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THE REDUCTION OF FUNGAL LACCASE AT HIGH pH

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SUMMARY

1. The nature and mechanism of the reduction of fungal laccase (*p*-diphenol: O_2 oxidoreductase, EC 1.10.3.2) obtained on an increase in pH have been studied by optical and electron paramagnetic resonance (EPR) spectroscopy and by measurements of O_2 concentration.

2. The decreases in the optical absorption and the EPR signal of the "blue" Type 1 Cu^{2+} at high pH indicate that this ion is reduced. This is confirmed by oxidation with hexachloroiridate(IV) which restores the blue color. The "nonblue" Type 2 Cu^{2+} remains divalent over the pH range studied, as seen from the EPR spectra.

3. Approximately one equivalent of hexachloroiridate(IV) is sufficient to restore the color of a pH-bleached protein which suggests that the reduction involves a single electron. A comparison between the optical spectra at pH 5 and 8 shows that the two-electron accepting unit, which at pH 5.5 is reduced concomitantly with the Type 1 Cu^{2+}, remains oxidized in the protein brought to high pH. This unit can be reduced at pH 8.3 by octacyanotungstate(IV), as shown by the fact that this reductant in anaerobic titrations is found to add about two electrons (and no more) to a protein already having the Type 1 copper reduced. Thus, an increase in pH introduces a difference in the reduction behavior of the electron acceptors in fungal laccase.

4. Oxygraph experiments show that there is no production of O_2 with an increase in pH, as would occur if water was oxidized by laccase. On the contrary, there is a continuous consumption of O_2 at both pH 5 and 8, indicating that the protein preparation contains a reducing substance which is responsible for the pH-dependent reduction.

INTRODUCTION

Fungal laccase (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2) is an enzyme which catalyzes the oxidation of various substances of diphenolic nature by O_2. The protein molecule contains four copper atoms[1] of at least three different kinds. In the oxidized form of the protein, the Type 1 copper is divalent, has an unusual

Abbreviation: EPR, electron paramagnetic resonance.

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electron paramagnetic resonance (EPR) spectrum and is responsible for the intense blue color of the protein[2]. This Cu is associated with an exceptionally high standard oxidation–reduction potential[3], and recent kinetic studies[4] have established its electron transfer role in the catalytic process. The Type 2 copper is also divalent in the oxidized protein. It exhibits no unusual physical properties, contributing little to the optical absorption in the region of 600 nm and having an EPR spectrum with parameters similar to those found for most small complexes of Cu^{2+}. It is essential for the enzymic activity[5], although its function has not been established.

The remaining two copper atoms in the molecule were earlier thought to be monovalent in the oxidized protein as they did not contribute to the paramagnetism[6,7]. However, recent studies[8,9] suggest that the two diamagnetic copper ions are present as a Cu^{2+}–Cu^{2+} pair which can accept two electrons. This two-electron accepting site has been associated with an intense absorption at approx. 330 nm (ref. 9).

This communication deals with the behavior of laccase at high pH. In 1965, MALMSTRÖM *et al.*[10] reported a reversible loss of blue color at pH values above 6, accompanied by a partial decrease in the total EPR intensity. These results suggested that Cu^{2+} was reduced at high pH. As electron donors, water or a specific reducing group of the protein were considered. The standard potential of laccase is so high[3] that on thermodynamic grounds water might well be the donor, and experiments on the exchange of atoms between water and O_2 in the presence of laccase[11] supported this view. However, measurements of O_2 concentration presented in this work show that no stoichiometric amounts of O_2 are produced on an increase in pH. This renders the oxidation of water by laccase unlikely. Other experiments clearly demonstrate that the reduction of laccase occurring at high pH involves only one electron which is taken up by the Type 1 Cu^{2+}. Thus, an increase in pH causes a difference in the reduction behavior of this copper and the other electron acceptors.

MATERIALS AND METHODS

Reagents and anaerobic titrations

Fungal laccase A was prepared by the method of FÅHRAEUS AND REINHAMMAR[12]. Potassium octacyanotungstate(IV) dihydrate was the same as that used earlier[8], and ammonium hexachloroiridate(IV) was obtained from Schuchardt Chemical Co., Munich. Reagent grade chemicals and deionized distilled water were used throughout. Buffers were extracted with dithizone (0.01 % in CCl_4). Anaerobic titrations were carried out as before[8].

Optical and EPR measurements

Absorbance measurements at a particular wavelength were made with a Zeiss PMQ-II spectrophotometer, and difference spectra were recorded with a Cary-15 spectrophotometer.

EPR spectra were recorded at 9.15 GHz and 77 °K with a Varian E-3 spectrometer. The relative intensities of the Types 1 and 2 Cu^{2+} were determined by the method of VÄNNGÅRD[13]. The pertinent g and A values used for these calculations were, for the Types 1 and 2 Cu^{2+}, $g_{||} = 2.190$ and 2.243, $g_{\perp} = 2.04$ and 2.04, $|A_{||}| = 88$ and 185 gauss, and $|A_{\perp}| = 10$ and 30 gauss, respectively.

Oxygraph experiments

Measurements of O_2 concentrations were made with a GME Model K oxygraph equipped with a Beckman 39065 oxygen sensor.

RESULTS

Effect of high pH on the spectroscopic properties of laccase

In the first report on the pH-dependence of the optical and EPR properties of laccase[10], the absorption at 610 nm and the total EPR intensity were measured as a function of pH. At pH 9 where the blue color was almost completely bleached, about 50 % of the EPR intensity remained. This was thought to be due to the presence of denatured protein molecules. The experiment has now been repeated, and the EPR signal has instead been analyzed in terms of contributions from Types 1 and 2 Cu^{2+}. The results are given in Fig. 1 which shows the absorption at 610 nm, the total EPR intensity and the intensities of the Types 1 and 2 Cu^{2+}, all as a function of pH. The EPR intensity of the Type 1 Cu^{2+} corresponds very closely to the blue color, and both parameters can be fitted to a curve, also shown in Fig. 1, calculated on the assumption that the loss of blue color and Type 1 EPR signal is associated with the dissociation of one proton from an acidic group having an apparent pK of 7.45. This value is in good agreement with the earlier reported number[10]. The EPR intensity of the Type 2 Cu^{2+} is relatively constant except at the extremes of the pH range where denaturation occurs. This leads to the appearance of an EPR signal[2] with parameters similar to those of Type 2 Cu^{2+}. The total EPR intensity drops to around 50 % at the higher pH values.

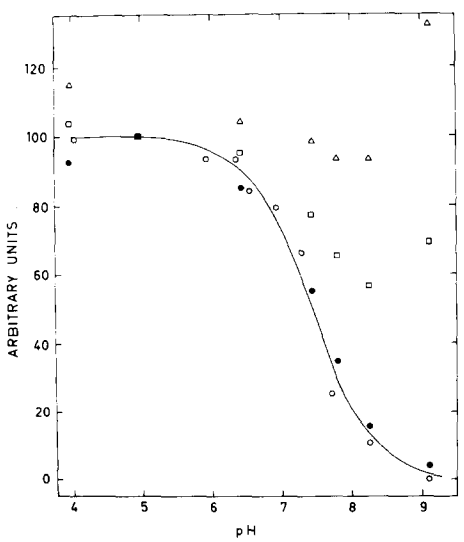

Fig. 1. The effect of pH on the spectral properties of laccase. ○, absorbance at 610 nm; □, total EPR absorption intensity; ●, intensity of the Type 1 Cu^{2+}; △, intensity of the Type 2 Cu^{2+}. Corrections for the optical absorption remaining at pH 9 have been made, and all values are normalized to 100 at pH 5. The concentration of laccase A was approx. 0.1 mM for the optical studies and 0.2 mM for the EPR measurements. Buffers and ionic strength were the same as previously described[10]. The solid line was calculated for a quantity, changing from 100 to 0 on the dissociation of one proton from an acidic group with an apparent pK of 7.45.

It is apparent from optical spectra given by MOSBACH[1] that reduction of laccase with ascorbate at pH 6 produces spectral changes over the entire visible and near-ultraviolet region. A recent study[9] has shown that the loss in blue color on such reduction is accompanied by the disappearance of an intense peak around 330 nm. In contrast, the published spectrum of the protein, bleached at high pH, indicates that the changes in the 380–500-nm region are rather small. A difference spectrum of the protein at pH 5 *versus* pH 7.8 is shown in Fig. 2. It is clearly seen that an increase of pH causes essentially no change of the optical absorption in the range from 320 to 490 nm. In the ultraviolet region changes occur, but these have not been studied in detail.

In one experiment a protein sample was brought to pH 8 and thereby partially bleached. The solution was made anaerobic, and the pH was adjusted back to 6. A return of blue color was observed.

Experiments involving oxidation and reduction of laccase at high pH

To get more information on the nature of the pH-dependent reduction we have performed experiments in which laccase bleached at high pH was treated with oxidizing or reducing agents. Fig. 3 shows the time-dependence of the absorption at 610 nm on rapid adjustment of the pH from 5 to 8.3 (Fig. 3A) and on subsequent additions of near equivalent amounts of hexachloroiridate(IV) to the same solution

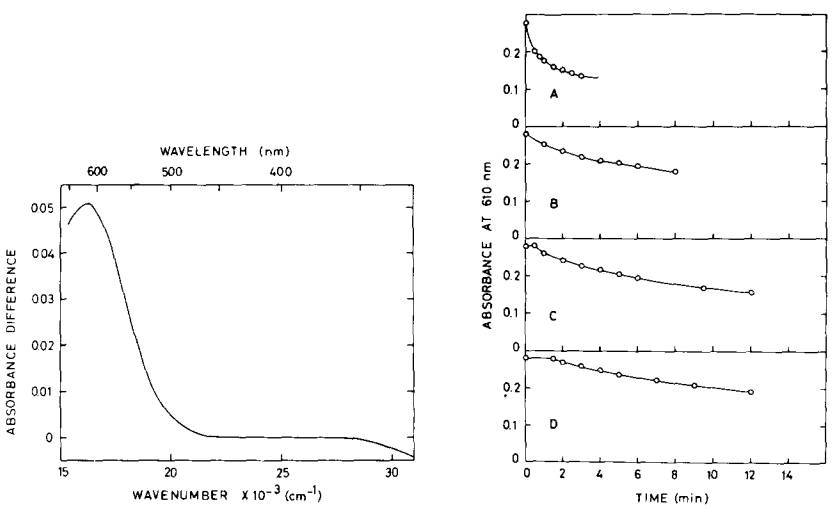

Fig. 2. Difference spectrum of laccase at pH 5 *vs.* pH 7.8. Dual compartment cuvettes with a total optical path of 1 cm were used. The sample cuvette contained 1.0 ml phosphate buffer (pH 7.8, $I = 0.1$) in one compartment and 1.0 ml acetate buffer (pH 4.97, $I = 0.1$) *plus* 0.1 ml 0.3 mM laccase A in the other compartment. In the reference cuvette the enzyme was placed in the high-pH buffer. The protein was mixed with the buffers and kept at room temperature for about 40 min before the spectrum was recorded.

Fig. 3. Reoxidation of laccase at high pH with hexachloroiridate(IV). Laccase A, 0.057 μmole, in 0.5 ml water was mixed with 0.5 ml phosphate–borate buffer (pH 8.26, $I = 0.1$) and the absorption change at 610 nm was observed as shown in A. After several minutes (at time zero in B) 1.3 electron equivalents of hexachloroiridate(IV) were added. There was an immediate and complete return of blue color followed by a slow decrease (tracing B). To the same solution 1.3 equiv. were again added (at time zero in C) followed by an addition of 2.6 equiv at time zero in D. Approx. 30 min were required to obtain stable readings at 610 nm.

(Figs. 3B–3D). It can be seen that 1.3 equiv of this oxidizing agent produce a complete return of color, followed by a slow decay which reproducibly occurs on successive additions of oxidant.

Anaerobic reductive titrations at pH 5.5 have shown that laccase can accept between three and four electrons[8]. In these experiments, octacyanotungstate(IV) proved to be a particularly useful titrant. It has an absorption band near 420 nm while its oxidized form does not, allowing one to observe the presence of titrant in the solution. An anaerobic titration has now been preformed of laccase bleached at pH 8.3. It was found that the absorption at 420 nm decreased due to a reduction of the protein absorption (*cf.* ref. 8) until about 2 electron equivalents of titrant had been added and increased upon further addition of titrant. This shows that the protein can accept at most two electrons at this pH and standard potential (0.5 V, *cf.* ref. 8).

Oxygraph and related experiments

An apparent O_2 dependence of the color loss was observed earlier[11]. We have now found that the rate of additional color loss that results on making a solution anaerobic depends on the particular preparation of the protein. Extensive dialysis against water and repassage over DEAE-Sephadex A-50 did not significantly change the behavior of a given preparation.

The O_2 concentration as a function of time in a laccase solution at different pH values is shown in Fig. 4. There is a continuous consumption of O_2 as the protein is kept at pH 5, brought to pH 8.3 and back again to the lower pH.

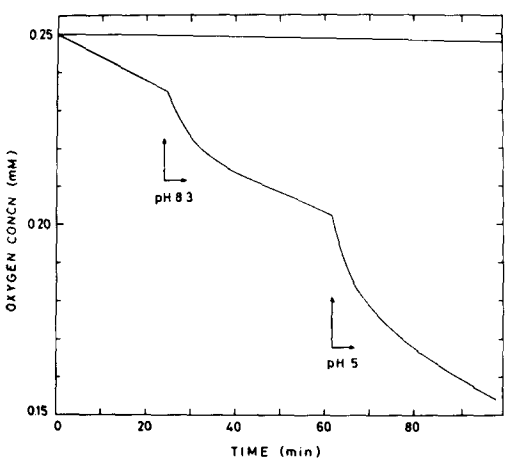

Fig. 4. The consumption of O_2 by a solution of laccase. A solution (0.125 mM) of laccase A, 130 mg in 16.5 ml of water (pH 5), was allowed to equilibrate for several hours with the atmosphere at room temperature. It was then placed in the cell of an oxygraph, the system was closed, and the solution was stirred rapidly. After 24 min 40 μl 1 M NaOH were added to change the pH to 8.3. The pH was returned to 5 at 62 min by the addition of 40 μl 1 M $HClO_4$. The initial slope corresponds to a consumption of 0.3 mole of O_2 per mole of laccase per h. The upper tracing indicates the O_2 consumption of the electrode alone.

DISCUSSION

State of laccase bleached at high pH

The data in Fig. 1 indicate that the Type 1 Cu^{2+} is reduced on an increase in pH as both the blue color and the EPR signal from Type 1 Cu^{2+} are decreased. Type 2 Cu^{2+}, on the other hand, remains divalent. That the Type 1 Cu^{2+} has actually been reduced is confirmed by the experiment with hexachloroiridate(IV) (Fig. 3), which shows that the blue color can be restored by the addition of an oxidizing agent. As approximately one electron equivalent is sufficient to bring the color back, the pH-dependent reduction seems to involve only one electron. In this respect it differs from the reduction obtained on the addition of reducing agents at lower pH values, in which the Type 1 Cu^{2+} is reduced concomitantly with a two-electron accepting unit[8,9]. The latter is associated in its oxidized form with an absorption band at 330 nm (ref. 9). It clearly remains oxidized in the protein at high pH, as an increase in pH produces no change in the absorption at this wavelength (Fig. 2). However, in an anaerobic titration of the pH-bleached protein with octacyanotungstate(IV) about two electrons are added to the protein molecule as was seen from the consumption of the titrant. These electrons apparently go into the two-electron accepting unit, which is consistent with the finding[9] that on titration with quinol at high pH the 330-nm band disappears on the uptake of two electrons.

Thus, an increase of the pH produces a difference in reducibility between the Type 1 copper and the two-electron accepting unit similar to the effect of fluoride at pH 5.5 (see ref. 9). The differentiation occurs somewhere in the pH range 5.5–7.5 and might be the cause of the loss of enzymic activity on the alkaline side which occurs at about pH 6 (A. LINDBERG, C. STRÖMBERG AND B. G. MALMSTRÖM, unpublished work, *cf.* ref. 15). The bleaching of laccase at high pH is due to a reduction of the Type 1 Cu^{2+} only by a substance with such properties that an apparent pK of 7.4 results.

The color increase obtained when an anaerobic solution was brought from pH 8 to 6 most likely is caused by a partial transfer of electrons from the Type 1 Cu^{+} to the two-electron accepting unit. As the latter is thought to accept electrons in pairs at equilibrium[9], electron transfer probably occurs between different protein molecules.

Mechanism for the reduction of laccase at high pH

In an earlier publication we suggested[11] that water could be the electron donor in the pH-dependent reduction, particularly as the standard oxidation–reduction potential of laccase determined at pH 6.2 is so high[3] that the oxidation of water is thermodynamically feasible at high pH, provided the standard potential of the protein is independent of pH. Furthermore, it was found that the enzyme can effect an exchange of the atoms of O_2 and water[11]. However, the hypothesis predicts that O_2 should be produced on increase of the pH of a laccase solution. Clearly this is not observed (Fig. 4). Although the details of the time-dependence of the O_2 concentration in Fig. 4 are not understood, there is a consumption of O_2 that continues even when the pH is raised. Thus, the oxidation of water seems to be excluded.

The results of Fig. 4 show that greater than stoichiometric amounts of oxidizable material is present in the laccase preparations. The nature of this substance

is not known. It cannot be dialyzed away which suggests that it has a high molecular weight or that it is tightly bound to the protein. It is possible that the carbohydrate portion of the protein[12] or denatured protein molecules are oxidized. The nature of the reducing substance and the reason for the observed pH-dependence of the reduction have not been investigated in detail, as the main question for this work was the state of laccase at high pH, particularly the changed redox behavior.

The findings reported in this work have some bearing on the reduction of azurin at high pH reported by BRILL *et al.*[16]. They suggested that the reduction may result from the oxidation of water to O_2 or H_2O_2. However, the redox potential of azurin[17] is so low compared to the potentials of the H_2O–O_2 or H_2O–H_2O_2 systems[18] that such reactions are excluded on thermodynamic grounds alone. A more plausible interpretation of the phenomenon is that the preparations contain material which can be oxidized at the higher pH values, leading to a reduction of the protein.

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