OXIDATIVE TITRATIONS OF RHUS VERNICIFERA LACCASE AND ITS SPECIFIC INTERACTION WITH HYDROGEN PEROXIDE

O. Farver, M. Goldberg, D. Lancet and I. Pecht
Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel

Received September 2,1976

SUMMARY

The reaction of oxidized Rhus vernicifera laccase and H_2O_2 leads specifically to the formation of a stable, high affinity complex. It is characterized by an absorption band at 325 nm and is most probably formed with the type 3 site. Oxidative titrations of laccase show a different pathway from the reductive ones. This is also expressed in different Nernst coefficients observed for each half of the redox cycle (2 for reduction, 1 for oxidation). Oxidation of the type 3 site by H_2O_2 proceeds in a bi-molecular reaction, whereas type 1 is oxidized in an indirect pathway.

INTRODUCTION

Rhus vernicifera laccase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase, E.C. 1.14.18.1) catalyses the reduction of molecular oxygen to water. The role of the three different redox sites of this oxidase in this process and its detailed pathway are subjects of much interest and research (1). No well defined intermediates have yet been characterized and different types of multiple electron transfers were proposed to account for this situation (2). Here we report the observation of a specific high affinity interaction between Rhus laccase and H_2O_2 which leads to a stable, spectroscopically distinct product. We have also carried out oxidative titrations of reduced laccase with different single and multi-electron acceptors. These titrations reveal that the electron distribution in the enzyme's sites is markedly different from that observed in reductive titrations.

The data suggest that the type 3 copper ions are the oxygen reducing site of Rhus laccase and that its high specificity for dioxygen is the result of the capacity of this ion pair in the reduced state to bind O_2 in an analogous manner to that of hemocyanins (3).

EXPERIMENTAL

Laccase was prepared according to Reinhammar (4) from acetone powder obtained from the lacquer of Rhus vernicifera. The purity of the protein

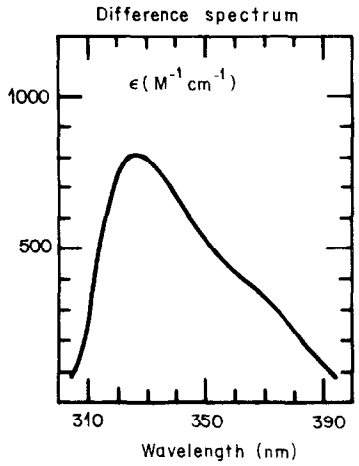

Fig. 1. Effect of hydrogen peroxide on oxidized Rhus laccase. Difference spectrum between oxidized laccase treated with an equimolar amount of H_2O_2 and native oxidized laccase in the near u.v. The spectrum was constructed from several experiments with laccase concentrations varying from 2 x 10^{-6} M to 5 x 10^{-4} M. No changes in the visible spectrum, (400-700 nm) were observed.

was determined by measurements of both its spectroscopic properties and its enzymatic activity with N,N-dimethyl-p-phenylenediamine and found to be similar to that reported earlier (4). The concentration of the protein was determined from the absorbance at 615 nm, using ε_{615} = 5700 M^{-1} cm^{-1}.

All chemicals used were of analytical grade. Doubly distilled water was used throughout. The concentration of the hydrogen peroxide stock solutions was determined iodometrically. All solutions were prepared in 0.1 M potassium phosphate buffer, pH = 7.0 (PPB).

Redox titrations of Rhus laccase were carried out in a specially constructed optical cell, described elsewhere (5). The solutions were freed from oxygen by alternative evacuation and flushing with water saturated argon. Traces of oxygen in the argon were removed by passing it through 4 columns with methylviologen. A slight excess pressure was kept inside the cell in order to prevent O_2 diffusion. The titrant was added with a micro-syringe through a serological cap. The solutions were stirred in the cell with a small magnetic bar.

The absorbance values were corrected for dilution and extinction due to titrant and for residual absorbance of the fully reduced chromophore. Absorption spectra and spectrophotometric titrations were recorded at 25°C on a Cary 15 equipped with a thermostatted cell holder. The kinetic experiments were carried out on a Gilford 240 spectrophotometer.

Electron paramagnetic resonance (EPR) spectra were made at liquid nitrogen temperature in a Varian E-3 spectrometer at about 9 GH_z (X-band) and in a Varian E-12 at about 35 GH_z (Q-band).

RESULTS

A. The addition of an equimolar amount of hydrogen peroxide to oxidized

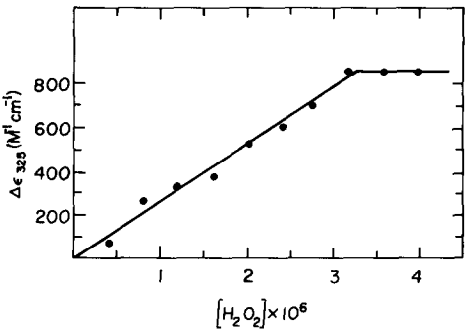

Fig. 2. Titration of oxidized Rhus laccase by hydrogen peroxide. Laccase, 27 ml 3.0 x 10^{-6} M, was titrated with aliquots of 10 µl 1.0 x 10^{-3} M H_2O_2 in a 10 cm cuvette at 325 nm. $\Delta\varepsilon_{325}$ was calculated from the difference in absorbance between hydrogen peroxide treated and oxidized laccase. The line is calculated with K = 1 x 10^9 M^{-1}.

laccase leads to spectral changes in the 300-400 nm region, a new band being formed with a maximum at 325 nm, $\Delta\varepsilon$ = 800 M^{-1} cm^{-1} (Fig. 1). The formation of this band occurs within minutes and even at micromolar concentrations of laccase and H_2O_2 less than 20 minutes are required for completion. From the titrations of oxidized laccase with H_2O_2 (Fig. 2) a lower limit for the affinity of interaction may be estimated $K \geq 10^8$ M^{-1} and the product is stable for days and independent of the presence of O_2. In order to check for the specificity of the spectral change of the interaction of the enzyme with H_2O_2, the effect of a number of other strong oxidants was examined. No change in the spectrum of the enzyme was observed with $PtCl_6^{2-}$, $S_2O_8^{2-}$ + Cu^{2+}, O_2, $MnEDTA^-$, $Mo(CN)_8^{3-}$ and tertiary butyl peroxide while $IrCl_6^{2-}$ or performate led to irreversible changes in the protein probably by non specific attack. Thus it seems that these spectral changes are specific for hydrogen peroxide. Fluoride ions did not inhibit the reaction between laccase and H_2O_2 even at fluoride concentrations as high as 10 mM. The EPR spectra of Rhus laccase show no shift in the hyperfine lines of type 2 copper upon addition of hydrogen peroxide neither in the X- nor in the Q-band. Such a shift has previously been reported for fungal laccase treated with excess H_2O_2 (6).

The effect of catalase and platinum-black, both known as specific and efficient catalysts for peroxide decomposition was examined on the new species formed by laccase and H_2O_2. Incubation with either of the two catalysts for 24 hours caused less than 10% decrease in the extra absorption at 325 nm. The formation of the new spectral band did not lead to any irreversible changes in the enzyme. Thus H_2O_2 treated laccase was reduced

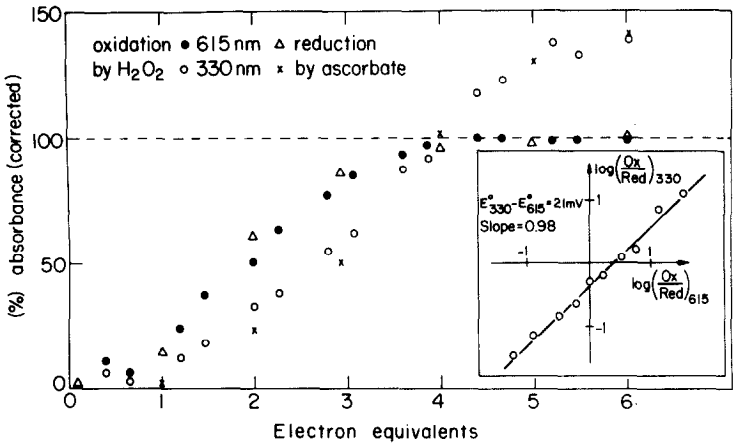

Fig. 3. Anaerobic titration of Rhus laccase.
Reduction by ascorbate ions of hydrogen peroxide treated laccase (Δ for type 1 and x for type 3 Cu). Oxidation of fully reduced laccase by H_2O_2 (• for type 1 and o for type 3 Cu). The corrected absorbance values have been calculated as $\frac{A-A_{red}}{A_{ox}-A_{red}}$ % where A is the measured absorbance, A_{red} and A_{ox} the absorbance of the fully reduced and the fully oxidized chromophore, respectively.

(Insert). Nernst plot of the type 3 Cu versus the type 1 Cu
Applying the Nernst equation to the distribution of electrons between the type 1 and type 3 site, $\log\left(\frac{Ox}{Red}\right)_{330}$ is plotted against $\log\left(\frac{Ox}{Red}\right)_{615}$ where Ox and Red stands for the concentration of the oxidized and the reduced chromophores, respectively. It should be stressed that for calculating Ox_{330} the extinction value at 330 nm for native oxidized laccase is used.

with excess ascorbate under anaerobic conditions and then exposed to air. The resultant spectrum was identical to that of oxidized laccase. In another experiment, H_2O_2 treated enzyme was left overnight with 0.01 μM catalase to remove any residual H_2O_2 and then titrated with ascorbate. Full reduction of this derivative of the enzyme required six electron equivalents (Fig. 3).

B. Oxidative titration of fully reduced laccase by H_2O_2 is shown in Figure 3. The absorption at 615 nm due to the type 1 Cu(II) as well as the absorption at 330 nm due to type 3 reach the values expected for fully oxidized enzyme after the addition of 4 redox equivalents (2 moles H_2O_2). However upon addition of a further mole of hydrogen peroxide, the band in the near UV increases up to the same value observed upon reacting oxidized laccase with H_2O_2. No further change in the 615 nm band was observed upon addition of the third mole of H_2O_2. In order to examine the electron

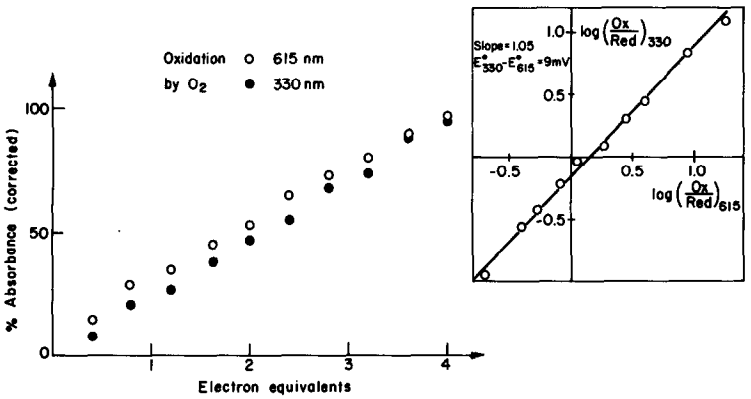

Fig. 4. Oxidation of reduced Rhus laccase with O_2.
Oxidation of fully reduced laccase with O_2 is represented by o for type 1 and • for type 3 Cu. The corrected absorbances have been calculated as described above in the legend to Fig. 3.

(Insert). Nernst plot of type 3 Cu versus type 1 Cu
For details see legend to insert in Fig. 3.

distribution between type 1 and type 3 copper sites the data from several titrations are presented in the insert of Figure 3 in the form of a Nernst plot. The experimental points clearly fall on a straight line with a slope of 1. This finding is in contrast to previously reported reductive titrations of oxidized laccase using one-electron donors, giving a straight line with slope=2 (7,8). The Nernst plot of the peroxide titration also reveals a significantly smaller difference in redox potentials between type 3 and type 1 sites (ΔE = 21 mV) compared to that reported for reductive titrations (35 - 40 mV) (8) and observed also in our preparations. Two other oxidants were employed for titrating reduced laccase. A titration by dioxygen is shown in Fig. 4, and from these data a Nernst plot with a slope of 1 is constructed (Fig. 4 insert). The shape of the titration curves presented in Fig. 4 is in agreement with that reported previously (9). $Mo(CN)_8^{3-}$ also oxidizes type 1 and type 3 Cu(I) at a fast rate. The use of this oxidant is complicated by its lability. Therefore the titration by this reagent was not quantitative. Still since the oxidation by Mo(V) was much faster than the reduction with CN^-, a Nernst plot could be derived giving again a straight line with slope=1.

C. The oxidation of both type 1 and type 3 copper by H_2O_2 at 10°C is slow enough so that the reaction can be followed at both wavelengths simultaneously by regular spectrophotometry. Laccase, fully reduced by

ascorbate was oxidized by successively adding aliquots of hydrogen peroxide and monitoring the absorbance at 615 and 330 nm. This was continued stepwise until full reoxidation of the enzyme had occurred. The appearance of type 3 absorption was found to be the faster reaction and is first order in reduced type 3 and in H_2O_2 concentrations, whereas that of type 1 absorption was a first order process and its specific rate was independent of reduced type 1 and hydrogen peroxide concentrations. At $10^{o}C$ the rate constants of the oxidation of type 3 and type 1 by H_2O_2 were 1.5×10^{3} M^{-1} s^{-1} and 1.0×10^{-2} s^{-1}, respectively, and independent of the stage of reduction of the protein. At $25^{o}C$ the oxidation of fully reduced laccase by H_2O_2 could be followed only at the 615 nm chromophore without using fast kinetics methods, while the oxidation of the 330 nm was too fast ($\tau_{\frac{1}{2}} < 10$ s) to be monitored on a recording spectrophotometer. The oxidation of the type 1 site was again found to be a first order process independent of the peroxide concentration, $k = 1.6 \times 10^{-2}$ s^{-1}.

Adding F^- up to 10 mM had no effect on the rate of reoxidation of the type 3 Cu, whereas the rate of appearance of the 615 nm band decreased by a factor of 5. Even after having added excess H_2O_2 the absorbance due to type 1 Cu(II) had increased to less than 50% of the value expected for full reoxidation. Moreover, even leaving the protein overnight exposed to O_2 did not change the absorbance at 615 nm. However, after extensive dialysis, the expected spectrum for fully oxidized laccase was obtained.

DISCUSSION

Our results demonstrate the formation of a new derivative of oxidized Rhus laccase upon its reaction with H_2O_2 which most probably is a complex formed with the type 3 site. Such a complex is likely to be formed during the reduction of O_2 to H_2O, and its observed high affinity can explain the undetectability of free H_2O_2 (10). The extra absorption band in the near u.v. is similar in shape to the transient observed in the reaction of fully reduced Rhus laccase with O_2 (11). The reaction between this enzyme and H_2O_2 is markedly different from that reported for fungal laccase where the complex was characterized by a much lower affinity and time stability (6).

Since there is no change in the EPR spectra of oxidized Rhus laccase upon the addition of H_2O_2 it seems very unlikely that H_2O_2 should bind to the type 2 copper as has been suggested for fungal laccase (6). This is further supported by the finding that F^- did not inhibit the formation of this derivative although F^- is known to bind strongly to the type 2 Cu (12).

The titrations of Rhus laccase clearly show that the electron distribution between the type 1 and type 3 sites is different in the reductive and

oxidative directions. This is found to be independent of the nature of the oxidant used, namely whether it is a single or multiple electron acceptor. The Nernst coefficients for the electron distribution between type 1 and type 3 is 1 for oxidation and 2 for reduction. This implies that while the oxidized type 3 site behaves as an obligatory 2 electron acceptor, the reduced site is a single electron donor. This is consistent with the structural concept of the site where the two Cu(II) ions form an antiferromagnetically coupled dimer (13). The inequivalence of the titration pathways is an example of hysteresis (14). Since there can be only one equilibrium pathway, at least one of the two parts of the redox cycle should consist of non-equilibrium states (14). This implies metastable conformational differences in the protein probably linked to the coupling-uncoupling of the type 3 pair.

The second order kinetics for the H_2O_2-oxidation of reduced type 3 indicates direct interaction between these species. This, and the observed binding of H_2O_2 to oxidized type 3 are in agreement with the direct involvement of this site in the reduction of O_2 and binding its reduction intermediates. The first order reaction of type 1 implies an indirect oxidation, either through type 2 or through type 3. The marked influence of F^- on this reaction suggests the former pathway.

REFERENCES

1. Holwerda, R.A., Wherland, S. and Gray, H.B. (1976) Ann. Rev. Biophys. Bioengin., 5, 363.
2. Malmström, B.G., Andréasson, L-E. and Reinhammar, B. (1975) The Enzymes (Boyer, P.D., ed), 12, 507, Academic Press, Inc., New York.
3. Freedman, T., Loehr, J. and Loehr, T. (1976) J. Am. Chem. Soc. 98, 2809.
4. Reinhammar, B. (1970) Biochim. Biophys. Acta 205, 35.
5. Goldberg, M. and Pecht, I. (1974) Proc. Nat. Acad. Sci. USA 71, 4684.
6. Brändén, R., Malmström, B.G. and Vänngård,T.I.(1971) Eur. J. Biochem. 18, 238.
7. Reinhammar, B.R.M. and Vänngård, T.I. (1971) Eur. J. Biochem. 18, 463.
8. Reinhammar, B.R.M. (1972) Biochim. Biophys. Acta 275, 245.
9. Makino, N. and Ogura, Y. (1971) J. Biochem. 69, 91.
10. Pecht, I. (1969) Abstr. Commun. 6th Meet. Fed. Eur. Biochem. Soc. Madrid p. 150.
11. Andréasson, L-E., Brändén, R. and Reinhammar, B. (1976) Biochim. Biophys. Acta 438, 370.
12. Brändén, R., Malmström, B.G. and Vänngård, T. (1973) Eur. J. Biochem. 36, 195.
13. Solomon, E.I., Dooley, D.M., Wang, R.H., Gray, H.B., Cerdonio, M., Moguo, F. and Romani, G.L. (1976) J. Am. Chem. Soc. 98, 1029.
14. Revzin, A., Neumann, E. and Katchalsky, A. (1973) J. Mol. Biol. 79, 95.