Oxygen effects in aqueous solutions of laccases: absorption spectra and reactivity

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The optical absorption spectra of laccases in aqueous solutions were found to undergo reversible changes in the presence of O_2. It was suggested that dioxygen is coordinated in the active center of the completely oxidized native enzyme. Abnormal behavior of superoxide radical anions upon variation of the laccase concentration was found by pulse radiolysis.

Key words: laccases, molecular oxygen, electron transfer, optical absorption spectrum, pulse radiolysis, superoxide radical anion.

The nature of the reaction sites and the mechanism of the elementary stages of redox catalysis with multielectron transfer in the processes involving copper-containing enzymes remain unclear in many respects, despite numerous and detailed studies (see, for example, Refs. 1—4). In particular, when considering the molecular mechanism of catalysis by laccases, the problem of conditions of electron transfer from a substrate to the primary acceptor site of the enzyme molecule is still avoided.[4,5] Moreover, it is assumed that the dioxygen molecule is included in the cycle only after the enzyme has undergone complete multistage reduction to give intermediates that ensure re-oxidation of the copper-containing centers and reduction of dioxygen to water. Meanwhile, the donor-acceptor interaction of dioxygen with monomeric compounds and polymeric systems has been studied both experimentally and theoretically.[6—8] In this work, we attempted to elucidate experimentally to what extent this interaction is significant for the kinetics of enzymatic oxidation.

For a biopolymer molecule, the extent of charge transfer to the O_2 molecule can vary, depending on the nature of the donor fragment of the macromolecule. As the ionization potential decreases, this ranges from weak charge transfer complexes (CTC) to structures characterized by complete charge transfer. Previously, assumptions concerning this point have been made and verified experimentally, in particular, by the optical absorption spectra of several metal complexes,[9—12] and used for interpretation of the mechanism of the primary stages of catalytic oxidation.[13] In this work, in addition to spectroscopic studies of the oxygen effect in aqueous solutions of laccases *Coriolus hirsitus* (Lch) and *Coriolus zonatus* (Lcz), we compared by pulse radiolysis their reactivities in aerobic and anaerobic media toward the primary products of radiolysis of aqueous solutions of the same composition.

Experimental

The laccase Lch (monophenol, dihydroxyphenylalanine: oxygenoxidoreductase KF 1.14.18.1) was isolated by a published procedure.[2] The molecular mass of Lch is 55 kDa: Lch is distinguished by high thermal stability. It has been found previously[14] that the enzymic activity of Lch is retained upon absorption of γ-radiation up to a dose of ~10 kGy. The laccase Lcz (molecular mass 60 kDa) was isolated by a known procedure.[15] The homogeneity of the enzyme preparations was checked by HPLC and electrophoresis. Solutions were prepared using potassium hydrogen and dihydrogen phosphates and sodium formate (Sigma), bidistilled water, and helium (spectral purity) (the latter for maintaining anaerobic conditions).

Spectral measurements were carried out using Specord M-40 and Hitachi-557 spectrophotometers.

The pulse radiolysis setup based on an Elektronika 003 linac was described in detail previously.[16] Pulses of accelerated electrons with a duration of 40 ns (energy 5 MeV, current 7.5 A) were used. The dose per pulse was varied from 5 to 20 Gy (rhodanide dosimetry). The data were recorded using an S9-8 digital oscillograph connected to an IBM PC, where the data were collected, stored, and subjected to primary processing. Pulse experiments were performed at 15±2 °C.

Results and Discussion

Laccase is the simplest of "blue" copper-containing oxidases, containing four copper ions, which are divided into three types in accordance with spectroscopic properties. T1 is the blue copper center with a characteristic spectral band at ~600 nm ($\varepsilon \approx 5000$ L mol^{-1} cm^{-1}) and

Published in *Izvestiya Akademii Nauk. Seriya Khimicheskaya*, No. 8, pp. 1471—1474, August, 2000.

1066-5285/00/4908-1464 $25.00

a relatively small HFC constant in the ESR spectrum, equal to ~40—70 cm^{-1}. T2 is the mononuclear copper center with a normal HFC constant of ~140 cm^{-1}, which exhibits no spectral transitions. T3 is the binuclear copper center in which the copper atoms are coupled antiferromagnetically through a bridging hydroxide ligand and which does not exhibit any ESR spectrum at 77 K but absorbs light at about 330 nm with $\varepsilon \approx 5000$ L mol^{-1} cm^{-1}. It should be emphasized that in the published data available to the authors, it is almost impossible to find out whether the data were collected under aerobic or anaerobic conditions. In any case, we do not know any studies of the influence of O_2 on the electronic spectra of laccases. In this work, we specially studied the effect of dioxygen on some properties of laccases in neutral aqueous solutions.

Optical absorption spectra. The optical absorption spectrum of Lch in a neutral air-saturated aqueous solution is shown in Fig. 1 (curve *1*). When the solution is saturated with helium, clear-cut changes are observed in the 360—600 nm spectral range (Fig. 1, curve *2*). This effect is strictly reversible, *i.e.*, after air has been passed, the absorption band is completely restored (Fig. 1, curve *3*). The optical absorption spectrum of an air-saturated aqueous solution of Lch is in good agreement with the spectra recorded previously under the same conditions.[4] Virtually the same spectral characteristics and the same oxygen effect were observed for an aqueous solution of Lcz (Fig. 2). These results of spectral measurements are retained on passing from neutral to weakly acidic solutions (pH ~5).

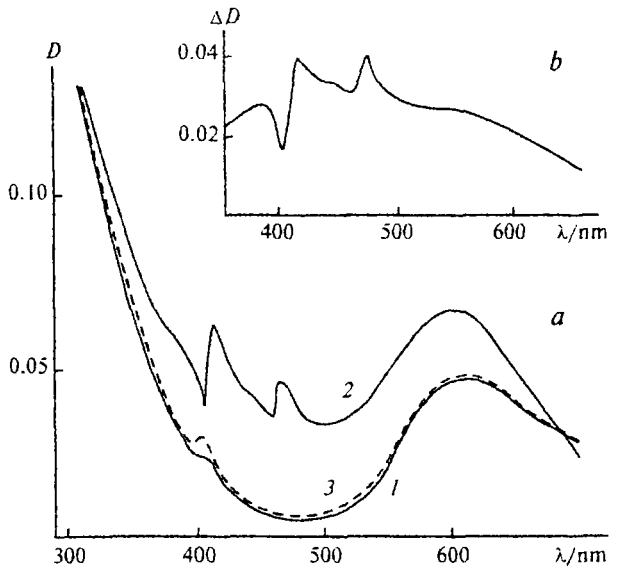

Fig. 1. *a*. Optical absorption spectra of an aqueous solution of Lch (7.8 μmol L^{-1}) at pH 6.8 and $T = 298 \pm 2$ K: air-saturated solution (*1*), a solution saturated with helium for 15 min (*2*), and the same solution saturated again with dioxygen (*3*). *b*. Differential spectrum.

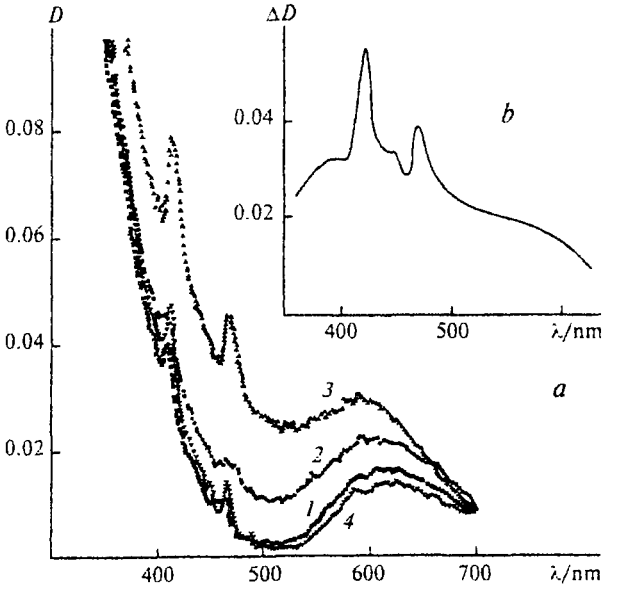

Fig. 2. *a*. Optical absorption spectra of an aqueous solution of Lcz (8.3 μmol L^{-1}) at pH 6.8 and $T = 288 \pm 3$ K: air-saturated solution (*1*), the solution immediately after the injection of air into the measuring cell (*2*), a solution saturated with helium for 15 min (*3*), and a solution saturated with air for 15 min (*4*). *b*. Differential spectrum.

Thus, our experiments showed that dioxygen definitely reacts with the laccase molecule. Since the effect is reversible, the interaction is weak and has, apparently, a donor-acceptor nature. At present, it is generally believed that the T2 center and the binuclear T3 center form an active cluster responsible for the reduction of O_2 to two H_2O molecules. It is commonly considered that the copper centers should be already reduced. However, the essence of the catalytic effect of the enzyme, *i.e.*, the reasons for the decrease in the activation energy for the substrate oxidation by dioxygen and for the transfer of an electron from the substrate to the T1 center, have not been discussed. If the effect that we found is actually related to the coordination of O_2, this coordination occurs most likely in the ligand field of the T1 center because it is characterized by substantial charge transfer from the S atom of the cysteine moiety to the Cu^{II} ion. The starting samples also can contain the reduced copper centers of the T1 type (≤10%). Interpretation of the structurized spectrum recorded under anaerobic conditions in the wavelength range of 340—600 nm requires additional studies and theoretical calculations.

The reactivity of laccases toward the intermediates formed in aqueous solutions upon radiolysis. The rate constants of the reactions of laccases with e_{aq}^- and with $^{\cdot}OH$, $CO_2^{\cdot -}$, $Bu^t{}^{\cdot}OH$, *etc.* radicals have been determined under anaerobic conditions.[17,18] These reactions have been found[17,18] to include at least two stages: the first (fast) stage requiring several microseconds gives intermediate adducts, while the second stage is a rela-

tively slow (up to several milliseconds) intramolecular transfer of an electron to the copper centers of the enzyme molecule. Since the published data on the rate constants are substantially different, we measured again the rate constants for the first stage of the reaction of e_{aq}^- with laccases in solutions containing $(1.2—25) \cdot 10^{-6}$ mol L^{-1} of Lch and Lcz saturated with helium at pH 7.1. The doses per pulse were 8±1 Gy. The found values ($(1.7\pm0.2) \cdot 10^{10}$ and $(2.0\pm0.2) \cdot 10^{10}$ L mol^{-1} s^{-1} for Lch and Lcz, respectively) are in satisfactory agreement with the data obtained previously.[18] In the presence of $\sim 2.5 \cdot 10^{-4}$ mol L^{-1} of O_2, the rate of consumption of e_{aq}^- would be mainly determined by the sum of the rate constants for its reactions with O_2 and Lc/Lc...O_2 (the formula Lc...O_2 designates symbolically the dioxygen laccase complex). Due to the limited speed of response of the recording equipment, we were able to estimate only the upper limit of the sum of the constants, which was $\sim 3 \cdot 10^{10}$ L mol^{-1} s^{-1}. More definite data on the oxygen effect cannot be obtained within the framework of this experiment. Nevertheless, it was found that important information can be gained by monitoring the behavior of another intermediate formed upon radiolysis of an aqueous solution of laccase under aerobic conditions, namely, the superoxide radical anion $O_2^{\cdot -}$. The following reactions significant for the analysis of the behavior of $O_2^{\cdot -}$ proceed on the microsecond time scale after completion of a pulse in an air-saturated solution of laccase containing phosphate buffer (pH 7.1) and 0.1 mol L^{-1} of sodium formate:

$$e_{aq}^- + O_2 \longrightarrow O_2^{\cdot -} \quad (k_1 = 1.9 \cdot 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}),^{19} \qquad (1)$$

$$^{\cdot}OH + HCO_2^- \longrightarrow CO_2^{\cdot -} + H_2O \quad (k_2 = 3.2 \cdot 10^9 \text{ L mol}^{-1} \text{ s}^{-1}),^{19} \qquad (2)$$

$$CO_2^{\cdot -} + O_2 \longrightarrow O_2^{\cdot -} + CO_2 \quad (k_3 = 2.0 \cdot 10^9 \text{ L mol}^{-1} \text{ s}^{-1}),^{20} \qquad (3)$$

$$e_{aq}^- + \text{Lc/Lc...O}_2 \longrightarrow \text{Adduct I } (k_4), \qquad (4)$$

$$CO_2^{\cdot -} + \text{Lc/Lc...O}_2 \longrightarrow \text{Adduct II } (k_5). \qquad (5)$$

The lifetime of $O_2^{\cdot -}$ under these conditions has been found[18] to be several microseconds. For this reaction scheme, the variation of the concentration of the superoxide radical anion upon variation of the Lc concentration can be written in a form convenient for experimental verification

$$[O_2^{\cdot -}]_f^0/[O_2^{\cdot -}]_f = 1 + (k_4 + k_5) \cdot [\text{Lc}]/(k_1 + k_3) \cdot [O_2], \qquad (6)$$

where $[O_2^{\cdot -}]_f^0$ and $[O_2^{\cdot -}]_f$ are the concentrations of radicals in the absence and in the presence of Lc, respectively, at the same time. The optical density measurements at 255 nm were carried out ~300 μs after completion of the pulse. Figure 3 presents the results of these experiments. It was found that for Lch, $k_4 + k_5 \approx 4.0 \cdot 10^{12}$ L mol^{-1} s^{-1}, and for Lcz, $k_4 + k_5 \approx 1.3 \cdot 10^{12}$ L mol^{-1} s^{-1}, which clearly exceeds the diffusion limit and, hence, contradicts the physical meaning. Direct monitoring of the decay of the hydrated electron in a solution containing laccase together with O_2 does not agree with this result either (see above).

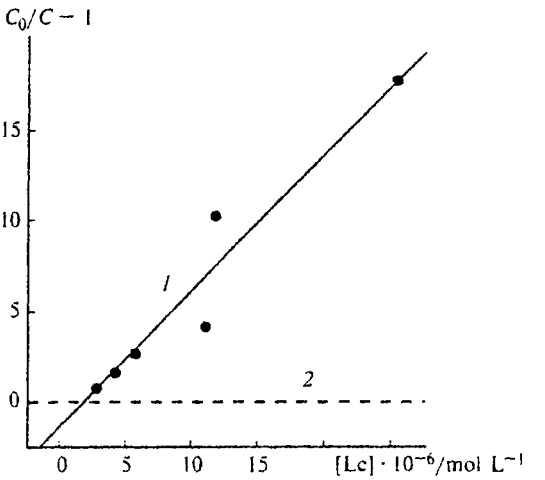

Fig. 3. Variation of the relative concentration of the superoxide radical anions formed in an air-saturated aqueous solution of Lch containing phosphate buffer (pH 7.1) and 0.1 mol L^{-1} of sodium formate on exposure to a pulse of fast electrons (8.5 Gy $pulse^{-1}$): (*1*) experimental dependence, (*2*) dependence calculated from Eq. (6) with the parameters $k_1 + k_3 = 2.1 \cdot 10^{10}$ L mol^{-1} s^{-1}, $k(e_{aq}^- + \text{Lch}) + k(CO_2^{\cdot -} + \text{Lc}) = 2.4 \cdot 10^{10}$ L mol^{-1} s^{-1}, $[O_2] = 2.5 \cdot 10^{-4}$ mol L^{-1}.

Such a sharp decrease in the concentration of the superoxide radical anions following an increase in the concentration of Lcz, which cannot be described in terms of a sensible formal kinetic model, might be due to a deficiency of dioxygen in the bulk solution caused by its binding by enzyme molecules, first of all, by its protein moiety in which the concentration of the donor centers can exceed the concentration of the copper centers by 2—3 orders of magnitude. However, saturation of the solution with dioxygen did not change fundamentally the behavior of superoxide radical anions upon variation of the laccase concentration; in addition, direct measurement of the content O_2 did not show its substantial deficiency (the difference was at most 15% for the laccase concentration of ~25 μmol L^{-1}).

Anyhow it is evident that the oxygen effects in aqueous solutions of laccases found in this study point to the necessity of refining the generally accepted view on the role of dioxygen in enzymatic oxidation.

Thus, the interaction of dioxygen with the enzyme having (apparently) the donor-acceptor nature was discovered in aqueous solutions of the laccases *Coriolus hirsutus* and *Coriolus zonatus*; in our opinion, this is the main reason for a decrease in the energy barrier to substrate oxidation. The O_2 molecule is presumably

coordinated in the region of the T1 center of the molecule.

The abnormal behavior of the superoxide radical anion upon variation of the laccase concentration is related neither to an increase in the laccase reactivity with respect to the hydrated electron (e_{aq}^-) and/or carboxyl radical anion under aerobic conditions nor to the deficiency of O_2 in the solution.

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Received August 6, 1999;
in revised form March 1, 2000