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Fluorescence Enhancement of Laccase Induced by Reduction of Cu(II) Sites

("blue" copper proteins/redox-related quenching)

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ABSTRACT **The intrinsic fluorescence of laccase (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2), emitted by its tyrosinyl and tryptophanyl residues, underwent significant enhancement upon reduction of the enzyme redox sites. The increase in quantum yield reached its maximum after the addition of approximately four reduction equivalents and depended on the wavelength of excitation:**

54% increase for λ_{ex} = 250 nm and 76% for λ_{ex} = 305 nm.

A linear correlation between this enhancement and the reduction of the type 1 Cu(II) was observed both by direct measurement and by calculation on the basis of added reductant.

The implications of the fluorescence enhancement and its correlation with the reduction of the type 1 copper are discussed in terms of the possible quenching mechanisms. The possibility of a redox-induced structural transition in the protein is suggested.

The spectroscopic properties of the copper ions of different copper proteins have been successfully used to study their environment and involvement in activity (1, 2). Notably optical absorption and electron paramagnetic resonance were applied to define the state and function of the different copper ions in the "blue" oxidases (3). No direct structural data are yet available on any of the above proteins, but studies involving magnetic resonance techniques (4, 5) as well as substitution of the copper by metal ions such as Co(II) or Hg(II) (6, 7) led to suggestions concerning the protein residues coordinated to the metal. In comparison with the detailed spectroscopic characterization of the different copper sites in the "blue" proteins, relatively little is known about the functional part played by the protein moiety itself, e.g., the nature of substrates' binding sites and possible structural changes involved in activity.

Intrinsic fluorescence of proteins, which is due to the emission of tyrosinyl and tryptophanyl residues, has been studied extensively; variations in its intensity and spectral behavior were very helpful in the investigation of protein structure (8–10).

Cupric ions were found to cause quenching of tryptophan and tyrosine fluorescence upon complex formation with these amino acids in aqueous solutions (11). In the "blue" copper proteins, azurin and stellacyanin, tryptophan emission was shown to be significantly quenched by the bound copper ions (7, 12). However, no difference was found in azurin between the quantum yields of the Cu(II) and Cu(I) states of the protein (7). This observation implies that the redox state of the protein is not involved in the quenching mechanism. Probing the protein part of a "blue" copper oxidase by following its intrinsic fluorescence is the subject of this report. The emission intensity of *Rhus* laccase (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2) was found to depend on the redox state of the enzyme. Moreover, a linear correlation between the quantum yield and the redox state of the type 1 copper was observed. This correlation may be due to a redox-related structural transition in the protein and offers a new approach to the study of its function.

MATERIALS AND METHODS

All solutions were prepared in doubly distilled water. Reagent grade materials were used without further purification. Ascorbic acid was Analar grade supplied by B.D.H., Poole, England. All measurements were carried out in 0.1 M potassium phosphate buffer, pH 7.5. Laccase and stellacyanin were prepared from the acetone extract of *Rhus vernicifera* lacquer supplied by Saito and Co. Ltd., Tokyo, Japan, according to the procedure previously described by Reinhammar (13). Azurin was isolated and purified by the method of Ambler (14) from *Pseudonomonas aeruginosa.* The purity of the proteins used was checked by measuring the absorption of the "blue" band relative to the absorption at 280 nm. The ratios obtained accorded well with the values cited in the literature.

Fluorometric titrations at 25 ± 1°C were carried out anaerobically in a specially constructed cell: To a fused quartz fluorescence and absorption cuvette (1 × 1 × 3 cm internal dimensions) a pyrex tube carrying two arms was attached; one end was closed by a glass stopcock and the second by a gas-tight serological cap. The solution was freed from oxygen by the following procedure: three ml of buffer solution were introduced into the cell and the serological cap was positioned in its place. The solution was then evacuated via the stopcock arm while stirred by a small magnetic bar in order to achieve smooth degassing. After four repetitive evacuations and flushings with argon (purified by a train of four washbottles containing methyl viologen), an aliquot of 10–30 μl concentrated solution of laccase was introduced with a microsyringe via the serological cap into the cell. One further cycle of gentle evacuation and argon flushing was found to be sufficient to remove residual O_2. Thus enzyme denaturation by foaming was kept to a minimum. Reductive titrations of the protein were performed by gradual additions of small aliquots (10–20 μl) of oxygen-free ascorbate solutions via the serological cap. A constant flow of purified, water saturated, argon was maintained through the cell to avoid leakage of O_2.

Titrations were carried out at protein concentrations of 8–12 μM. These were found to be the lowest concentrations at which interference from traces of oxygen was negligible. At these protein concentrations, excitation had to be performed on either side (305 and 250 nm) of the protein absorption maximum (ϵ_{280} = 93500 M^{-1} cm^{-1}) (15) in order to avoid high optical

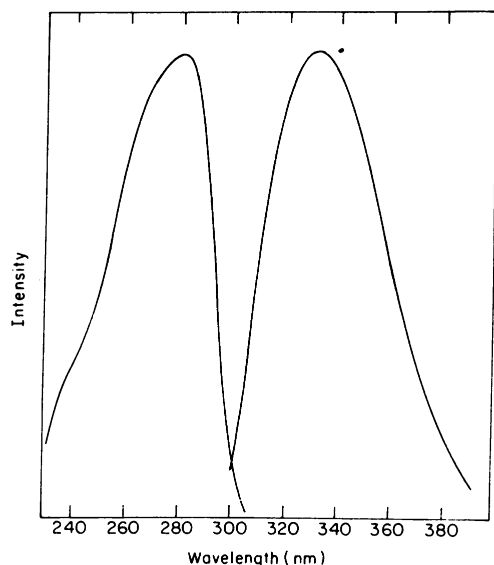

FIG. 1. Corrected excitation and emission spectra of *Rhus vernicifera* laccase. The excitation maximum is 280 nm; emission maximum, 330 nm. The solution was 2 μM laccase in 0.1 M potassium phosphate buffer, pH 7.5. Excitation band width was 6 nm; emission band width, 8 nm.

densities. The required corrections in fluorescence intensity due to the absorption at the emission wavelengths (residual tryptophan absorption) were found to be within the experimental error.

Corrected fluorescence spectra were measured (at 90° to the incident exciting beam) on a Hitachi-Perkin Elmer MPF-3 spectrofluorometer. Absorption measurements were made on a Zeiss PMQ II or a Gilford 2400-S spectrophotometer. Due to the low concentration of protein, rates of reduction were slow and care had to be taken to allow completion of the reduction after each addition of reductant. Ascorbate was used as reductant, since it contributed only negligibly to the absorption of the solutions in the spectral regions investigated.

The quantum yields were obtained from the areas of the emission spectra of the protein and *N*-acetyltryptophanamide, NATA, a standard of known quantum yield, and from the absorbances *A* at the exciting wavelength.

$$Q = Q_{NATA} \cdot \frac{(\text{area})_{Prot}}{(\text{area})_{NATA}} \cdot \frac{A_{NATA}}{A_{Prot}}$$

A quantum yield of 0.13 has been reported for NATA (twice-recrystallized) in aqueous solution at 25°C (16).

RESULTS

The corrected excitation and emission spectra of oxidized *Rhus* laccase are shown in Fig. 1. The maxima of excitation and emission are at 280 and 330 nm, respectively. Excitation at 250 nm led to the same emission maximum at 330 nm, whereas excitation at 305 nm caused a small red shift to 338 nm. The quantum yields for excitation at 280 nm and 305 nm were found to be 0.022 and 0.011, respectively, based on *N*-acetyl-tryptophanamide as standard. Upon reduction of the enzyme, its emission intensity gradually increased (Fig. 2) and reached a maximal value after the addition of about four reduction equivalents. The maximal enhancement was about 76% for excitation at 305 nm and about 54% for excitation at 250 nm. Reoxidation of the reduced enzyme by oxygen re-

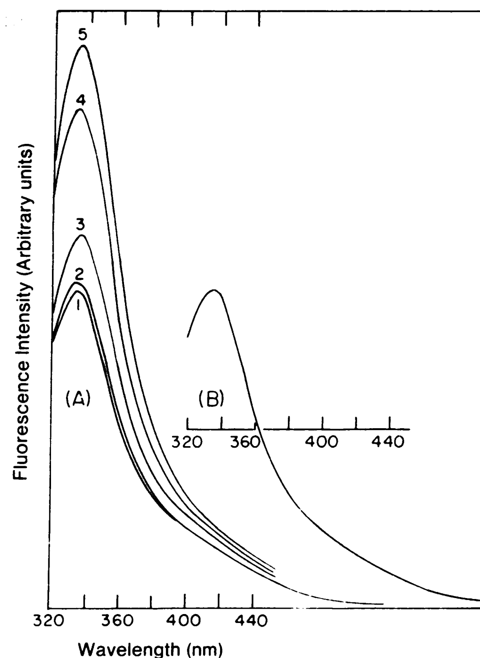

FIG. 2. Anaerobic redox titration of laccase as reflected in its fluorescence intensity. Excitation wavelength was 305 nm for the solution of 8 μM laccase in 0.1 M potassium phosphate buffer, pH 7.5. (A) Stepwise addition of oxygen-free ascorbate solution (1 mM, freshly prepared and containing 10 μM EDTA). (B) Following reoxidation by oxygen.

stored the original emission spectrum of oxidized laccase (Fig. 2).

The relative fluorescence change as a function of added reduction equivalents is graphically presented in Fig. 3. It is evident that the observed relative changes in fluorescence intensity follow closely the extent of reduction of the type 1 Cu(II) as calculated on the basis of the data of Reinhammar and Vänngård (17).

In a further series of titrations the absorbances of the redox active sites of the oxidase at 614 and 330 nm were measured in parallel to the measurements of the emission spectra. The enhancement in emission was found to change linearly with the reduction of type 1 Cu(II) as measured at 614 nm (Fig. 4). No such simple relationship between the fluorescence en-

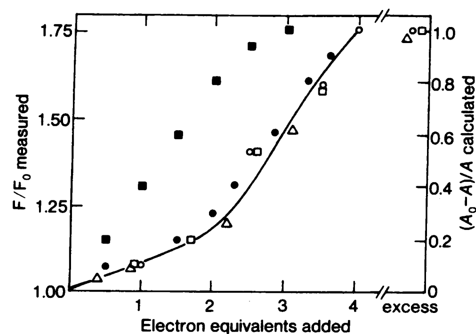

FIG. 3. Fluorescence enhancement of laccase upon reduction. Excitation wavelength was 305 nm. ○,△,□, fluorescence enhancement data from three different sets of titrations (8–12 μM laccase). ●,■, extent of reduction of type 1 Cu(II) and type 3 Cu(II), respectively, calculated from data of Reinhammar and Vänngård (17).

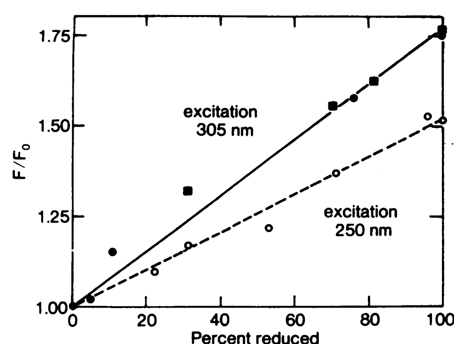

FIG. 4. Plot of fluorescence enhancement versus fraction of type 1 Cu(II) reduced. ●,■, data from titrations at 9 μM laccase. O, data from titrations at 8 μM laccase. Percent reduced type 1 Cu(II) was calculated from the measured absorbance at 614 nm after correction for the absorbance of the reduced protein.

hancement and the absorbance decrease of the 330-nm band could be observed.

The fluorescence spectra of azurin and stellacyanin in both oxidized and reduced states were measured. In agreement with previous observations (7) no difference in quantum yields could be observed for azurin. Essentially no difference was found between the quantum yields of the two states of stellacyanin.

DISCUSSION

Rhus vernicifera laccase contains six tryptophanyl and 24 tyrosinyl residues (13). The fluorescence emission of the protein results largely from tryptophans which are either excited directly or by energy transfer from tyrosines. This is indicated by the dependence of the emission spectra on the excitation wavelengths: Excitation at 305 nm, where only tryptophan absorbs, leads to an emission maximum at 338 nm. But excitation at 280 nm, where both tryptophan and tyrosine absorb, or at 250 nm, where essentially only tyrosine absorbs, causes a small shift in the emission to 330 nm, along with a 2-fold increase in efficiency. Direct emission of tyrosines may also contribute to this behavior. Upon reduction of the oxidase the emission intensity increases without change in the shape of the spectrum or in the position of the maximum, and irrespective of the excitation wavelength. These observations preclude any specific assignment of the emitting residues undergoing enhancement. The finding that the difference in maximum enhancement at the two excitation wavelengths is small is worth noting and may imply that in both cases the same tryptophan residues are involved.

The linear correlation of the increase in protein fluorescence with the reduction of the type 1 Cu(II) is a somewhat surprising result in view of the pronounced absorption band of the oxidized protein centered at 330 nm. This chromophore may be a potential acceptor for nonradiative energy transfer from the tryptophans which emit in the same spectral region (10). In fact, the emission of the copper-containing oxygen-carrying protein hemocyanin has been found to be quenched by this mechanism (18). The so-called copper band present in the hemocyanin–O_2 complex at 340 nm was shown to serve as an acceptor for a Förster type energy transfer from the excited tryptophan residues. The extent of energy transfer was found to depend linearly on the degree of oxygen binding to the protein and was used to study this binding equilibrium extensively.

Our observation that the fluorescence quenching is linearly correlated with the redox state of the type 1 Cu(II) of laccase and that no such correlation could be found with the redox site associated with the 330-nm absorption band indicates that a different mechanism is operative in the quenching in this case. Interestingly, the two "blue" single-copper proteins, azurin and stellacyanin, do not show any significant changes in their intrinsic fluorescence intensity or spectra as a result of valence changes of the copper center, although it has been suggested that in both cases a tryptophan is in close proximity to it (7, 19). It is noteworthy that the binding of copper ion, independent of its oxidation state, causes very pronounced quenching of the apoprotein (4.7-fold for stellacyanin (6) and 6-fold for azurin (A. Grinvald, J. Schlessinger, I. Z. Steinberg, and I. Pecht, unpublished results). In contradistinction, no difference in quantum yields could be observed between apo and holo hemocyanin, i.e., the copper ions do not affect the emission in this case (17). The lack of correlation between the absorbance changes at the 330-nm band and the fluorescence enhancement makes a nonradiative energy transfer to the former band rather unlikely.

The alternative mechanisms responsible for the changes in fluorescence intensity stem from the sensitivity of tryptophans to their environment. Thus both quenching by charged or paramagnetic groups as well as changes in the polarity of neighboring residues would lead to the observed effects. The insensitivity of the emission intensity of azurin and stellacyanin to the redox state of the copper ion is at least a good indication that it is not the paramagnetic Cu(II) state that causes the quenching. This mechanism, however, cannot be excluded completely on these grounds. Another plausible explanation would be one involving a structural change in the protein triggered by the change of the redox state of type 1 copper ion. It is conceivable that the interdependence of the different redox sites of laccase, indicated by the effect of ligand binding on redox potentials (17) and by the kinetics of its reduction (20, 21), involves a conformational transition. Such a transition would lead to changes in the nature or location of the amino acid residues (e.g., charged carboxylate or ammonium groups) in contact with the tryptophans and, therefore, cause the variation in their emission intensity.

Changes in tryptophan fluorescence intensity have been used to monitor activity of a variety of proteins, e.g., binding of substrates to enzymes or of haptens to antibodies (16, 22). The pronounced enhancement of laccase fluorescence which is caused by its reduction may be an additional way of following its function, notable in kinetic studies.

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