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PEROXIDE BINDING TO THE TYPE 3 SITE IN RHUS VERNICIFERA LACCASE DEPLETED OF TYPE 2 COPPER

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SUMMARY: The interaction between hydrogen peroxide and oxidized Rhus vernicifera laccase from which the type 2 copper has been removed, was investigated. For that end, the circular dichroic spectrum of the modified enzyme has been measured in the presence of increasing concentrations of hydrogen peroxide. The characteristic band observed upon binding peroxide to native laccase is also observed for the type 2 copper depleted enzyme. However, there are several quantitative differences in the latter one. First, the intensity is lower and band width is larger. Secondly, from the titrations, it becomes apparent that the affinity for H_2O_2 is markedly lower than that of the native enzyme. While the affinity for the native enzyme is higher than 10^8 M^{-1}, it decreases to $1 \cdot 10^4$ M^{-1} for the type 2 depleted enzyme.

INTRODUCTION

The reversible removal of the type 2 copper from Rhus vernicifera laccase (1-3) produces an enzyme derivative which is of significant value for investigating the functional and structural nature of its different copper sites and their interactions (2-5). However, conflicting reports have been published about spectroscopic features of this enzyme derivative prepared by essentially the same procedure (2-5). Thus, while the 330 nm band characteristic of the oxidized type 3 site has been reported to be absent in the type 2 copper depleted laccase (T2D) and the 615 nm band remained unaffected (2), another report claimed essentially the reverse; namely, that no change takes place at the 330 nm band,

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yet a marked decrease in extinction occurs at 615 nm (4). More recently, an attempt has been made to resolve this controversy by providing evidence that the type 3 site in T2D is reduced, and at least the spectral features at 330 nm can be recovered by oxidation with hydrogen peroxide (5). In contrast, other studies indicated that the type 3 site of T2D laccase is in an oxidized state (2,6).

The type 3 copper binding site of laccase, which is a coupled binuclear one, has been proposed as the dioxygen binding and reduction site (7-11). In its native, oxidized form, this site has been shown to bind hydrogen peroxide with rather high affinity and specificity (7,8,10). The role of that peroxy-laccase complex in the dioxygen reduction mechanism is an interesting and important issue, and has been examined in some detail (9-11). In the above mentioned study (5) of T2D laccase oxidation by H_2O_2, the high affinity peroxide binding has been reported to be lost upon removal of type 2 copper. In order to examine the effect of the latter modifications on the enzyme in its interaction with peroxide, we have monitored the circular dichroic spectrum of that system during the course of its titration The near ultra violet CD spectrum has earlier been shown to provide a characteristic evidence for the peroxide binding to the binuclear copper site in laccase as well as other proteins like hemocyanins and tyrosinase (7-13). Hence, it enables a clear distinction between the oxidation of copper site and its binding of peroxide. We have found that although the affinity of the T2D laccase for peroxide is markedly decreased compared to that of the native enzyme, it still is capab!. of forming a peroxy-complex as evidenced by the characteristic CD band.

MATERIALS AND METHODS

Laccase solutions were made up in 0.1 M HEPES buffer, pH 7.0, and the protein concentrations were determined from the

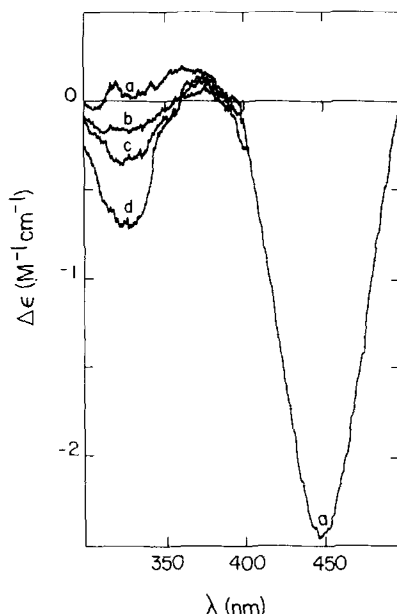

Figure 1. CD spectra of T2D laccase (23 μM) titrated with hydrogen peroxide. a) no peroxide; b) 23 μM; c) 115 μM; and d) 1150 μM of H_2O_2 added to solution a. All other conditions as in the text.

absorbance at 615 nm (ε = 5700 M^{-1} cm^{-1}). Type 2 copper depleted laccase was prepared according to the method of Graziani et al (1-3). Its copper content was determined spectrophotometrically with biquinoline. The two, independently prepared samples of T2D laccase studied in this work contained 2.8 and 2.9 Cu ions per mole protein, respectively. In view of changes caused in the molar extinction at 280 nm of T2D laccase, protein concentration was determined by the Lowry method. This showed that while essentially no change occurs in the intensity of the 615 nm band, that at 280 nm increases to ∿99700 M^{-1} cm^{-1} (6). T2D laccase preparations were oxidized by excess ferricyanide which was removed by extensive dialysis or gel filtration. Protein concentrations examined ranged from 23 to 70 μM. CD spectra were measured in 1 cm cuvettes on a Roussel Jouan Dichrograph III. Data were collected by means of a Tektronix minicomputer averaging 200 measurements per nm.

RESULTS AND DISCUSSION

The circular dichroic spectrum of oxidized T2D laccase in the near UV region is similar to that of the native enzyme. Namely, both the negative (447 nm) and positive (375 nm) bands are present with close to the same intensity (cf. also ref. 2). The shorter wavelength features, though not too well resolved, do look similar. Upon addition of H_2O_2, changes in the region

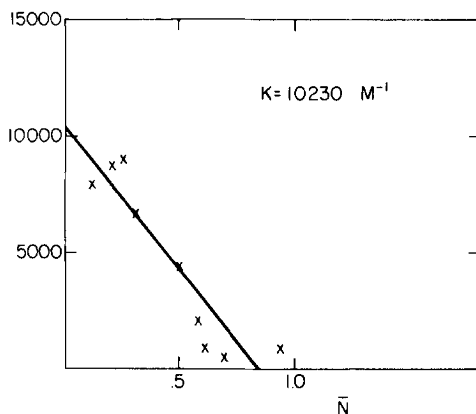

Figure 2. Scatchard plot derived from titrations of T2D laccase with hydrogen peroxide. L is the concentration of free ligand (H_2O_2), and $\bar{N}$ is the fraction of complex divided by total enzyme concentration. $\bar{N}$ is determined at $(\Delta\varepsilon_o - \Delta\varepsilon_x)/ \Delta\varepsilon_o - \Delta\varepsilon_N)$ where $\Delta\varepsilon_o$ is the intensity of the 330 nm band for T2D laccase without peroxide, $\Delta\varepsilon_N$ is the intensity of the same band after addition of a 100 fold excess of H_2O_2, and $\Delta\varepsilon_x$ denotes the intensities of the various intermediate peroxide concentration.

300-400 nm emerge. Figure 1 provides typical illustration of these changes. A prominent band appears at 330 nm, while that at 442 nm is unaffected. The intensity of the 330 nm band increases with peroxide concentration, reaching a maximum value of 0.75 M^{-1} cm^{-1} in the presence of a hundred fold excess of H_2O_2 From several such titrations, done on two independently prepared T2D laccase samples, a Scatchard plot was constructed and is shown in Figure 2.

The formation of the peroxy complex is reversible. This has been shown by the regeneration of both the original CD as well as the near UV and visible electronic and EPR spectrum of oxidized T2D laccase (6) upon reduction by excess ascorbate, followed by extensive dialysis against ferricyanide and and buffer.

The negative CD-band formed at ∿330 nm upon addition of hydrogen peroxide to oxidized T2D laccase bears close resemblance to the one produced when 1 mole equivalent of H_2O_2 is added to oxidized native laccase, and shown to be a peroxy-laccase

complex (7,8,11). Conspicuous differences are a lower intensity, increased width, and the requirement of a rather large excess of peroxide in order to produce that band with T2D laccase. Still, the appearance of the (-)330 nm CD band in a concentration dependent and specific manner, clearly indicates that a T2D laccase-peroxy complex is indeed produced. This spectral feature is exhibited by the native form of laccase as well as by the binuclear copper sites in other proteins like hemocyanins and tyrosinase (12,13).

The possibility that the new band is formed as a result of reoxidation of a partly reduced enzyme can be excluded for several reasons: Prior to the peroxide titration, the T2D laccase was dialyzed against a large excess of ferricyanide which was removed by gel filtration just before the titration with H_2O_2 monitored via the CD spectra. We have shown in other experiments that even reduced T2D laccase is fully reoxidized under these conditions (6). Secondly, as can be seen in Figure 1, the intensity of the 450 nm band is not affected by the addition of peroxide, and neither are the bands at higher wave length (not shown), demonstrating that at least the type 1 copper is fully oxidized at the beginning. Finally, anaerobic titrations of a similar sample of T2D laccase with benzohydroquinon (6) or ascorbate (14) has shown that three electron equivalents are required for the complete reduction of the 615 nm band.

Taking the maximum $\Delta\varepsilon = -0.75\ M^{-1}\ cm^{-1}$ as a measure for full formation of a T2D laccase-peroxide complex, a linear Scatchard plot could be constructed. This yielded a stability constant of 10200 M^{-1} for a 1:1 complex between the reactants (Figure 2). In comparison, the stability constant for the native laccase-peroxide complex was estimated as $\geqslant 10^9\ M^{-1}$ (7,8). Hence,

the affinity of laccase towards peroxide decreases drastically when the type 2 copper is missing.

It is interesting to note that while at wavelengths longer than 400 nm, the CD spectrum of T2D laccase is essentially identical with that of the native oxidized enzyme, in the near UV region the positive CD band at 375 nm is significantly smaller for T2D laccase compared with the intact protein [$\Delta\varepsilon$ = -0.7 M^{-1} cm^{-1} for native laccase (8,9,11), and -0.2 M^{-1} cm^{-1} for T2D laccase.] The same is the case for the peroxy complexes ($\Delta\varepsilon$ = -2.6 M^{-1} cm^{-1} vs. -0.75 M^{-1} cm^{-1} for the native and T2D laccase-peroxide complex, respectively).

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