 330 nm. (B) Emission wavelength = 465 nm. (C,D) The reaction mixture contained 146 μM *P. versicolor* laccase in 0.5 M ionic strength sodium phosphate buffer (pH 7.0). Slit widths = 8 nm. (C) Excitation wavelength = 330 nm. (D) Emission wavelength = 440 nm.

4. DISCUSSION

The data presented above characterize previously undetected emissions from both *R. vernicifera* and *P. versicolor* laccases. Each enzyme exhibits both fluorescence (near 420 nm) and phosphorescence (in the 440–465 nm range). It is possible that the observed fluorescence and phosphorescence do not come from the same chromophore. However, the fact that the major excitation peaks are identical in the fluorescence and phosphorescence spectra of both enzymes, suggests that the same center is involved in both emissions. It is not yet clear why the phosphorescence excitation spectra appear to contain some contribution from aromatic amino acids while the fluorescence excitation spectra do not. This may result from the different temperatures used for the two measurements (293 K for fluorescence *vs.* 77 K for phosphorescence).

The absence of 420 nm emission from proteins, such as plastocyanin and stellacyanin, that contain only type 1 Cu, suggests that the emission does not arise from the type 1 Cu of laccase. Similarly, the removal of at least 80% of the type 2 Cu from *R. vernicifera* laccase without any loss of 420 nm fluorescence makes it unlikely that type 2 Cu is involved. A process of elimination leaves the binuclear type 3 Cu center as the most likely candidate to play a role in the 420 nm fluorescence. This suggestion is strengthened by the close correspondence between the absorbance maximum for the type 3 Cu and the excitation maximum for the newly-discovered fluorescence and phosphorescence. Additional support for this hypothesis comes from the observations (reported above) that reduction of the type 3 Cu, either by addition of ascorbate or during preparation of T2D laccase, causes a large decrease in the 420 nm fluorescence. Although it is quite possible that the type 3 Cu center itself is the emitting species, the above arguments are not meant to imply that the 420 nm emission necessarily comes directly from the binuclear type 3 Cu center per se. It is possible, for example, that the emitting species is an aromatic amino acid residue, with spectroscopic properties strongly perturbed by interactions with the type 3 Cu.

After this work had been completed and this manuscript was in preparation, a manuscript appeared [17] reporting a similar fluorescence emission (emission maxima: 415–445 nm; excitation maxima: 325–345 nm, lifetime < 10 ns) from 3 proteins that contain binuclear Cu centers (tyrosinase, ceruloplasmin and hemocyanin). These observations strengthen our hypothesis that the emission is associated with type 3 Cu centers.

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